Generation of mTert-GFP mice as a model to identify and study tissue progenitor cells

David T. Breault*,†, Irene M. Min*, Diana L. Carlone*, Loredana G. Farilla*, Dana M. Ambroz*, Daniel E. Henderson*, Selma Algra†, Robert K. Montgomery†, Amy J. Wagers‡, and Nicholas Hole§

Divisions of *Endocrinology and †Gastroenterology, Children’s Hospital Boston, Harvard Medical School, Boston, MA 02115; §Section on Developmental and Stem Cell Biology, Joslin Diabetes Center and Harvard Stem Cell Institute, Boston, MA 02215; and ‡School of Biological and Biomedical Sciences, Durham University, Durham DH1 3LE, United Kingdom

Communicated by Patricia K. Donahoe, Massachusetts General Hospital, Boston, MA, May 23, 2008 (received for review August 9, 2007)

Stem cells hold great promise for regenerative medicine, but remain elusive in many tissues in part because universal markers of “stemness” have not been identified. The ribonucleoprotein complex telomerase catalyzes the extension of chromosome ends, and its expression is associated with failure of cells to undergo cellular senescence. Because such resistance to senescence is a common characteristic of many stem cells, we hypothesized that telomerase expression may provide a selective biomarker for stem cells in multiple tissues. In fact, telomerase expression has been demonstrated within hematopoietic stem cells. We therefore generated mouse telomerase reverse transcriptase (mTert)-GFP-transgenic mice and assayed the ability of mTert-driven GFP to mark tissue stem cells in testsis, bone marrow (BM), and intestine. mTert-GFP mice were generated by using a two-step embryonic stem cell-based strategy, which enabled primary and secondary screening of stably transfected clones before blastocyst injection, greatly increasing the probability of obtaining mTert reporter mice with physiologically appropriate regulation of GFP expression. Analysis of adult mice showed that GFP is expressed in differentiating male germ cells, is enriched among BM-derived hematopoietic stem cells, and specifically marks long-term Brdu-retaining intestinal crypt cells. In addition, telomerase-expressing GFP+ BM cells showed long-term, serial, multilineage BM reconstitution, fulfilling the functional definition of hematopoietic stem cells. Together, these data provide direct evidence that mTert-GFP expression marks progenitor cells in blood and small intestine, validating these mice as a useful tool for the prospective identification, isolation, and functional characterization of progenitor/stem cells from multiple tissues.

Results

Generation of mTert-GFP-Transgenic Mice. Given that previous attempts to generate mTert-GFP mice (using the same 4.4-kb promoter fragment used in this article) failed to show reporter gene expression in adult tissues (15), we used an alternative method to generate transgenic mice with the goal to screen for optimal levels of GFP expression before the production of live animals. Because ES cells normally express telomerase at high levels (5), the use of ES cell transgenesis (rather than pronuclear oocyte injection) allowed for the selection of maximal transgene expression (1st screen) and exclusion of clones that underwent transgene silencing upon genomic integration. G418-resistant ES cell clones expressing high levels of GFP fluorescence were


The authors declare no conflict of interest.

1To whom correspondence should be addressed. E-mail: david.breault@childrens.harvard.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0804800105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA
visualized by using standard epifluorescence microscopy (Fig. 1a). Of 100 G418-resistant colonies examined, only half demonstrated GFP expression, ranging from low to high. Fourteen clones demonstrating the highest level of GFP fluorescence were expanded for further analysis.

Because ES cells down-regulate telomerase expression upon in vitro differentiation (5), each clone was secondarily screened by using embryoid body (EB) formation to ensure proper down-regulation of GFP expression and to prevent selection of clones demonstrating constitutive or dysregulated mTert-GFP expression (2nd screen). Upon differentiation, a decrease in GFP expression could be visualized in cells forming the outer layer of primitive endoderm (Fig. 1b and c).

Of the 14 clones studied, 9 showed appropriate down-regulation of the GFP transgene upon differentiation, and the 2 brightest (clones 14 and 22) were selected for blastocyst injection. Chimeric mice were generated and bred to germ-line heterozygosity. Male and female mice from both lines are healthy, are fertile, demonstrate normal longevity, and can be bred to homozygosity. In addition, Southern blot analyses revealed 1-3 transgene copies for both lines (data not shown). Extensive analysis of both lines confirmed an overlapping GFP expression profile.

**mTert-GFP Expression in Testis.** To validate transgene expression in a tissue known to express telomerase at high levels, (16), GFP expression was analyzed in testis. GFP was detected in both lines 14 and 22 by Western blot analysis of whole testis protein extract by using an anti-GFP antibody. Protein extracts from wild-type and constitutively expressing Actin-GFP-transgenic mice served as negative and positive controls, respectively (Fig. 2a). The amount of total protein loaded from the Actin-GFP control was empirically reduced to allow for a more direct comparison with the less abundant GFP signal present in the mTert-GFP lanes (Fig. 2a).

To establish transgene expression within the germ cell population, we used flow cytometry. Single-cell suspensions were purified from transgenic and wild-type seminiferous tubules, labeled with Hoechst dye 33342, and analyzed by flow cytometry based on DNA content [i.e., haploid (1N), diploid (2N), or tetraploid (4N)] (Fig. 2b). Analysis of both lines demonstrated GFP+ cells in each cell fraction, with the largest percentage of GFP+ cells (as compared with GFP- cells) found in the 4N population (line 22, 72.9 ± 2.0%; line 14, 73.3 ± 9.8%), indicating that a substantial fraction of meiotically active primary spermatocytes express GFP (Fig. 2b). A smaller fraction of 2N cells (line 22, 29.6 ± 2.1%; line 14, 47.4 ± 10.5%), representing spermatogonia, secondary spermatocytes, and/or somatic cells, and 1N cells (line 22, 21.1 ± 2.2%; line 14, 19.9 ± 2.5%), representing spermatids, was observed to be GFP positive (Fig. 2b). Given that 4N cells represented the smallest fraction of the isolated cells (9.4 ± 2.3%) when compared with 2N (14.6 ± 1.3%) or 1N (67.2 ± 2.2%) cells, the fraction of GFP+ cells as a function of isolated germ cells was 4N (line 22, 8.3 ± 0.7%; line 14, 4.9 ± 1.6%), 2N (line 22, 40.0 ± 0.7%; line 14, 66.6 ± 0.6%), and 1N (line 22, 14.2 ± 1.6%; line 14, 13.8 ± 1.5%). These data are consistent with previous reports demonstrating telomerase activity in each germ cell population (16) and validate GFP expression as a marker of male germ cells.

To further localize GFP expression, in situ hybridization (ISH) (Fig. 2c) and immunohistochemistry (Fig. 2d) were performed. GFP mRNA and protein were localized within the seminiferous tubules and corresponded to primary and secondary spermatocytes, consistent with the flow-cytometry analysis. Surprisingly, the majority of spermatogonial stem cells lining the basement membrane (arrowhead) did not express GFP (Fig. 2c and d), suggesting that most slowly cycling spermatogonial stem cells do not express mTert-GFP at baseline. In addition, GFP was not detected within the interstitial (non-germ cell) compartment (Fig. 2c and d). No GFP expression was detected using WT control testis (Fig. 2d). These data indicate that GFP is differentially expressed in male germ cells during meiosis.

**mTert-GFP Expression in BM and Peripheral Blood.** To establish mTert-GFP expression in a second tissue where stem and progenitor cells have been shown to express telomerase (6, 7), whole BM was analyzed for GFP expression. Flow-cytometric analysis revealed ~2% of BM cells to be GFP+ (see Figs. 3 and 4 for phenotypic and functional analysis). In addition, both lines of mice display stable expression of GFP in a small subset (~1%) of peripheral blood cells (PBCs), which include B cells (~45%),
analyzed by using multicolor flow cytometry. Although only
These circulating GFP
GFPhi populations by using multicolor FACS analysis of LT HSCs. Pooled results
(mean ± SEM) from two independent experiments; Student’s t test indicated.
(b) Increased frequency of GFPhi cells within the LT HSC population compared with the
ST HSC population. Pooled results from two independent experiments (mean ± SEM). Student’s t test indicated.

T cells (~25%), and myeloid cells (~25%) (data not shown). These circulating GFP+ PBCs express CD45 (data not shown),
allowing them to be readily distinguished from GFP-expressing putative tissue stem cells.

Phenotypic Analysis of GFP Expression in HSCs. To establish that
GFP is expressed specifically within the HSC, previously shown to express telomerase (6), whole BM from mTert-GFP mice was
analyzed by using multicolor flow cytometry. Although only
long-term (LT) HSCs have unlimited self-renewal potential (17),
additional hematopoietic cells [e.g., transiently self-renewing short-term (ST) HSCs, hematopoietic progenitor cells, and
lymphoid cells] also express telomerase (6, 7). To determine at
the single-cell level the expression of mTert in these hematopoietic subsets, BM cells were analyzed based on the presence or
absence of two distinct cell surface markers (CD34 or Flk2) within the cKit+“Sca1”Lineage− (KSL) subset of hematopoietic
progenitor cells. CD34+“KSL” and Flk2−“KSL” cells correspond to
LT HSCs, whereas CD34−“KSL” and Flk2+“KSL” cells correspond to
ST HSCs (18–20). Extensive analysis of lines 14 and 22 demonstrated similar GFP expression profiles, and so, for
simplicity, results from only line 22 are presented here.

To determine whether GFP fluorescence intensity correlated with self-renewal capacity, LT HSCs were gated into GFPhi, GFPlo,
and GFP− populations [see supporting information (SI) Fig. S1a for
representative FACS plots], and the frequency of cells within each
population was determined. A 2-fold enrichment of LT HSCs was
detected within the GFPhi population compared with the GFP−
population: CD34+ KSL (0.44 ± 0.02% vs. 0.20 ± 0.04%; P = 0.004)
(Fig. 3a). No enrichment of LT HSCs was observed in the GFPlo
population compared with the GFP− population (data not shown).
We also assessed the frequency of myeloid progenitor cells (MPCs)
(cKit+“Sca1”Lineage−), a population of lineage-committed progenitors
without self-renewal potential (21) within the GFPhi and
GFP− populations. The results showed a significant enrichment
of MPC in the GFP− subset of BM cells compared with GFPlo cells
(0.93 ± 0.18% vs. 6.99 ± 2.23%; P = 0.04) (Fig. 3a). Together,
these results indicate that the GFPhi subset of BM cells in mTert-GFP
mice is enriched for self-renewing LT HSCs and contains few
non-self-renewing MPCs. Thus, in mTert-GFP BM, fluorescence
intensity correlates with self-renewal capacity, consistent with a
previous report that telomerase activity is highest in LT HSCs and
increases with hematopoietic cell differentiation (6).

To analyze the fraction of HSCs expressing GFP and the relative
intensity of GFP expression, BM cells were gated into LT or ST
HSC populations and then plotted to display GFP fluorescence. A
2-fold higher frequency of GFPhi cells was detected within LT
HSCs, compared with ST HSCs: (CD34+ “KSL” vs. CD34− “KSL,”
7.7 ± 1.4% vs. 3.8 ± 0.3%; P = 0.04; Flk2+ “KSL” vs. Flk2− “KSL,” 8.2 ±
1.0% vs. 4.3 ± 0.1%; P = 0.02) (Fig. 3b). No differences were
observed between GFPlo and GFP− populations (data not shown).
Representative FACS plots for LT and ST HSCs illustrate the gates
corresponding to the three GFP populations: GFPhi, GFPlo, and
GFP− (Fig. S1a). These results suggest that GFP marks a subset of
highly self-renewing HSCs.

Failure to detect GFP in all LT HSCs may be due to additional
regulation of telomerase activity within these cells. One possible
explanation is that telomerase activity is regulated by cell cycle
progression (22). To assess whether GFP expression is induced in
HSCs during the proliferative phase of the cell cycle, we
performed cell cycle analysis on BM cells using Hoechst dye
labeling, followed by multicolor FACS analysis. Cells were gated
into LT HSCs, ST HSCs, or MPCs and fractionated into Go/G1 (enriched
for noncycling cells) or S/G2-M (proliferating cells) according to their DNA content (2N vs. 4N, respectively).
Each group was then analyzed according to GFP fluorescence
intensity (Fig. S1b). This analysis confirmed our previous finding that
the frequency of GFPhi cells is greatest among LT HSCs when
compared with either ST HSCs or MPCs. Despite a trend toward
an increased frequency of GFPhi cells in the proliferating fractions,
no statistical differences were observed between Go/G1 and S/G2-M within a given population. No differences
were observed between GFPlo and GFP− populations (data not
shown). To further define whether GFP is preferentially
expressed during the proliferative phase of the cell cycle, we used
Ki67 staining (expressed during late G1–S/G2–M, but not in Go)
in conjunction with flow cytometry; ~30% of the GFPhi
population stained positive for Ki67, which was no different from
the control population (Fig. S2). Therefore, taken together, these

Fig. 3. Phenotypic Analysis of mTert-GFP expression in HSCs. (a) Increased
frequency of LT HSC and decreased frequency of myeloid progenitors among
GFPhi populations by using multicolor FACS analysis of LT HSCs. Pooled results
(mean ± SEM) from two independent experiments; Student’s t test indicated.
(b) Increased frequency of GFPhi cells within the LT HSC population compared with the
ST HSC population. Pooled results from two independent experiments (mean ± SEM). Student’s t test indicated.

Fig. 4. Functional analysis of mTert-GFP expression in HSCs. (a) FACS scatter
plot of BM from a male mTert-GFP mouse demonstrating GFP+ cells above the
dotted line (set using WT BM). GFP* or GFP− cells were sorted according to the
gates shown and transplanted into sublethally irradiated female recipient
mice. (b) FACS analysis of BM from female recipient mice 5 months after
transplant. GFP* cells were sorted and transplanted into secondary female recipients. (c) FACS analysis of PBCs 5 months after serial BMT confirm serial LT
engraftment of GFP+ cells. (d) TRAP assay was performed by using isolated
GFP* and GFP+ BM cells (Fig. 4a) to determine telomerase activity. Heat
inactivation samples were used as a negative control. (e) FISH for Y chromo-
some demonstrated engraftment into both myeloid (Gr-1+ and Mac-1+) and
lymphoid (B220+ and CD4+) lineages 2 months after serial BMT. (f) FACS
analysis of GFP* PBCs 5 months after transplantation confirm GFP in both
myeloid (Mac-1+) and lymphoid (B220+, CD4+) lineages at levels comparable
to donor animals.
results demonstrate that GFP expression in LT HSCs is not a function of cell cycle regulation.

Functional Analysis of GFP Expression in HSCs. To validate that mTert-GFP expression marks a functionally significant subset of HSCs, we assessed the capacity of GFP+ BM cells to exhibit long-term, serial, multilineage reconstitution, the functional definition of the HSC. BM was harvested from adult mTert-GFP male mice and sorted by FACS into GFP+ and GFP− fractions (Fig. 4a). The cell-sorting gate for GFP+ cells corresponds to the GFP+ population defined to be enriched for HSCs (Fig. S1a). An equal number of GFP+ or GFP− cells were injected into the tail vein of prewarmed, sublethally irradiated isogenic (129/SvImJ) female mice (GFP− recipients, n = 4; GFP+ recipients, n = 5). Long-term engraftment, defined as the persistence of donor cells >5 months after BM transplantation (BMT), was demonstrated in 50% of recipient mice receiving GFP+ cells by FACS analysis of BM and PBCs (data not shown). In contrast, recipient mice receiving GFP− BM cells showed no evidence for engraftment as late as 3.5 months after BMT (data not shown). Together, these data support the conclusion that (i) mTert-GFP+ HSCs maintain their capacity to compete for the HSC niche, (ii) give rise to long-term engraftment, and (iii) are enriched in the GFPSi population. These data do not, however, rule out the presence of HSCs within the GFP− population, as demonstrated by the phenotypic analysis (see Fig. 3).

To further demonstrate that GFP marks HSCs in mTert-GFP mice, we next performed BMT into secondary female recipients using GFP+ BM cells obtained 5 months after the initial BMT (Fig. 4b). FACS-purified GFP+ cells (10,000 or 40,000) were injected into lethally irradiated isogenic female recipients (to establish the capacity of GFP+ cells for radioprotection). The secondary recipient receiving 10,000 GFP+ cells survived for 28 days, compared with control mice, which died at 13–15 days (data not shown). In contrast, the female recipient receiving 40,000 GFP+ cells demonstrated long-term HSC engraftment, surviving 5 months after 2° BMT, when she was killed for analysis of blood chimerism. Multilineage reconstitution was assessed at 2 and 5 months after 2° BMT (Fig. 4c). At 2 months, PBCs were sorted into myeloid (Mac-1+ and Gr-1+) or lymphoid (CD4+ and B220+) populations, followed by FISH analysis for the Y chromosome, to confirm that cells were derived from the original male donor. The results demonstrated chimerism for both myeloid (9.8% ± 0.8) as well as lymphoid cells (11.4% ± 2.4) (Fig. 4c). The presence of Y chromosome-containing myeloid cells 2 months after serial BMT (7 months after the initial BMT) demonstrates GFP is expressed within the HSC because any transplanted myeloid progenitors would be exhausted by this time (21). Chimerism also was confirmed at 5 months (10 months after the initial BMT) by demonstrating GFP+ myeloid (Mac-1+) and lymphoid (CD4+ and B220+) cells in the peripheral blood (Fig. 4f) at levels comparable to the frequency of GFP expression in these subsets in unmanipulated donor mice (data not shown). These results demonstrate the capacity of GFP+ BM cells to support long-term, multilineage reconstitution in 2° transplants, and thus functionally establish mTert-GFP expression as a marker for the HSC.

Analysis of GFP Expression in Intestinal Stem Cells. For >30 years, it has been accepted that the continuously renewing intestinal epithelium is maintained by pluripotent stem cells located in the intestinal crypts (23). Despite this well established model, intestinal stem cells (ISCs) have remained highly elusive (24). To determine whether GFP expression could serve as a marker for ISCs, we used the only established method to label these cells, long-term (LT) BrdU retention (25). mTert-GFP mice were administered a BrdU pulse during intestinal regeneration, followed by a period of chase. Immunohistochemical analysis of sequential sections from small intestine revealed single GFP+BrdU+ cells in the lower portion of the intestinal crypts (Fig. 5, arrowhead), consistent with previous reports demonstrating single, LT BrdU-retaining cells in the lower crypt (25). Analysis of ~15,700 crypts demonstrated 1 in 157 to contain single GFP+ cells and 1 in 25 to contain single LT BrdU+ cells, similar to prior estimates of intestinal stem cell frequency (25). Of the identified GFP-expressing crypt cells, 17% coexpressed LT BrdU, suggesting that GFP is marking a subpopulation of ISC’s. Expression of GFP within single cells provides us with a useful model system in which to isolate viable cells and functionally characterize this elusive stem cell population.

Telomerase Activity Colocalizes with GFP-Expressing Cells. Finally, to ensure that GFP expression in vivo correlates with telomerase activity, isolated cells from BM, testis, and intestine were fractionated into GFP+ and GFP− populations by FACS and assayed for telomerase activity by using the Telomeric Repeat Amplification Protocol (TRAP) (Fig. 4d and Fig. S3). Telomerase activity was detected within each GFP+ cell fraction, whereas low or undetectable levels of activity were observed in each GFP− cell fraction, indicating that mTert-GFP is a valid biomarker for mTert activity.

Discussion
We have generated mTert-GFP-transgenic mice as a model system to facilitate the identification, isolation, and functional analysis of adult stem cells. To ensure adequate levels of transgene expression in adult mice, we used a two-step ES cell-based visual screening method, which resulted in the efficient generation of two informative transgenic founder lines. GFP expression was then shown to mark three well accepted stem cell populations (male germ cells, HSCs, and ISCs). These results illustrate the power of this model system to identify and enrich for putative stem cell populations and to subsequently validate their functional significance.

Using multiple techniques, mTert-GFP-transgene expression was demonstrated in male germ cells at various stages of spermatogenesis, with the greatest percentage of GFP+ cells demonstrated within meiotically active primary spermatocytes, followed by secondary spermatocytes. Haploid spermatids showed the lowest percentage of GFP+ cells. These results are consistent with previous reports describing telomerase expression in meiotically active male germ cells (16). The expression of GFP within spermatids, previously suggested to be mTert-negative (26), may be explained by the long half-life of GFP (~24 h) or by aberrant transgene expression within these cells.

Fig. 5. GFP expression in intestinal stem cells. Immunohistochemical analysis for GFP and BrdU using serial sections from mTert-GFP mice having undergone BrdU pulse-chase. (a) Staining with anti-GFP antibody, VIP (dark purple) chromagen substrate. (b) Staining with anti-BrdU antibody, DAB (dark brown) chromagen substrate. (Magnification: ×40.)
The slight increase in the percentage of GFP+ cells in the 2N population from line 14, compared with line 22, may be explained by the site of transgene integration and/or by differential expression in the somatic cell compartment.

GFP expression also was shown to mark the LT HSC using both phenotypic and functional definitions. The functional analysis confirmed the presence of LT HSCs within the GFP+ population based on the capacity of these cells to give rise to long-term, serial, multilineage BM reconstitution. Analysis of mTert-GFP-expressing BM cells, however, is complicated by the fact that other hematopoietic cells, in addition to the HSC, express telomerase (e.g., ST HSCs and lymphocytes) (6, 7). Therefore, the finding that ~2% of whole BM cells express GFP was not surprising.

Phenotypic analysis of BM cells using multicolor flow cytometry demonstrated an enrichment of LT HSCs within the GFP+ population, as well as the reciprocal, an enrichment of GFP− cells within the LT HSC population compared with the ST HSC population. In contrast, no enrichment of LT HSCs was observed in the GFP− population when compared with the GFP+ population, suggesting a correlation between GFP fluorescence intensity and self-renewal potential. This observation is consistent with a previous report demonstrating a correlation between the frequency of telomerase-expressing cells within a given population and their degree of self-renewal potential (6). Furthermore, the decreasing frequency of GFP+ cells in populations of progressively differentiated cells is consistent with a decreasing role for telomerase as cells mature.

The low overall percentage of GFP+ LT HSCs was unanticipated and led us to speculate that mTert-GFP might be differentially regulated throughout the cell cycle. Previous reports have indicated that quiescent stem cells might be telomerase-negative (6, 9) whereas proliferative stem cells may be telomerase-positive (22, 27). To address this hypothesis, we performed cell cycle analysis using Hoechst dye on HSCs harvested from mTert-GFP mice, but we were unable to demonstrate a statistically higher percentage of GFP+ cells in the proliferative (S/G2-M) phase of the cycle compared with the (largely) noncycling (G0/G1) phase. These results may be complicated by coanalysis (via Hoechst staining) of G0/G1 and G2-M phase HSCs particularly if telomerase expression is induced during late G2 phase. To further assess whether GFP was preferentially expressed during the proliferative phase of the cycle, we performed Kit67 staining on LT HSCs, which showed no difference between groups. These results demonstrate that the regulation of mTert-GFP expression in LT HSCs is not a function of cell cycle. Alternatively, it remains possible that (i) the promoter fragment used to generate these mice may not contain all of the necessary regulatory elements required for mTert expression in HSCs, and/or (ii) stem cell heterogeneity may explain differential telomerase expression. For example, it has recently been shown that ES cells display marked heterogeneity in their expression of key pluripotency factors, demonstrating a level of regulation not previously appreciated (28). Consistent with this notion, our recent analysis of mTert-GFP expression after the generation of induced pluripotent stem (iPS) cells revealed significant heterogeneity in GFP expression along with other markers (29). These results suggest that heterogeneity of mTert-GFP expression within stem cell compartments may be explained by, as yet undefined, self-renewal mechanisms.

To identify the highly elusive ISC, we used LT BrdU retention and demonstrated GFP coexpression within this population. The ability to mark single intestinal crypt cells in this highly regenerative tissue, in contrast to the multiple cell types marked in BM and testis, where the telomerase expression profile is complicated, demonstrates the utility of this model system. The capacity to express GFP within this intestinal population provides us with a unique opportunity, not previously possible, to prospectively isolate and functionally validate ISCs.

To summarize, we have generated mTert-GFP-transgenic mice, a reporter system to provide a straightforward method for the identification, prospective isolation, and functional validation of adult stem cells from multiple tissues. In addition, this model can be used to seek out novel progenitor/stem cells in tissues where their presence remains to be established and to potentially investigate mechanisms underlying stem cell heterogeneity. These mice also may be useful to investigate other phenomena related to telomerase activation, such as oncogenesis, where GFP might serve as a sensitive tool to study the initial stages of tumor formation, identify cancer stem cells, or establish minimal residual disease status after specific treatment regimens.

Methods

Generation of Mice. Linearized mTert-GFP transgene (5) was electroporated into mouse (J1) ES cells, selected with G418, and screened for GFP expression (1st screen). EBs were generated in suspension culture in the absence of LIF for 7 days ± 1% DMSO as described previously (5). Nuclei were counterstained with DAPI, and images were obtained by using a Nikon Eclipse E800.

Mice were maintained on a pure 129S1/SvImJ background. All animal procedures were approved by the Children's Hospital Institutional Animal Care and Use Committee.

Western Blot Analysis. Western blotting was performed by using 50 μg (WT and mTert-GFP) or 2.5 μg (Actin-GFP) of whole testis protein extract. Immunoblotting was performed by using a rabbit anti-GFP antibody (Abcam 6556) and the ECL reagent (Bio-Rad). The blot was stripped and reblotted by using anti-Actin antibody (Cell Signaling) as a loading control.

FACS Analysis of Germ Cells. Seminiferous tubules were digested with collagenase (100 IU/ml, Worthington), washed to remove interstitial cells, dissociated to obtain germ cells, incubated with verapamil (Sigma) and Hoechst 33342 (Invitrogen) as described previously (30), and sorted by using a FACS VantageSE flow cytometer.

Histochemistry. In situ hybridization was performed as described previously (31). Briefly, 10-μm frozen sections of adult testes were hybridized with 3S-labeled anti-sense or sense cRNA GFP riboprobes (~350 bp). For testis, immunohistochemistry was performed on 10-μm frozen sections by using rabbit anti-GFP antibody (MBL), Vectastain ABC kit (Vector Laboratories), VIP substrate reagent (Vector Laboratories), and 1% methyl green. For intestine, immunohistochemistry was performed on 4-μm sequential paraffin sections by using rabbit anti-GFP (Abcam 6556), Vectastain ABC kit, VIP substrate reagent, and 1% methyl green. BrdU staining used the BrdU in situ detection kit (BD PharMingen) as recommended, with DAB and hematoxylin. Light and dark field images were obtained by using a Nikon Eclipse E800.

FACS Analysis of BM and PBCs. Whole BM and PBCs were isolated from adult mice as described previously (32). Briefly, mature cells were depleted from BM by using lineage markers CD3 (clone KT3.1.1, C4D [GK.1.5]), CD5 (53-7.8), CD8 (53-6.7), B220 (EB2), Mac-1 (M1/70), Gr-1 (8C5), and Ter119, followed by magnet bead (Dynal) separation. Depleted cells were stained with goat anti-rat PE/Cy5 to visualize lineage marker staining. HSCs and progenitors were stained with PE/Cy7-conjugated c-KIT (2B8) and APC-conjugated ScA1 (D7) with either biotinylated CD34 or biotinylated Fik2, followed by PE-conjugated streptavidin. Cells were analyzed by using a Dako-Cytometry MoFlo and/or a BD FACSARia flow cytometer. Data were analyzed by using FlowJo (Treestar), with frequencies of CD34− or Fik2− KSL (kit− ‘scA1+ ’ lineage ) HSCs and CD34+ or Fik2+ KSL progenitors determined after gating on live (PI−) cells. For cell cycle analysis, lineage-depleted BM was stained additionally with Hoechst 33342 and verapamil (32).

BM Transplantation. Primary BM recipient were x-irradiated (12 Gy, delivered as a split dose 3 h apart) before BM of ~1.8 × 106 GFP+ or GFP− cells by tail vein injection. 2° BMT recipients received γ-irradiation (11 Gy, delivered as a split dose 3 h apart) before BM of 1 × 106 or 4 × 106 GFP+ cells by retro-orbital injection. Mice were maintained in autoclaved cages with acidified (pH 2.5), Baetrin-treated water.

Fluorescence In Situ Hybridization. For FISH analysis of PBC nuclei, FACS-isolated cells were treated with hypoton solution and fixed in methanol/ acetic acid before slide preparation as described previously (33). The Y chromo...
some FISH probe (kindly provided by E. Gussoni, Children’s Hospital, Boston) was labeled with digoxigenin-11-dUTP as described previously (33) and standardized on male and female control cells with a hybridization efficiency of >90%. Slides were examined by using a Zeiss Axioshot microscope.

**BrdU Labeling of ISCs.** LT (pulse–chase) BrdU labeling was performed by using a modification of Potten et al. (25). Adult mice received a single dose of γ-irradiation (10 Gy) in the morning, followed by administration of BrdU for 48 h. During the day, mice received BrdU IP (100 mg/kg every 6 h) beginning immediately after the dose of radiation. During the overnight (active) period, BrdU was provided in the drinking water (1 mg/ml in water). Intestines were harvested for analysis 10 days after irradiation.

**Telomerase Activity in BM.** Whole BM was sorted into GFP− and GFP+ populations, and 2,500 cells from each group were assayed for telomerase activity by using the TRAPEze Telomerase Detection Kit (Chemicon) per the manufacturer’s protocol. Heat-inactivated samples were used as controls.

**TRAP Assay, RT-PCR, and Ki67 FACS Analysis.** These procedures are described in **SI Methods.**

**ACKNOWLEDGMENTS.** We thank J. A. Majzoub, E. Gussoni, S. Bonner-Weir, M. Thompson, and R.A. DePinho for key insights; H. Ye, Y. Zhou, H. El Toimi, A. Payne-Tobin, and A. Flint for expert assistance; and S. Mayack, F. Kim, J. LaVecchio, and G. Buruzula at the Joslin-HSCI Flow Core. This work was supported by National Institutes of Health Grants ST32 DK007699, K08 DK066305, JDFR 5–2005–216, and 1–2007–116 (to D.T.B.); R37 DK32658 (to R.K.M.), and 2T32 DK07260 (to I.M.M.); a Harvard Stem Cell Institute Seed Grant (to D.T.B.); National Institute of Child Health and Human Development Grant P30 HD18655 (to D.T.B.); the Timothy Murphy Fund; a Burroughs Wellcome Fund Career Award (to A.J.W.); a Smith Family New Investigator Award (to A.J.W.), and the Leukemia Research Foundation (to N.H.).

18. Payne-Tobin, and A. Flint for expert assistance; and S. Mayack, F. Kim, J. LaVecchio, and G. Buruzula at the Joslin-HSCI Flow Core. This work was supported by National Institutes of Health Grants ST32 DK007699, K08 DK066305, JDFR 5–2005–216, and 1–2007–116 (to D.T.B.); R37 DK32658 (to R.K.M.), and 2T32 DK07260 (to I.M.M.); a Harvard Stem Cell Institute Seed Grant (to D.T.B.); National Institute of Child Health and Human Development Grant P30 HD18655 (to D.T.B.); the Timothy Murphy Fund; a Burroughs Wellcome Fund Career Award (to A.J.W.); a Smith Family New Investigator Award (to A.J.W.), and the Leukemia Research Foundation (to N.H.).
Supporting Information

Breault et al. 10.1073/pnas.0804800105

Methods

TRAP Assay. Telomeric Repeat Amplification Protocol assays were performed using either the TeloTAGGG Telomerase PCR ELISA (Roche) or the Trapeze Detection Kit (Chemicon) to analyze telomerase activity in the testis or intestine, respectively. Briefly, cells were isolated from testis as described in Methods and from intestine using a chelation protocol (1) and sorted into GFP⁺ and GFP⁻ populations using either a FACSAria or a FACS VantageSE flow cytometer. Telomerase activity was assessed using cell extracts from 1,000–2,000 GFP⁺ or GFP⁻ cells, as indicated.

RT-PCR. Total RNA was extracted from FACS purified GFP⁺ and GFP⁻ male germ cells or intestinal cells from adult mTert-GFP mice using TRizol reagent (Sigma–Aldrich) per the manufacturer’s protocol. RNA was then concentrated using RNeasy MinElute columns (Qiagen) and first strand cDNA synthesis was performed using the iScript Select cDNA Synthesis Kit (Bio-Rad). One tenth the volume (2 μl) of cDNA was used to amplify either mTert or β-actin transcripts yielding 107-bp and 390-bp products, respectively, using the following primer sets: mTert forward, 5’-TCAGGAACTGATGAGAAAG-3’; mTert-reverse, 5’-GATCCTCTCCCTCAGACGTT-3’; β-actin-forward, 5’-GCCCTAGAAGTCAGAGAAGGCA-3’; β-actin-reverse, 5’-CAGTGGAGCGAGATTAGAGC-3’. All PCRs were performed using an annealing temperature of 60°C and 40 cycles of amplification.

Ki67 FACS Analysis. Total bone marrow cells were isolated from mTert-GFP or wild-type mice and stained for LT-HSCs (Flk2⁺ KSL) as described in Methods. Cells were then fixed with 4% PFA-PBS, permeabilized in 0.1% Triton-PBS, refixed, and stained with a Ki67 rabbit monoclonal antibody (diluted 1:1; NeoMarker catalog no. # RM-9106-R7). Pacific Blue goat anti-rabbit Ig (1:100; Invitrogen) was used as a secondary antibody. Data were collected using a FACSAria flow cytometer and subsequently analyzed using FlowJo.

Fig. S1. Phenotypic analysis of GFP expression and cell cycle regulation in mTert-GFP mice. (A) Representative FACS plots for LT HSC [c-Kit⁻ Sca-1⁻ Lin⁻ (KSL) and CD34⁻ or Flk2⁻] and ST HSC (KSL and CD34⁺ or Flk2⁺) illustrate the gates corresponding to the three GFP populations: GFP⁻, GFPlo, GFPhi. (B) Cell cycle analysis was performed by using Hoechst dye labeling followed by multicolor FACS analysis. Pooled results (mean ± SEM) are from two independent experiments (n = 4 for each group; ANOVA, P < 0.003); results from post hoc Fisher’s (PLSD) analysis indicated.
Fig. S2. No difference in proliferation status (Ki67⁺) between mTert-GFP⁺ and control LT-HSCs (Flk2⁻KSL). (A) Flow-cytometry analysis of lineage-negative bone marrow cells from mTert-GFP or WT mice. Long-term reconstituting hematopoietic stem cells, defined as c-Kit⁺Sca-1⁻Lin⁻ (KSL) and Flk2⁻, were analyzed for either Ki67 (Upper Right) or for GFP⁺ expression followed by Ki67 (Lower Right). (B) Comparison of percent Ki67⁺ LT HSCs in control (Flk2⁻ KSL) vs. GFP (GFP⁺ Flk2⁻ KSL) populations. Data presented are from two experiments performed in duplicate or triplicate; mean ± SEM, *P* = 0.3, Student's t test.
Fig. S3. Telomerase expression and activity colocalize with GFP⁺ testis and intestinal cells. (A) Analysis of mTert and actin mRNA levels in FACS-purified GFP⁺ or GFP⁻ testis cells from adult mTert-GFP mice by RT-PCR. (B) Analysis of telomerase activity in 1000 FACS-purified GFP⁺ or GFP⁻ testis cells performed by using the TeloTAGGG Telomerase PCR ELISA (Roche) according to the manufacturer’s instructions. Telomerase-expressing 293 cell extract served as a positive control and heat inactivated (HI) 293 cell extract served as a negative control. The data shown are from 2–4 experiments, each performed in duplicate. Comparison between absorbance values (mean ± SEM) from GFP⁺ and GFP⁻ populations was performed by using Student’s t test, *P < 0.001. (C) Analysis of mTert and actin mRNA levels in FACS-purified GFP⁺ or GFP⁻ intestinal cells from adult mTert-GFP mice by RT-PCR. (D) Analysis of telomerase activity in FACS-purified GFP⁺ or GFP⁻ intestinal cells performed by using the Trapeze Detection Kit (Chemicon) according to the manufacturer’s instructions. Telomerase-expressing 293 cell extract served as a positive control, and HI 293 cell extract along with CHAPS buffer ± HI served as a negative control. The number of cells used in each lane is indicated. IC corresponds to an internal PCR control band. Heat inactivation controls for each GFP⁺ and GFP⁻ sample were negative for telomerase activity (data not shown).