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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-CD45pos EpCAMpos, SCA1pos, CD117neg, CD138neg, MHCIIneg cells as Foxn1-expressing BM cells.

The online version of this article contains supplemental material.

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 viaramium. Because we have shown in the previous study that both lines nonself-renewing, and therefore the thymus relies on the bone marrow (BM) for a continuous supply of progenitors to maintain T lymphopoiesis (9, 10). The multipotent progenitor (MPP) population in the BM is thought to contain the precursor to thymic ETP (11, 12). With age, the number of MPP declines, suggesting that thymic involution is initiated in the BM (13).

Using a Foxn1 transgenic mouse (Foxn1Tg) model, we demonstrated that age-associated thymic involution is attenuated (14). In aged Foxn1Tg, the frequency of ETP is not reduced, and the number of ETP is higher than in aged wild type (Wt), resulting in a higher number of thymocytes (14). Because overexpression of Foxn1 attenuates the decline in ETP number with age, we were compelled to interrogate a potential function of Foxn1 in maintaining the ETP precursors, which are the MPP in the BM.

In this study, we report that Foxn1 is expressed in the BM of Wt mice and overexpressed in the BM of Foxn1Tg. Foxn1-expressing cells were found within a novel population of Lin-CD45pos EpCAMpos, SCA1pos, CD117neg, CD138neg, MHCIIneg 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: 5583–5593.
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 mice 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 × 10^3) 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 25°C for 1 h.

**BM adoptive transfers**

FACS-sorted Lineage^- Sca1^+ c-kit^+ (LSK) cells (8–16 × 10^3) 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 BM colony assay**

BM cells (10^6) from Wt and Foxn1Tg (1–4 and 19–25 mo) were cultured in MethoCult methylcellulose (Stem Cell Technologies, Vancouver, Canada) at 37°C with 5% CO2. After 9 d, the total number and type of colonies were counted.

**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 D11 and D44 in sorted cells; primers are listed in Table II. Expression of Hprt was used as control.

**Immunohistochemistry**

Sternums were fixed for 48 h in Zamboni solution (4% paraformaldehyde with picric acid) and decalcified in 15% sucrose containing 2% acetic acid (pH 6.8) and transgene Foxn1 mice. The age-associated changes in HSC and MPP populations 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). 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 = 0.036). Furthermore, the sizes of the MP 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). 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 = 0.036). 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.

**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– 21- and 24– 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.
FIGURE 1. Changes in HSC and MPP number with age in Wt and Foxn1Tg mice. One to two million (1-2 x 10^6) BM nucleated cells from Wt and Foxn1Tg were analyzed using flow cytometry to determine percentages that were then used to calculate the total number of MPP and HSC per two tibias and two femurs. HSC were defined as Lin^− Sca1^pos CD117pos CD135neg and MPP as Lin^− Sca1pos CD117pos CD135pos. (A) Total number of MPP in Wt and Foxn1Tg from three age groups. The p values were from Student t test, *p < 0.001 Wt 24–25 mo versus Wt 1–4 mo, †p = 0.002 Wt 20–21 mo versus Foxn1Tg 20–21 mo, ‡p = 0.03 Foxn1Tg 24–25 mo versus Wt 24–25 mo. (B) Total number of HSC in Wt and Foxn1Tg. *p = 0.004 Wt 20–21 mo versus Wt 1–2 mo, †p = 0.036 Wt 24–25 mo versus Wt 1–4 mo, ‡p = 0.036 Foxn1Tg 1–4 mo versus Wt 1–4 mo, §p < 0.001 Foxn1Tg 20–21 mo versus Foxn1Tg 1–4 mo, #p = 0.01 Foxn1Tg 20–21 mo versus Wt 20–21 mo, §§p = 0.01 Foxn1Tg 24–25 mo versus Wt 24–25 mo. (C) Representative flow cytometry profiles showing changes with age in the frequencies of LSK in Wt and Foxn1Tg. LSK cells were identified as Lin^− Sca1pos and CD117pos. Numbers represent the average plus or minus SD. Numbers in parentheses denote the number of mice.

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 Foxn1 Tg, and Wt and Foxn1 Tg Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> cells also expressed CD45 and SCA1; however, these cells were negative for CD117 and MHCII. In agreement with this observation, we detected Foxn1 transcripts by RT-PCR in sorted CD45<sup>−/−</sup> but not in the CD45<sup>+/+</sup> BM cell fraction (Supplemental Fig. 3A). Sorted HSC and MPP from Wt and Foxn1 Tg mice did not express Foxn1 (Supplemental Fig. 3B).

FACS-sorted Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> cells from aged Foxn1 Tg 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 Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> CD138<sup>−/−</sup> (or Syndecan-1, a common marker of plasma cells) and stained for Foxn1. Between 23 and 40% of the Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> were negative for CD138 in Wt and Foxn1 Tg, respectively (Fig. 4B, middle panels, data not shown). Notably, the Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> CD138<sup>−/−</sup> population was greatly enriched for Foxn1pos cells; on average, 45 and 68% of this subset were positive for Foxn1 in Wt and Foxn1 Tg, respectively (data not shown). The frequencies of Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> CD138<sup>−/−</sup> Foxn1pos cells per 100,000 BM nucleated cells were calculated to be 3 ± 1 in old Wt and 95 ± 49 in old Foxn1 Tg (Fig. 4C). Additionally, Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> CD138<sup>−/−</sup> BM cells from Foxn1 Tg 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 Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> CD138<sup>−/−</sup> BM cells from Foxn1cre-Rosa26-Lac Z mice (24 mo) stained positively for β-galactosidase and showed identical morphology as compared with Foxn1pos within the Lin<sup>−/−</sup> EpCAM<sup>−/−</sup> population of Foxn1 Tg and Wt (Fig. 4B, bottom two panels). 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).

Foxn1pos cells are present in the BM of Wt and Foxn1 Tg

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 endosteal or the vascular niches (18). Foxn1pos cells appeared as single cells in the central marrow cavity with proximity to sinusoids 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 Foxn1 Tg, Foxn1pos cells were readily detectable within the vicinity of sinusoids, 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 Foxn1 Tg, the Foxn1pos cell number per section increased; however, there were more Foxn1-expressing cells in aged Foxn1 Tg compared with aged Wt (Fig. 3C). We also detected keratin 14pos cells with an identical morphology to the Foxn1pos cells in both Wt and Foxn1 Tg 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 Foxn1 Tg mice, BM cells were analyzed by flow cytometry for EpCAMpos. EpCAMpos cells were present in both Wt and Foxn1 Tg BM among the Lin<sup>−/−</sup>EpCAM<sup>−/−</sup> cells (Fig. 4A). Both Wt and Foxn1 Tg Lin<sup>−/−</sup>EpCAM<sup>−/−</sup> cells also expressed CD45 and SCA1; however, these cells were negative for CD117 and MHCII. In agreement with this observation, we detected Foxn1 transcripts by RT-PCR in sorted CD45<sup>−/−</sup> but not in the CD45<sup>+/+</sup> BM cell fraction (Supplemental Fig. 3A). Sorted HSC and MPP from Wt and Foxn1 Tg mice did not express Foxn1 (Supplemental Fig. 3B).
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 21–25-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; 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 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 \((p = 0.002, \text{ANOV A})\) (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>−/−</sup>CD45<sup>+</sup>CD117<sup>−</sup>EpCAM<sup>+</sup>CD138<sup>−</sup>BM cells express Notch ligands that are responsible for the commitment to T lineage progenitors. We sorted Lin<sup>−/−</sup>CD45<sup>+</sup>CD117<sup>−</sup>EpCAM<sup>+</sup>CD138<sup>−</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>−/−</sup>CD45<sup>+</sup>EpCAM<sup>+</sup>SCA1<sup>+</sup>CD117<sup>−</sup>CD138<sup>−</sup> MHCIi<sup>−</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
number of MPP significantly declines with age in Wt mice, their number was not reduced but maintained, even in Foxn1Tg mice. 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 Foxn1Tg 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 5.** Differences in the ability of aged BM microenvironment of Wt and Foxn1Tg mice to promote the generation of MPP from HSC. LSK were sorted from CD45.1pos mice, and 8–16 × 10³ cells were transferred into aged Wt or Foxn1Tg hosts via the retro-orbital route. After 10 wk, the frequencies of donor CD45.1pos 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 D4, the physiological ligand for Notch in T lymphopoiesis (31). Notch signals through Foxn1pos-expressing D4 may prime MPP to differentiate toward the T lineage as well as limit B lineage cell 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 Foxn1cre-Rosa26-foxn1Tg host thymus expresses high levels of Foxn1, 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). This notion is supported by the finding that Foxn1pos BM cells express D4, the physiological ligand for Notch in T lymphopoiesis (31). Notch signals through Foxn1pos-expressing D4 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 MMP 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 MMP. 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 MMP 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 MMP 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 MMP (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 MMP with age.

The finding that Foxn1 is expressed in Wt Lin−/− EpCAMpos CD138pos/CD38pos BM cells supports the notion that expression of the Foxn1Tg is not ectopic. Approximately 68% of the Lin−/−low EpCAMpos CD138pos/CD38pos 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 Lin−/− EpCAMpos CD138pos 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 Foxn1\textsuperscript{pos} cells are plasma cells because the majority of the Foxn1\textsuperscript{pos} cells are CD138\textsuperscript{neg}; furthermore, these Foxn1\textsuperscript{pos} cells also express keratin 14, suggesting epithelium in nature.

### Table II. List of primers used in the study

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Forward and reverse sequences of primers used to measure gene expression.
We reason that the identification of Foxn1pos 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 Foxn1Tg 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 VEXpos 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 Foxn1Tg 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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We thank Pat Simms for technical skills in cell sorting, Dr. Jiwang Zhang and Mary Kay Olsen for help with the histology studies, and Dr. Dixit for and Mary Kay Olsen for help with the histology studies, and Dr. Dixit for

Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental
Figure 1

A

Total Number of Nucleated Cells (10^9)

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B

1-4 m

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19-28 m

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Supplemental Figure 3

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A: 

B: 

Wt Foxn1Tg

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Figure Legends

Supplemental Figure 1. A Total number of nucleated cells in the BM of young and aged Wt and Foxn1Tg. Two tibias and femurs were flushed and the total number of nucleated cells in Wt, (closed circles 1-4 mo and closed squares 24-25 mo), and Foxn1Tg (open circles 1-4 mo and open squares 24-35 mo) were counted using a hemocytometer. P values were from t-test; #p=0.01 Wt 1-2 mo vs. Wt 24-25 mo. Each symbol represents result from one individual animal from 3-4 experiments. Error bars are SD. B. In vitro generation of CFU-M, CFU-GM, CFU-G, and CFU-E colonies in young and aged Wt and Foxn1Tg BM. Total number of CFU-M, CFU-GM, CFU-G and CFU-E in Wt and Foxn1Tg 1-4 (white bars) and 19-28 (black bars) months of age. Numbers in parentheses denotes the number of mice. Error bars are SD.

Supplemental Figure 2: K14 expression in the BM of WT and Foxn1Tg mice. Keratin 14 expressing cells in the BM as determined by immunohistochemistry assay. Sternums from Wt and Foxn1Tg mice were fixed, embedded in paraffin blocks, and sectioned at 5 μm. Antigen retrieval was performed on rehydrated tissue sections prior to staining with either rabbit anti-mouse K14 (or rabbit IgG at 2!-g/ml. Primary antibodies were incubated overnight at 4°C. Dako Universal LSAB biotinylated antibody cocktail or Donkey anti-rabbit biotin (6 !-g/ml) followed by streptavidin-HRP was used for detection of primary antibody. Sections were developed with AEC for 1.5 minutes and counterstained with hematoxylin. Pictures were taken using a Leitz Diaplan microscope.
with Retiga 2000R camera. A-D: Wt with anti-K14 antibody (A-C) and control IgG (D). E, F: Foxn1Tg with anti-K14 antibody (E) and IgG (F). G-J: Sorted Lin neg EpCAM pos CD138 neg cells from Foxn1Tg BM stained with anti-K14 (G-I) or rabbit IgG control (J).

Supplemental Figure 3: Foxn1 is expressed in CD45 pos hematopoietic cells but is not expressed in HSC and MPP. A) Tibias and femurs from a Foxn1Tg mouse were flushed and BM cells were sorted for CD45 pos and CD45 neg subsets. RT-PCR was used to measure if Foxn1 expressed in CD45 neg and CD45 pos cells. Vertical lines represent repositioning of gel. B) HSC and MPP were electronically sorted from the BM of Wt and Foxn1Tg mice and RT-PCR was used to determine if HSC and MPP express Foxn1.

Supplemental Figure 4: Assessment of CTP and CIP: A-D, Changes in number and cell cycle profile with age; E, generation of CTP and CIP populations after adoptive transfer into Wt and Foxn1Tg hosts. Flow cytometry was used to determine the total number of CTP and CIP in the BM of young (2-4 mo) and aged (21-25 mo) Wt and Foxn1Tg mice. A) Flow cytometry gating of CTP and CIP. BM CTP were identified as Lin neg CD90 pos CD2 neg and CIP as identified as Lin neg CD90 pos CD2 pos. CTP and CIP frequencies were used to calculate the total number of each population based on the number of nucleated cells isolated from 2 tibias and 2 femurs. B) Total CTP and CIP in young (2-4 mo) and aged (21-25 mo) Foxn1Tg and Wt mice; *p<0.001 CIP in Wt vs. Foxn1Tg. The total numbers of CTP and CIP increased with age in both Wt and
Foxn1Tg (p<0.001 Wt CTP 2-4 mo vs. Wt CTP 21-25 mo; p=0.006 Foxn1Tg CTP 2-4 mo vs. Foxn1Tg CTP 21-25 mo; p<0.001 Wt CIP 2-4 mo vs. Wt CIP 21-25 mo; p = 0.002 Foxn1Tg CIP 2-4 mo vs. Foxn1Tg CIP 21-25 mo). Aged Foxn1Tg 21-25 mo had fewer CTP than Wt 21-25 mo, ‡p=0.002 vs. Foxn1Tg. C) Ratio of CIP over CTP in Wt and Foxn1Tg; †p=0.047 and §p<0.001 were comparisons of Wt vs. Foxn1Tg. D) Electronically sorted CTP and CIP from young (2-4 mo) and aged (21-25 mo) Wt and Foxn1Tg were analyzed for DNA content using propidium iodine and flow cytometry. Data represent the percentages of cells in S, G2/M phase. *p=0.002 CTP from Foxn1Tg 2-4 mo vs. Wt 2-4 mo, †p=0.054 CTP from Foxn1Tg 21-25 mo vs. Wt 21-25 mo. Numbers in parentheses denote number of mice in each age group. Error bars are SD; p values were from t-test. E) Data were obtained from adoptive transfer experiments described in Figure 5 and analyzed for the frequencies of donor CTP, CIP and ratios of CIP/CTP. Each symbol represents result from one mouse. Error bars are SD.