Corrections

BIOCHEMISTRY. For the article “An allosteric model of calmodulin explains differential activation of PP2B and CaMKII,” by Melanie I. Stefan, Stuart J. Edelstein, and Nicolas Le Novère, which appeared in issue 31, August 5, 2008, of Proc Natl Acad Sci USA (105:10768–10773; first published July 31, 2008; 10.1073/pnas.0804672105), the authors note that incorrect versions of Figs. 3 and 4 were inadvertently incorporated during the final stages of production. The correct figures, as submitted with the original manuscript, and their legends, appear below.

Fig. 3. Comparison between simulation results and experimental results reported by Crouch and Klee (16). Moles of calcium bound per mole of calmodulin are shown as a function of calcium concentration. Diamonds, data points measured by Crouch and Klee; dashed line, curve used in Crouch and Klee to fit experimental data points; solid line, steady-state results of simulations at different initial calcium concentrations. Calmodulin concentration was $2 \times 10^{-7}$ M.

Fig. 4. Increased affinity of calmodulin for calcium in the presence of a target protein. Upper dotted line, $R$ state only; lower dotted/dashed line, $T$ state only; dashed line, combined $R$ and $T$ states in the absence of target; solid line, combined $R$ and $T$ states in the presence of CaMKII. All lines are steady-state results of simulations at different initial calcium concentrations. Calmodulin concentration was $2 \times 10^{-7}$ M.

MEDICAL SCIENCES. For the article “Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics,” by Paul P. Szotek, Henry L. Chang, Kristen Brennand, Akiiro Fujino, Rafael Pieretti-Vanmarcke, Cristina Lo Celso, David Dombkowski, Frederic Preffer, Kenneth S. Cohen, Jose Teixeira, and Patricia K. Donahoe, which appeared in issue 34, August 26, 2008, of Proc Natl Acad Sci USA (105:12469–12473; first published August 18, 2008; 10.1073/pnas.0805012105), the authors note that the following should be added to the Acknowledgments: “P.P.S. and H.L.C. were supported by Massachusetts General Hospital/National Institutes of Health (NIH) T32 Training Program in Cancer Biology Grant 2T32CA071345-11. David T. MacLaughlin and P.K.D. were funded by Brigham and Women’s Specialized Program of Research Excellence (SPORE) Grant 5P05CA105009-03, Harvard Stem Cell Institute Grant DP-0010-07-00, and NIH/National Cancer Institute Grant 5R01CA017393-33.”

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Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics

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Ovulation induces cyclic rupture and reparative repair of the ovarian coelomic epithelium. This process of repeated disruption and repair accompanied by complex remodeling typifies a somatic stem/progenitor cell-mediated process. Using BrdU incorporation and doxycycline-inducible histone2B-green fluorescent protein pulse–chase techniques, we identify a label-retaining cell population in the coelomic epithelium of the adult mouse ovary as candidate somatic stem/progenitor cells. The identified population exhibits quiescence with asymmetric label retention, functional response to estrous cycling in vivo by proliferation, enhanced growth characteristics by in vitro colony formation, and cytotoxic mechanisms by enrichment for the side population. Together, these characteristics identify the label-retaining cell population as a candidate for the putative somatic stem/progenitor cells of the coelomic epithelium of the mouse ovary.

The ovarian coelomic epithelium covers the ovary as a layer of squamous or cuboidal cells. Folliculogenesis in the adult ovary is characterized by extensive architectural remodeling that culminates in disruption of the coelomic epithelium and extrusion of the ovum at ovulation (1, 2). After disruption, a series of molecular events initiates and executes repair of the epithelial wound (3, 4). The cyclic pattern of repeated disruption and repair with complex remodeling associated with ovulation leads one to intuit the existence of a population of somatic stem/progenitor cells that would be responsible for these processes. Additionally, previous studies of the coelomic epithelium (CE) have implicated cyclic re-epithelialization as the source of accrued mutations leading to ovarian cancer (5).

Somatic stem cells are a subset of normal tissue cells that, through asymmetric division, have the ability to self renew and produce lineage committed daughter cells responsible for tissue regeneration and repair (6, 7). Injury-responsive stem cells and their niches have been described in a variety of tissues, such as skin and hair follicle (6, 8, 9), mammary gland (10, 11), and intestine (6, 12). In some tissues, slow-cycling somatic stem cells were initially identified by their ability to retain label for long periods of time, whereas asymmetrically derived lineage committed daughter cells dilute out label during rapid proliferation and terminal differentiation (9, 13–18). These studies, as well as the recent identification of label-retaining cells (LRCs) in the uterine endometrial stroma and myometrium (19), used BrdU, 3H-Thymidine, or histone2B-green fluorescent protein (H2B-GFP) labeling to identify candidate somatic stem cells. The functional capacity of the identified candidate cells could subsequently be confirmed evaluating in vivo and in vitro behavior. Characterization of these cells could then lead to the discovery of tissue-specific surface markers.

Additionally, somatic and cancer stem cells from various tissues have been identified by their ability to efflux Hoechst 33342 dye through ATP-binding-cassette transporters, such as Abcg2/Bcrp1 (11, 20–27), including our recent identification of these “side population” (SP) cells as potential tumor-initiating cells in ovarian cancer (28). It has been postulated that this chemical-effluxing capability contributes to the cytopreservation necessary for the longevity attributed to stem/progenitor cells (29). Thus, label retention and Hoechst dye efflux are two distinct methods that can be used to identify candidate somatic stem cells.

Using BrdU and H2B-GFP transgenic mice as models, we have identified a population of long term LRCs in the coelomic ovarian surface epithelium that were studied further for functional characteristics as defined in vivo by functional proliferative response to estrous cycle and in vitro by robust colony formation and enrichment of GFP cells in the SP.

Results

Identification of BrdU and H2B-GFP Label Retaining Cells in the Coelomic Epithelium. We used pulse–chase labeling with BrdU and tetracycline-regulated (doxycycline responsive) H2B-GFP fusion protein in female mice to identify a slow-cycling LRC population in the mouse ovary [supporting information (SI) Fig. S1A]. Rosa26-rTTA; teto-H2B-GFP (H2B-GFP) mice were generated and provided by Brennand and colleagues (30). The H2B-GFP animals were pulsed during the embryonic period (E0–P42) or as an adult (6 wk–10 wk) to determine which one gave the better ovarian staining (see Fig. S1B) and found the adult pulsed animals resulted in better labeling. BrdU animals were pulsed as adults (6 wk–7 wk).

To validate the ability of H2B-GFP mice to identify somatic stem cells, flow cytometry was used to compare bone marrow GFP LRCs to the hematopoietic stem cell phenotype Lin−cKit+Sca-1+ (31, 32). Analysis of H2B-GFP whole bone marrow indicated that Lin−cKit+Sca-1+ cells preferentially retain the H2B-GFP label (Fig. 1, Fig. S2). These findings confirm that the H2B-GFP model can identify somatic stem cells.

Ovarian H2B-GFP and BrdU labeling was evaluated by immuno-fluorescence. PBS injected (no BrdU) and naive (no doxycycline) H2B-GFP mice were used as negative controls (not shown). Significant variability in whole ovary pulse labeling was observed by computerized image analysis of all three experiments performed in this study (see Fig. 1A, Fig. S3A) (embryonic pulse H2B-GFP = 26.0 ± 2.5%, adult pulse H2B-GFP = 82.5 ± 7.9%, adult pulse BrdU = 46.9 ± 8.9%). Despite this initial pulse labeling variation,


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the differences were less marked (Fig. S3B) (adult pulse H2B-GFP = 5.5%, embryonic pulse H2B-GFP = 5.1%, and adult pulse BrdU = 2.2%) after 3 months of chase. After 3 months chase, gross observation of H2B-GFP ovaries demonstrated epifluorescent LRCs on the surface epithelium (not shown). The three distinct labeling experiments performed in this study identified a consistent population of LRCs (see Fig. 1B–D) in the CE. Colocalization of BrdU and H2B-GFP was consistently observed throughout the chase (see Fig. 1E–L).

**Phenotypic Characterization of Ovarian LRCs and their Microenvironment.** To understand better the identified LRC population, we evaluated a series of known stem, epithelial, mesenchymal, bone marrow, germ cell, and ovarian cancer cell markers. Immunofluorescence of H2B-GFP LRCs in tissue and separated CE LRCs in vitro demonstrate that these cells express cytokeratin 8 (Fig. 2A), β-catenin (Fig. 2B and C), vimentin (Fig. 2C), E-cadherin, collagen IV, PTEN, gata-4, and P13K (not shown), and are negative for EpCam (Fig. 2 D and E), α-smooth muscle actin (Fig. 2F), c-kit, CD90, CD45, and CD31 (not shown). Additionally, neither H2B-GFP (Fig. 2G) nor BrdU (Fig. 2H) LRCs colocalized with VASA+/c-Kit+ oocytes (arrows) with either H2B-GFP or BrdU at 3 and 2 months chase, respectively, was not observed. Figures representative of n = 3.

**LRCs Replicate in Response to the Estrous Cycle in Vivo.** To assess the coelomic LRCs as functional candidate stem/progenitor cells, we explored the capacity of LRCs to proliferate in response to physiologic estrous cycling using stage-specific short-term Ido-deoxyuridine (IdU) injections after long term chases. Pre- and postovulatory H2B-GFP 12 week chase mice were timed, staged by vaginal cytology, and injected with IdU 2 to 4 h before sacrifice to identify actively mitotic cells. Preovulatory ovaries showed no colocalization of IdU with H2B-GFP CE cells before sacrifice to identify actively mitotic cells. Preovulatory ovaries showed no colocalization of IdU with H2B-GFP CE cells. Following ovulation, we identified colocalization of IdU with H2B-GFP LRCs in the interfollicular clefts at 25, 50, and 100 µm from the edge of the corpus lutea (see Fig. 3B and C) and on either side of the repairing epithelium (see Fig. 3D and E) indicating that coelomic LRCs are mitotically responsive to the estrous cycle postovulation in these regions.

**Coelomic LRCs Show Increased Growth Potential in Vitro.** A CFU assay was used to detect ovarian CE cells capable of generating colony growth. After 1 to 3 months of chase, H2B-GFP CE cells were released by collagenase treatment and plated. After plating the unsorted population and incubating for 14 days, the average colony growth. After 1 to 3 months of chase, H2B-GFP CE cells were released by collagenase treatment and plated. After plating the unsorted population and incubating for 14 days, the average
number of Giemsa-stained colonies observed was 10 ± 5 CFUs per 1 × 10^4 plated cells (n = 9) at each of the chase time-points (not shown).

Confocal microscopy showed that colony-forming H2B-GFP LRCs maintain a three-dimensional structure as their dividing daughter cells proliferate and dilute the H2B-GFP signal (Fig. 4A, B). Quantification of H2B-GFP signal intensity loss (n = 3) with replication was determined to be exponential and a function of distance from the brightest LRC (Fig. 4C).

To elucidate further the growth potential of LRCs, CE cells from 4 month chase H2B-GFP mice (n = 3) were separated, sorted into GFP+ LRCs and GFP-non-LRCs before being plated in the described CFU assay at a density of 1 × 10^4 cells per well. Label-retaining GFP cells showed increased growth potential after 14 days as measured by colony formation density when compared to non-GFP cells (35% versus 14%, P < 0.05, n = 3) (Fig. 4D).

**SP Enrichs for H2B-GFP LRCs in the Coelomic Epithelium.** Chase H2B-GFP ovarian CE cells were isolated by collagenase treatment and subjected to SP analysis (1 month n = 3, 2 month n = 3, 3 month n = 1). We identified a verapamil-sensitive SP within the normal CE in adult H2B-GFP mice (Fig. 5A and B). Intensity gates were set using wild type epithelial cells. Evaluation of 2 month chase SP cells for H2B-GFP expression demonstrated that 56.5 ± 4.1% SD of SP+ cells are H2B-GFP+ and that 67.7 ± 8.1% of these cells are classified as bright LRCs (Fig. 5C). Given that less than 15% of the ovary is expected to be GFP or BrdU positive after a 2 month chase (Fig. S3A), these findings demonstrate a significant enrichment for LRCs by the SP.

**Discussion**

By using H2B-GFP and BrdU pulse–chase experiments, we have identified a population of label-retaining ovarian coelomic epithelial cells that are quiescent, slow-cycling, and may undergo asymmetric division (see Fig. 1A–D). If all of the cells in the coelomic epithelium contributed equally to postovulation repair, one would expect homogenous dilution of label with wash out after a chase period, as is the case in other tissues (30). Rather, we identify cells that retain label after up to 4 months of chase, indicating that these cells divide much less frequently than the surrounding tissue. This difference in cell turnover suggests that these LRCs have a biologic behavior distinct from their surrounding nonlabel retaining counterparts.

Despite the initial labeling variability between the different H2B-GFP and BrdU, we are confident that the LRCs identified after chase represent the same quiescent population for two reasons. First, the differences in initial labeling among the three pulse schedules did not persist after the chase period. Second, we observed consistent colocalization between the BrdU and H2B-GFP throughout the pulse–chase experiments (see Fig. 1 E–L). The initial labeling variability likely represents poor Rosa26-rtTA expression in immature granulosa cells, the accepted but shorter labeling period for BrdU (8, 33), and the inability of BrdU to label nondividing cells.

Furthermore, we demonstrate that the identified coelomic LRCs are functionally responsive to estrous cycling in vivo. The pattern of IdU incorporation before and after ovulation indicates that the CE LRCs respond to the estrous cycle by replication. The lack of IdU incorporation in the CE LRCs before ovulation (see Fig. 3C) demonstrates that these cells are quiescent before ovulation. Localization of the mitotically active LRCs at the interfollicular clefts (see Fig. 3C) and at the edge of the epithelial wound (see Fig. 3D) after ovulation suggests that the LRCs are involved in the repair and remodeling that necessarily occur after this process and respond to estrous cycling.
LRCs comprise 56% of the SP at 2 month chase, representing an almost threefold enrichment of label from the expected 15% or less GFP retention (see Fig. S3A) in the whole CE at that point. The majority of LRCs exhibit this chemical-effluxing cytoprotective capacity. The enrichment of LRCs by SP gives one confidence in using the SP as a selection marker for identifying and isolating cell populations that would significantly overlap with the population identified and isolated by H2B-GFP label retention in circumstances where these transgenic models would be unfeasible.

Surface marker analysis demonstrated that although the coelomic LRCs have an epithelial lineage in vivo and in vitro (cytokeratin-8+, β-catenin+, E-cadherin+), they are also vimentin+ (see Fig. 2 A–C), which lends support to the dual epithelial/mesenchymal potential ascribed to the ovarian coelomic epithelium (34, 35). Interestingly, whereas ovarian cancer cells express the surface marker EpCam (36, 37), the CE LRCs were negative (see Fig. 2 D and E). Although we evaluated a wide range of known cellular markers, we were unable to identify a marker signature that uniquely identified the CE LRCs aside from the SP, as described above. More work needs to be done to characterize a cellular marker profile, which would definitively identify the CE LRC population as well as further characterize their niche.

Collectively, our findings identify a candidate putative somatic stem cell population within the mouse ovarian coelomic epithelium that exhibits the properties of quiescence (label retention), functional response to estrous cycling in vivo (GFP-IdU colocalization), enhanced growth in vitro (colony formation), and cytotoxic protection (SP).

The notion that cancer is derived from tissue stem cells is over 100 years old, but only recently has this hypothesis been validated (29, 38) and insight provided into the mechanisms by which mutations are accumulated, passed on to differentiating daughter cells, and ultimately lead to tumor progression. Accumulating evidence suggests that somatic stem cells in niche microenvironments may ultimately undergo mutagenic transformation into cancer stem cells (6, 7, 29). Alternatively, aberrant regulatory signals from the niche microenvironment might also lead to tumorigenesis (6, 7, 29). Because many of the same functional properties that define somatic stem cells also define cancer cells, our identification of candidate somatic stem cells in the adult mouse ovary makes it attractive to suggest that these hypotheses might also apply to the generation of ovarian cancer. Elucidation of the elements that lead to malignant transformation will be made more feasible if we have the normal somatic stem cell for comparison.

Our findings indicate that a better understanding of the molecular mechanisms that regulate the identified somatic stem cells within their niche microenvironment is required. Ultimately, it is the comparison of somatic stem cells and their niche to cancer stem cells that may direct the development of treatments targeted toward chemotherapeutic elusives cancer stem cells, particularly in ovarian cancer.

**Experimental Procedures**

**Animal Housing, Estrous Staging and BrdU/IdU Labeling.** All protocols involving animal experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Animals were housed, estrous cycle staged, and pulsed with BrdU and IdU (see SI Experimental Procedures).

**Transgenic Mice and H2Bj-GFP Label and Chase.** H2Bj-GFP mice purchased from Jackson Laboratories (JAX GEMM Strains, Stock Tg(tetO-HIST1H2BJ/ GFP)47Efu/J, Stock Number 005104) were crossed with M2-Rosa26-rtTA mice, generously provided by Konrad Hochedlinger (Boston, MA), for near ubiquitous expression of H2Bj-GFP in the presence of doxycycline (9, 30, 39). To induce expression of H2Bj-GFP, embryonic mice were fed doxycycline (2 mg/ml, 5% sucrose drinking water) from E0 to P42 or from 6 to 10 weeks of age. At the end of the pulse period, doxycycline withdrawal suppressed H2Bj-GFP transgene expression. Colocalization of H2Bj-GFP with known hematopoietic stem cell surface markers (positive) and transgenic mice not receiving doxycycline served as controls for autofluorescence (see “Quantification of Labeling” and “Flow Cytometry” below). Embryonic pulsed bone marrow and ovaries were

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**Fig. 5.** EeEEnrichment of H2B-GFP LRCs by coelomic epithelial SP cells. (A and B) The H2B-GFP CE has a verapamil-sensitive SP (2.46 ± 0.27%; n = 10). (C) Wild type coelomic epithelial cells (blue line) were used to establish a GFP+ intensity gate of ≥103 and a GFPBright intensity gate of ≥104. At 2 months chase, 56.5 ± 4.1% of SP cells were GFP+ and 67.7 ± 8.1% of GFP+/SP cells were GFPBright.

The growth characteristics exhibited by the LRCs in vitro are consistent with those expected of somatic stem/progenitor cells. Even after disruption of the cellular microenvironment and 14 days of incubation and proliferation in vitro, strongly labeled GFP cells can still be identified in a number of colonies (see Fig. 4 A and B). Additionally, the identified GFP+ colonies exhibit an intensity dilution pattern of exponential signal loss, which is consistent with asymmetric label retention (see Fig. 4 C). Most convincingly, the H2B-GFP LRCs after 4 months of chase showed significantly more growth and colony formation when compared with nonlabel retaining cells (see Fig. 4 D). Taken together, the in vitro growth characteristics indicate that LRCs have distinct biologic characteristics consistent with somatic stem/progenitor cells.

The side population phenomena of Hoescht 33342 dye efflux has been used to identify a variety of somatic as well as cancer stem cells in various tissues (20–28). The ability to efflux a variety of chemicals is postulated to be a defense mechanism, which leads to the longevity required of somatic stem cells and the chemoresistance characteristic of cancer stem cells (29). We show that the H2B-GFP LRCs comprise 56% of the SP at 2 month chase, representing an
harvested at pulse day 42 (sexually mature, n = 3), chase 1 week (n = 2), 2 weeks (n = 2), 1 month (n = 3), 2 months (n = 3), 3 months (n = 4), and 6 months (n = 1), and evaluated for GFP expression. Adult pulsed ovaries were harvested at pulse 1 month (n = 3), chase 1 month (n = 3), chase 2 months (n = 3), chase 3 months (n = 1), and 4 months (n = 3). In some instances (n = 3) mice were injected with BrdU or IdU (Sigma; 1 mg/ml) 2 h before killing to correlate mitotic cells with H2B-GFP LRCs.

**Immunofluorescence.** Immunofluorescence and BrdU detection was performed as described (28). Primary and secondary antibodies (see SI Experimental Procedures) were diluted in 1% BSA/PBS and incubated in a humidified chamber for 1 h. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) 1:20,000 Vectashield (Vector Labs) mounting media. Images were captured using a Nikon 80i epifluorescence scope, SPOT RT-K/ Camera, and Spot Advance Software (Diagnostic Instruments). Confocal images at 2 cell images in 2 ml of Murine MesenCult Media (StemCell Technologies) at 37°C, 5% CO2 for 14 days to obtain an optimal plating number of 1 x 10^6 for subsequent studies. Plated cells were routinely observed and imaged for GFP expression and colony formation during incubation. For live imaging, cells were incubated and imaged under the BD Pathway live cell confocal imager (Becton Dickinson) using BD Attovision imaging software. Data analysis was performed using ImageJ software. All experiments were performed in triplicate.

**Quantification of Labeling.** Quantification of BrdU and H2B-labeled nuclei was performed either by flow cytometry or by image acquisition (camera settings: Exp. 5000ms; Gamma ~ 0.95; Gain ~ 4), and analyzing using ImagerJ software (National Institutes of Health) with the nucleus counting plugin (see SI Experimental Procedures). Quantification of BrdU and H2B-labeled nuclei was performed on the BD Pathway imaging using BD Attovision software. Cell nuclei were electronically gated as regions of interest. The individual nuclei of greatest intensity was designated as the point from which loss of signal was measured and sequential elliptical rings of equal numbers of cells were established to estimate the loss of signal intensity. Experiments were performed in triplicate.

**Flow Cytometry.** To validate the ability of the H2B-GFP model to identify stem cells, we analyzed the bone marrow for colocalization with the known hematopoietic stem cell phenotype of Lin−/c-Kit+/Sca-1+ (38, 39) over the course of the chase by flow cytometry (see SI Experimental Procedures). H2B-GFP LRC colocalization and enrichment in the Hoechst 33342 excluding SP phenotype was evaluated in the Flow Cytometry Laboratory of the Department of Pathology and the Center for Regenerative Medicine according to their published protocols (25) and as previously described (28). H2B-GFP coelomic epithelial single cell suspensions were generated (see SI Experimental Procedures), cells were stained with Hoechst 33342 (5 μg/ml) dye for 90 min at 37°C, washed, and resuspended in PBS containing 2% FCS for analysis of GFP and SP colocalization using the LSRIII flow cytometer (Becton Dickinson). At least 2.5 x 10^5 events were collected for each analysis and analyzed using FlowJo version 8.1.1 software.

**Functional Colony Forming Assay and BD Pathway Live Imaging.** Coelomic LRCs were isolated by collagenase treatment after a given chase (see SI Experimental Procedures), counted, and 2 x 10^5, 1 x 10^5, 5 x 10^4, 2.5 x 10^4, 1 x 10^4, and 5 x 10^3 cells cultured in 2 ml of Murine MesenCult Media (StemCell Technologies) at 37°C, 5% CO2 for 14 days to obtain an optimal plating number of 1 x 10^6 for subsequent studies. Plated cells were routinely observed and imaged for GFP expression and colony formation during incubation. For live imaging, cells were incubated and imaged under the BD Pathway live cell confocal imager (Becton Dickinson) using BD Attovision imaging software. Data analysis was performed using ImageJ software. All experiments were performed in triplicate.

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Supporting Information

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SI Experimental Procedures

Animal Housing, Estrous Staging, and BrdU/IIdU Labeling. Animals were housed in a controlled environment at 24°C, 12 h dark/light cycle, with food and water ad libitum. Vaginal smear cytology was performed using ImageJ software (National Institutes of Health). The quantification analysis was standardized using the whole surface area percentage coverage.

Immunofluorescence. Primary antibodies and dilutions were as follows: BrdU-Alexa488 (1:20), BrdU-Alexa 555 (1:20), BrdU-Alexa 647 (1:20), GFP-Alexa488 (1:200; Molecular Probes), Cytokeratin-1 (1:50), CD105 (1:10), Connexin 32 (1:10; Iowa Studies Hybridoma Bank); c-Kit receptor (1:100, R & D Systems); α-smooth muscle actin (1:5000), β-Catenin (1:1500, Sigma), Gata-4 (1:100, Santa Cruz Biotechnology); EpCam, CD31 (PECAM), CD45, GR-1-PE, Mac-1-PE, Ter119-PE, pan-NK-PE, CD90, CD34 (all 1:50, Becton Dickinson), VASA/MVH (1:1000, generous gift from T. Noche, Mitsubishi Kagaku Institute of Life Sciences), NG2 (1:250, Chemicon), PDGFRβ (1:25, E-Bioscience), and CD44 (1:10, Caltag). Secondary antibodies were as follows: AlexaFluor 488 donkey-anti-(goat, rabbit, rat), AlexaFluor 555 donkey-anti-(goat, rabbit, rat), and AlexaFluor 647 donkey-anti-(goat, rabbit, rat) (1:1000, Molecular Probes).

Image Processing, Quantification, and Colony Density Analysis. Images were obtained as described and quantification of images was performed using ImageJ software (National Institutes of Health). The quantification analysis was standardized using Adobe Photoshop software to set a threshold level of 60 (determined by images of transgenic mice not receiving doxycycline as a baseline control for “leaky” GFP) on each greyscale image. The ImageJ nucleus counting plugin was set to count particle sizes from 4 to 500 pixels with K-means clustering to obtain the nuclei count data. Grayscale images were evaluated from at least three images per animal and three animals per time-point.

Colony density analysis was performed on scanned photomicrographs of Giemsa stained CFU plates using ImageJ software. Individual well images were converted to grayscale by RGB splitting the images and using the green channel image. The images were standardized to a threshold of 115 in black and white. Particle analysis was performed with a size range of 0.0009 to 1.00 inches², with a circularity setting of 0.00 to 1.00. The area fraction given after analysis was then converted to a percentage and used as the well surface area percentage coverage.

Coelomic Epithelial Single Cell Suspension. Ovaries were dissected, cleared of their bursa, and enzymatically digested at 37°C in 0.2% (b/w) collagenase type II (Gibco/BRL type II in DMEM) for 30 to 45 min followed by collagenase inactivation with F12 + 20% FBS and transfer of the ovaries to a six-well plate for mild trituration to disperse the more superficial coelomic. The ovaries were removed and the remaining media collected, centrifuged at 1,500 × g for 5 min, and the cells resuspended in PBS, filtered through a 70-μm mesh (BD Falcon), centrifuged again, and resuspended in 2-ml ammonium chloride RBC lysis solution on ice for 3 min. After addition of 10-ml HBSS + 2% FBS, the cells were centrifuged, then resuspended in DMEM + 2% FCS, and counted.

Flow Cytometry. Bone Marrow. GFP expression in whole bone marrow and the Lin⁻/c-Kit⁺/Sca-1⁺ fraction was assessed in the control mice receiving no doxycycline (GFP-negative gate) and in embryonic (E0-P42) doxycycline pulsed mice, at the end of the pulse and following 1 week, 2 weeks, 1 month, 2 months, and 6 months of chase (no doxycycline).

Bone marrow was harvested by crushing femoral bone shafts in PBS with heat inactivated 2% FBS. Cells were stained with lineage-specific biotinylated antibodies (CD3, CD8, CD4, GR-1, Mac-1, Ter119, and B220), Sca1 PE-conjugated, and cKit APC-conjugated (all BD PharMingen) for 20 min at 4°C. Cells were washed twice and then stained with streptavidin PerCP-conjugated (BD PharMingen) antibody for 20 min at 4°C. Cells were washed and resuspended in PBS for immediate analysis or fixed with 2% paraformaldehyde for analysis within 24 h. Flow cytometric analysis was performed using a FACScalibur (BD Biosciences) flow cytometer. Live cells were gated based on forward-scatter and side-scatter parameters. Wild type bone marrow was analyzed to set the GFP high/low/- threshold gates. At least 2.5 × 10⁵ events were collected. Data were analyzed on FloJo version 8.1.1 analysis software.
Fig. S1. Schematic of Rosa26-rtTA; tetO-H2Bj-GFP and pulse-chase timelines. (A) Rosa26-rtTA; tetO-H2Bj-GFP doxycycline responsive mouse construct and mechanism of GFP expression with the presence of doxycycline removing the EGFP repressor element. (B) Pulse-chase timelines with H2Bj-GFP timelines in green (adult pulse) and red (embryonic pulse) and BrdU animals in blue (all adult pulse).
Fig. S2. Characterization of Lin-cKit+Sca-1+ hematopoietic stem cell label retention. Flow cytometry was used to identify LRC in various hematopoietic populations. (A–C) Gating strategy used to identify total bone marrow cells (A), lineage negative cells (B) and lineage negative, Sca positive and Kit positive cells (LKS cells, a population enriched for hematopoietic stem cell) (C). (D–E) GFP expression was analyzed in all gated populations. Representative plots of cells obtained from pulsed and 1-month chase animals are shown. (D) total bone marrow cells. (E) LKS cells. (F–G) Graphs representing the percentage of GFP bright cells in total bone marrow (F) and LKS cells (G) at each time point considered. Line and asterisk in (E) indicate the GFP bright gate. Error bars in (F,G) are standard deviation.

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Fig. S3. Ovarian Pulse Labeling Efficiency. (A) H2B-GFP and BrdU whole ovary pulse chase labeling demonstrated significant variability in pulse whole ovary labeling between techniques (* P < 0.05). After 3 months chase there was a significant difference in whole ovary label retention when comparing BrdU to the adult and embryonic pulse H2B-GFP labeling methods (*, P = 0.016 and P = 0.010). There was no significant difference noted at 3 months chase between the two H2Bj-GFP pulse-labeling methods (P = 0.12). (B) Quantification of coelomic/subcoelomic labeling efficiency demonstrated no significant difference between the two H2B pulse periods (91% and 88%, P = 0.43), while a significant difference between H2B-GFP and BrdU pulses remained (*, P = 0.0016 and P = 0.00011). Likewise, no significant difference was noted in label retention after 3 months chase in the H2Bj-GFP animals (P = 0.794), while the difference in label retention between the H2B-GFP and BrdU pulses remained significant (B; P = 0.021 and ** P = 0.041). Green — adult pulse H2Bj-GFP; Blue — adult pulse BrdU; Red — embryonic pulse H2Bj-GFP; identical colors plus stripes in B denotes 3 month chase animals.