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Cell Injury, Repair, Aging and Apoptosis

Fate of Bone Marrow Stem Cells Transplanted into the Testis

Potential Implication for Men with Testicular Failure

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To assess adult stem cell differentiation in the testis, we injected bone marrow cells from adult green fluorescent protein (GFP) transgenic mice into the seminiferous tubules and the testicular interstitium of busulfan-treated wild-type or c-kit mutant (W/W^v) mice. Ten to 12 weeks after transplantation, we examined the fate of the transplanted bone marrow cells and found that they survived in recipient testes. In both the busulfan-treated and W/W^v mice, some of the GFP-positive donor cells had a Sertoli cell appearance and expressed follicle-stimulating hormone receptor within the seminiferous tubules. In addition, GFP-positive donor cells were found in the interstitium of recipient testes, and they expressed the cytochrome P450 side chain cleavage enzyme (P450scc). In the seminiferous tubules of busulfan-treated mice, GFP-positive donor cells had the appearance of spermatogonia or spermatocytes and expressed VASA. However, this was not found in the seminiferous tubules of W/W^v mice. We conclude that adult bone marrow cells, in a favorable testicular environment, differentiate into somatic and germ cell lineages. The resident neighboring cells in the recipient testis may control site-appropriate stem cell differentiation. This clinically relevant finding raises the possibility for treatment of male infertility and testosterone deficiency through the therapeutic use of stem cells. ***(Am J Pathol 2007, 170:899–908; DOI: 10.2353/ajpath.2007.060543)***

Testes have two main functions in mammals: they produce androgens and gametes. Spermatogenesis occurs in the seminiferous tubules, an immunoprivileged site, and requires functionally competent Sertoli and Leydig cells. Spermatogonial stem cells are undifferentiated cells defined by their ability to both self-renew and differentiate into mature spermatozoa. When spermatogonial stem cells are harvested from donor testes and transplanted into a sterilized recipient testis, morphologically and functionally normal spermatogenesis is re-established.[1,2] Transplantation of Leydig stem cells isolated from mice can restore the serum testosterone concentration in luteinizing hormone receptor knockout mice.[3] Transplantation of wild-type Sertoli cells into infertile Steel (SI) testes creates a permissive testicular microenvironment for restoration of spermatogenesis.[4,5] In general, homeostatic cell replacement and tissue regeneration in the adult is dependent on tissue-resident stem cells. The resident stem cell in adult proliferates and differentiates into mature cell types that correspond to their tissue of origin and do not cross tissue boundaries to generate cell types of different lineages.[6] However, a growing body of literature has challenged this notion and demonstrated stem cells crossing tissue boundaries in response to a variety of microenvironmental regenerative cues.[7,8] Recent studies have demonstrated that spermatogonial stem cells from either neonatal mouse testes[9] or adult mouse testes[10] possess pluripotency and have the potential to differentiate into embryonic stem cells.

Adult stem cell plasticity and its capacity to transdifferentiate create a huge promise for understanding and treating diseases. Studies of bone marrow stem cell

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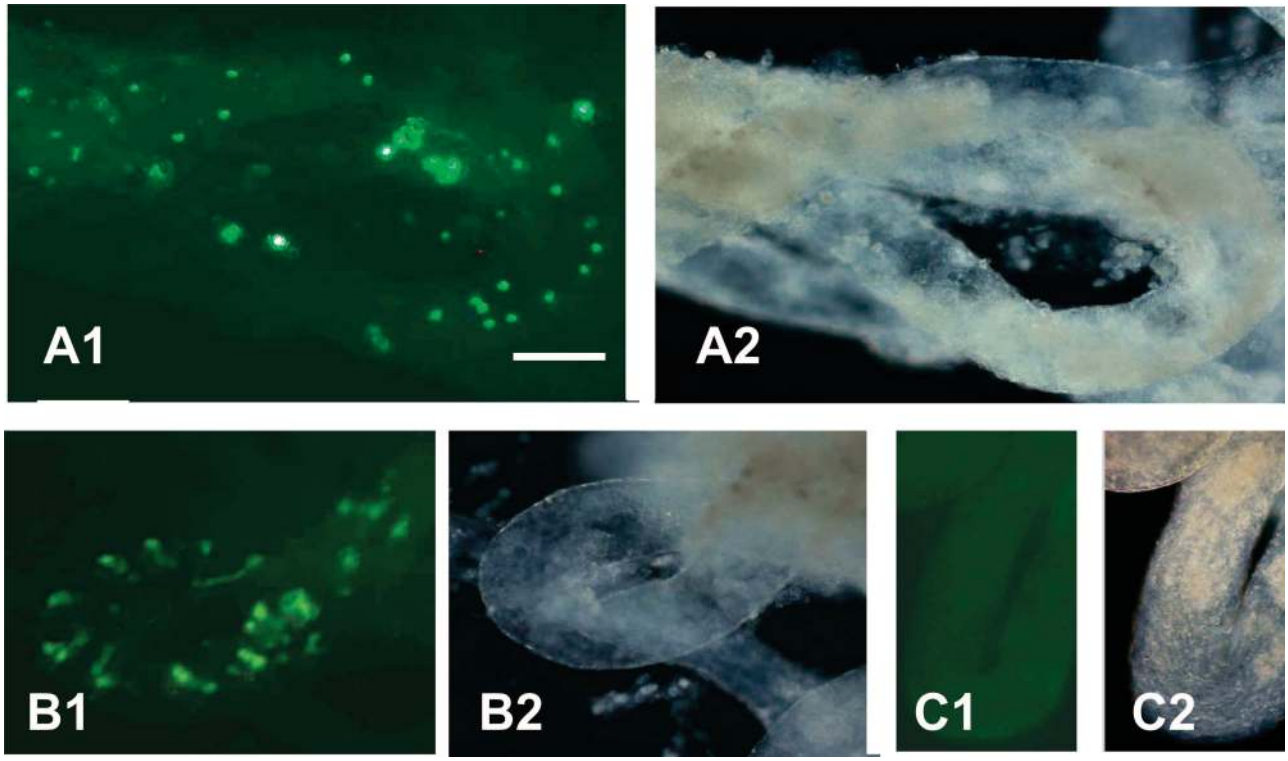

Figure 1. Micrographs of seminiferous tubules observed under florescence microscopy. Seminiferous tubules of a busulfan-treated recipient testis show adult bone marrow-derived cells as GFP-positive (**A1**, fluorescence microscopy; **A2**, transillumination). GFP-positive cells in seminiferous tubules exhibit a spatial and morphological pattern typical for Sertoli cells in recipient testis (**B1**, fluorescence microscopy; **B2**, transillumination). Busulfan-treated wild-type mouse testis without transplantation under fluorescence (**C1**) and transillumination (**C2**) microscopy (negative controls). GFP-positive cells in the interstitium are not readily visible. Scale bar = 0.2 mm.

transdifferentiation in adopted tissues have been controversial,[7,11] Recent literature suggests that bone marrow stem cells transferred into recipient mice can contribute to multiple nonhematopoietic tissues including myocytes, hepatocytes, lung epithelial cells, and neurons.[12–14] Research on bone marrow cells delivered to the ovaries via the blood stream giving rise to bona fide oocytes in mice[15] has been challenged by a new study.[16] In male mice, a recent study has demonstrated that bone marrow stem cells are able to differentiate into primordial germ cells and spermatogonia both *in vitro* and *in vivo*.[17] In addition, another study has demonstrated that adult stem cells derived from bone marrow stroma can differentiate into Leydig cells in rat testes.[18] In this study, we provide evidence showing that adult bone marrow cells injected into seminiferous tubules or interstitial spaces are not only able to differentiate into germ cells (spermatogonia and spermatocytes) as well as Sertoli and Leydig cells. This finding may be of clinical relevance to the understanding of testicular pathology and may lead to unique treatment of male infertility and testosterone deficiency.

Materials and Methods

Animal Preparation

Male wild-type (C57BL/6), green fluorescent protein (GFP) transgenic breeder mice (C57BL/6-Tg-UBC-GFP) and c-kit mutant homozygous (W/W^v) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Adult GFP transgenic male mice were generated from our colony and used as bone marrow cell donors. These GFP transgenic mice express GFP under the direction of the human ubiquitin C promoter. These mice express GFP in all tissues examined (Figure 8). We used two kinds of recipient mice: busulfan-treated mice and W/W^v mice (homozygous), which both have been used as standardized recipients for germ cell transplantation.[19,20] Busulfan is a chemotherapeutic agent that can eliminate spermatogenesis and induce male infertility. For our study, at 4 weeks of age, recipient wild-type mice were given a single dose of busulfan (50 mg/kg body weight) by intraperitoneal injection to destroy endogenous spermatogenesis. Recipients were then used for transplantation 4 weeks after the busulfan injection.[21] We also used recipient W/W^v mice that have no germ cells as a result of mutations in the c-kit receptor. Animal handling, experimentation, and the bone marrow and testicular tissue harvesting protocol were in accordance with the recommendations of the American Veterinary Medical Association and approved by the Institutional Animal Care and Use Review Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center.

Study Design

To determine whether bone marrow stem cells can differentiate into somatic or germinal cells, we inserted bone

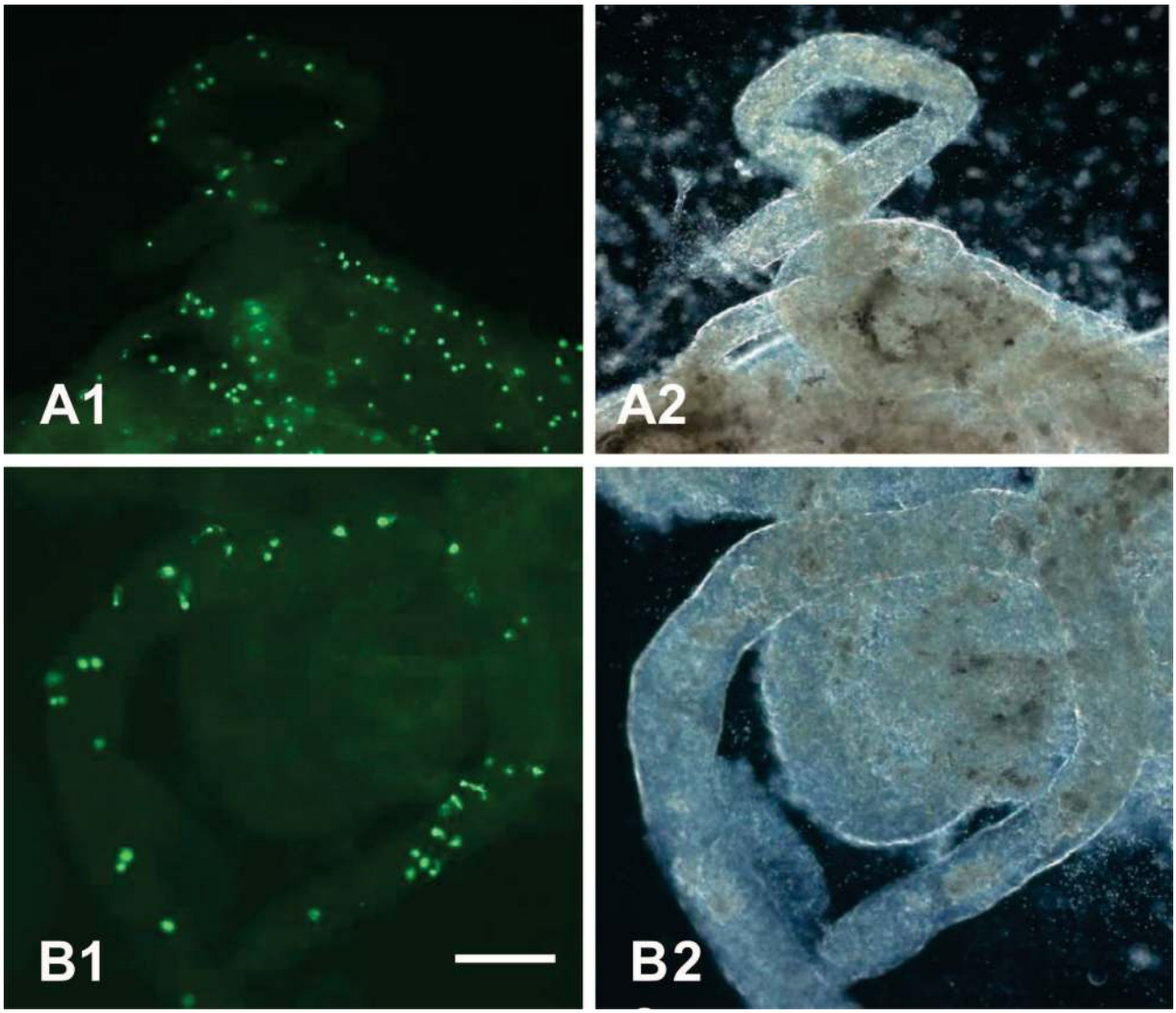

Figure 2. Seminiferous tubules from W/W^v recipient testis show adult bone marrow-derived cells as GFP-positive (**A1**, fluorescence microscopy; **A2**, transillumination). GFP-positive cells in seminiferous tubules exhibit a spatial and morphological pattern typical for Sertoli cells in recipient testis (**B1**, fluorescence microscopy; **B2**, transillumination). GFP-positive cells in the interstitium are not readily visible. Scale bar = 0.2 mm.

marrow cells isolated from GFP transgenic mice directly into the seminiferous tubules and interstitial space of recipient testes in groups of eight busulfan-treated wild-type and W/W^v mice. Recipients were sacrificed at 10 and 12 weeks after transplantation, and the results were evaluated. One side of the testes was dissected out and decapsulated. Seminiferous tubules were dispersed gently in 2 ml of Hanks' balanced salt solution held in a Petri dish and the live tissue visualized under Zeiss Axioskop 40 fluorescence microscope (Zeiss, Thornwood, NY). The contralateral testis from each animal was fixed in Bouin's solution, embedded in paraffin, and sectioned for immunohistochemistry to detect cells with GFP alone or GFP co-localized with Sertoli, Leydig, and germ cell markers. The Sertoli cell marker used was follicle-stimulating hormone receptor (FSH-R); the Leydig cell marker used was P450scc, and the germ cell marker used was VASA. Co-staining of GFP with various cell markers was detected by double-immunofluorescence technique in combination with confocal laser-scanning microscopy.

Donor Cell Preparation and Transplantation

Donor bone marrow cells were isolated from 6- to 8-week-old GFP transgenic mice by flushing dissected femurs and tibias with phosphate-buffered saline (PBS) (pH 7.4). The cells were pelleted by centrifugation at 600 $\times$ *g* for 5 minutes, after which a single cell suspension was obtained at 34°C by gentle digestion in calcium- and magnesium-free Hanks' balanced salt solution, which contained 0.05 μg of collagenase/ml (Life Technologies, Inc., Grand Island, NY), 0.05 mg/ml DNase (Sigma, St. Louis, MO), and 0.025% trypsin (Life Technologies, Inc.). After adding trypsin inhibitor (Sigma), centrifuging, and washing with Dulbecco's modified Eagle medium (Invitrogen Corp. Carlsbad, CA),

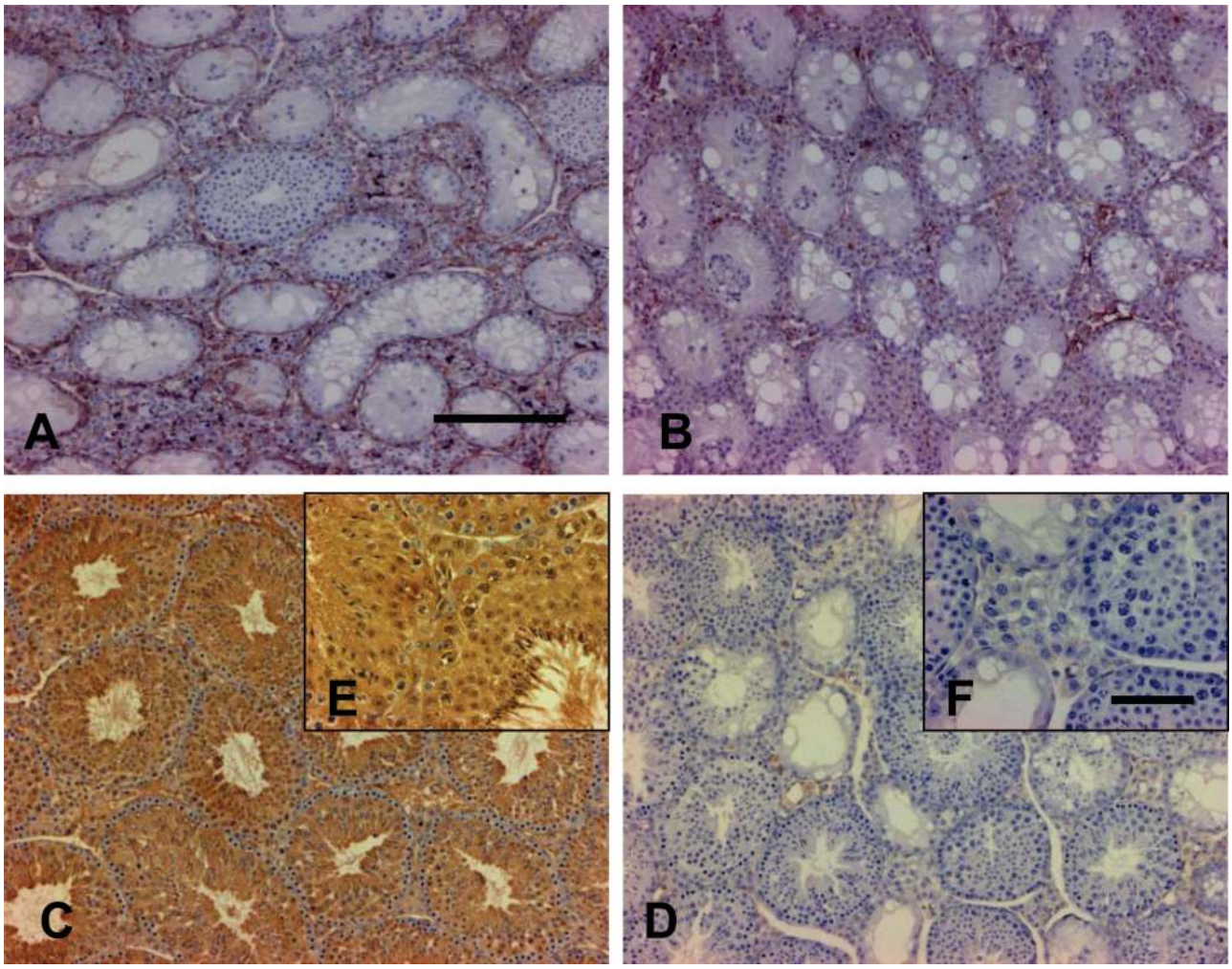

Figure 3. Immunohistochemistry of GFP (shown in dark brown) in recipient testicular sections from busulfan-treated (**A**) and W/W^v mice (**B**). GFP staining of germ, Sertoli, and Leydig cells in GFP transgenic mouse testes was used as the positive control (**C** and **E**). Busulfan-treated mouse testes that did not receive donor cells were used as the negative control (**D** and **F**). Scale bars: 0.2 mm (**A–D**); 0.05 mm (**E**, **F**).

the cells were then counted, pelleted by centrifugation at 600 × *g* for 5 minutes, and resuspended in injection media with 0.04% trypan blue stain (Invitrogen Corp.)[1,22] at a concentration of 5 to 15 million cells/ml.

Microinjection needles were constructed from 20-μl glass micropipettes (catalog 53432-740; VWR, West Chester, PA) drawn on a pipette puller (model P-97; Sutter Instruments, Novato, CA). The tip of each pipette was grounded to a sharp beveled point on a microbeveler (model 48000-F; World Precision Instruments, Sarasota, FL). The injection procedure was a modification of the efferent duct injection procedure previously described.[19,20] A small incision was made with a sterile 30-gauge needle ~3 mm from the efferent bundles' junction with the testis. The tip of the injection pipette was inserted into the bundle and then gently pushed toward the rete testis. As the tip entered the area of the rete, 10 μl of the cell suspension was injected under constant pressure. In addition to transplanting the cells into the seminiferous tubules, they were directly injected into the interstitium via rete testis puncture.

Immunohistochemistry for Detecting Bone Marrow-Derived Cells

Immunohistochemistry was performed on Bouin's fixed and paraffin-embedded testicular sections from recipient mice as previously described.[23] Testicular sections were briefly deparaffinized, hydrated by successive series of ethanol, rinsed in distilled water, and then incubated in 2% H_2O_2 to quench endogenous peroxidases. Sections were blocked with 5% normal horse serum for 20 minutes to prevent nonspecific binding of IgG and subsequently incubated with a 1:500 dilution of a monoclonal anti-GFP antibody (sc-9996; Santa Cruz Biotechnology, Santa Cruz, CA).[15] Immunoreactivity was detected using biotinylated anti-mouse IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex visualized with diaminobenzidine tetrahydrochloride (DAB) as per the manufacturer's instructions (Mouse UniTect ABC immunohistochemistry detection system; Calbiochem, La Jolla, CA). Slides were counterstained with

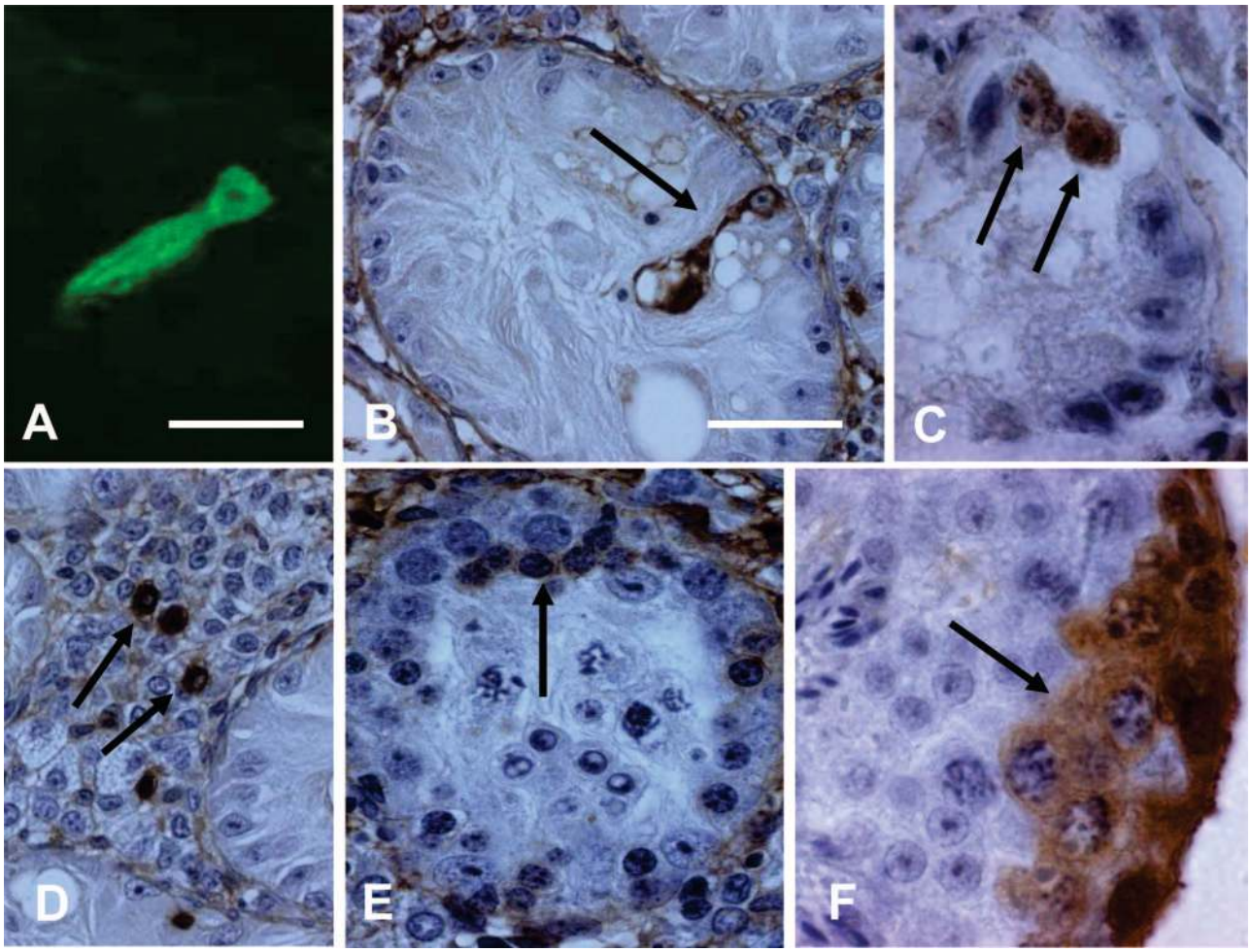

Figure 4. Immunohistochemistry of GFP in Bouin's fixed, paraffin-embedded testicular sections. GFP-positive Sertoli cell (**arrow**) in recipient testis was shown in **A–C**. Note of typical morphology of Sertoli cell with both nuclear and cytoplasm staining. **A:** GFP-positive Sertoli cell was detected by using fluorescent secondary antibody. **E:** GFP-positive preleptotene spermatocytes are shown. **F:** A clone of germ cells including spermatogonia and spermatocytes was embedded in endogenous spermatogenesis. **D:** GFP-positive Leydig cells (**arrow**) were found in the testicular interstitium. Scale bars: 20 μm (**A**, **C**, **F**); 50 μm (**B**, **D**, **E**).

hematoxylin and reviewed with a Zeiss Axioskop 40 microscope. Busulfan-treated testes without transplantations and testes from either wild-type or W/W^v mice were processed identically as negative controls. Testes from GFP mice were used analogously as a positive control.

Immunofluorescence and Confocal Analysis for Co-Localization of GFP and Cell-Specific Markers in the Testis

Bouin's fixed testicular sections were used for immunohistochemistry to detect cells with co-localized expression of GFP (green, 1:500) and Sertoli, Leydig, or germ cell markers. The Sertoli cell marker used was FSH receptor (FSH-R), the Leydig cell marker used was P450scc, and the germ cell marker used was VASA. We did not find any immunostaining of FSH-R, P450scc, and VASA in isolated bone marrow cells before transplantation. The specificity of the primary antibodies has been previously described.[3,24,25] After deparaffinization and rehydration, tissue sections were treated with 2% H_2O_2 in PBS for 10 minutes followed by 20 minutes of incubation with blocking serum (5% normal horse serum) at room temperature. After washing the slides three times in PBS (pH 7.4), sections were incubated with a 1:500 dilution of a monoclonal anti-GFP antibody (Santa Cruz Biotechnology) for 1 hour and then incubated with goat anti-mouse Alexa Fluor 488 (green)-labeled secondary antibody (Molecular Probes, Eugene, OR) for 30 minutes. Then the sections were incubated with one of the following antibodies for 1 hour: FSH-R goat polyclonal antibody (1:100; Santa Cruz Biotechnology, Inc.), P450scc rabbit polyclonal antibody (1:100; Chemicon Inc., Temecula, CA), or

Table 1. GFP-Positive Bone Marrow-Derived Cells in Recipient Testes

	Busulfan-treated mice	W/W^v mice
Leydig cells/10^6 μm	40.2 ± 17.8*	11.4 ± 1.98
Sertoli cells/10^6 μm	10.2 ± 1.4*	4.2 ± 1.0
Germ cells/10^6 μm	6.6 ± 1.8	None

Values are the mean ± SEM.
*Significant at $P < 0.05$.
Note: Germ cells, Sertoli cells, and Leydig cells were characterized based on their morphologic criteria.[37] Germ cells included spermatogonia and spermatocytes.

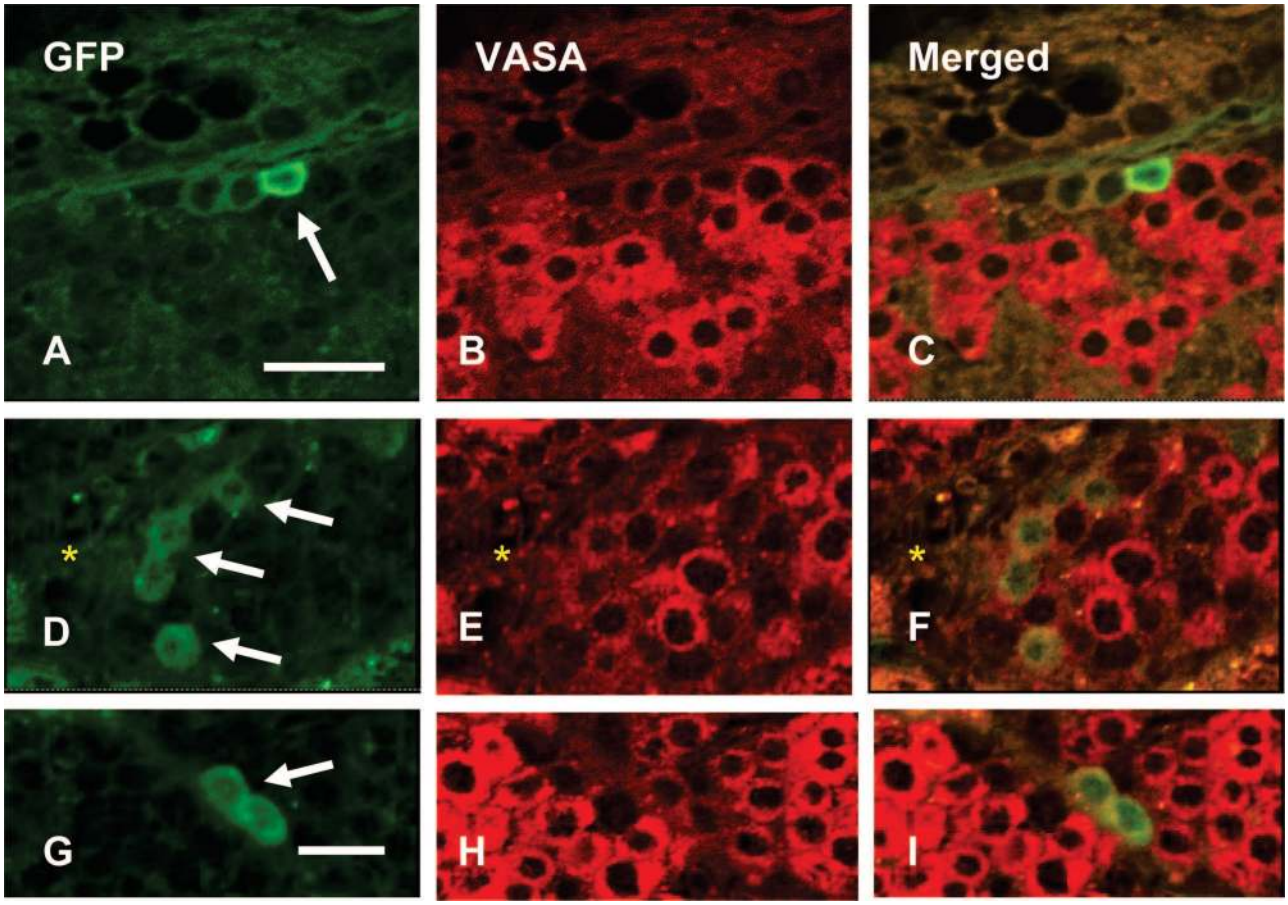

Figure 5. Confocal images show GFP-positive spermatogonia (green; **A**, **D**, and **G**), VASA expression (red; **B**, **E**, and **H**), and co-localization of GFP with VASA (greenish yellow; **C**, **F**, and **I**). **Yellow asterisks** indicate interstitial space. Scale bars = 10 μm.

VASA (DDX4/MVH) rabbit polyclonal antibody (1:100; Abcam Inc., Cambridge, MA). The slides were then treated with another fluorescent secondary antibody for 30 minutes at room temperature. Goat anti-rabbit Alexa Fluor 594 (red)-labeled secondary antibody (Molecular Probes) was used for P450scc and VASA; donkey anti-goat Alexa Fluor 594 (red)-labeled secondary antibody (Molecular Probes) was used for FSH-R. Slides were washed and then mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). For negative controls, sections were processed without the primary antibody, and no signals were detected. Confocal imaging was performed using a TCS-SP-MP confocal microscope (Leica Corp., Deerfield, IL).

Morphometric Assessment of GFP-Positive Cells in Testes

The method used for germ cell quantitation was similar to that described previously.[26,27] In brief, testicular sections were examined with an American Optical Microscope (Buffalo, NY) with a ×40 objective and a ×10 eyepiece. A square grid fitted within the eyepiece provided a reference area of 62,500 μm^2. GFP-positive Leydig cells, Sertoli cells, and germ cells within 40 grids of testicular sections from each animal were counted.

Statistical Analysis

Statistical analyses were performed using the SigmaStat 2.0 program (Jandel, San Rafael, CA). Results were tested for statistical significance using a *t*-test. Differences were considered significant if $P < 0.05$.

Results

Bone Marrow-Derived Cells Were Detected in the Live Testicular Tissue 10 to 12 Weeks after Transplantation

We found that GFP-positive bone marrow-derived cells survived in both busulfan-treated (Figure 1) and W/W^v testes (Figure 2) for at least 12 weeks after transplantation. GFP-positive cells were observed within seminiferous tubules and in the interstitium in both busulfan-treated (Figure 1A1) and W/W^v (Figure 2A1) recipient testes. In some of the seminiferous tubules, the green florescent cells extended from the basal lamina toward the luminal compartment and demonstrated a spatial and morphological pattern characteristic of typical Sertoli cells (Figures 1B1 and 2B1).

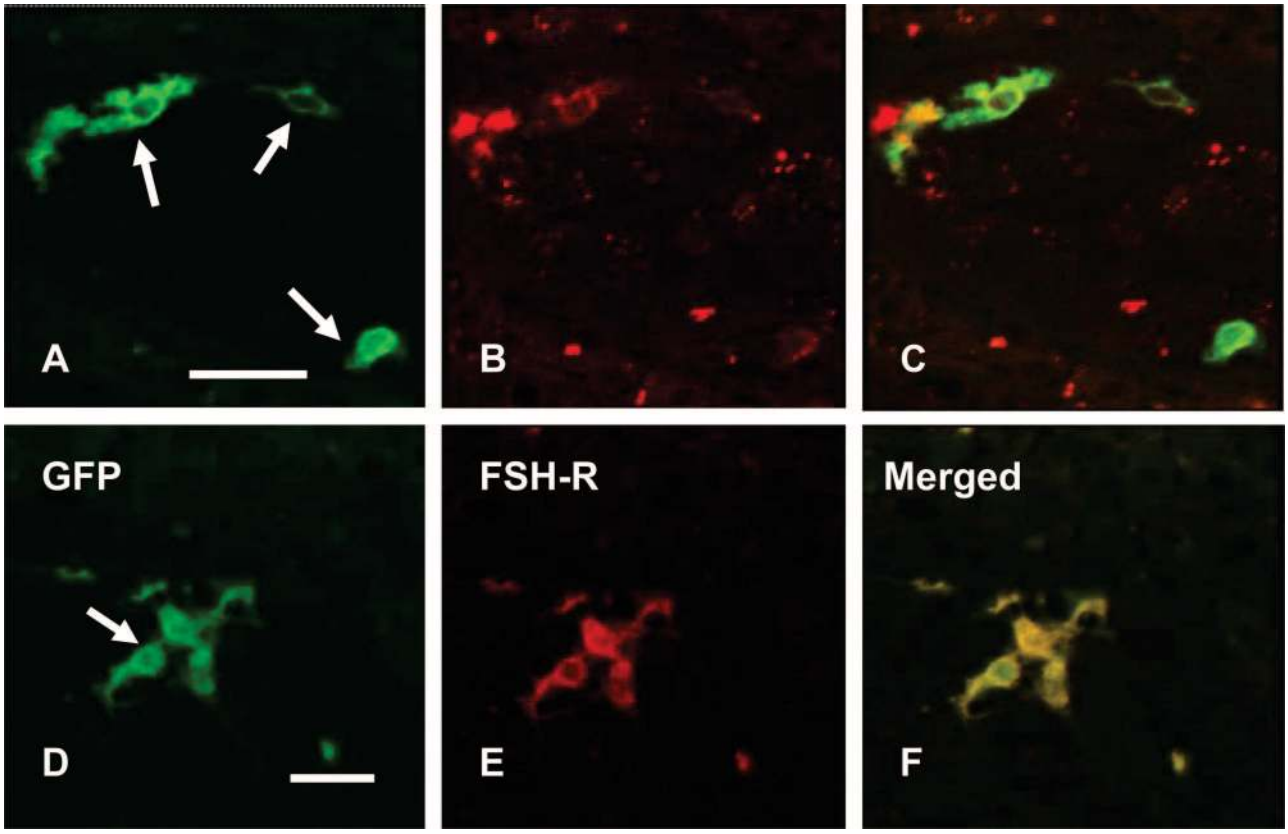

Figure 6. Confocal images show GFP-positive donor-derived Sertoli cells (green; **A** and **D**), FSH-R expression (red; **B** and **E**), and co-localization of GFP with FSH-R (yellow; **C** and **F**). Scale bars = 10 μm.

The Bone Marrow-Derived Cells Were Detected as Sertoli, Male Germ, and Leydig Cells by Morphological Assessment and Immunohistochemistry

Bone marrow-derived GFP-positive donor cells were further examined by immunohistochemistry and were present in recipient testicular sections from busulfan-treated (Figure 3A) and W/W^v (Figure 3B) mice. Further morphological examination showed that some of these GFP-positive cells in the busulfan-treated recipient testis had a Sertoli cell appearance (Figure 4, A–C) characterized by an irregular nucleus containing a tripartite nucleolus located near the basal lamina as well as cytoplasm extending from the basal lamina toward luminal compartment. GFP staining was found in both nuclear and cytoplasm of bone marrow cell-derived Sertoli cells. Some of these GFP-positive cells in seminiferous tubules exhibited as a clone consisting of interconnected preleptotene and/or pachytene spermatocytes (Figure 4, E and F). In busulfan-treated

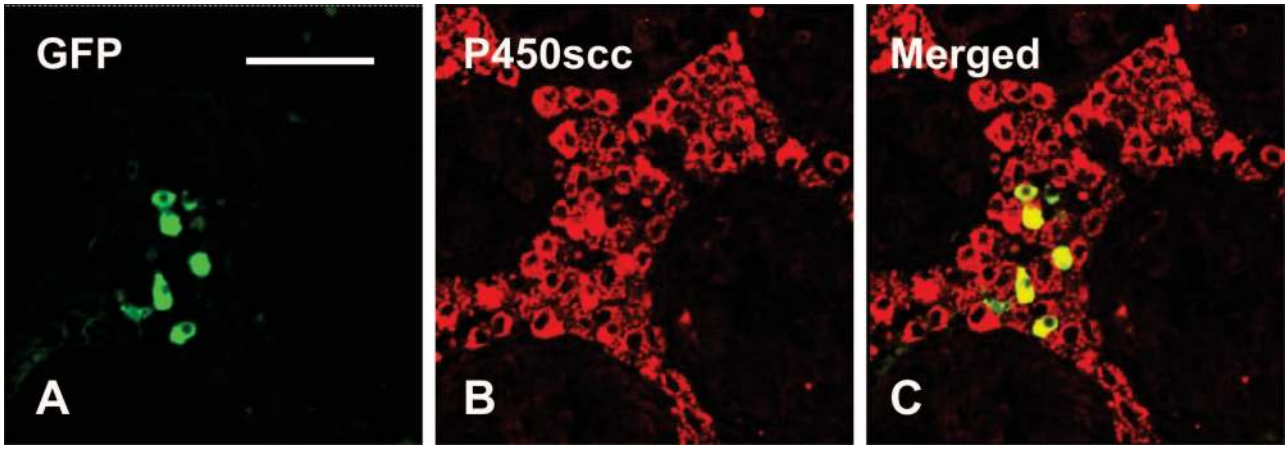

Figure 7. Confocal images show GFP-positive donor-derived Leydig cells (green, **A**), P450scc expression (red, **B**), and co-localization of GFP with P450scc (yellow, **C**). Scale bar = 50 μm.

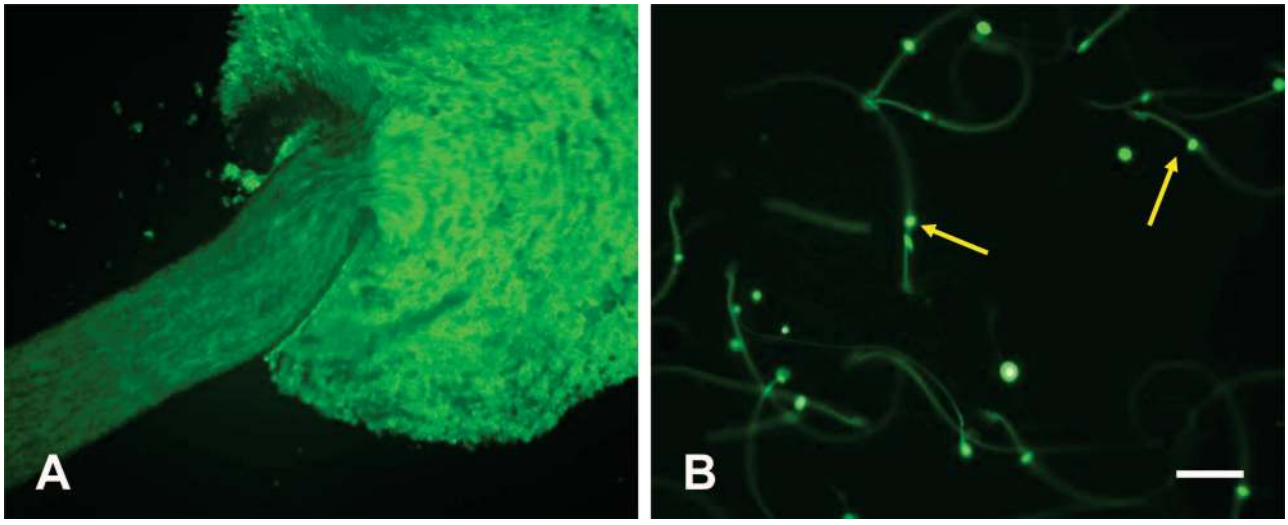

Figure 8. A segment of seminiferous tubule squashed from UBC-GFP transgenic mouse shows GFP-positive cells (**A**) and spermatozoa (**B**) visualized under fluorescent microscope. Note green cytoplasmic droplets on spermatozoa (**arrows**). Scale bars: 0.2 mm (**A**); 10 μm (**B**).

mice, the donor-derived germ cells were surrounded and embedded in recovered and endogenous spermatogenesis. No GFP-positive round spermatids were found in the seminiferous tubules of testicular sections examined. In the interstitium, the GFP-positive Leydig cells were readily found embedded in the native Leydig cells in the interstitium (Figure 4D) of busulfan-treated and W/W^v mice. Quantitative data (Table 1) of GFP-positive Leydig, Sertoli, and germ cells show significantly lower differentiation rates of bone marrow-derived cells in W/W^v mice when compared with busulfan-treated mice. Testicular serial sections under confocal microscopy showed that GFP-positive donor-derived Sertoli, Leydig, and germ cells have a single nucleus.

The GFP-Positive Donor-Derived Cells Co-Localized with Cell-Specific Markers in the Testis

Confocal microscopy demonstrated co-localization of GFP-positive donor-derived germ cells with VASA, a germ cell-specific marker in the testis (Figure 5). In bone marrow cell-derived germ cells, GFP was expressed in both the cytoplasm and nucleus. VASA protein was detected in both endogenous and donor-derived germ cells in the busulfan-treated recipient testis. Bone marrow cell-derived GFP-positive Sertoli cells expressed FSH-R (Figure 6). In the interstitium, GFP-positive donor-derived Leydig cells expressed P450scc (Figure 7), which is a Leydig cell marker in the testis. Twelve weeks after engraftment in W/W^v recipient testes, GFP-positive donor cells expressed FSH-R in the seminiferous tubules and P450scc in the interstitium (Figure 8). However, donor-derived germ cells were not observed in the seminiferous tubules in W/W^v recipient testes. A few GFP-positive donor cells with macrophage appearance were occasionally found in the center of the seminiferous tubules in W/W^v recipient testes.

Discussion

We demonstrated that donor-derived GFP-positive cells were present in seminiferous tubules and in the interstitium, indicating that bone marrow-derived cells survive in recipient testes for at least 12 weeks after transplantation. The donor cells used in this study were unfractionated bone marrow cells containing hematopoietic stem cells, endothelial stem/progenitor cells, mesenchymal stem cells, and multipotent adult progenitor cells. Thus, we do not know which type of bone marrow stem cells generated the testicular cells. Previous reports demonstrated that bone marrow-derived mesenchymal stem cells are capable of differentiating into germ cells and Leydig cells in the testis.[17,18]

We intended to use busulfan-treated and W/W^v mice as our recipients. Busulfan treatment induces chemical injury of spermatogenesis, leading to infertility in male mice. In busulfan-treated recipient testes, we found GFP-positive Sertoli cells, spermatogonia, and early spermatocytes in the seminiferous tubules and Leydig cells in the interstitium. The GFP-positive germ cells were halted at the early spermatocyte stage without further differentiation into spermatids. The mechanisms of donor-derived germ cells that failed to go through meiosis remain unknown. We speculate that donor-derived germ cells arrest at the spermatocyte stage because of their inert genetic imprinting or they are incompatible with the support by Sertoli cells. In the W/W^v recipient testes, which are devoid of endogenous germ cells as a result of mutations in the c-kit receptor, we did not find germ cells. A major difference in the two recipient mice is the complete absence of endogenous germ cells in W/W^v mice and the presence of spontaneously recovered endogenous germ cells along with donor-derived germ cells in busulfan-treated mice. We found donor-derived germ cells embedded in or surrounded by the spontaneously recovered endogenous germ cells. Donor-derived Sertoli and Leydig cells were also observed among endogenous Sertoli and Leydig cells, respectively. Our observation

suggests an essential role of recovering endogenous germ cells in inducing transdifferentiation of donor bone marrow cells into germ cells in the microenvironment of the seminiferous tubules. Endogenous Sertoli and Leydig cells may also play a role in inducing the differentiation of GFP-positive donor-derived Sertoli and Leydig cells because the numbers of GFP-positive cells were significantly higher in busulfan-treated recipient than W/W^v mice. Based on this observation, we conclude that on a proper migration of donor stem cells, the resident neighboring cells in the recipient testis may control site-appropriate stem cell differentiation. We found GFP-positive donor-derived cells had a single nucleus in each cell, but we cannot completely exclude the possibility of donor cell fusion with native germ, Sertoli, or Leydig cells.

The percentage of GFP-positive germ, Sertoli, and Leydig cells were low in the recipient testes. The functional status of donor-derived germ, Sertoli, and Leydig cells remains to be determined. In future experiments we will use flow cytometric analysis for quantitative evaluation of donor-derived cells from both interstitial space and seminiferous tubules of recipient testes with cell-specific markers.[28,29] To increase the uptake and transdifferentiation of bone marrow cells, we plan to inject cultured and/or isolated and enriched adult stem cells alone with growth factors such as glial cell line-derived neurotrophic factor, stem cell factor, and insulin-like growth factors. Isolation of hematopoietic stem cells has been achieved.[30] Clonogenic *in vivo* and *in vitro* assays suggest a high level of purity (~85 to 95%) is attainable for these cells.[31] Multipotent marrow stromal cells, which give rise to multiple mesenchymal lineages, can also be isolated from bone marrow.[32,33]

The molecular mechanism of adult stem cell plasticity is not completely understood. The testis creates a unique microenvironment for donor stem cell migration, proliferation, differentiation, and apoptosis. The testis is protected from immunological influences by the blood-testis barrier allowing the recipient to host donor cells without rejection. By transplanting adult stem cells isolated from gene knockout or transgenic mice into wild-type mice, or vice versa, we will be able to study the effect of gene mutations on stem cell biology.

The pathogenesis of male infertility is attributable either to the failure in germ cell proliferation and differentiation or to somatic cell dysfunction. In many cases, germ cells are present. The presence of donor-derived somatic cells is critical because both Leydig and Sertoli cells support spermatogenesis. Defects in these cells have been believed to contribute to abnormal spermatogenesis.[34] The possibility of beneficial hormonal effects of Leydig cell transplantation independent of their support of spermatogenesis also exits. Because Leydig cells are responsible for testosterone production, stem cell transplantations may replace the need of life-long testosterone supplementation in male hypogonadism or aging.[35,36] Additional studies are required to demonstrate such hormone benefits. Thus, the present finding may have a major impact in understanding reproductive physiology and recovery from testicular pathology and also may have the potential for novel future therapies in patients with testicular failure.

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