

Oocyte Generation in Adult Mammalian Ovaries by Putative Germ Cells in Bone Marrow and Peripheral Blood

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Summary

It has been suggested that germline stem cells maintain oogenesis in postnatal mouse ovaries. Here we show that adult mouse ovaries rapidly generate hundreds of oocytes, despite a small premeiotic germ cell pool. In considering the possibility of an extragonadal source of germ cells, we show expression of germline markers in bone marrow (BM). Further, BM transplantation restores oocyte production in wild-type mice sterilized by chemotherapy, as well as in ataxia telangiectasia-mutated gene-deficient mice, which are otherwise incapable of making oocytes. Donor-derived oocytes are also observed in female mice following peripheral blood transplantation. Although the fertilizability and developmental competency of the BM and peripheral blood-derived oocytes remain to be established, their morphology, enclosure within follicles, and expression of germ-cell- and oocyte-specific markers collectively support that these cells are bona fide oocytes. These results identify BM as a potential source of germ cells that could sustain oocyte production in adulthood.

Introduction

A central dogma of reproductive biology has been that females of most mammalian species lose the capacity for oocyte production during fetal development, leading to the endowment of a finite stockpile of oocytes, enclosed by somatic cells in structures referred to as follicles, at or shortly after birth (Zuckerman, 1951; Zuckerman and Baker, 1977). Only a small fraction of the postnatal oocyte pool survives to be ovulated at some point in reproductive life, as the fate of the vast majority of follicles that house the oocytes is atretic degeneration (Tilly, 2001). For example, in humans, it has been estimated that less than 3×10^5 of the original pool of about 1×10^6 nondegenerative oocytes present

at birth survive at puberty. This number continues to decline throughout adulthood to the point of exhaustion around age 50, driving menopause (Richardson et al., 1987). Proportionately similar reductions in oocyte numbers occur in mice during postnatal life (Perez et al., 1999; Canning et al., 2003), with complete depletion of the oocyte pool also noted long before death due to chronological age (Gosden et al., 1983).

A recent study has, however, challenged this dogma by showing in mice that production of oocytes (oogenesis) and their enclosure within somatic cells (folliculogenesis) persist in juvenile and adult life (Johnson et al., 2004). Similar events occur in female flies, which maintain oocyte production as adults via a specialized cell population referred to as germline stem cells (GSCs) (Deng and Lin, 2001). Although this concept has now been extended to mammals (Johnson et al., 2004), widespread acceptance that adult mammalian females are indeed capable of producing new oocytes is slow in coming (Gosden, 2004). This likely stems, at least in part, from a lack of direct evidence for the existence of mammalian female GSCs and their progenitors, which would be manifest by, among other things, the ability of such cells to rescue and sustain oocyte production following transplantation.

Here we show that adult mouse ovaries can produce hundreds of new oocytes within 24 hr, reinforcing the primary conclusion from our earlier work that initially challenged the dogma of a fixed complement of oocytes at birth (Johnson et al., 2004). Moreover, based on gene expression analyses and bone marrow transplantation (BMT) experiments using chemotherapy-sterilized recipients, a putative GSC reservoir that supports oogenesis in adult female mice appears to reside in bone marrow (BM). Additional transplantation studies using peripheral blood (PB) harvested from transgenic females with germline-restricted green fluorescent protein (GFP) expression show that GFP-positive oocytes form in chemotherapy-treated recipient females, suggesting that putative germ cells in BM release progenitor cells into the peripheral circulation that travel to the ovaries for oocyte production. Although the BM- and PB-derived cells exhibit many fundamental characteristics of bona fide oocytes (e.g., morphology, presence within follicles, expression of germ-cell- and oocyte-specific markers), we have not yet determined whether these oocytes can fertilize normally and develop into viable offspring. Nonetheless, these results identify BM and PB as potential sources of female germ cells that could sustain oocyte production in adulthood.

Results

Oogenesis and Folliculogenesis in Adulthood

The reported existence of mammalian female GSCs (Johnson et al., 2004) implies an inherent capacity of the ovaries to generate new oocyte-containing follicles in a regulated fashion. To address this, we injected adult female mice with doxorubicin to synchronize oo-

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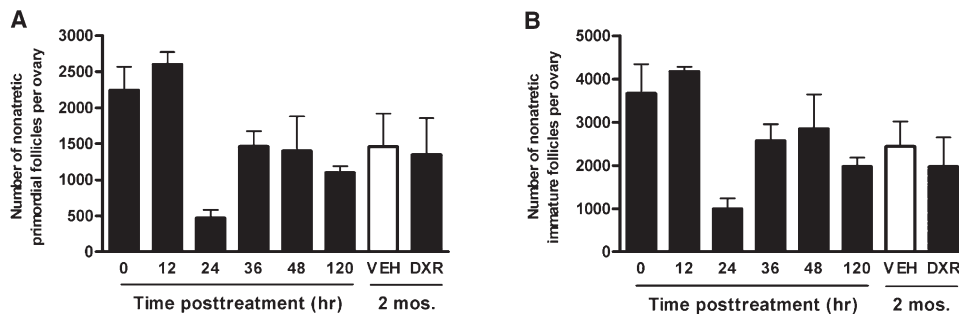


Figure 1. Spontaneous Regeneration of Oocyte-Containing Follicles in Adult Female Mice

Number of nonatretic primordial (A) or total immature (primordial, primary, small preantral [B]) follicles in ovaries of adult female mice at the indicated times following doxorubicin (DXR) injection (5 mg/kg). Follicle numbers in vehicle (VEH)- versus DXR-treated mice at 2 months (2 mos.) postinjection are also shown. Values are the mean \pm SEM from an analysis of four to five mice per treatment group.

cyte death (Perez et al., 1997), and ovaries were analyzed at multiple intervals afterwards. A rapid and extensive loss of resting (primordial) and early-growing follicles occurred within the first 24 hr after doxorubicin treatment (Figure 1). However, spontaneous regeneration of the immature follicle pool was observed between 24 and 36 hr posttreatment, and the number of follicles stabilized thereafter such that, by 2 months posttreatment, there were no differences between vehicle- and doxorubicin-treated mice (Figure 1). These data, along with results from other experiments (see Figure S1 with the Supplemental Data available with this article online), support our earlier work on the occurrence of oogenesis and folliculogenesis in adult female mammals (Johnson et al., 2004).

Characterization of Adult Ovary-Derived Germ Cells

An earlier study suggested that non-follicle-enclosed germ cells on the ovarian surface were logical candidates for GSCs (Johnson et al., 2004). However, the number of these cells drops precipitously as female mice transit through puberty (63 ± 8 versus 6 ± 3 cells per ovary at postpartum day 30 versus day 40, respectively; mean \pm SEM, $n = 4$ mice per group), suggesting that these cells could not represent adult GSCs. In considering other strategies to potentially identify GSCs, we focused on stage-specific embryonic antigen-1 (SSEA1), a carbohydrate expressed at high levels on primordial germ cells (PGCs) (Marani et al., 1986). Immunohistochemical analysis of SSEA1 expression in adult mouse ovaries revealed a cluster of SSEA1-positive (SSEA1⁺) cells in the medulla (Figures S2A–S2D). Outside of a low level of immunoreactivity occasionally observed in some scattered granulosa cells, SSEA1 was not expressed in any other area of the ovary or in any cell type of known origin, including oocytes (Figure S2A).

Since SSEA1 is expressed by some somatic stem cell lineages in adults (Capela and Temple, 2002), we subjected dispersed adult mouse ovaries to SSEA1-antibody-based magnetic bead sorting (Geijsen et al., 2004) to determine if the SSEA1⁺ cell pool detected in vivo contains germ cells. Cells within the SSEA1⁺

fraction expressed the POU domain transcription factor *Oct4*, which plays a key role in stem cell pluripotency and early germ cell development (Schöler et al., 1989), as well as the germline markers mouse Vasa homolog (*Mvh*) (Fujiwara et al., 1994; Noce et al., 2001), *Dazl* (Cooke et al., 1996) and *Stella* (Saitou et al., 2002) (Figure S2E). As expected, all four germline markers were also expressed in the residual cell fraction, which contains oocytes (Figure S2E). However, SSEA1⁺ cells did not express synaptonemal complex protein 3 (*Scp3*), which marks meiotic entry in germ cells (Cohen and Pollard, 2001). Moreover, we failed to detect expression of several oocyte markers, including histone deacetylase 6 (*Hdac6*; Figure S3), growth differentiation factor 9 (*Gdf9*; McGrath et al., 1995), and zona pellucida protein 3 (*Zp3*; Philpott et al., 1987), in the SSEA1⁺ cells (Figure S2E). These data, and the finding that oocytes do not express SSEA1 (Figure S2A), indicate that the SSEA1⁺ cell fraction contains germ cells that are not oocytes.

Expression of Germline Markers in BM of Adult Mice

If GSCs were represented by SSEA1⁺ germ cells in the ovary, the relatively small size of this cell pool would be incongruous with the ability of adult mouse ovaries to generate hundreds of new oocytes in only 24 hr (Figure 1, Figure S1). Accordingly, we next considered the possibility that a GSC reservoir exists somewhere outside of the ovaries. During embryogenesis, PGCs and hematopoietic stem cells (HSCs) are known to originate from the same region—the proximal epiblast (Lawson and Hage, 1994). Early HSCs then colonize the aorta-gonad-mesonephric region of the developing embryo prior to migration into the fetal liver (Medvinsky and Dzierzak, 1996), at roughly the equivalent time that PGCs enter the same region of the embryo to colonize the fetal gonads (Molyneaux and Wylie, 2004). In postnatal life, the hematopoietic system is maintained by stem cells that home to and reside in the BM (Morrison et al., 1995). This information, along with the reported ability of PGCs to generate primitive HSCs in vitro (Rich, 1995) and the increasing number of studies demonstrating the multilineage potential of BM-derived

cells (Herzog et al., 2003; Grove et al., 2004; Heike and Nakahata, 2004), prompted us to examine if germ cells could be identified in adult female BM.

Initial experiments demonstrated expression of *Oct4*, which in adult mice is reportedly restricted to germ cells (Schöler et al., 1989; Yoshimizu et al., 1999), as well as *Mvh*, *Dazl*, *Stella*, and a fifth germline marker gene termed *Fragilis* (Saitou et al., 2002), in BM isolated from adult female mice (Figures 2A–2D). In addition, expression of the female-germ-cell-specific homeobox gene *Nobox* (Suzumori et al., 2002), which is critical for directing expression of *Oct4* and *Gdf9* in primordial oocytes as well as for folliculogenesis (Rajkovic et al., 2004), was detected in BM of adult females (Figure 2A). Independent confirmation of our findings that multiple germline markers are expressed in mouse and human BM was provided by analysis of public microarray databases. For example, expression of *Mvh*, *Dazl*, *Stella*, and *Fragilis* has been identified in mouse BM (<http://genome.ucsc.edu>; Benson et al., 2004; Su et al., 2004), and *STELLA* expression has been identified in human BM (<http://genome.ucsc.edu>; GenBank Accession Number CV414052 from Dias Neto et al. [2000]). Consistent with results from this latter study, we also detected germline markers in BM obtained from human female donors between the ages of 24 and 36 (Figure S4).

Given the large number of studies documenting the germ-cell-restricted nature of *Vasa* expression throughout the animal kingdom (Noce et al., 2001), we selected *Mvh* to quantitatively assess potential changes in germline marker expression in BM during the female reproductive cycle. Using real-time PCR, marked estrous-cycle-related changes in *Mvh* expression in BM of adult female mice were uncovered, with a 9.52-fold difference noted between estrus (nadir) and metestrus (peak) (Figure 2E). A parallel evaluation of ovarian germ cell dynamics revealed a positive correlation between the cycle-related changes in BM *Mvh* expression and oocyte numbers, with ovaries at metestrus containing over 800 more primordial follicles than ovaries at estrus (Figure 2F). We then examined the effect of ovariectomy, without or with subsequent ovarian steroid supplementation, on BM *Mvh* levels. Removal of the ovaries caused a near-complete elimination of *Mvh* expression in BM, and replacement of estrogen, progesterone, or the two steroids together did not alter BM *Mvh* levels in ovariectomized females (Figure 2G). In parallel studies, we observed that *Mvh* levels in BM of adult females at metestrus were 1.6% of those detected in adult ovaries (Table S1), which contain thousands of *Mvh*-expressing oocytes (Fujiwara et al., 1994; Figure 2D). Interestingly, *Mvh* expression was also detected in BM of adult male mice, with a level of expression slightly less than 20% of that detected in BM of adult females at metestrus (Table S1).

We next sorted BM samples based on cell surface stem cell markers and quantitatively analyzed the resultant cell fractions for *Mvh* levels. After removal of differentiated cells committed to hematolymphoid lineages by negative selection (Spangrude et al., 1988), *Mvh* expression was retained in the lineage-depleted (*lin*[−]) cell fraction (Figure 3A). Subsequent separation of *lin*[−] cells based on cell surface expression of Sca-1 (Van de Rijn et al., 1989) or c-Kit (Okada et al., 1991) showed

that the majority (82.9%) of the *Mvh*-expressing *lin*[−] cells were negative for expression of Sca-1 but positive for c-Kit (Sca-1[−]/c-Kit⁺) (Figure 3A). Moreover, expression of *Dazl*, *Stella*, and *Fragilis* cosegregated with *Mvh* in the *lin*[−]/Sca-1[−]/c-Kit⁺ cell fraction (Figure S5). In parallel experiments, in vitro culture of adult female BM-derived cells under conditions that permit the progressive enrichment of stem cells from BM (Meirelles Lda and Nardi, 2003) demonstrated that all of the germline markers present in freshly isolated BM were expressed by the adherent cell fraction and remained so following multiple serial passages over a 6 week period (Figure 3B).

BMT Rescues Oocyte Production in Sterile Adult Females

To assess if putative BM-derived germ cells could generate oocytes, BM was isolated from adult wild-type female mice and transplanted into recipient adult females sterilized by pretreatment with cyclophosphamide and busulfan to destroy the existing pre- and postmeiotic germ cell pools (Shiromizu et al., 1984; Johnson et al., 2004). Ovaries were then collected, and the presence of oocytes was determined by their morphological appearance, their enclosure within histologically normal follicles, and, in some experiments, expression of germ-cell- and oocyte-specific markers. Two months after treatment, very few, if any, immature oocytes or follicles were detected in the ovaries of those females given cyclophosphamide and busulfan alone (Figures 4A–4C, Figures S6A–S6B). However, ovaries of mice receiving BMT 1 or 7 days after chemotherapy possessed several hundred oocyte-containing follicles at all stages of development, including the resting primordial stage (Figures 4A and 4D, Figure S6C). Histological evaluations showed that the chemotherapy regimen essentially destroyed the ovaries—which, after treatment, were composed of little more than stromal and interstitial cells with an occasional cystic follicle or old corpus luteum (Figure 4C, Figure S6B). By comparison, ovaries of mice receiving BMT—even when the transplants were given a week after inflicting the damage to the tissue—possessed a complete spectrum of immature and mature oocyte-containing follicles as well as corpora lutea indicative of a resumption of normal ovulatory cycles (Figure 4D, Figure S6C). Furthermore, oocytes and follicles were found in ovaries of chemotherapy-sterilized females more than 11 months after the initial transplantation (Figures 4E and 4F), indicating that the putative BM-derived germ cells are capable of sustaining long-term oocyte production.

To further establish that putative germ cells present in BM can generate oocytes, BMT was next performed in adult female mice with a targeted disruption in the ataxia telangiectasia-mutated (*Atm*) gene. This line of mutant mice was selected based on past studies showing that *Atm*-deficient mice lack the ability to produce mature germ cells due to the critical role that this kinase plays in early meiotic progression (Barlow et al., 1998). In females, neonatal *Atm* mutants are devoid of oocytes and remain so throughout postnatal life (Di Giacomo et al., 2005). However, consistent with the fact that premeiotic germ cell development occurs normally

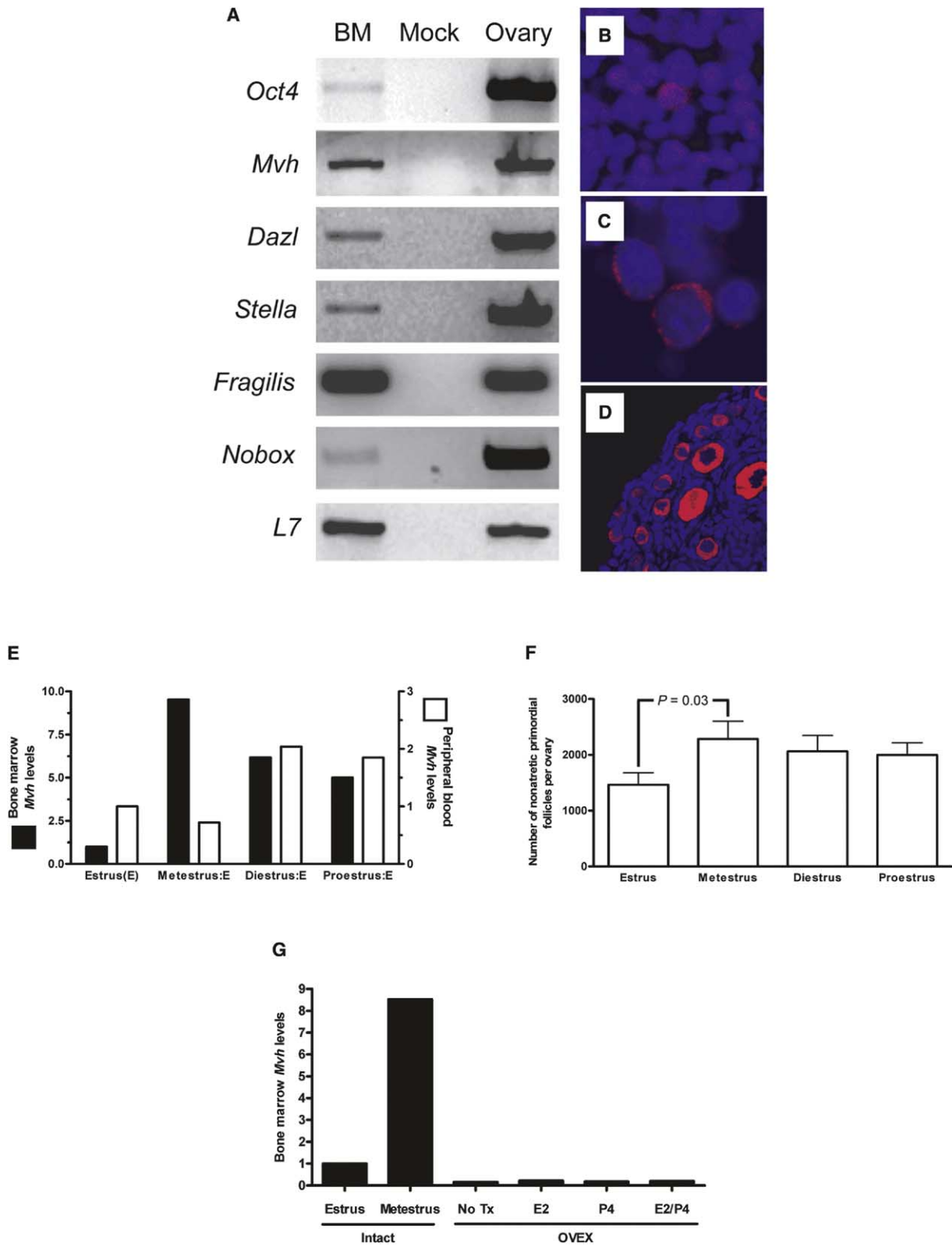


Figure 2. Ovarian-Regulated Expression of Germline Markers in BM

(A) Germline marker expression in BM of adult wild-type female mice. As a positive control for expression of germline markers, adult mouse ovary RNA was analyzed in parallel. *Oct4* mRNA was low in abundance and not consistently detected in all BM samples. *L7*, “housekeeping” gene. Mock, mock reverse-transcribed BM RNA samples.

in the absence of *Atm*, analysis of adult *Atm*-deficient ovaries revealed expression of *Oct4*, *Mvh*, *Dazl*, and *Stella* (Figure S7). Although mutant females are incapable of generating oocytes from early germ cells, *Atm* null female mice were nonetheless conditioned with cyclophosphamide and busulfan to eliminate the possibility of host germ cell contribution to oocyte production following BMT. In contrast to the expected and complete absence of oocytes in nontransplanted *Atm* mutants (Figure 5B; no oocytes or follicles were observed in ovaries of ten animals total), oocyte-containing follicles were found in the transplanted mutant females and remained detectable more than 11 months after the initial BMT (Figures 5C–5G, Table S2).

Generation of Oocytes by Peripheral Blood Cell Transplantation

If BM houses female GSCs, then the progeny from these putative germ cells would likely utilize the PB supply for travel to the ovaries. We therefore next determined if peripheral blood cell transplantation (PBCT) could contribute to oocyte production in transplanted female recipients. In the first of three experiments, we used transgenic female mice with GFP expression driven by an 18 kb fragment of the *Oct4* promoter in which the proximal enhancer region has been inactivated (GOF18- Δ PE or TgOG2) (Yeom et al., 1996; Yoshimizu et al., 1999; Szabo et al., 2002) as donors for PBCT. Past studies have reported that endogenous *Oct4* expression in adult animals is restricted to germ cells (Schöler et al., 1989; Yoshimizu et al., 1999), and the introduction of deletions in the proximal enhancer of the *Oct4* promoter (Δ PE) leads to exclusive expression of the transgene in the germline even during embryogenesis (Yeom et al., 1996). As controls, GFP expression was detected only in primordial and growing oocytes of transgenic females (Figures 6A and 6B), and the GFP signal was absent in oocytes of wild-type females prior to PBCT (Figure 6C). However, primordial follicles with highly GFP-positive (GFP⁺) oocytes were detected in the ovaries of chemoablated adult wild-type female mice within 28–30 hr of PBCT (Figures 6D–6F; see also Figure 7).

Similar findings were obtained when the experiments were repeated using chemoablated *Atm* null female mice as recipients (Figures 6G and 6H), thus excluding the possibility of a nonspecific “restorative” effect of

PBCT on endogenous oocyte production in the host females. Moreover, transplantation of PB-derived mononuclear cells harvested from adult male TgOG2 mice, which also exhibit abundant expression of the transgene in germ cells (Figures S8A–S8C), did not result in the production of GFP⁺ oocytes in female recipients (Figures S8D–S8F), ruling out the possibility that the oocytes observed following transplantation of female PB developed as a result of cell fusion. Further, the GFP⁺ cells contained within follicles of hosts following transplantation of PB collected from adult female TgOG2 mice expressed MVH (Figures 7A–7F), HDAC6 (Figures 7G–7L), NOBOX (Figures 7M–7O), and GDF9 (Figures 7P–7R), supporting their status as germ cells (MVH, Noce et al. [2001]) and oocytes (HDAC6, Figure S3; NOBOX, Suzumori et al. [2002]; GDF9, McGrath et al. [1995]). In a final experiment, doxorubicin-treated mice (Figure 1) were used as recipients for transplantation of PB collected from adult transgenic female mice with ubiquitous GFP expression (Hadjantonakis et al., 1998). Despite the fact that different conditioning and different transgenic donor cells were used, similar results were obtained in that primordial follicles containing GFP⁺ oocytes were observed shortly after PBCT (Figure S9).

Irrespective of the model, the number of follicles containing GFP⁺ oocytes in the recipient ovaries at 28–30 hr posttransplantation was variable (mean of 13 ± 3 , ranging between 3 and 33 per ovary; $n = 9$). In light of the striking estrous-cycle-related changes in BM *Mvh* expression discussed earlier (Figure 2E), one aspect of the PBCT procedure that may impact on the number of donor-derived oocytes generated is the stage of the donor female’s reproductive cycle during which blood is harvested for transplantation. Accordingly, PB was collected from adult female mice during estrus, metestrus, diestrus, and proestrus and analyzed by real-time PCR for *Mvh* expression. These experiments demonstrated that *Mvh* levels in PB, like those in BM, varied during the estrous cycle (Figure 2E; see also Figure S10). Lastly, like that observed for human female BM, we also detected expression of germline markers in PB of human females between the ages of 23 and 33 (Figure S4).

Discussion

We recently provided evidence demonstrating the continued production of oocytes and follicles in postnatal

(B and C) Analysis of MVH immunoreactivity (red, with nuclei highlighted by propidium iodide in blue) in BM of adult wild-type female mice. (D) Mouse ovary analyzed in parallel as a positive control for the immunostaining shown in (B) and (C), demonstrating a restricted expression of MVH (red) to germ cells (oocytes).

(E) Real-time PCR analysis of *Mvh* levels in BM or PB of adult female mice during the indicated stages of the estrous cycle. The data shown represent the combined results from an analysis of three to four mice per group, with mean levels at estrus set as the reference point for comparisons to other stages of the cycle following normalization of the data against β -actin for sample loading. For mice in estrus, *Mvh* expression in bone marrow was detected during linear amplification in only one of the three samples analyzed. See Table S1 for additional results.

(F) Number of nonatretic primordial oocyte-containing follicles in adult female mice at the indicated stages of the estrous cycle (mean \pm SEM, $n = 4$ mice per group).

(G) Quantitative PCR analysis of *Mvh* levels in BM of adult female mice 2 weeks following ovariectomy (OVEX) without (no treatment, No Tx) or with subsequent supplementation of estrogen (E2, 100 ng/mouse) and/or progesterone (P4, 2 mg/mouse) for the final 24 hr before BM collection. The OVEX data shown represent the combined results from an analysis of three mice per group, with mean BM *Mvh* levels in ovary-intact female mice at estrus set as the reference point for comparisons (BM *Mvh* levels in ovary-intact metestrus stage female mice are also provided for reference) following normalization of the data against β -actin to control for sample loading.

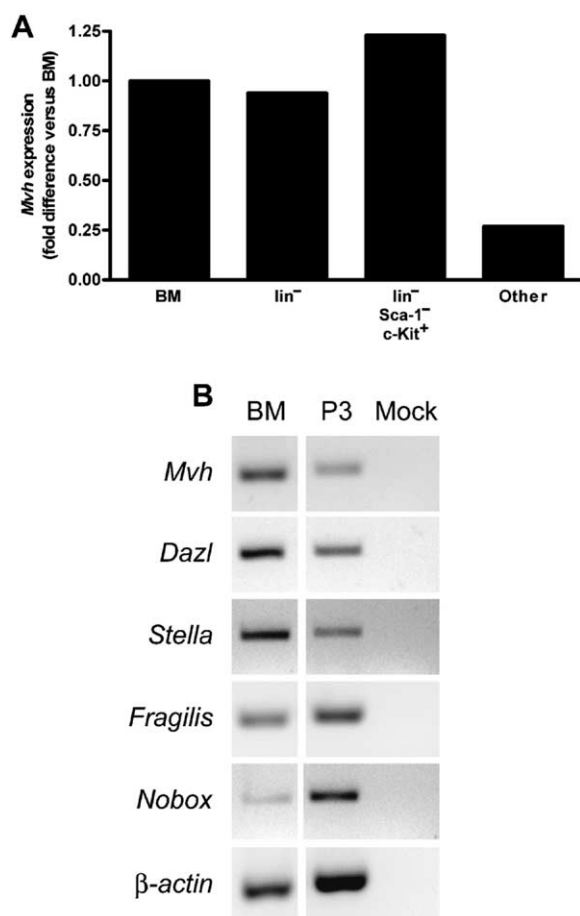


Figure 3. Properties of Putative Germ Cells in Adult Female BM

(A) Quantitative analysis of *Mvh* levels in crude BM and lineage-depleted (lin⁻) BM samples without or with further fractionation by FACS based on cell surface expression of Sca-1 or c-Kit. All remaining lin⁻ cells not represented in the Sca-1⁻/c-Kit⁺ cell fraction of the were pooled and analyzed together (Other). The data shown represent the combined results from an analysis of three adult female mice, with mean *Mvh* levels in the crude BM sample set as the reference point for comparisons following normalization of the data against β -actin for sample loading. See Figure S5 for additional data.

(B) Germline marker expression in adherent BM-derived cells following a total of three serial passages (P3) over a 6 week period in vitro. BM, freshly isolated bone marrow. β -actin, housekeeping gene. Mock, mock reverse-transcribed BM RNA samples.

mouse ovaries, thus challenging a long-held belief that female mammals are born with a finite and nonrenewing oocyte reserve (Johnson et al., 2004). The present studies confirm and extend this earlier conclusion and raise the possibility that GSCs in adult female mammals may actually reside in BM. In this regard, the fractionation strategies provide initial insight into some of the basic characteristics of these putative BM-derived germ cells. First, *Mvh*-expressing cells were retained following negative selection against cell surface markers characteristic of differentiated cells of the hemato-lymphoid lineage (Spangrude et al., 1988). More importantly, however, further sorting of the *Mvh*-expressing lin⁻ cells based on cell surface expression of stem cell markers revealed that these putative BM-derived germ

cells express c-Kit but not Sca-1, thus classifying them as entities distinct from HSCs, which express both markers (Van de Rijn et al., 1989; Okada et al., 1991, 1992; Wognum et al., 2003). The finding that *Mvh*-expressing cells were retrieved from lin⁻ cells by positive selection for c-Kit is fully consistent with past studies documenting the critical role of c-Kit in early germ cell development (Sette et al., 2000). Of note, other germline-associated genes were also expressed in the lin⁻/Sca-1⁻/c-Kit⁺ cell fraction, suggesting that a common pool of cells in BM expresses this gene profile. This conclusion was bolstered by in vitro culture of adult female BM-derived cells, which demonstrated that our panel of germline markers cosegregated with the adherent cell fraction and remained so following serial passage.

Although the expression of germline markers in BM and PB was initially a surprise, several lines of evidence indicate that these results support the existence of extragonadal germ cells in adult females. First and foremost, mononuclear cells derived from either source were capable of generating oocytes in sterilized female recipients (see also discussions below). In addition, we detected multiple germline markers, not just a single germline-associated gene, in BM and PB, and at least one of these genes (*Mvh*) has been consistently identified as a specific germ cell marker (Noce et al., 2001). Further, the levels of *Mvh* expression in BM and PB of adult female mice were dramatically influenced by the estrous cycle. This finding is even more striking when considered with the estrous-cycle-related shifts in primordial follicle numbers identified in the same females. In a study conducted in mice more than 80 years ago, Allen (1923) similarly concluded that 400–500 new oocytes are produced in adult females during each estrous cycle, with the highest and lowest numbers of immature oocytes also observed during metestrus/diestrus and estrus, respectively. The results from the ovariectomy experiments lend further support to the existence of a novel communication loop between the ovaries and BM that may regulate the extent of de novo oocyte production each cycle. This may serve to counterbalance the constantly high level of oocyte loss normally observed in adult animals and thus allow the ovaries to remain functional for more than a few weeks (Johnson et al., 2004). Interestingly, the ovarian-derived signal(s) that regulates the level of BM *Mvh* expression is neither estrogen nor progesterone—arguably the two most prominent hormones secreted by the female gonads in an estrous-cycle-stage-specific manner.

In addition to BMT, we showed that PB is capable of generating oocytes following transplantation into adult female recipients. That these oocytes are derived from cells in the donor blood is supported by a number of observations. First, the Oct4-GFP (TgOG2) transgenic line used to provide “marked” donor cells has been well-characterized in terms of transgene expression, which by virtue of the Δ PE mutation in the Oct4 promoter is restricted to the germline (Yeom et al., 1996; Yoshimizu et al., 1999). Thus, the appearance of GFP⁺ oocytes in recipient ovaries following PBCT is fully consistent with the specificity of transgene expression in the donor animals. Second, GFP⁺ oocytes were detected following PBCT in wild-type female recipients

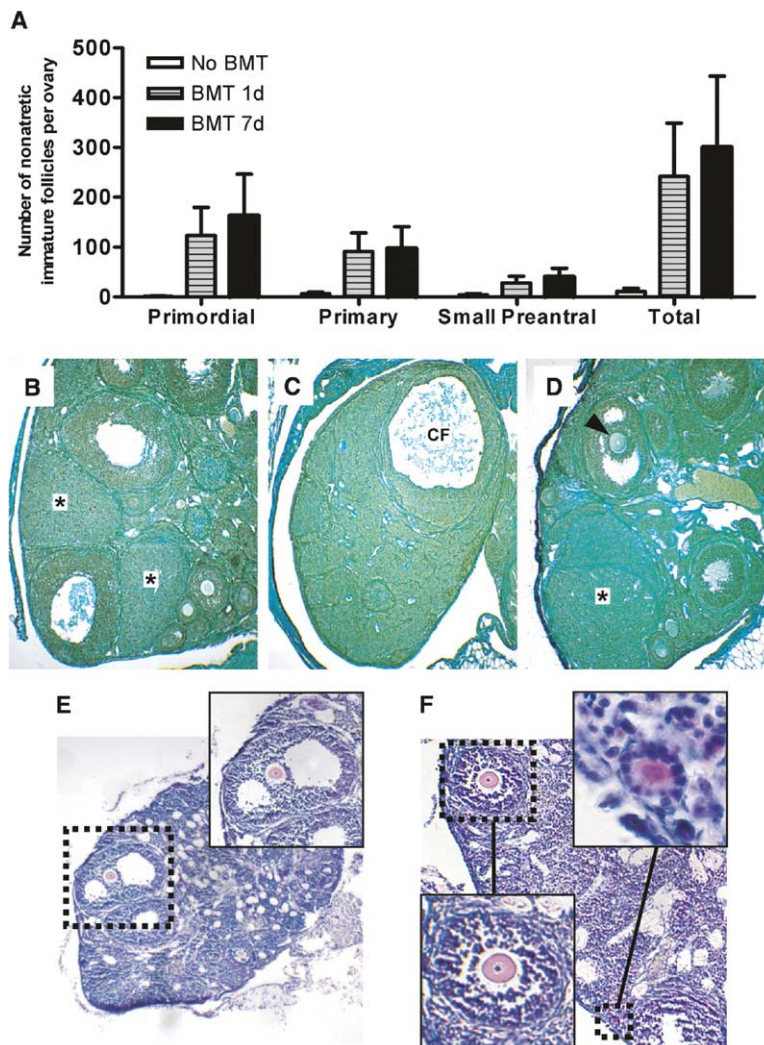


Figure 4. BMT Rescues Oocyte Production in Chemotherapy-Sterilized Female Mice

(A) Number of nonatretic immature follicles in wild-type female mice 60 days after treatment with cyclophosphamide and busulfan on day 42 postpartum without or with BMT 1 or 7 days (d) later. Values are the mean \pm SEM from analysis of five mice per group, with four of the five chemoablated mice not receiving BMT completely lacking immature oocytes.

(B–D) Representative ovarian histology in adult female mice treated with vehicle and no BMT ([B], control), combination chemotherapy without BMT ([C]; CF, cystic follicle), or combination chemotherapy with BMT performed 7 days later (D), as described in (A). Asterisks denote corpora lutea in (B) and (D), whereas the arrowhead in (D) points to a large healthy oocyte contained within a mature antral follicle. See Figure S6 for additional examples and results.

(E and F) Ovarian histology of adult wild-type female mice 11.5 months after combination chemotherapy followed by BMT on day 42 postpartum. Follicles at various stages of maturational development are highlighted (insets).

that had received prior conditioning with a combination chemotherapy regimen known to be extremely cytotoxic to both pre- and postmeiotic germ cells (Shiozumi et al., 1984; Johnson et al., 2004). Since cell fusion is dependent on the presence of differentiated cells of a given lineage—in this case, germ cells—in the host for donor cells to combine with (Herzog et al., 2003), it is unlikely that cell fusion could explain oocyte production in the chemotherapy-conditioned females following PBCT (or BMT). Third, similar findings were obtained when chemoablated *Atm*-deficient females were used as recipients for PBCT. If one considers that these mutant mice are genetically incapable of producing oocytes during postnatal life (Barlow et al., 1998; Di Giacomo et al., 2005), the possibility that the GFP⁺ oocytes observed in recipient ovaries following PBCT were generated as a result of some nonspecific protective or restorative effect of PB on host-germ-cell-supported oogenesis can be excluded. Fourth, PBCT using male TgOG2 mice as donors, which also express high levels of GFP in the germline, was completely ineffective in producing GFP⁺ oocytes in recipient females, further eliminating the possibility that cell fusion is involved.

It is important to emphasize that we do not yet know if the oocytes produced as a result of BMT or PBCT are competent for fertilization, embryonic development, and the generation of viable offspring. Until experiments have been completed to rigorously test this, we feel it is reasonable to refer to the BM- and PB-derived cells that generate what appear to be oocytes as putative germ cells. However, it is equally important to emphasize that the vast majority of oocyte-containing follicles produced by the ovaries under normal conditions die off and are not used directly for procreation (Tilly, 2001). Nonetheless, these follicles are still functional up until the point of their demise in that they play a critical role in the propagation of each ovarian cycle. Keeping these points in mind, the donor-derived oocytes formed following BMT or PBCT meet several other well-established criteria that support their status as bona fide oocytes.

First, from a histological perspective, follicle assembly is one of the most widely accepted and time-tested hallmarks for deeming when a female germ cell is an oocyte (Peters, 1969; Hirshfield, 1991). In fact, both folliculogenesis and the subsequent maturational devel-

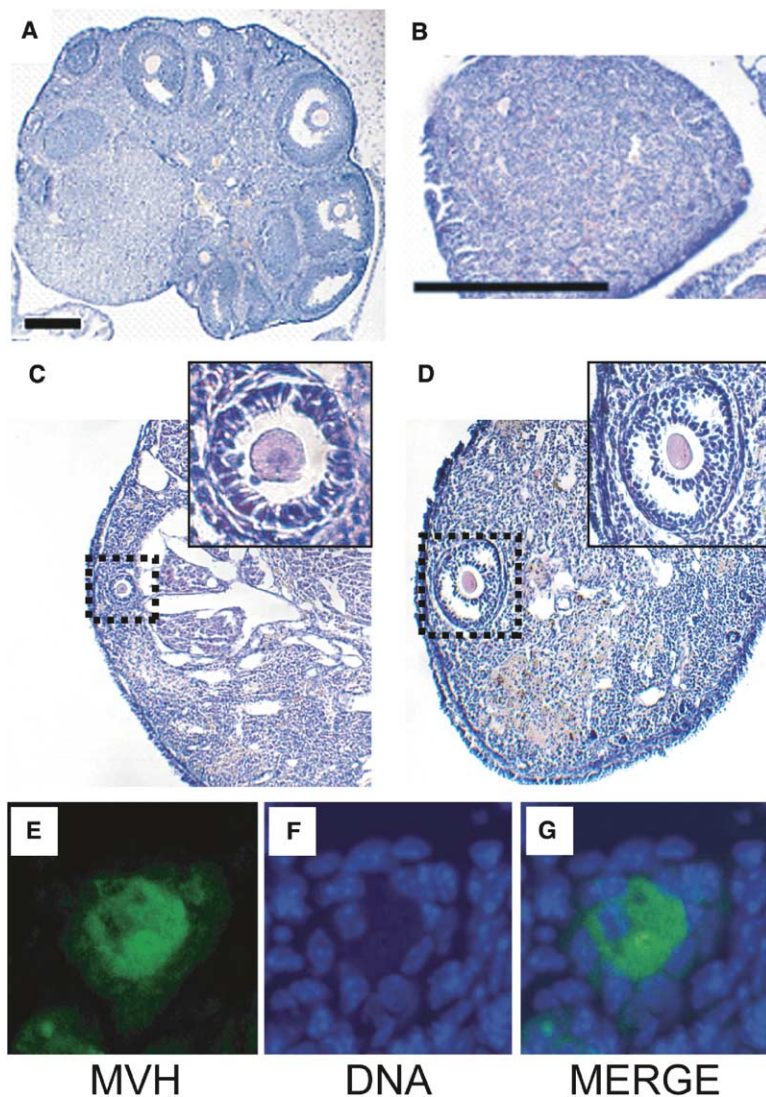


Figure 5. Wild-Type BM Generates Oocytes in *Atm* Mutant Females

(A and B) Histology of adult wild-type (A) and *Atm*^{-/-} (B) mouse ovaries. Scale bars are provided to highlight large difference in ovary size between the two genotypes.

(C and D) Oocyte-containing follicles in ovaries of chemoablated *Atm*^{-/-} female mice at 11.5 months following transplantation with wild-type BM at 6 weeks of age. Follicles in hatched black boxes are highlighted (insets).

(E–G) Oocyte-containing follicle in an *Atm* mutant mouse 2.5 months after BMT, as viewed by MVH immunofluorescence (green; nuclear DNA highlighted by TO-PRO-3 in blue).

opment of follicles, once formed, require active participation by functional oocytes (Erickson and Shimasaki, 2000; Matzuk et al., 2002). Mutant mice lacking *Gdf9* (Dong et al., 1996) and *Nobox* (Rajkovic et al., 2004) are particularly illustrative of the critical role played by oocytes and their specific gene repertoire in follicular dynamics. Accordingly, and in keeping with the findings that the donor-derived oocytes were enclosed within histologically normal follicles, these cells were shown to express markers of not just germ cells (MVH) or primordial oocytes (HDAC6) but of functional oocytes (NOBOX, GDF9) that have fulfilled their intrinsic contribution to follicle formation, survival, and growth.

Additional evidence that the oocytes produced following transplantation are bona fide functional oocytes is provided by the striking differences in the overall histological architecture of the ovaries in the chemoablated females without or with subsequent BMT. Specifically, ovaries of female mice receiving BMT after combination chemotherapy were histologically indistinguishable from those of untreated controls in that the ovaries of both

groups contained a full spectrum of immature and mature oocyte-containing follicles as well as corpora lutea indicative of normal ovulatory cycles. It is unlikely that this outcome simply reflects a nonspecific “protective” effect of BM on the gonads since chemotherapy-induced ovarian failure was reversed by BMT irrespective of whether the transplant was performed shortly after (1 day) or well after (7 days) the damage had been inflicted. Furthermore, BMT was ineffective at rescuing radiation-induced ovarian failure (Figures S6D and S6E), despite past studies showing that depletion of the oocyte pool in adult female mice by ionizing radiation can be prevented using other approaches aimed directly at protecting the resident germ cells (Morita et al., 2000). Therefore, BMT not only rescues production of what appear to be bona fide oocytes and follicles but also, as a consequence, the functional properties of an organ that without BMT would have otherwise exhibited permanent failure.

Interestingly, we also detected expression of *Mvh*, as well as *Dazl* and *Stella* (data not shown), in BM of adult

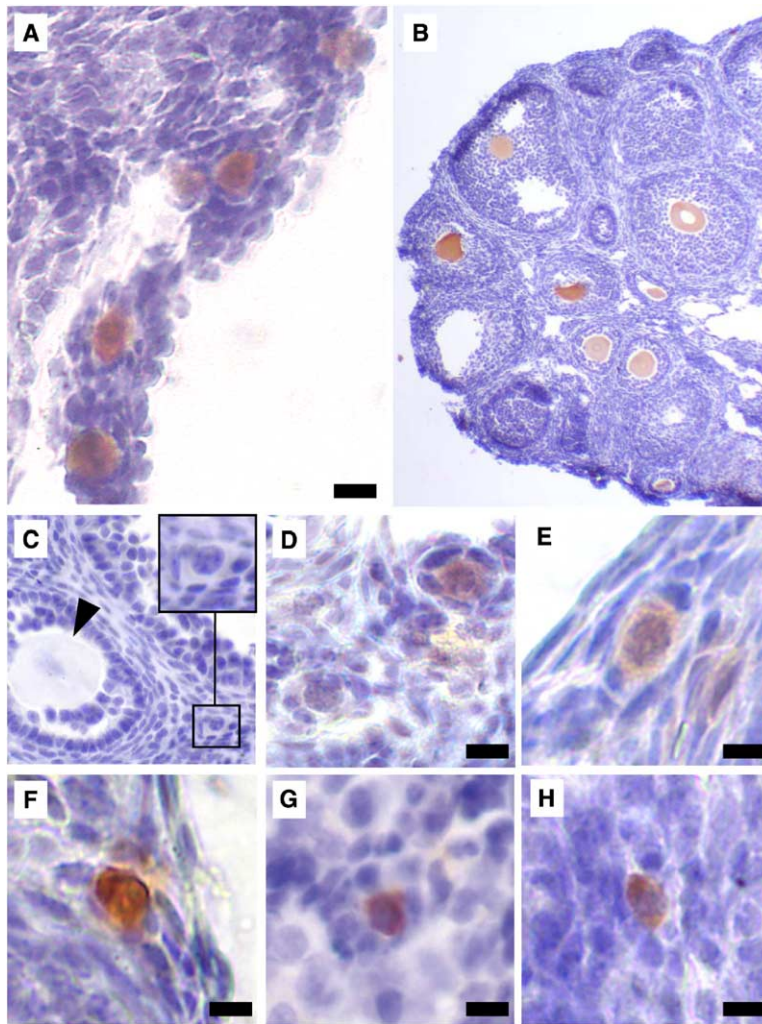


Figure 6. Female PB Generates Oocytes following Transplantation

(A and B) Follicles containing GFP-positive (brown) oocytes in ovaries of adult *Oct4*-GFP transgenic mice ([A], multiple primordial oocytes are highlighted). Scale bar, 10 μ m.

(C) Oocytes (arrowhead or boxed) in a wild-type ovary prior to PBCT using *Oct4*-GFP (TgOG2) females as donors, showing a lack of GFP signal (inset, primordial oocyte).

(D–F) Primordial follicles containing GFP-positive oocytes in ovaries of wild-type female mice 28–30 hr after PBCT, using adult TgOG2 transgenic females as PB cell donors (see also Figure 7). Scale bars, 10 μ m.

(G and H) GFP-positive primordial oocytes in ovaries of *Atm*-deficient females 30 hr after PBCT using adult TgOG2 transgenic females as donors. Scale bars, 10 μ m.

male mice. Although the levels of BM *Mvh* expression in males were less than 20% of those in metestrus-stage females analyzed in parallel, such results were nonetheless unexpected, since it is known that male GSCs reside in the testes (Brinster, 2002). In the only past study we could find that has directly examined the prospects of extragonadal support of adult gamete production, it was reported that spermatogenesis in male rats sterilized by radiation could not be rescued by BMT. Further, there was no spontaneous resumption of spermatogenesis in male rats when the radiation was directed only at the testes, thus sparing any potential extragonadal germ cells from radiation-induced death (Green and Bernstein, 1970). However, we similarly observed that oocyte production and ovarian function in adult female mice exposed to radiation could not be rescued by BMT (Figures S6D and S6E), collectively suggesting that radiation-induced damage to the gonads is so broad and severe that the ability of germ cell progenitors to engraft in the tissue and function following irradiation is disrupted to the point of failure. Accordingly, it may prove informative to repeat the BMT studies in males using a model for inducing spermatogenic failure, such as busulfan exposure, which is ame-

nable to rescue by male GSC transplantation (Brinster et al., 2003).

Experimental Procedures

Animals

Wild-type C57BL/6 female mice were obtained from Charles River Laboratories (Wilmington, Massachusetts). Mutant mice lacking *Atm* (129S6/SvEvTac-*Atm*^{tm1-Awb}) and transgenic mice with ubiquitous GFP expression (STOCK TgN[GFPU]5Nagy) were obtained from Jackson Laboratories (Bar Harbor, Maine). *Oct4*-GFP transgenic mice (TgOG2) were obtained from J.R. Mann through K.J. MacLaughlin (University of Pennsylvania, Kennett Square, Pennsylvania). Estrous cyclicity was determined by daily monitoring of vaginal cytology (Cooper et al., 1993). The institutional animal care and use committee of Massachusetts General Hospital approved all animal procedures.

Human Subjects

Cryopreserved BM or PB samples from female donors between 24 and 36 (BM) or 23 and 33 (PB) years of age were utilized for these studies, following approval of the protocol (#2004-P-001665/1) by the human research committee of Massachusetts General Hospital.

Ovarian Histology and Oocyte Counts

After fixation, ovaries were sectioned and stained for light microscopic evaluation. In some experiments, serial ovarian sections

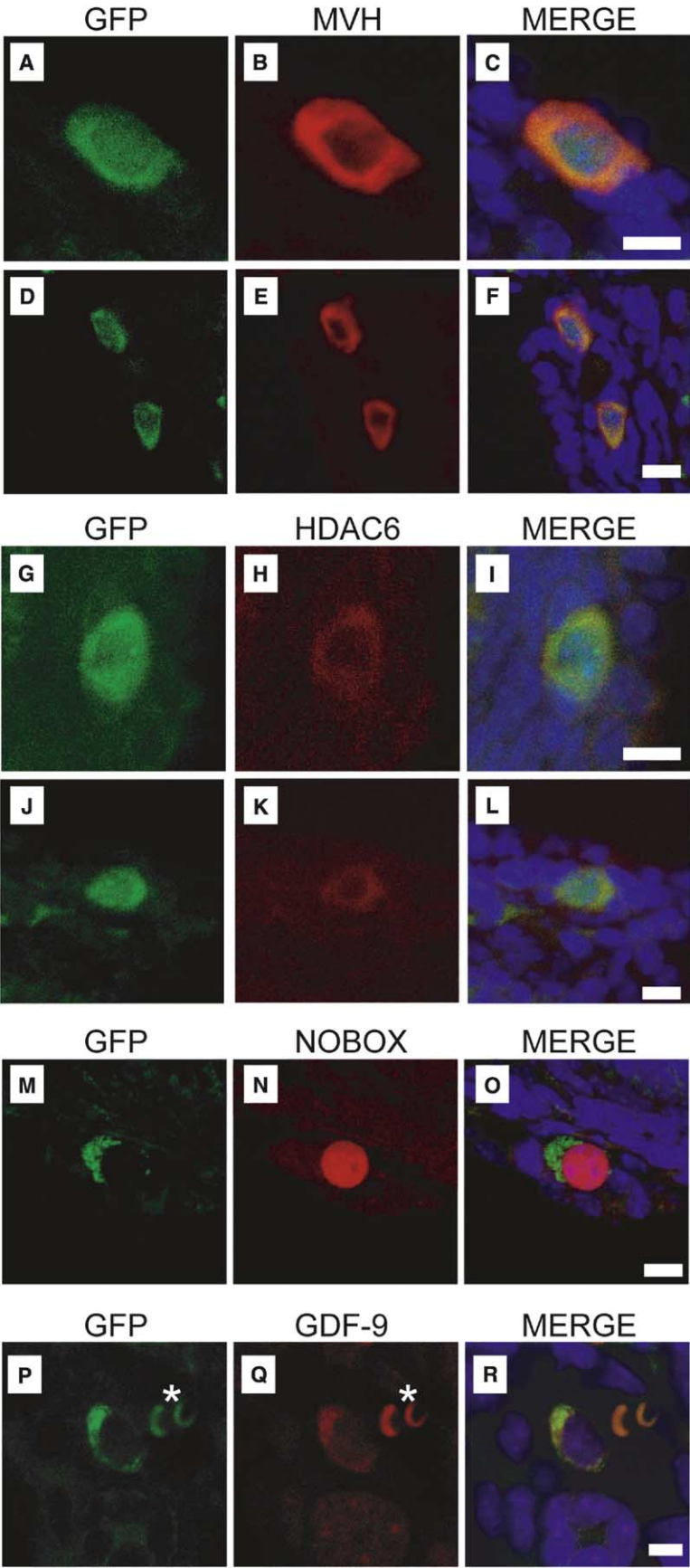


Figure 7. PBCT-Derived Ovarian Follicular Cells Express Germline and Oocyte Markers
Dual immunofluorescence analysis showing coexpression of GFP (green) and MVH (red) (A–F), GFP (green) and HDAC6 (red) (G–L), GFP (green) and NOBOX (red, note the nuclear localization) (M–O), or GFP (green) and GDF9 (red) (P–R) in oocytes of immature follicles within ovaries of recipient female mice 28–30 hr after transplantation with PB harvested from adult *Oct4*-GFP (TgOG2) transgenic females (see Figure 6 for controls). Asterisks in (P) and (Q) denote auto-fluorescent red blood cells. All cell nuclei are highlighted by TO-PRO-3 iodide staining (blue) in the merged panels. Scale bars, 10 μ m.

were analyzed by histomorphometry to determine the number of nonatretic or atretic oocyte-containing follicles per ovary (Tilly, 2003; Johnson et al., 2004).

Immunohistochemistry

Ovaries, testes, and bones (femurs) were fixed in 4% neutral-buffered paraformaldehyde, and bones were then decalcified for 72 hr in formic acid-EDTA. The tissues were sectioned for immunohistochemical analysis using antibodies specific for SSEA1 (MAB4301; Chemicon, Temecula, California), MVH (generously provided by T. Noce; Fujiwara et al., 1994), HDAC6 (2162; Cell Signaling Technology, Beverly, Massachusetts), NOBOX (generously provided by A. Rajkovic; Suzumori et al., 2002), GDF-9 (AF739; R&D Systems, Minneapolis, Minnesota) or GFP (sc-9996; Santa Cruz Biotechnology, Santa Cruz, California) after high-temperature antigen unmasking, as recommended by each supplier. For the studies involving transgenic mice with ubiquitous GFP expression, antigen detection was visualized after tyramide amplification (PerkinElmer, Boston, Massachusetts). For immunofluorescence-based antigen detection, sections were mounted with propidium iodide (Vectashield; Vector Labs, Burlingame, California) or TO-PRO-3 iodide (Molecular Probes, Eugene, Oregon), and images were captured using a Zeiss LSM 5 Pascal Confocal Microscope.

Isolation of SSEA1⁺ Cells

Ovaries from female mice between 6 and 9 weeks of age were cleaned of adherent tissue, minced, and incubated in Dulbecco's modified Eagle's Medium (DMEM) containing 1 mg/ml collagenase for 45 min at 37°C. The digested tissue was then spun through a 40 μ m cell strainer at 1000 \times g for 10 min, after which the supernatant was removed and the pelleted cells were resuspended in DMEM. Anti-mouse IgM beads (Dynabeads M-450; Dynal Biotech) precoated with mouse monoclonal SSEA1 antibody (MAB4301; Chemicon) were then added to the cell suspension, and separation of SSEA1⁺ cells was performed according to the manufacturer's protocol.

FACS and Serial Passage Fractionation of BM

Bone marrow was isolated from adult female mice and sorted using a BD Biosciences FACSscalibur cytometer based on cell surface expression of Sca-1 (Van de Rijn et al., 1989) and/or c-Kit (Okada et al., 1991, 1992) following an initial fractionation step to obtain lineage-depleted cells (Spangrude et al., 1988; Spangrude and Scollay, 1990). For enrichment of BM-derived stem cells in vitro (Meirelles Lda and Nardi, 2003), BM isolated from adult female mice was placed on plastic in DMEM (Fisher Scientific, Pittsburgh, Pennsylvania) with 10% fetal bovine serum (Hyclone, Logan, Utah), penicillin, streptomycin, L-glutamine, and amphotericin-B. Forty-eight hours after the initial plating, the supernatants containing nonadherent cells were removed and replaced with fresh culture medium, and the cultures were then maintained and passed once confluence was reached for a total of three times over the span of 6 weeks.

Reverse Transcription-PCR Analysis

Total RNA was extracted from each sample, and 1 μ g was reverse transcribed (Superscript II RT; Invitrogen, Carlsbad, California) using oligo-dT primers. Amplification via 28–45 cycles of PCR was then performed using *Taq* polymerase and Buffer-D (Epicentre, Madison, WI) with primer sets specific for each gene (Table S3). For each sample, RNA encoded by the ribosomal gene *L7* (mouse studies), β -actin (mouse studies), or the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*; human studies) was amplified and used as a loading control. All PCR products were isolated, subcloned, and sequenced for confirmation. For quantitative analysis of *Mvh* levels, PCR was performed using a Cepheid Smart Cycler II and primers specific for amplification of *Mvh* (FAM-labeled LUX Fluorogenic Custom Primers, Invitrogen; forward 5'–3': CAC CTCAGAGGGTTTCCAAGCGAGGG; reverse 5'–3': CCTCTTCT GAACGGGCGCTGA) and β -actin (LUXPrimer Sets for Housekeeping Genes 101M-01; Invitrogen). Expression ratios were calculated using the method of Pfaffl (2001), with *Mvh* levels in bone marrow at estrus set as the reference point (1.0) for comparisons.

Transplantation Studies

Bone marrow was harvested from wild-type C57BL/6 female mice between 6 and 10 weeks of age, and $2\text{--}5 \times 10^7$ cells were injected intravenously via the tail vein into recipients using standard procedures. To prepare recipients, wild-type or *Atm* null female mice received 0.5 mg anti-CD4 antibody (GK1.5; Dialynas et al., 1984) and 1 mg anti-CD8 antibody (2.43; Sarmiento et al., 1980) 1 week prior to injection of 120 mg/kg cyclophosphamide (Cytoxan; Bristol-Meyers Squibb, New York, New York) and 12 mg/kg busulfan (Sigma) at 6 weeks of age. BMT was performed 1 or 7 days later, and ovaries were collected at indicated times following BMT. In other experiments, wild-type female mice were subjected to whole-body irradiation (0.5 Gy total, at a rate of 89 rads/min from a ¹³⁷cesium source) followed by mock transplantation or BMT 1 day later, and ovaries were collected 2 months afterward for analysis.

For PBCT, blood was harvested from adult transgenic female mice with *Oct4*-specific or ubiquitous expression of GFP (see Animals above), or from adult male *Oct4*-GFP transgenic mice, and layered on Ficoll-Paque Plus (Amersham Biosciences/GE Healthcare, Piscataway, New Jersey). The samples were centrifuged at 800 \times g for 15 min at 4°C, and mononuclear cells were collected from the Ficoll-buffer interface. The cells were washed and resuspended in PBS at a final concentration of $2\text{--}4 \times 10^7$ cells/ml. In some experiments, recipient adult wild-type or *Atm* null female mice were conditioned with chemotherapy as described above for BMT, followed by PBCT (0.5 ml of cells per mouse, via the tail vein) 24 hr later. In other experiments, recipient adult wild-type female mice were injected with doxorubicin (5 mg/kg) 25 hr prior to PBCT. In all cases, ovaries were collected 28–30 hr after PBCT and analyzed for GFP expression by immunohistochemistry. For experiments involving PBCT using males as donors, recipient ovaries were fixed, serially sectioned, and screened in their entirety for GFP-expressing oocytes.

Supplemental Data

Supplemental Data include supplemental text, ten figures, and three tables and can be found with this article online at <http://www.cell.com/cgi/content/full/122/2/303/DC1/>.

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