

The primordial follicle reserve is not renewed after chemical or γ -irradiation mediated depletion

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Abstract

Reports indicate that germ-line stem cells present in adult mice can rapidly generate new oocytes and contribute to the primordial follicle reserve following conditions of ovotoxic stress. We further investigated the hypothesis that adult mice have the capacity to generate new oocytes by monitoring primordial follicle numbers throughout postnatal life and following depletion of the primordial follicle reserve by exposure to doxorubicin (DXR), trichostatin A (TSA), or whole-body γ -irradiation. We show that primordial follicle number remains stable in adult C57BL/6 mice between the ages of 25 and 100 days. However, within 2 days of treatment with DXR or TSA, primordial follicle numbers had declined to 65 and 51% respectively ($P < 0.05$ – 0.01 when compared to untreated controls), with no restoration of follicle numbers evident after 7 days for either treatment. Furthermore, ovaries from mice subjected to sterilizing doses of γ -irradiation (0.45 or 4.5 Gy) revealed complete ablation of all primordial follicles 5 days after treatment, with no indication of follicular renewal. We conclude that neo-folliculogenesis does not occur following chemical or γ -irradiation mediated depletion of the primordial follicle reserve.

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Introduction

The female germ-line is generated during fetal development and then stored in the ovary in the form of primordial follicles, which are comprised of nongrowing, nondividing oocytes, enclosed by a single layer of squamous granulosa cells. Throughout reproductive life, the number of primordial follicles found in the ovary progressively declines as a consequence of recruitment toward further development and eventual ovulation, or through mechanisms of cell death (McGee & Hsueh 2000). Once the pool of primordial follicles reaches a critical minimum threshold, fertility and ovarian function can no longer be supported and reproductive senescence, or menopause, ensues (Hansen *et al.* 2008).

The widely accepted notion of a fixed, nonrenewable pool of primordial follicles was questioned after reports of germ-line stem cells giving rise to new oocytes in the adult ovary (Johnson *et al.* 2004, 2005). These findings were based on the quantitative analyses of healthy and atretic follicles in the ovaries of mice showing the restoration of primordial follicle numbers within 36 h of depletion with the antineoplastic agents, doxorubicin (DXR) and trichostatin A (TSA).

We have shown that the size of the primordial follicle reserve pool remains stable in adult C57BL/6 mice between the ages of 25 and 100 days (Kerr *et al.* 2006). This plateau in numbers may indicate a very slow rate of primordial follicle recruitment, and limited loss of primordial follicles through death, such that a decline cannot be detected with the number of animals used or the statistical analysis applied (Faddy & Gosden 2007). Alternatively, stable primordial follicle numbers, despite continuous loss through recruitment and death, may be evidence of neo-folliculogenesis in the adult ovary (Kerr *et al.* 2006, Skaznik-Wikiel *et al.* 2007).

To further investigate these possibilities, we extended our earlier studies of follicle numbers in adult mice by increasing the number of ovaries analysed and subjecting the data to rigorous statistical analyses. Additionally, we tested the hypothesis that new oocytes can be generated *de novo* by depleting the primordial follicle population in C57BL/6 mice using DXR, TSA, or γ -irradiation, and then monitoring ovarian morphological and follicle numbers using unbiased and assumption-free stereological methods (Myers *et al.* 2004, Kerr *et al.* 2006).

Results

The number of primordial follicles is stable in the postnatal mouse ovary

Consistent with our previous findings (Kerr *et al.* 2006), the mean (\pm s.e.m.) number of nongrowing germ cells (naked oocytes and primordial follicles) were highest in prepubertal mice from postnatal day 1 to 3 (Fig. 1a). Mean primordial follicle numbers declined significantly by day 7 to 12 ($P < 0.01$), but then remained unchanged from day 25 to 110 ($P > 0.33$; Fig. 1a). A second dramatic decline in mean primordial follicle numbers per ovary was observed between day 110 and 200 to day 300 ($P < 0.01$; Fig. 1a).

The relationship between primordial follicle number per ovary and age was further examined by linear

regression, showing that there was no significant difference in primordial follicle number from day 25 to 110 ($R^2 = 0.001$, $y = 1351 - 0.76x$ age, $n = 95$; $P = 0.75$; Fig. 1b). Thus, primordial follicle numbers remain stable in mice between day 25 and 110.

Primordial follicle numbers are not restored after DXR-induced depletion

Control mice showed normal follicle distributions and morphology (Fig. 2a). By contrast, morphological analysis revealed the presence of primordial follicles comprising dysmorphic oocytes with condensed nuclei as early as 1 day following DXR treatment (Fig. 2b and d). Empty follicles, devoid of morphologically distinguishable oocytes, were also observed (Fig. 2d). At 2 and

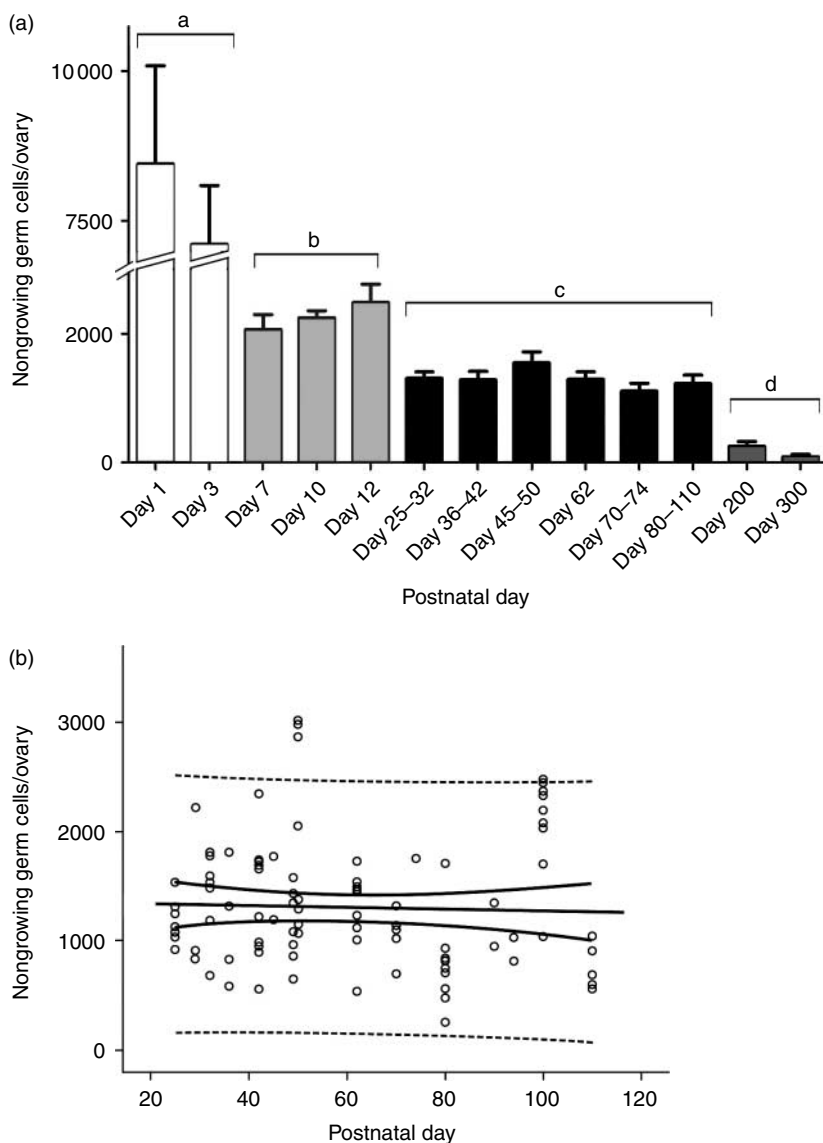


Figure 1 Primordial follicle numbers remain stable in PN 25–110 ovaries. (a) Mean (\pm s.e.m.) follicle numbers per ovary at indicated ages was estimated using optical disector/fractionator stereological methods. Age groups with same letters a, b, c and d are significantly different ($P < 0.05$ – 0.01). (b) Subjected to regression analysis, primordial follicle numbers in individual ovaries show no significant ($P = 0.75$, $R^2 = 0.001$) decline with age between PN 25 and 110 (regression equation is $1351 - 0.76$ per day). Solid curved lines are 95% confidence intervals for the regression line; dashed lines are 95% prediction intervals.

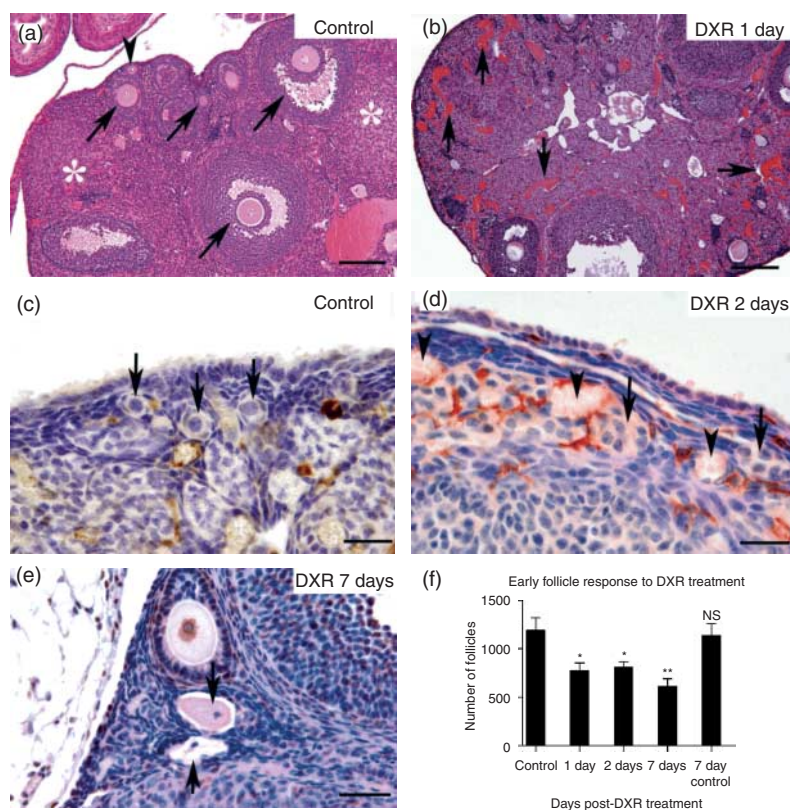


Figure 2 DXR depletes primordial follicle number. (a and c) Control and (b, d and e) DXR-treated ovaries. (a) Six-week-old control ovary (H&E) exhibits early (arrowhead) and growing follicles (arrows) and corpora lutea (asterisks). Bar = 100 μ m. (b) One day post-DXR. Blood vessels (arrows) are numerous and dilated (H&E). Bar = 100 μ m. (c) Primordial follicles (arrows) in control ovary; F4/80 stain. Bar = 30 μ m. (d) Two days post-DXR (F4/80 stain) showing shrunken follicles lacking oocytes (arrows), numerous macrophages (orange stain), and capillaries with erythrocytes (arrowheads). Bar = 20 μ m. (e) Seven days post-DXR (PCNA stain) with two degenerated follicles containing remnants of the oocyte (arrows). Bar = 40 μ m. (f) Primordial follicle numbers per ovary in control mice (6 weeks of age) and 1, 2, and 7 days after DXR injection, and in age-matched control mice (7 weeks of age) estimated using optical disector/fractionator stereological methods. Single and double asterisks indicate significant differences ($P < 0.05$, $P < 0.01$ respectively) compared to both control groups. NS indicates no significant difference compared to 6 week control group. Data are presented as means \pm S.E.M. ($n = 7$ all groups).

7 days after DXR treatment, structures resembling collapsed follicles were commonly noted in the ovarian cortex (Fig. 2d and e). Because macrophages are found in the stroma of ovaries, F4/80 immunostaining was also used in order to accurately distinguish macrophages from oocytes (Fig. 2c and d).

These morphological observations were confirmed by stereological analyses. Mean primordial follicle numbers per ovary remained unchanged in control ovaries over the 7-day treatment period (1189 ± 131 vs 1130 ± 126 , $P = 0.89$; Fig. 2f). However, 1 day after DXR treatment, the mean primordial follicle number per ovary had declined to 65% of the mean number in control mice (770 ± 83 vs 1189 ± 131 , $P < 0.05$ respectively). No further reduction in follicle numbers was observed at 2 days (774 ± 59). However, at 7 days after DXR treatment the mean number of primordial follicles fell to 51% (608 ± 62 vs 1189 , $P < 0.01$; Fig. 2f). The reduction observed at 7 days indicates that there was no restoration of primordial follicle numbers after DXR treatment.

To further confirm these findings, multiple DXR treatments were performed on 6-week-old mice and follicle numbers were assessed 4 weeks later. Stereological analysis provided no evidence of restoration of primordial follicle number (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Primordial follicle numbers are not restored after TSA-induced depletion

Ovaries from control mice showed normal follicle distributions and morphology (Fig. 3a and c). At 1 day post-TSA treatment, morphologically normal primordial and primary follicles were observed (not shown). At 2 and 7 days post-TSA treatment, primordial follicle death became apparent by the appearance of empty follicular-type structures (Fig. 3b, d and e).

Immunostaining for the proliferative marker, proliferating cell nuclear antigen (PCNA) was used to identify proliferating germ-line stem cells. PCNA was detected in granulosa, theca, and interstitial cells but was not detected in oocytes in control and treated ovaries (Fig. 3e). Potential germ-line stem cells, defined as PCNA-positive cells that could not be classified as theca, granulosa, or oocytes morphologically, were not detected. Germ cell nuclear antigen (GCNA), a marker of prediplotene oocytes, was used in order to identify newly generated oocytes (Fig. 3b). However, all oocytes in control and treated ovaries were GCNA negative. Thus, no evidence of germ-line stem cells was provided by use of these markers.

The inability to morphologically or immunohistochemically detect the presence of germ-line stem cells was supported by stereological analysis of treated ovaries. Between 1 and 7 days after TSA treatment (Fig. 3f), mean

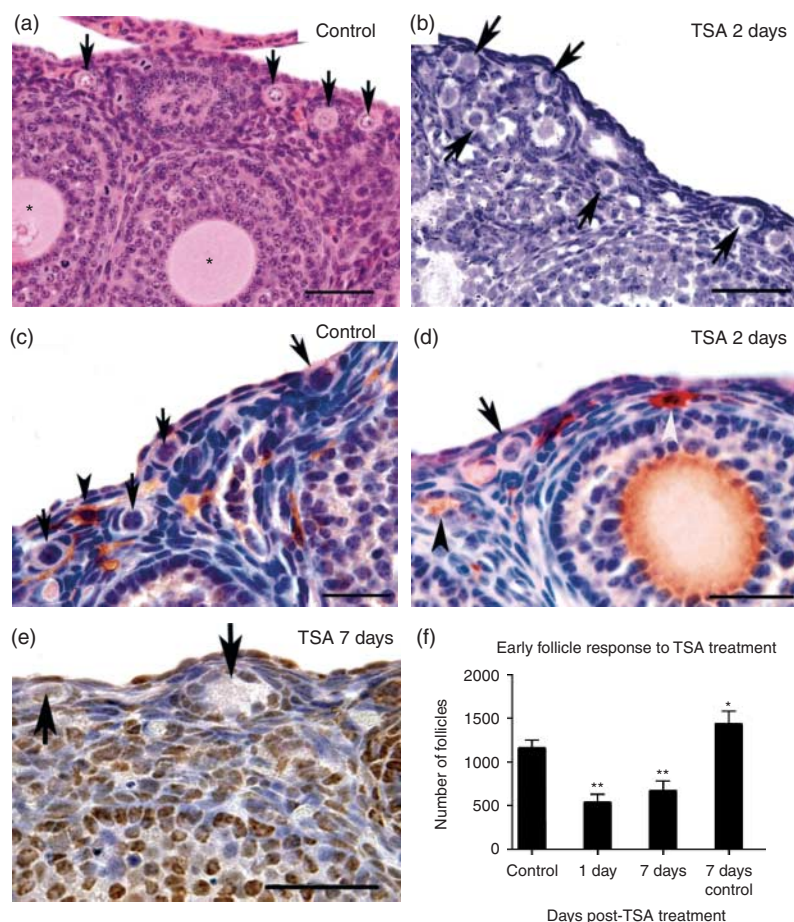


Figure 3 TSA depletes primordial follicle number. (a and c) Control and (b, d and e) TSA-treated ovaries. (a) Twenty-five day control ovary (H&E) with early (arrows) and growing follicles (asterisks). Bar = 50 μ m. (b) Two days post-TSA (GCNA stain) with follicles (arrows) showing oocytes with no GCNA label. Bar = 50 μ m. (c) Primordial follicles (arrows) and macrophages (arrowhead) in control ovary (F4/80 stain). Bar = 35 μ m. (d) Two days post-TSA (F4/80 stain) showing one primordial follicle (arrow) and a follicle lacking an oocyte (arrowhead). Macrophages (white arrowhead) stained orange. Bar = 45 μ m. (e) Seven days post-TSA (PCNA stain) empty or collapsed follicles lacking intact oocytes (arrows) are evident. Bar = 40 μ m. (f) Primordial follicle numbers per ovary in control mice (25 days of age) and 1 and 7 days after TSA injection, and in age-matched control mice (32 days of age) estimated using optical disector/fractionator stereological methods. Double asterisks indicate significant difference ($P < 0.01$) compared to both control groups. Single asterisk indicates significant difference ($P < 0.05$) compared to 25 day control group. Data are presented as means \pm S.E.M. ($n = 7$ all groups).

primordial follicle numbers per ovary declined significantly ($P < 0.01$) to 51% (605 ± 96) and 61% (720 ± 118) compared to the mean number in ovaries from day 25 control (untreated) mice (1173 ± 77). Thus, by 7 days after TSA treatment, there was no evidence of a renewal in primordial follicle numbers. Furthermore, F4/80 immunostaining was performed in order to ensure that macrophages were not mistaken for oocytes (Fig. 3c and d).

Primordial follicle numbers are not restored after γ -irradiation-induced ablation

In untreated control ovaries, primordial, primary, and secondary follicles were abundant at PN 5 and 10 (Fig. 4a). By contrast, primordial follicles could not be found in ovaries from mice that had been γ -irradiated (0.45 or 4.5 Gy) at 5 days of age and analysed at 10 days (Fig. 4c). Primary follicles were rarely observed in irradiated ovaries, but secondary follicles were commonly detected (not shown). Comparative quantitative estimation of follicle numbers after γ -irradiation confirmed the complete elimination of primordial follicles (Fig. 4d). There was no evidence of survival or restoration of primordial follicle numbers after either dose of γ -irradiation.

Discussion

We previously reported that in a stereological analysis of 28 ovaries of C57BL/6 mice, the primordial follicle pool remains stable from day 7 to 100 of postnatal life and thereafter declines significantly to $\sim 10\%$ of that level by day 200 (Kerr *et al.* 2006). In the present study, using the same unbiased, assumption-free disector/fractionator method, we quantified additional ovaries to augment our earlier results. We confirmed a period of apparent stability of the primordial follicle pool from day 25 to 110 ($n = 95$ ovaries) by regression analysis. A similar plateau of follicle supply in the human ovary has also recently been reported (Hansen *et al.* 2008). As primordial follicles are continuously lost from the pool through recruitment and/or death, the plateau of primordial follicle numbers occurring during adult life may be evidence of primordial follicle regeneration from germ-line stem cells (Kerr *et al.* 2006, Skaznik-Wikiel *et al.* 2007). We therefore conducted a series of primordial follicle depletion experiments and carefully monitored ovarian morphology and primordial follicle numbers in order to further investigate the potential for neo-folliculogenesis in the postnatal mouse ovary.

Johnson *et al.* (2005) reported an extensive (80%) loss of primordial and early-growing follicles within 24 h of

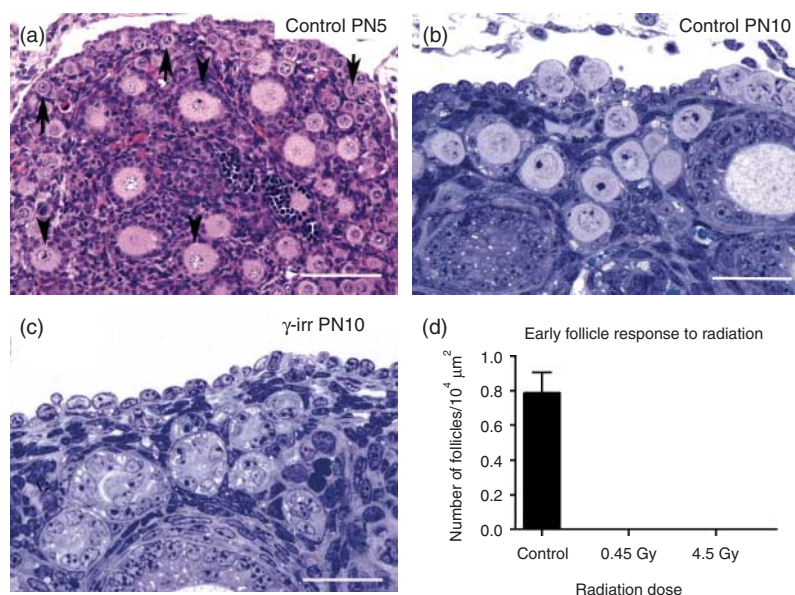


Figure 4 γ -Irradiation depletes primordial follicle number. (a) Five day control ovary (H&E) showing primordial (arrows) and growing follicles (arrowheads). Bar = 80 μm . (b) Ten day control ovary (toluidine blue) showing primordial follicles in the tunica albuginea and in the ovarian cortex. Bar = 35 μm . (c) Ten day ovary (toluidine blue) 5 days after 0.45 Gy γ -irradiation showing empty primary follicles and absence of primordial follicles. Bar = 35 μm . (d) Primordial follicle numbers per ovary (expressed per $10^4 \mu\text{m}^2$ section area) at 10 days of age in untreated control mice and mice receiving either 0.45 or 4.5 Gy γ -irradiation on day 5. Primordial follicles containing oocytes are undetectable following γ -irradiation. Data are presented as means \pm S.E.M. ($n = 6$ all groups).

DXR treatment, followed by rapid regeneration of primordial follicle numbers 36 h after treatment, with complete restoration of primordial follicle numbers at 2 months. However, using the same strain of mice, DXR dose, and similar timing of analysis as in this earlier paper, we are unable to show any restoration of primordial follicles after treatment. In support of our findings, Kujjo *et al.* (2011) also replicated the studies reported by Johnson *et al.* (2005) and using the C57BL/6 strain, found a significant primordial follicle depletion from 36 to 120 h after DXR, with a further decline to almost zero at 2, 4, and 6 months posttreatment (Kujjo *et al.* 2011). The reasons for the discrepancy between Johnson *et al.* (2005) and these two more recent studies are not clear.

Interestingly, Johnson *et al.* (2004, 2005) could not detect follicle renewal 2 months after treatment of C57BL/6 mice with ovotoxicins, cyclophosphamide and busulfan. One possibility for the failure of cyclophosphamide and busulfan to induce spontaneous follicle renewal is that these particular treatments may destroy germ-line stem cells in addition to oocytes. This argument is supported by a study reporting the derivation of germ-line stem cells from bone marrow, in which transplantation of bone marrow from untreated donors rescued primordial follicle numbers after cyclophosphamide and busulfan exposure (Johnson *et al.* 2005).

Our observations on follicle numbers after TSA treatment also differ from those of Johnson *et al.* (2005). They found that in day 13 mice exposed to the histone deacetylase inhibitor, TSA, primordial follicle numbers were increased by 52% compared to vehicle-treated control animals within 24 h. More striking results were reported in older animals (241 days of age), in that treatment with TSA doubled primordial follicle numbers in ovaries within 24 h. Furthermore, the observed

increases in primordial follicles could not be attributed to a reduced rate of primordial follicle recruitment or a reduced incidence of follicle death. However, using a similar experimental design with day 25 mice, we noted a consistent and persistent decrease in primordial follicles, with no restoration of follicle numbers during the period of observation (24 h, 2 days, and 7 days posttreatment).

If new primordial follicles are replenished after TSA treatment, it is expected that their oocytes would express the prediplotene germ cell marker, GCNA, as they progress through the first meiotic prophase. There was no evidence of GCNA-positive germ cells following TSA treatment were observed in the current study. Furthermore, immunostaining for the proliferative marker PCNA would be expected if new oocytes were to arise by proliferation of existing intraovarian germ-line stem cells. There was no evidence of PCNA-positive germ cells were detected in this study.

Similar to previous reports for fetal or neonatal rats (Mazaud *et al.* 2002, Guigon *et al.* 2003, 2005, Mazaud Guittot *et al.* 2006), we observed total elimination of primordial follicle numbers in mice after ionizing radiation. Interestingly, Johnson *et al.* (2005) reported that bone marrow transplantation after exposure to γ -irradiation was not able to rescue the ovarian failure (Johnson *et al.* 2005). The doses of γ -irradiation used in our study (0.45 or 4.5 Gy) and by Johnson *et al.* (2005) (0.5 Gy) do not cause fatal failure of hemopoiesis or irreversible damage to the intestines because these mice survive for months posttreatment. Therefore, it would be expected that if the hypothesis of Johnson *et al.* is correct, there should be available sufficient stem cells from the bone marrow to restore primordial follicle numbers in the ovary after exposure to γ -irradiation, particularly after bone marrow transplantation.

If follicle regeneration in the adult ovary does not occur, what is the explanation for the plateau in primordial follicle numbers observed in adult life? The unbiased and assumption-free method we used for estimating the numbers of primordial follicles is mathematically robust and widely accepted (Miller *et al.* 1997, 1999, Sonne-Hansen *et al.* 2003, Wang *et al.* 2005, Charleston *et al.* 2007, McTavish *et al.* 2007, Walters *et al.* 2007, Hansen *et al.* 2008). Nevertheless, the extent of variation between animals in ovarian primordial follicle number may make very small declines in follicle loss through low rates of recruitment and death difficult to detect statistically, unless very large numbers of animals are examined (Faddy & Gosden 2007). In the present study, empty or degenerating primordial follicles were only rarely observed in the 141 untreated C57BL/6 ovaries analysed. Combined with our follicle number data, this observation suggests a very low rate of follicle loss through death (Tingen *et al.* 2009). However, to gain further insight, two questions must be explored in future studies: i) what is the rate of primordial follicles loss from postnatal mice ovaries; and ii) what are the mechanisms for their disposal?

In conclusion, our own data, in addition to the experimental and theoretical arguments of others (Byskov *et al.* 2005, Telfer *et al.* 2005, Eggan *et al.* 2006, Begum *et al.* 2008, Kujjo *et al.* 2011), provide accumulating evidence against the hypothesis of neo-folliculogenesis in the adult mouse ovary (Johnson *et al.* 2004, 2005, Lee *et al.* 2007, Skaznik-Wikiel *et al.* 2007). If neo-folliculogenesis was a physiologically important and relevant mechanism employed to maintain oocyte number, then one might expect substantial *de novo* recovery after depletion of the primordial pool of oocytes by treatments that reduce the size of the pool. We found no evidence of full or even partial *de novo* renewal of the follicle pool after depletion with two different antineoplastic treatments, DXR or TSA, or by exposure to whole-body γ -irradiation. These interventions either substantially or completely depleted the primordial follicle pool in a permanent manner. Thus, our data support the hypothesis that a fixed number of oocytes are generated during ovarian development and that no new oocytes are made once the pool of primordial follicles is established.

Materials and Methods

Animals and ovary collection

Wild-type female C57BL/6 mice, supplied by Monash Animal Services or The Walter and Eliza Hall Institute mouse breeding facility, were kept in controlled conditions of 12 h light:12 h darkness, with free access to water and mouse chow in pathogen-free conditions. Animal procedures were approved by the animal ethics committee at Monash University and the Walter and Eliza Hall Institute were carried out in accordance

with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Ovaries for immunohistochemistry were fixed in 10% formalin and processed into paraffin and 5 μ m sections were cut. Ovaries for morphological analysis were fixed in 2.5% cacodylate-buffered glutaraldehyde, postfixed in 2% cacodylate-buffered osmium tetroxide, processed into an Epon-Araldite mixture and 1 μ m sections stained with toluidine blue. Ovaries for stereology were fixed in Bouin's solution and processed into hydroxyethyl methacrylate resin (Technovit 7100; Kulzer and Co., Friedrichsdorf, Germany), serially sectioned at 20 μ m with a Leica RM2165 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and stained with periodic acid-Schiff and counterstained with hematoxylin.

Assessment of primordial follicle numbers in mice

Ovaries (97 in total) were collected from mice aged 3–300 days old. Stereological data were obtained as previously described in detail (Myers *et al.* 2004, Kerr *et al.* 2006) using a $\times 100$ oil immersion objective on an Olympus BX51 microscope equipped with an Autoscan stage (Autoscan Systems Pty Ltd, Melbourne, Victoria, Australia) in conjunction with the CASTGRID stereological system (CAST 2002; Olympus, Albertslund, Denmark). The present data were combined with previously reported data (44 in total, Kerr *et al.* 2006). Stereological methods (optical disector/fractionator), the animal colony, and mouse strain (C57BL/6) were identical between the two studies.

Primordial follicle depletion

Experiment 1: DXR treatment

Mice (6 weeks old) received 5 mg/kg DXR in 100 μ l saline (>98% purity, Sigma-Aldrich) via a single tail vein injection (the i.v. route was used to mimic clinical administration) and ovaries were collected at 1, 2, and 7 days posttreatment ($n=7$ /day). Control mice (6 weeks old) received saline only and ovaries were collected at 12 h ($n=7$) and at 7 days ($n=7$) after treatment. To test the hypothesis that multiple DXR treatments would result in oocyte regeneration, mice (6 weeks old) received 5 mg/kg per 100 μ l saline DXR ($n=7$) or saline alone ($n=7$) via tail vein injection, with repeated doses at 7 and 8 weeks. Ovaries were collected at 10 weeks. Dose of DXR was identical to Johnson *et al.* (2004).

Experiment 2: TSA treatment

Mice (25 days old) received 10 mg/kg TSA in 100 μ l DMSO (>98% purity, Sigma-Aldrich) ($n=7$) or DMSO alone ($n=14$) via a single s.c. injection as described in mice (Fenic *et al.* 2008, Nasu *et al.* 2008). Ovaries were collected at 1, 2, and 7 days after treatment. Dose of TSA was identical to Johnson *et al.* (2004, 2005).

Experiment 3: whole-body γ -irradiation

Mice (5 days old) were whole-body γ -irradiated with 0.45 or 4.5 Gy ($n=6$) or untreated ($n=6$) and ovaries collected at

10 days old. Both of these doses are sublethal and allow recovery of the hemopoietic and intestinal tissue compartments since these irradiated mice in our colony survive for up to 12 months. Doses were selected because they had been previously shown to result in complete elimination of the primordial follicle pool (Suh *et al.* 2006).

Quantification of follicles

Primordial follicle depletion experiments 1 and 2

Primordial follicles numbers (means \pm S.E.M.) were counted using the fractionator/optical disector stereological technique as previously described in C57BL/6 mice by this laboratory (Myers *et al.* 2004, Kerr *et al.* 2006). Only those follicles with morphologically normal oocyte nuclei were counted. A total of 8283 sections from 141 ovaries (35 controls for antineoplastic experiments and 106 additional ovaries from normal mice aged between 1 and 300 days of age) were prepared and analysed.

Primordial follicle depletion experiment 3

Irradiated ovaries were not examined using the fractionator/optical disector technique because primordial and primary follicle numbers with oocytes were close to zero. Instead, Bouins' fixed ovaries from γ -irradiated or control mice were processed into paraffin and 5 μ m serial sections of each ovary were stained with hematoxylin and eosin. From each ovary, the middle section and two sections located 100 μ m on either side were selected for semiquantitative estimation of primordial follicles using morphological criteria previously described (Myers *et al.* 2004, Kerr *et al.* 2006). Primordial follicles with an oocyte nucleus were counted in the three selected sections per ovary and the area (in μ m²) of the ovarian sections was measured with image analysis software (Infinity Analyzer v5; Lumenera, Ottawa, ON, Canada). Mean (\pm S.E.M.) follicle numbers per ovary were expressed per 10 000 μ m².

Immunohistochemistry

Proliferating cells were detected with a MAB to PCNA (Oktay *et al.* 1995) using a PCNA staining kit (Zymed Laboratories, South San Francisco, CA, USA). The presence of prediplotene/early diplotene oocytes was assessed by immunostaining for GCN (1:200, supplied by Dr George Enders, KUMC, KS, USA). Macrophages were detected with a MAB to mouse F4/80 antigen (F4/80, 1:200; Serotec, Oxford, UK). Antigen retrieval was performed by boiling slides in 0.01 M citrate buffer (pH 6) for 10 min in the microwave. Sections were permeabilized with 0.1% Triton X and peroxidases quenched in 2% H₂O₂. Following blocking for 1 h with 10% normal rabbit serum or 1% BSA, sections were incubated with primary antibody for 1 h at room temperature. Sections were incubated with biotinylated secondary antibody (anti-rat IgG 1:200) for 1 h. Antibodies were detected by incubation for 1 h with avidin–biotin–peroxidase, followed by exposure to diaminobenzidine reaction. Sections were counterstained with hematoxylin and mounted with Histomount.

Statistical analysis

Data for primordial follicle numbers are presented as means \pm S.E.M. and statistical analysis of follicle numbers was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data were normally distributed and analysed by one-way ANOVA and the significance determined by the Newman–Keuls *post hoc* multiple comparison test. Differences were considered significant when $P < 0.05$. Data for primordial follicles between day 25 and 110 were further examined by regression analysis (SPSS GmbH Software, Munich, Germany) and expressed as a function of age. Data from the follicle depletion experiments are presented as means \pm S.E.M. and statistical analysis of follicle numbers was performed as described above.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0430>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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