ARTICLES

Ovulated oocytes in adult mice derive from non-circulating germ cells

Kevin Eggan[1], Sara Jurga[2], Roger Gosden[3], Irene M. Min[2] & Amy J. Wagers[2]

Decades of research in reproductive biology have led to the generally accepted belief that in female mammals, all surviving germ cells enter meiosis at the end of fetal development and as a result, the postnatal ovary harbours a limited supply of oocytes that cannot be replenished or regenerated if lost to injury or disease. However, recent reports have challenged this view, suggesting instead that oocyte production is maintained through continual seeding of the ovary by circulating, bone-marrow-derived germ cells. To test directly the physiological relevance of circulating cells for female fertility, we established transplantation and parabiotic mouse models to assess the capacity of circulating bone marrow cells to generate ovulated oocytes, both in the steady state and after induced damage. Our studies showed no evidence that bone marrow cells, or any other normally circulating cells, contribute to the formation of mature, ovulated oocytes. Instead, cells that travelled to the ovary through the bloodstream exhibited properties characteristic of committed blood leukocytes.

The view that all oocytes capable of participating in female reproduction are present in the ovary at birth has been challenged recently by the surprising observation that the female gonad may exhibit unexpected regenerative activity into adulthood[1]. Subsequently, it was suggested that bone marrow and peripheral blood may serve as reservoirs of cells responsible for this regenerative capacity[2]. These reports have led to the proposal that bone marrow or peripheral blood cell transplantation might provide novel treatments for premature menopause or chemotherapy-induced sterility[3]; however, these findings remain controversial due to residual concerns over the physiological relevance of putative marrow-derived or circulating germ cell precursors and their functional capacity to enter into the pool of mature, ovulated oocytes[4].

Using acute, intravenous injection of peripheral blood or bone marrow cells from transgenic donor mice, one study[2] recently reported that oocytes could be restored in the ovaries of chemically or genetically[5] sterilized female mice within as little as 30 h. It was concluded from these studies that mammalian oocytes are produced postnatally from germline progenitor cells that reside in the bone marrow and travel to the ovaries via the peripheral circulation[2,3]. To investigate directly the capacity of naturally circulating peripheral blood cells to engraft in the ovary and contribute to oogenesis, we examined ovulated oocytes from adult female mice surgically joined by parabiosis (Fig. 1a). Parabiotic mice develop a common, anastomosed circulatory system within 2–3 days of joining and exhibit continuous, rapid exchange of cells and other circulating factors through the bloodstream[6,7]. Thus, parabiosis allows direct tracking of genetically marked cells supplied continuously and at physiological levels through the circulation and provides a powerful approach[7–10] to determine whether blood-borne factors (either humoral or cellular) normally contribute to ovarian function or repair.

Circulating cells do not generate ovulated oocytes

Parabiotic pairs were created by joining wild-type mice with transgenic animals that ubiquitously expressed green fluorescent protein (GFP) under control of the β-actin promoter[7,11] (Fig. 1a). GFP-transgenic mice were joined to non-transgenic partners at 4–8 weeks of age, and remained joined for 6–8 months before further analysis. As expected[7–10], all parabiotic pairs exhibited high-level peripheral blood chimaerism (on average, ~65% GFP^+ blood leukocytes in both partners; $n = 4$ pairs, Supplementary Table 1), indicating that a common circulatory system had been established and maintained.

We predicted that if naturally circulating germ cells or their precursors were present in the bloodstream and capable of colonizing the ovary, then as we observed in the blood, GFP^+ cells from the transgenic parabionts would cross into their non-transgenic partners and colonize their unlabelled ovaries with GFP-labelled oocytes. Similarly, unlabelled germ cells from the non-transgenic animal would cross into the transgenic partner, resulting in GFP-negative oocytes in an otherwise green ovary (Fig. 1b). To test this prediction, we superovulated parabiotic pairs that had been joined for 6–8 months, collected metaphase II oocytes from the oviduct of each partner, and analysed them for expression of GFP (Fig. 1a). In contrast to the substantial chimaerism evident in the peripheral blood of long-term parabionts (Supplementary Table 1), we observed no chimaerism of oocytes in these mice (Fig. 1c and Supplementary Table 1). All oocytes (80 out of 80, $n = 4$ mice) collected from GFP-transgenic partners robustly expressed GFP when examined by direct fluorescence microscopy, whereas none of the oocytes (0 of 66, $n = 4$ mice) collected from non-transgenic partners expressed GFP. Although we occasionally observed GFP^+ cells associated with the ovulated oocytes in the cumulus masses, staining with antibodies specific to the pan-haematopoietic marker CD45 revealed that these were in fact circulating blood cells (Supplementary Fig. 1). Thus, although circulating cells have the capacity to enter the ovary and to associate with ovulated oocytes, these cells maintain haematopoietic fates in this environment and do not contribute to the production of ovulated oocytes during normal homeostasis.

[1]The Stowers Medical Institute, Harvard Stem Cell Institute and The Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, USA. [2]Section on Developmental and Stem Cell Biology, Joslin Diabetes Center and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02215, and Harvard Stem Cell Institute, Cambridge, Massachusetts 02138, USA. [3]Center for Reproductive Medicine and Infertility, Weill Medical College, Cornell University, New York, New York 10021, USA.

No oocytes from circulating cells after ovarian damage

Our analysis of 146 ovulated oocytes collected from animals that shared a common blood circulation for most of their reproductive lifespan revealed no evidence of cross-engraftment of cells capable of generating mature oocytes. Nonetheless, it remained possible that damage to the bone marrow, ovary, or germ cells might be required to enable the ovarian engraftment of circulating cells. To test whether such an injury would enhance cross-engraftment of circulating oocyte precursors in parabiotic mice, we pre-treated non-transgenic mice with the ovotoxins cyclophosphamide (Cytoxan, Cy) and busulfan (Bu)[2,12–15], and then joined them 1 day later with untreated GFP-transgenic partners (Fig. 2a). These animals were maintained as parabionts for ~2 weeks ($n = 4$ parabiotic pairs) or 2 months ($n = 2$ pairs), then induced to superovulate, euthanized for oocyte retrieval, and analysed for chimaerism of oocytes and haematopoietic tissues. As a control we also examined oocytes and blood from parabiotic mice that had been joined for the same length of time, but in which neither partner had been pre-treated by chemotherapy ($n = 2$–4 pairs).

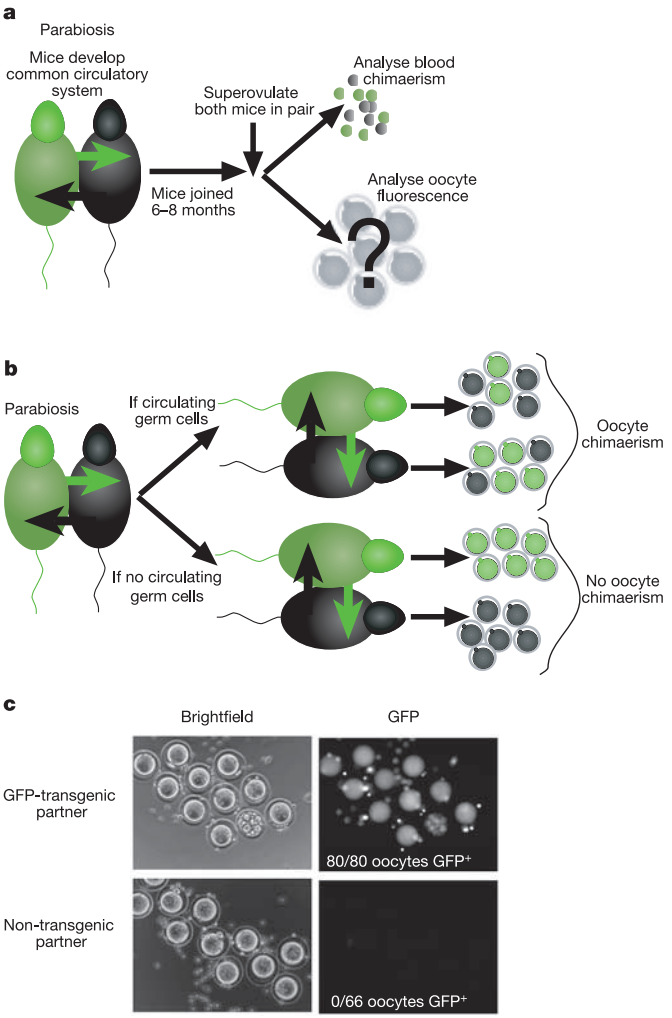

Figure 1 | Circulating cells do not give rise to mature oocytes in long-term parabionts. a, Experimental design. GFP-transgenic and non-transgenic C57BL/Ka mice were surgically joined by parabiosis such that they developed a common, anastomosed vasculature. After 6–8 months of shared circulation, pairs were treated with PMS and HCG, and analysed for blood chimaerism and for the presence of cross-engrafting oocytes. **b**, Predictions of the experiments. If circulating germ cells contribute to oocyte formation in adult females, then the population of ovulated oocytes collected from parabiotic mice will contain both host-derived and partner-derived cells (that is, both GFP^+ and GFP^- oocytes). If circulating cells do not give rise to oocytes, then all ovulated oocytes collected from parabiotic mice will exhibit the phenotype of the host (that is, GFP^+ in GFP-transgenic parbionts and GFP^- in their non-transgenic partners). **c**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected from the GFP-transgenic or non-transgenic partners of pair 9 (Supplementary Table 1). Total numbers of GFP^+ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 4$ parabiotic pairs). All oocytes from GFP-transgenic parabionts expressed GFP, whereas no oocytes from their non-transgenic partners expressed GFP.

In treated and untreated parabiotic pairs, we observed extensive leukocyte chimaerism in the peripheral blood of both partners (Supplementary Table 2). We also detected significant chimaerism among total bone marrow cells and within the rare subset of blood-forming haematopoietic stem cells (HSCs) in both treated and untreated mice. Consistent with the notion that chemoablative damage by Cy/Bu treatment can facilitate engraftment by circulating stem cells, we observed an increase in the level of cross-engraftment of GFP-expressing HSCs into the bone marrow of Cy/Bu-treated non-transgenic mice relative to that observed in the bone marrow of untreated parabiotic controls (Supplementary Table 2). However, in stark contrast to the cross-engraftment evident in the haematopoietic lineages, we found no evidence for cross-engraftment of oocytes in these same animals (Fig. 2 and Supplementary Table 2). Every oocyte (281 out of 281, $n = 12$ mice) collected from GFP-transgenic partners robustly expressed GFP when examined by epifluorescence microscopy. Similarly, none of the oocytes collected from untreated non-transgenic partners (0 out of 154, $n = 6$ mice) or Cy/Bu-treated non-transgenic partners expressed GFP (0 out of 80, $n = 4$, for mice treated 2 weeks before analysis, and 0 out of 4, $n = 2$, for mice treated 2 months before analysis) (Fig. 2b, d and Supplementary Table 2).

In total, we analysed 665 mature, ovulated oocytes from chemotherapy treated and untreated parabiotic pairs and found that they always (665 of 665) possessed the phenotype of the animal from which they were retrieved (Supplementary Tables 1 and 2). In both injured and uninjured non-transgenic parabiotic partners, all GFP^+ cells associated with the ovulated cumulus complexes expressed CD45 (Supplementary Figs 1 and 3), indicating commitment to the blood cell lineage. Taken together, these data argue against the hypothesis that cells from the bone marrow seed the ovary through the circulation and contribute to functional adult oogenesis, either in the steady state or in response to overt damage to the ovary. Although cells derived from circulation may be found within the ovaries of parabiotic animals, these engrafted cells exhibit exclusively haematopoietic fates, and probably represent circulating blood cells known to infiltrate all tissues in their role as immune responders.

Chemotherapy does not fully deplete oocyte reserves

Although we observed in parabiosis experiments a significant decline in the total numbers of oocytes ovulated by Cy/Bu-treated animals over time, this chemoablative regimen did not cause complete depletion of oocyte reserves in the treated parabionts (Fig. 2c, e and Supplementary Table 2). In parabiotic mice treated with Cy/Bu 2 weeks before superovulation, the numbers of oocytes ovulated were only slightly decreased compared to untreated controls (on average, 20 ± 6 versus 32 ± 13) (Fig. 2c). Similarly, in animals treated with Cy/Bu 2 months before superovulation, ovulated oocytes were substantially reduced in number compared to untreated controls (on average, 2 ± 1 versus 14 ± 4), but not fully eliminated (Fig. 2e). Notably, we found no difference in the number of oocytes ovulated by Cy/Bu-treated mice joined by parabiosis to untreated partners when compared to the number of oocytes ovulated by control (non-parabiosed) animals also treated with Cy/Bu (Supplementary Fig. 2a). These data indicate that Cy/Bu-induced damage, which results in reduced ovulation, is not rescued by factors present in the circulation and supplied through parabiosis.

Incomplete depletion of oocytes in Cy/Bu-treated animals was also apparent upon histological analysis of ovaries retrieved from

these animals (Supplementary Fig. 2b–g). Ovaries retrieved from untreated control and parabiotic animals contained follicles at many stages of maturation (Supplementary Fig. 2b, f). In contrast, 2 months after treatment with Cy/Bu, the number of follicles was markedly reduced and in some sections, no follicles could be found (Supplementary Fig. 2c, g). However, although Cy/Bu-induced damage to the ovaries was apparently widespread, maturing and mature follicles were observed in some histological sections (Supplementary Fig. 2d, e). The ovaries of both Cy/Bu-treated control and Cy/Bu-treated parabiotic mice showed substantial damage, consistent with the impairment of ovulation observed in these animals (Supplementary Fig. 2c, g).

No ovulated oocytes from transplanted bone marrow cells

Together, these data argue that Cy/Bu-mediated destruction of germ cells is not substantially ameliorated by cells and/or factors naturally present in the circulation (and therefore provided by parabiosis); however, it remained possible that direct intravenous transplantation of bone marrow cells introduces cells into the circulation that are not normally present in the bloodstream but are capable of contributing to or stimulating oogenesis. To evaluate directly the capacity of transplanted bone marrow cells to contribute to the pool of ovulated oocytes, we examined ovulation efficiency in wild-type mice that had been treated with Cy/Bu and then intravenously infused 24 h later with 4×10^7 unfractionated bone marrow cells. Because our parabiosis experiments indicated that Cy/Bu treatment does not lead to a complete depletion of the oocyte pool, we also examined animals that were sterilized by low-dose total body irradiation (0.5 Gy)—as in the case of ref. 2—and subsequently transplanted with bone marrow cells. Two months after chemotherapy- or radiation-induced damage and bone marrow transplantation, experimental and control animals were induced to superovulate, euthanized for oocyte retrieval, and

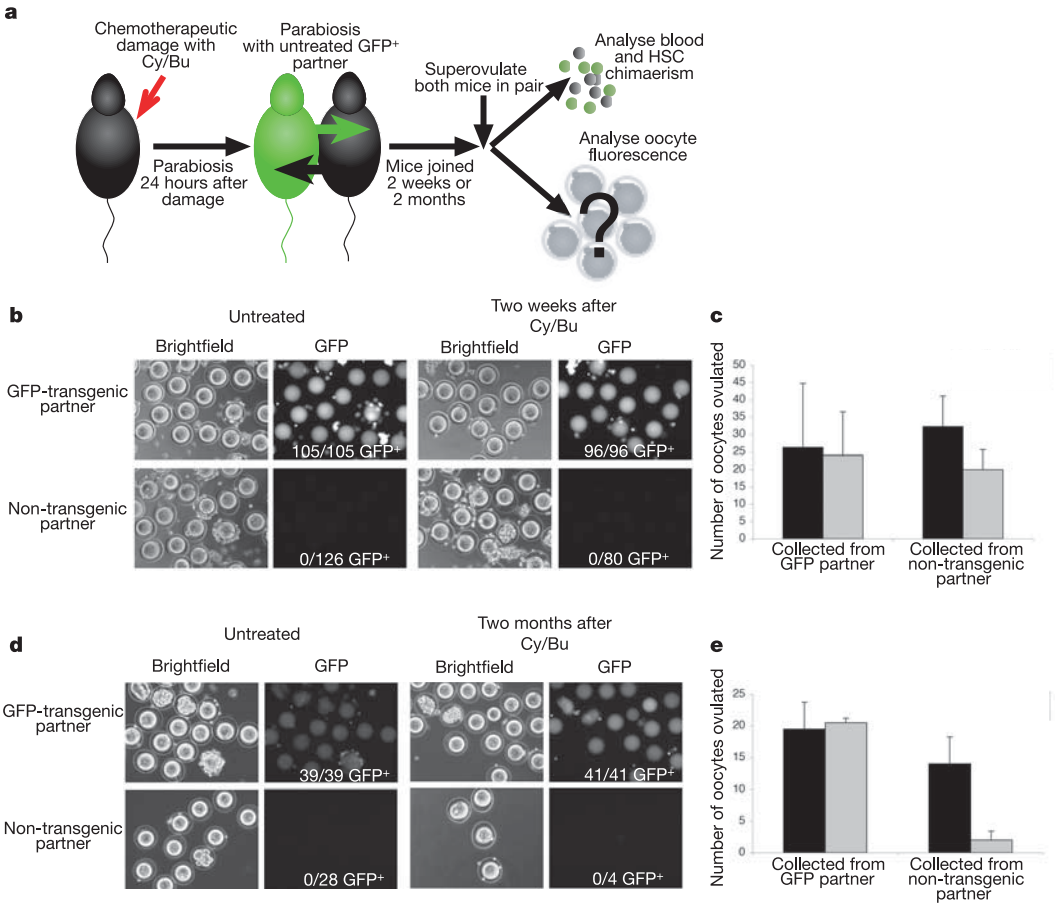

Figure 2 | Circulating cells do not give rise to mature oocytes in injured parabionts. **a**, Experimental design. GFP-transgenic and non-transgenic C57BL/Ka mice were surgically joined by parabiosis 1 day after treatment of the non-transgenic partner only with the ovotoxic agents cyclophosphamide (Cy) and busulfan (Bu). After 10 days or 2 months of shared circulation, pairs were treated with PMS and HCG, and analysed for the presence of cross-engrafting oocytes and/or germ cells. **b**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected after 2 weeks of parabiosis from the GFP-transgenic or non-transgenic partners of untreated pair 5, in which the non-transgenic partner was left untreated, or Cy/Bu-treated pair 8, in which the non-transgenic partner was pre-treated with Cy/Bu (Supplementary Table 2). Total numbers of GFP^+ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 4$ parabiotic pairs). **c**, Average number of oocytes (±s.d.) collected from parabiotic mice 2 weeks after injury and parabiosis. Black bars represent data from pairs in which neither partner received Cy/Bu treatment; grey bars represent data from pairs in which the non-transgenic partner only received Cy/Bu treatment. All oocytes from GFP-transgenic parabionts expressed GFP, whereas no oocytes from their non-transgenic partners expressed GFP (see Supplementary Table 2). **d**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected after 2 months of parabiosis from the GFP-transgenic or non-transgenic partners of untreated pair 1, in which the non-transgenic partner was left untreated, or pooled from Cy/Bu-treated pairs 1 and 2, in which the non-transgenic partner was pre-treated with Cy/Bu (Supplementary Table 2). Total numbers of GFP^+ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 2$ parabiotic pairs). **e**, Average number of oocytes (±s.d.) collected from parabiotic mice 2 months after injury and parabiosis. Black bars represent data from pairs in which neither partner received Cy/Bu treatment; grey bars represent data from pairs in which the non-transgenic partner only received Cy/Bu treatment. All oocytes from GFP-transgenic parabionts expressed GFP, whereas no oocytes from their non-transgenic partners expressed GFP (see Supplementary Table 2).

analysed for chimaerism in haematopoietic tissues and among ovulated oocytes (Fig. 3a).

Low-level haematolymphoid chimaerism was observed in recipient animals receiving GFP-transgenic bone marrow cells and pre-conditioned by either irradiation or cytotoxic drugs (Cy/Bu), but was not observed in untreated recipients (Fig. 3d). These data are consistent with the notion that productive engraftment of transplanted haematopoietic stem cells depends on the immediate availability of 'empty' bone marrow niches, which are rare in untreated animals, but increase in frequency after chemotherapy or irradiation[16]. However, in contrast to the haematopoietic engraftment evident in mice transplanted with bone marrow cells expressing GFP, we saw no evidence of GFP^+ oocytes in these same animals (Fig. 3b, c, $n = 6$).

Equivalent numbers of oocytes were collected from untreated mice (whether untransplanted, transplanted with GFP-transgenic bone marrow, or transplanted with non-transgenic marrow), whereas animals pre-treated with irradiation or cytotoxic drugs showed a marked impairment in their competency to ovulate (Fig. 3e). Consistent with previous findings[2], after superovulation no oocytes were found in the oviducts of animals pre-conditioned by 0.5 Gy irradiation, whether or not they received bone marrow transplants. Also consistent with previous results[2,12–15], a subset of animals (4 out of 10) pre-treated by Cy/Bu injection produced a small number (on average 2 ± 3) of ovulated oocytes; however, neither the frequency of mice ovulating nor the total number of ovulated oocytes was increased in these animals as a result of bone marrow transplantation (Fig. 3e). Significantly, and as in our parabiosis studies, the ovulated oocytes in Cy/Bu-treated mice (7 out of 7) possessed the phenotype of the animal from which they were retrieved; we found no evidence for the development of GFP^+ oocytes derived from transplanted cells (Fig. 3c).

Taken together, our data indicate that, unlike low-dose irradiation, Cy/Bu chemotherapy does not completely sterilize female mice and allows a small population of residual endogenous oocytes and/or germ cells to survive and maintain ovulation[12–15]. Importantly, circulating and transplanted bone marrow cells neither contribute directly to the formation of ovulated oocytes in these animals nor do

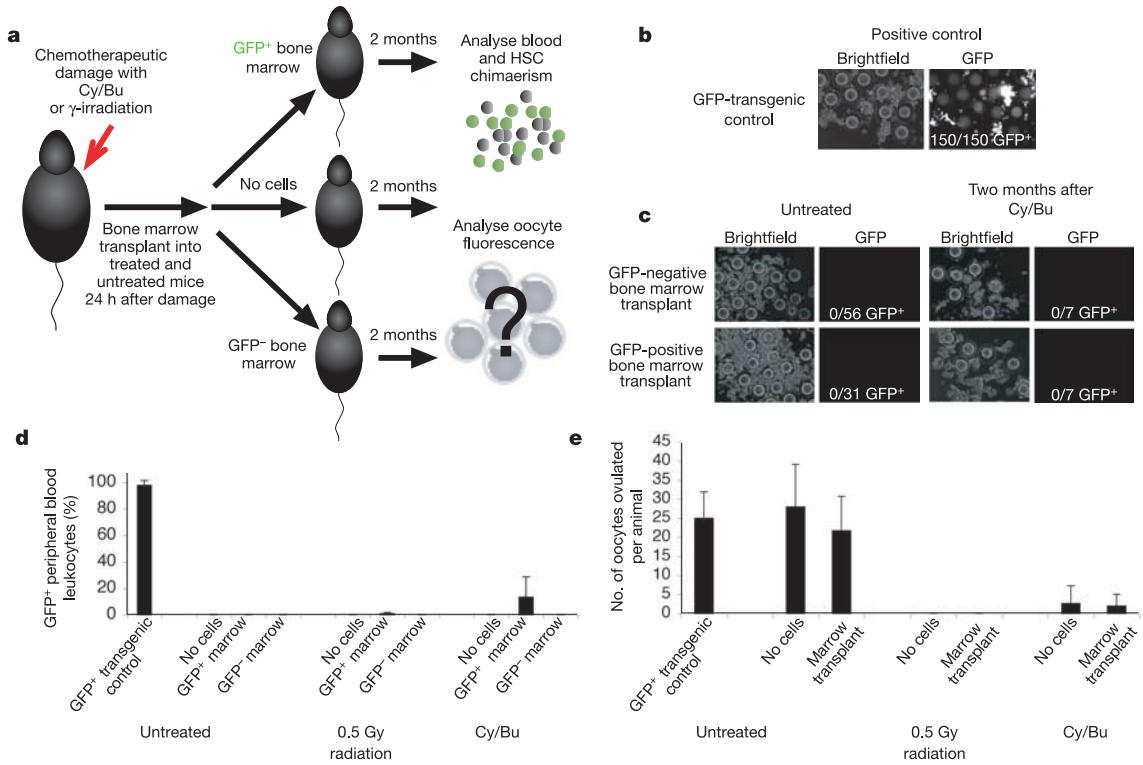

Figure 3 | Bone marrow cells do not give rise to oocytes and do not enhance ovulation of endogenous oocytes in transplanted mice. **a**, Experimental model. Wild-type C57BL/Ka recipients either were pre-conditioned 1 day before transplant by injection of Cy/Bu or by total body irradiation (0.5 Gy), or were left untreated. Mice from each treatment group then received by intravenous injection 4×10^7 GFP-transgenic bone marrow cells or wild-type (GFP^-) bone marrow cells, or received no cells as a control. Two months later, animals were euthanized for chimaerism analysis of haematopoietic cells and oocytes. **b**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected from GFP-transgenic controls. Total numbers of GFP^+ oocytes per total oocytes examined are summarized on the epifluorescence panel; all oocytes collected from GFP-transgenic animals were GFP^+. **c**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected from non-transgenic animals 2 months after transplantation of GFP^- or GFP^+ bone marrow cells. Recipients were untreated before transplant or pre-treated one day previously with Cy/Bu. Total numbers of GFP^+ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 2$–6). No GFP^+ oocytes were detected in either untreated or Cy/Bu-treated mice receiving GFP^- or GFP^+ bone marrow cells. **d**, Chimaerism (average $\pm$ s.d.) of peripheral blood cells in control mice receiving no cells, or in transplanted mice receiving GFP^+ bone marrow cells or wild-type (GFP^-) bone marrow cells. Animals were untreated or pre-conditioned by low-dose total body irradiation (0.5 Gy) or intraperitoneal injection of Cy/Bu 1 day before transplantation. Analysis of control GFP-transgenic blood cells demonstrates expression of GFP by 98–100% of circulating leukocytes. **e**, Effects of bone marrow transplant on ovulation in chemotherapy- or radiation-treated animals. Total numbers (average $\pm$ s.d.) of ovulated oocytes retrieved from untreated ($n = 4$–9) or radiation (0.5 Gy, $n = 2$–4) or chemotherapy (Cy/Bu, $n = 3$–7) pre-treated animals that either received no cells ($n = 2$–9) or bone marrow cells ($n = 4$–7) from GFP-transgenic or non-transgenic donors 1 day after treatment are shown. All control GFP-transgenic and untreated animals ovulated, whereas no irradiated animals ovulated. A subset of animals pre-treated with Cy/Bu ovulated (1 out of 3 receiving no cells and 3 out of 7 receiving bone marrow cell transplant). Differences were significant ($P < 0.05$) in comparisons of untreated versus 0.5 Gy- or Cy/Bu-treated groups, but differences in Cy/Bu-treated animals that did (bone marrow cell transplant) or did not (no cells) receive transplants were not significant ($P > 0.05$).

they enhance endogenous recovery after chemotherapy at the time points examined. Instead, it appears that circulating or bone-marrow-derived cells found in association with ovulated oocytes maintain haematopoietic lineage commitment. It remains a possibility that such circulating or bone-marrow-derived cells that travel to the ovary may in some cases co-express or co-stain with some markers typically associated with female germ cells[2]; however, the inability of these cells to form ovulated oocytes demonstrates that any such cells that might exist do not contribute to oocyte pools available for fertilization. Finally, the failure of bone marrow transplant and parabiosis to rescue ovulation in Cy/Bu-treated mice indicates to us that bone marrow transplantation is unlikely to reverse or significantly ameliorate premature menopause or chemotherapy-induced sterility. Thus, the findings presented here are consistent with the prevailing theory[4,17–20] that oocytes in adult mammals are derived from a pool that cannot be regenerated by circulating germ cell progenitors.

METHODS

Mice and antibodies. C57BL/Ka and C57BL/Ka-β-actin/eGFP mouse strains were bred and maintained at Joslin Diabetes Center or at the Harvard School of Public Health. The generation of GFP-transgenic mice has been described elsewhere[11,21]. GFP-transgenic mice used in these studies were backcrossed for at least 15 generations to C57BL/Ka mice. For parabiosis, animals were joined at 4–8 weeks of age. The monoclonal antibodies used in these studies included OX-7 (anti-Thy1.1, phycoerythrin (PE) conjugate, BD Pharmingen), 2B8 (anti-c-kit, PE–Cy7 conjugate, Ebioscience) and E13-161.7 (anti-Sca-1, Ly6A/E, allophycocyanin conjugate, Ebioscience). The cocktail of haematopoietic lineage (Lin) marker antibodies included KT31.1 (anti-CD3), GK1.5 (anti-CD4), 53-7.3 (anti-CD5), 53-6.7 (anti-CD8), Ter119 (anti-erythrocyte-specific antigen), 6B2 (anti-B220), 8C5 (anti-Gr-1) and M1/70 (anti-Mac-1).

Parabiosis. Parabiosis surgery was performed exactly as described previously[7,10], and in accordance with the guidelines established by the Joslin Diabetes Center IACUC for the humane care and use of animals. Where indicated, C57BL/Ka partners were pre-treated by intraperitoneal injection of cyclophosphamide ($120\,mg\,kg^{-1}$, Sigma) and busulfan ($12\,mg\,kg^{-1}$, Sigma), as in ref. 2. Treated mice were parabiosed to GFP-transgenic partners 1 day after Cy/Bu treatment.

Bone marrow transplantation. C57BL/Ka recipients were pre-conditioned as in ref. 2, 1 day before transplantation, by intraperitoneal injection of cyclophosphamide ($120\,mg\,kg^{-1}$) and busulfan ($12\,mg\,kg^{-1}$) or by 0.5 Gy total body irradiation (delivered from a J.L Shepard 6810 sealed source ^{137}Cs irradiator). Bone marrow cells for transplantation were flushed from the bones of either non-transgenic C57BL/Ka or GFP-transgenic C57BL/Ka-β-actin/eGFP donors into cold Hank's Buffered Saline Solution, containing 2% FBS, using a 21-gauge needle and 1-ml syringe. Cy/Bu-treated, irradiated, or untreated recipients were left untransplanted, or were transplanted intravenously, by retro-orbital injection, with 4×10^7 GFP^+ or GFP^- bone marrow cells. Recipient animals were euthanized 2 months after transplantation for analysis of oocyte and haematopoietic cell chimaerism.

Collection of oocytes. Mice were injected intraperitoneally with 5 international units (IU) of pregnant mares serum (PMS) (Calbiochem) and 48 h later with 5 IU of human chorionic gonadotropin (HCG) (Calbiochem). At 14–15 h after HCG administration, mice were euthanized and oviducts removed. Ovulated oocytes were released from the ampullary region of the oviduct into M2 medium (Speciality Media) supplemented with 0.1% bovine testicular hyaluronidase (Sigma). After dissociation of the cumulus cell–oocyte complexes in hyaluronidase, oocytes were collected into KSOM media (Speciality Media) for epifluorescence analysis and somatic cells from the complexes were subjected to flow cytometric analysis.

Flow cytometry. Haematopoietic chimaerism of parabiotic pairs was determined by flow cytometric analysis of peripheral blood and bone marrow cells after ammonium-chloride-mediated lysis of red blood cells. Chimaerism of oocyte-associated cells and debris collected from the oviduct was also assayed by flow cytometry, without ammonium chloride lysis. For analysis of GFP expression by peripheral blood cells, and of cells from the oviduct, total leukocytes were gated by forward and side scatter characteristics, and dead cells were excluded by staining with propidium iodide. To visualize HSCs, nucleated cells were isolated from whole bone marrow after ammonium chloride lysis of red blood cells and stained first with biotinylated lineage cocktail (Lin, described above), followed by PE–Texas red-conjugated streptavidin (Caltag), PE-conjugated OX-7, allophycocyanin-conjugated E13-161.7, and PE–Cy7-conjugated 2B8. Data were collected on a Coulter Epics cytometer (Beckman Coulter) maintained at the Diabetes and Endocrinology Research Center (DERC) Flow Cytometry Core Facility at Joslin Diabetes Center or on a FACSAria cytometer (Becton Dickinson) maintained at the Harvard Stem Cell Institute (HSCI) Flow Cytometry Core Facility at the Joslin Diabetes Center. Data were analysed using FlowJo software (Tree Star), and are presented as contour plots of relative fluorescence intensity or tabulated as either the percentage of GFP^+ leukocytes among total live cells for blood or the percentage of GFP^+ HSCs among total c-$kit^+Thy1.1^{lo}Lin^-Sca\text{-}1^+$ HSCs.

Epifluorescence analysis. Epifluorescence analysis was performed on ovulated oocytes using an inverted Olympus IX-51 with epifluorescence powered by a super high-pressure mercury lamp. Sequential brightfield and fluorescence images (using a FITC filter) of oocytes were acquired at ×20 magnification using an Olympus U-CMAD3 CCD camera (Olympus) and PictureFrame v2.1 software. Images were saved as high-resolution TIFF files and imported into Adobe Photoshop or InDesign for display purposes.

Histological analysis. Ovaries were fixed in 4% paraformaldehyde at room temperature for 1 h, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Quantification and statistics. Oocytes were counted by visual inspection under brightfield and epifluorescence illumination. Data were analysed for statistical significance using Student's *t*-test (Microsoft Excel).

Received 30 March; accepted 22 May 2006.
Published online 14 June 2006.

1. Johnson, J., Canning, J., Kaneko, T., Pru, J. K. & Tilly, J. L. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* **428,** 145–150 (2004).
2. Johnson, J. *et al.* Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122,** 303–315 (2005).
3. Johnson, J. *et al.* Setting the record straight on data supporting postnatal oogenesis in female mammals. *Cell Cycle* **4,** 1471–1477 (2005).
4. Telfer, E. E. *et al.* On regenerating the ovary and generating controversy. *Cell* **122,** 821–822 (2005).
5. Barlow, C. *et al.* Atm deficiency results in severe meiotic disruption as early as leptonema of prophase I. *Development* **125,** 4007–4017 (1998).
6. Bunster, E. & Meyer, R. K. An improved method of parabiosis. *Anat. Rec.* **57,** 339–343 (1933).
7. Wright, D. E., Wagers, A. J., Gulati, A. P., Johnson, F. L. & Weissman, I. L. Physiological migration of hematopoietic stem and progenitor cells. *Science* **294,** 1933–1936 (2001).
8. Conboy, I. M. *et al.* Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* **433,** 760–764 (2005).
9. Sherwood, R. I., Christensen, J. L., Weissman, I. L. & Wagers, A. J. Determinants of skeletal muscle contribution from circulating cells, bone marrow cells, and hematopoietic stem cells. *Stem Cells* **22,** 1292–1304 (2004).
10. Wagers, A. J., Sherwood, R. I., Christensen, J. L. & Weissman, I. L. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* **297,** 2256–2259 (2002).
11. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. & Nishimune, Y. "Green mice" as a source of ubiquitous green cells. *FEBS Lett.* **407,** 313–319 (1997).
12. Shiromizu, K., Thorgeirsson, S. S. & Mattison, D. R. Effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. *Pediatr. Pharmacol.* **4,** 213–221 (1984).
13. Burkl, W. & Schiechl, H. The growth of follicles in the rat ovary under the influence of busulphan and endoxan. *Cell Tissue Res.* **186,** 351–359 (1978).
14. Mattison, D. R., Chang, L., Thorgeirsson, S. S. & Shiromizu, K. The effects of cyclophosphamide, azathioprine, and 6-mercaptopurine on oocyte and follicle number in C57BL/6N mice. *Res. Commun. Chem. Pathol. Pharmacol.* **31,** 155–161 (1981).
15. Meirow, D., Epstein, M., Lewis, H., Nugent, D. & Gosden, R. G. Administration of cyclophosphamide at different stages of follicular maturation in mice: effects on reproductive performance and fetal malformations. *Hum. Reprod.* **16,** 632–637 (2001).
16. Bhattacharya, D., Rossi, D. J., Bryder, D. & Weissman, I. L. Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. *J. Exp. Med.* **203,** 73–85 (2006).
17. Byskov, A. G., Faddy, M. J., Lemmen, J. G. & Andersen, C. Y. Eggs forever? *Differentiation* **73,** 438–446 (2005).
18. Franchi, L. L., Mandl, A. M. & Zuckerman, S. *The Development of the Ovary and the Process of Oogenesis* (ed. Zuckerman, S.) (Academic Press, New York, 1962).
19. Peters, H., Levy, E. & Crone, M. DNA synthesis in oocytes of mouse embryos. *Nature* **195,** 915–916 (1962).
20. Zuckerman, S. The number of oocytes in the mature ovary. *Recent Prog. Horm. Res.* **6,** 63–108 (1951).
21. Wright, D. E. *et al.* Cyclophosphamide/granulocyte colony-stimulating factor causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. *Blood* **97,** 2278–2285 (2001).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements The authors wish to thank J. LaVecchio and the Joslin Diabetes Center DERC and HSCI Flow Cytometry Cores for FACS analysis, J. O'Keefe for animal management, J. Stockton for provision of the dissecting microscope, C. Lesser for helpful comments, and D. Mathis, D. Egli and E. Trish for critical reading of the manuscript. This work was supported in part by a Burroughs Wellcome Fund Career Award to A.J.W., and NIH grants to I.M.M. and K.E.

Author Contributions A.J.W., K.E. and R.G. designed the experiments. A.J.W. performed parabiosis surgeries and transplantations, collected tissues, and analysed haematopoietic chimaerism. K.E. collected oocytes and analysed oocyte chimaerism. I.M.M. and S.J. assisted with flow cytometry and tissue analysis. A.J.W. and K.E. wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.J.W. (amy.wagers@joslin.harvard.edu).