Sex-specific differences in fetal germ cell apoptosis induced by ionizing radiation

Marie-Justine Guerquin1,2,3, Clotilde Duquenne1,2,3, Hervé Coffigny1,2,3, Virginie Rouiller-Fabre1,2,3, Romain Lambrot1,2,3, Mariana Bakalska4, René Frydman5,6,7, René Habert1,2,3, and Gabriel Livera1,2,3,8

1CEA, DSV/DRR/SEGG/LDRG, Laboratory of Differentiation and Radiobiology of the Gonads, Unit of Gametogenesis and Genotoxicity, F-92265 Fontenay aux Roses, France 
2Univ. Paris 7—Denis Diderot, UFR of Biology, UMR-S 566, F-92265 Fontenay aux Roses, France 
3INSERM, U566, F-92265 Fontenay aux Roses, France 
4Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, Sofia, Bulgaria 
5Univ Paris-Sud, Clamart F-92140, France 
6AP-HP, Service de Gynécologie-Obstétrique et Médecine de la Reproduction, Hôpital Antoine Béclère, Clamart F-92141, France 
7INSERM, U782, Clamart F-92140, France 
8Correspondence address. Unit of Gametogenesis and Genotoxicity, CEA, Université Paris 7, INSERM U566, CEA/DSV/DRR/SEGG/LDRG, Route du Panorama-BP6, 92265 Fontenay aux Roses Cedex, France. Tel: +33-1-46-54-99-36; Fax: +33-1-46-54-99-06; E-mail: gabriel.livera@cea.fr

BACKGROUND: We have previously shown that male human fetal germ cells are highly radiosensitive and that their death depends on p53 activation. Male germ cell apoptosis was initiated with doses as low as 0.1 Gy and was prevented by pifithrin α, a p53 inhibitor. In this study, we investigated the radiosensitivity of early female and male fetal proliferating germ cells.

METHODS AND RESULTS: Both male and female fetal germ cells displayed a similar number of γH2AX foci in response to ionizing radiation (IR). In organ culture of human fetal ovaries, the germ cells underwent apoptosis only when exposed to high doses of IR (1.5 Gy and above). Accumulation of p53 was detected in irradiated male human fetal germ cells but not in female ones. Inhibition of p53 with pifithrin α did not affect oogonia apoptosis following irradiation. IR induced apoptosis similarly in mouse fetal ovaries in organ culture and in vivo during oogonial proliferation. Germ cell survival in testes from p53 knockout or p63 knockout mice exposed to IR was better than wild-type, whereas female germ cell survival was unaffected by p53 or p63 knockout.

CONCLUSIONS: These findings show that pre-meiotic male and female fetal germ cells behave differently in response to a genotoxic stress—irradiation—with oogonia being less sensitive and undergoing p53-independent apoptosis.

Key words: gonocyte / oogonia / ionizing radiation / apoptosis / p53

Introduction

Mammalian germ cells acquire sex-specific features early during fetal life (McLaren, 1995). In humans, primordial germ cells (PGC) colonize the morphologically undifferentiated gonadal anlage during the fourth week post-conception (pc) (McKay et al., 1953). Once in the gonad, PGC are termed gonocytes and proliferate actively. They remain very similar in male and female gonads until about the end of the first trimester. At this stage, some female germ cells initiate the prophase of the first meiotic division (Gondos et al., 1986; Bendsen et al., 2006). The same events occur during mouse development but do so much faster with PGC colonizing the gonads about 10.5 days post-coitus (dpc) and meiosis beginning as early as 13.5 dpc (Bowles and Koopman, 2007).

There is diverse evidence that fetal germ cells are most susceptible to damage during these early steps of extensive proliferation. In the female, the depletion of the oogonia stock induces premature ovarian failure (Mazaud et al., 2002; Hanoux et al., 2007). In the fetal testis, early germ cells are the most sensitive to many physiological and pollutant agents such as retinoic acid and DES (Livera et al., 2000; Lassurguere et al., 2003).

DNA is under constant assault from various endogenous and exogenous DNA-damaging agents. DNA double-strand breaks (DSBs) are the most dangerous lesion and result from exposure to genotoxic agents such as ionizing radiation (IR), doxorubicin, UV and heavy metals. Usually proliferating cells respond to limited DNA damage by activating a complex signaling network leading to transient cell cycle arrest and allowing the lesions to be repaired (Houtgraaf et al., 2006). When the damage is too extensive and cannot be repaired, apoptosis is induced. The choice between apoptosis and DNA repair is mostly orchestrated by the tumor suppressor gene p53 and its related family members p63 and p73 (Fei and El-Deiry, 2008).
visually on the basis of their limbless phenotype. The genotypes of the fetuses were removed from uterine horns. Gonads with their ovaries were present in the human ovaries. Targeted disruption of murine p63 and p53 genes has been described elsewhere (Allemand et al., 1999; Mills et al., 2000). The Antoine Béclère Ethics Committee approved this study. The sex of the fetus was determined from the presence of a vaginal plug. The 12.5 dpc stage was chosen as mice ovaries contained only oogonia at this age and these gonads are therefore equivalent to the human fetal ovaries in term of germ cell population.

Organotypic culture
All tissues (human or murine gonads) were cultured on Millicell-CM Biopore membranes (pore size 0.4 μm; Millipore, Billerica, MA, USA) in 0.3 ml of Ham F12/DMEM (1:1) containing 80 μg/ml gentamicin and 1% fetal calf serum in a humidified atmosphere containing 95% air—5% CO₂. The medium was changed every 48 h. For human tissue cultures, each gonad was cut into small pieces and all pieces were placed separately on 2 or 4 Millicell membranes as required. Some pieces were used as controls and the others were irradiated. For murine tissue cultures, contra-lateral gonads were placed on different Millicell membranes; one was irradiated and the other served as a control (subjected to the same conditions but not irradiated). For organ culture, the medium was changed immediately after irradiation. To investigate the involvement of the p53 pathway, 5 μM of pifithrin α (Pif α, Sigma-Aldrich, St Louis, MO, USA), an inhibitor of p53 activity, was added to the medium 1 h before irradiation and maintained until the end of the culture period; the vehicle (dimethyl sulfoxide, DMSO) was similarly included in the culture medium of the paired control.

Irradiation
Gamma irradiation was applied with a 137Cs source (IBL 637; CIS bio International, Gif-sur-Yvette, France). For dose/effect studies with human ovaries, gonads were irradiated with various doses between 0.2 and 5 Gray (Gy). For all other experiments, human and murine gonads were irradiated with 1.5 Gy. For in vitro irradiation, fetal gonads were directly irradiated in tissue culture dishes, 1 day (human) or 1 h (mouse) after explantation. For in vivo irradiation, pregnant mice were exposed to a whole-body irradiation at 12.5 dpc, and gonads were collected at 18.5 dpc.

Electron microscopy
Ovaries were fixed in 2.5% glutaraldehyde in cacodylate buffer, post-fixed in 1% OsO₄, then dehydrated in graded alcohol solutions and finally propylene oxide. They were embedded in Durcupan (Fluka). Ultrathin sections were cut and counterstained with uranyl acetate and lead citrate, and examined under a Jeol–1010 electron microscope.

Materials and Methods

Collection of human fetal gonads
Human fetal gonads were obtained from pregnant women referred to the Department of Obstetrics and Gynecology at the Antoine Béclère Hospital, Clamart (France), for legally induced abortion in the first trimester of pregnancy, i.e. between the 6th and the 10th weeks pc, as previously described (Lambrot et al., 2006). The Antoine Béclère Ethics Committee approved this study. The sex of the fetus was determined from the morphology of the gonads. Ovaries were thinner and more closely associated with the mesonephros than testes. The fetal age was evaluated by measuring the length of limbs and feet (Evtouchenko et al., 1996). At these stages, histological analysis confirmed that only oogonia were present in the human ovaries.

Mice
All animal studies (NMRI, p53 and p63 transgenic mice) were conducted in accordance with the guidelines for the care and use of laboratory animals of the French Ministry of Agriculture. Mice were housed in controlled photoperiod conditions (lights on from 08:00 to 20:00) and were supplied with commercial food and tap water ad libitum. Adult males were caged with adult females overnight, and the presence of a vaginal plug was examined the following morning. On the assumption that mating occurred between 00:00 and 02:00, the next midday was defined as 0.5 dpc. p63−/− and p53−/− embryos were produced by intercrossing heterozygous p63Brm2 or Tg-Hp33 mice with a C57/BL6 genetic background. Targeted disruption of murine p63 and p53 genes has been described elsewhere (Jacks et al., 1994; Mills et al., 1999), p63−/− mice were identified visually on the basis of the limbless phenotype. The genotypes of the other embryos (p63+/+, p53+/+ and p53−/−) were determined by PCR using DNA extracted from tail biopsy samples as previously described (Allemand et al., 1999; Petre-Lazar et al., 2006).

Pregnant females were killed by cervical dislocation at 12.5 dpc and their fetuses were removed from uterine horns. Gonads with their mesonephros were isolated from fetuses under a binocular microscope and kept in Ham F12/DMEM (1:1) (Life Technologies, Inc., Grand Island, NY, USA) until explantation. The sex of the fetus was determined by the morphology of the gonad. The 12.5 dpc stage was chosen as mice ovaries contained only oogonia at this age and these gonads are therefore equivalent to the human fetal ovaries in term of germ cell population.
count per gonad. We then used the Abercrombie formula to correct for any double counting due to single cells appearing in two successive sections and thereby obtained a ‘true count’ (TC): TC = CC × S/(S + D), where CC is the crude count, S the section thickness (μm) and D the mean diameter of the gonocyte nuclei (Abercrombie, 1946). D is also equal to the average nuclear diameter, as measured on the section (Dn) divided by \( \sqrt{4} \) to correct for the over-representation of smaller profiles in sections through the spherical particles. We measured Dn in each gonad studied, by means of at least 200 random determinations. For human fetal oocytes, the number of germ cells counted in each fragment was normalized by dividing the number by the area to obtain a germ cell density.

### Immunohistochemistry

Immunohistochemistry, based on peroxidase activity, was performed using commercially available primary antibodies: anti-PS3 (working dilution 1:50, Cell Signaling, Beverly, MA, USA), anti-Cleaved Caspase-3 Asp 175 (1/100, Cell Signaling), anti-Cleaved Caspase-9 Asp 353 (1/100, Cell Signaling) and anti-DDX4/MVH (1/500, Abcam, Cambridge, UK). Five tissue sections per gonad were mounted on glass slides, dewaxed and boiled for 10 min in 10 mM Tris pH 10.6 for immunostaining of cleaved Caspases-3 and -9, or in 10 mM citrate pH6.0 for immunostaining of PS3, and MVH. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The sections were washed with PBS (for cleaved caspases-3 and -9 and MVH) or TBS (for PS3), and blocked for 30 min with 5% normal goat serum (NGS) and 10% of bovine serum albumin. Slides were incubated overnight at 4°C with the primary antibody and 5% NGS. We detected bound primary antibody with a biotinylated goat anti-rabbit secondary antibody in 5% of NGS and the avidin–biotin-peroxidase complex (Vectorstain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized using 3,3′-diaminobenzidine (DAB) as substrate.

### Measurement of cell proliferation

The percentage of cells in S-phase was evaluated by measuring the BrdU (5-bromo-2′-deoxyuridine) incorporation, by immunohistochemical methods, using the Cell Proliferation Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s recommendations. BrdU (1%) was either added at the end of the culture period or 3 h before tissue fixation. Briefly, five randomly selected sections were mounted, rehydrated, incubated for 1 h with anti-BrdU antibody and with a peroxidase-linked antirabbit secondary IgG. Peroxidase activity was then detected with DAB. The BrdU incorporation index was determined by blindfolded counting of at least 500 stained and unstained gonocytes.

### Immunofluorescence

Fixed gonad sections were incubated for 2 h with the primary antibodies at room temperature. The primary antibodies used were mouse anti-γ-H2AX (1/200, Upstate, Temecula, CA, USA), rabbit anti-DDX4/MVH (1/500) and mouse anti-D2-40 (prediluted, Abcam). After washing in PBS–Triton 0.1% (Triton X100, Sigma-Aldrich), sections were incubated for 45 min with a donkey anti-mouse IgG-Cy3 (1/800, Jackson ImmunoResearch Laboratories), donkey anti-mouse IgG–FITC (1/50, Jackson ImmunoResearch Laboratories) or with a donkey anti-rabbit–FITC (1/50, Jackson ImmunoResearch Laboratories). Slides were mounted with Vectashield with or without Dapi (Vector Laboratories) and analyzed by conventional immunofluorescence microscopy using a ProvisAX70 Olympus microscope and a Leica confocal microscope.

### Data analysis

Each data point represents the mean ± SEM of at least three independent experiments. Images show one representative of at least three experiments.

Data were analyzed using Graphpad Instat 3.0, by one-way ANOVA followed by the Tukey–Kramer multiple comparisons test or paired or unpaired Student’s t-test.

### Results

#### γ-H2AX foci formation following irradiation in male and female fetal germ cells

First, we compared DNA damage induced by IRs in male and female germ cells. DSB formation was monitored using γ-H2AX staining in human and murine fetal gonads. Human fetal ovaries and testes were γ-irradiated with a 1.5 Gy dose and cultured for one additional hour. Human germ cells were recognized by D2-40 staining (Fig. 1A). Prior to irradiation, hardly any γ-H2AX foci was observed in the nuclei of germ cells, whereas a strong staining was observed in both male and female germ cells 1 h following irradiation. γ-H2AX staining in human irradiated ovaries and testes was identical. Similarly, pregnant mice were irradiated at 12.5 dpc and DSB formation was monitored. In mice, germ cells were recognized by MVH-staining (VASA). IRs induced the appearance of similar number of γ-H2AX foci in male and female fetal germ cells during the first hours post-irradiation (Fig. 1B and C). From 3 h, γ-H2AX foci decreased faster in female germ cells and at 24 h most γ-H2AX foci have disappeared.

#### Effect of irradiation on human fetal female germ cells in vitro

After explantation, human ovaries were γ-irradiated with 0.2–5 Gy or sham irradiated and cultured 1 day. They were then examined by electron microscopy. From 1.5 Gy, there was an increase in germ cell apoptosis appearing as the loss of local junctions with bordering cells, large nuclear condensation, cellular fragmentation (Fig. 2A) and a relative decrease in germ cell density (data not shown). We used immunohistochemistry to determine the percentage of cleaved caspase-3-positive germ cells in irradiated ovaries to evaluate apoptosis, and the percentage of BrdU-positive germ cells to evaluate proliferation. Low-dose irradiation (0.2 and 0.5 Gy) had no effect on germ cell apoptosis; there was a significant dose-dependent increase in the percentage of cleaved caspase-3-positive germ cells from 1.5 Gy (Fig. 2B). Irradiation had no effect on BrdU incorporation into germ cells, except that germ cell proliferation in ovaries irradiated with 0.2 Gy was significantly higher than that in controls (Fig. 2C). After 14 days in culture, the number of germ cells was unchanged in untreated controls; following a 1.5 Gy dose of irradiation, the germ cell number halved, whereas a low dose (0.2 Gy) of γ-rays had no effect on the germ cell number (Fig. 2D). Apoptosis was induced similarly whatever the age of the ovaries for the period studied (6.5–10 weeks pc).

To study the effect of γ-irradiation on human female germ cells through time, we irradiated human ovaries with 1.5 Gy and cultured them for 9, 24 and 48 h. In control ovaries, basal germ cell apoptosis did not change during the 48 h of culture. Nine hours after irradiation,
there was a significant increase in the percentage of cleaved caspase-3-positive germ cells (3.7 ± 0.9% for control versus 6.8 ± 2.0% for irradiated), and the percentage was even higher after 48 h (Fig. 3A). Similarly, the percentage of cleaved caspase-9-positive germ cells doubled 9 h after irradiation (3.0 ± 0.9% in the control and 6.8 ± 2.3% in the irradiated samples) (Fig. 3B). The rate of BrdU incorporation was 28.3 ± 4.5% at the beginning of culture and was not modified during the 48 h of culture either in control or in irradiated ovaries.

Figure 1  Number of γH2AX foci in male and female fetal germ cells following irradiation. For A, pieces of human fetal gonads from 8- to 10-week-old fetuses were irradiated with a dose of 1.5 Gy and cultured for 1 h and the other pieces were conserved as the control. DNA DSB in the germ cells was detected by co-staining for γ-H2AX (red) and D2-40 (green). For B and C, pregnant mice were irradiated with a 1.5 Gy dose at 12.5 dpc. At 0, 15, 30 min, 1, 3, 6 or 24 h after irradiation, mice were sacrificed and gonads were dissected out from fetuses and fixed. The number of γ-H2AX foci per germ cell was determined by co-staining for γ-H2AX (red) and MVH (green). For C, means ± SEM of 3–5 values are shown. ***p < 0.01 and ****p < 0.005, comparing ovary and testis in unpaired Student’s t-test. Arrowheads indicate germ cells. Bars represent 5 μm.
Involvement of p53 pathways in radiation-induced germ cell apoptosis in human ovary in vitro

To investigate the production and stabilization of p53 in irradiated ovary, we used p53 immunohistochemistry in human ovary 3 h after a 1.5 Gy irradiation. As previously described, in the testis p53 was present in germ cells after irradiation. In contrast, in the irradiated ovary, we could not detect p53 protein (Fig. 4A). We measured the percentage of cleaved caspase-3-positive germ cells 24 h after a 1.5 Gy irradiation in ovaries with or without pifithrin α, an inhibitor of p53 (Fig. 4B). Pifithrin α had no effect on germ cell apoptosis in either control or irradiated ovaries.

Figure 2 Effect of various doses of γ-irradiation on the apoptosis, proliferation and number of human female germ cells in vitro.
Human ovaries from 6- to 10-week-old fetuses were dissected and cultured for 1 day (A–C) or 14 days (D). Three pieces of each ovary were cultured as control samples (0 Gy) and the other pieces were irradiated with various doses: 0.2, 0.5, 1.5, 3 and 5 Gy. Germ cell apoptosis was observed by electron microscopy (A, bar represents 1 μm) and the percentage of apoptotic germ cells was measured by immunohistochemical staining for cleaved caspase-3. The germ cell proliferation index was determined by BrdU immunostaining. The number of germ cells was counted after 14 days of culture (D). Representative photographs of immunostained sections of 24 h cultured ovaries are shown in B and C (right panel). Stained germ cells are indicated with a black arrowhead and unstained germ cells with a white arrowhead. Germ cells are indicated with a black arrow in D (right panel). Means ± SEM of 3–9 values are shown; the number of ovaries analyzed for each condition is indicated in brackets. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control values in unpaired Student’s t-test. For B, C and D, bars represent 10 μm.

Figure 3 Effect of γ-irradiation on the apoptosis and proliferation rate of human female germ cells at various subsequent times.
For each human fetal ovary, three or four pieces were irradiated at a dose of 1.5 Gy and cultured for 0, 9, 24 and 48 h; the other pieces served as controls. Early and late germ cell apoptosis was measured by immunohistochemical staining of the cleaved caspase-3 (A) and cleaved caspase-9, respectively (B and D). Germ cell proliferation was determined by immunostaining for BrdU incorporation (C). In (D), germ cells immunostained for cleaved caspase-9 are indicated with black arrowheads and unstained germ cells with white arrowheads. For A, B and C, data are expressed as percentages with the value of control ovary defined as 100%. Means ± SEM of five values for A and four values for B and C are shown. *P < 0.05 and **P < 0.01, in paired statistical comparisons with the corresponding control values. Bars represent 10 μm (D).
Effect of irradiation on murine fetal female germ cells in vitro and in vivo

After explantation, 12.5 dpc murine ovaries were γ-irradiated, or not, at a dose of 1.5 Gy and cultured for 24 and 48 h. As in irradiated human ovary, electron microscopy revealed germ cells presenting the typical features of apoptosis 24 h post-irradiation (Fig. 5A). Germ cell apoptosis indicated by immunostaining of cleaved caspase-3 was unchanged in control ovaries after 48 h of culture. The number of apoptotic germ cells in irradiated ovaries 24 h after irradiation was three times higher than that in control ovaries (2.5 ± 0.5% for control and 7.4 ± 1.5% for irradiated) and had further increased 48 h post-irradiation (from 2.5 ± 0.4% to 10.7 ± 1.8%) (Fig. 5B). Irradiation had no significant effect on germ cell proliferation (Fig. 5C). The number of germ cells decreased significantly after irradiation from 6244 ± 1131 germ cells in the controls versus 2489 ± 1030 in the irradiated samples after 24 h and from 5283 ± 590 in the control to 1706 ± 406 in the irradiated ovaries after 48 h (Fig. 5D). Consistent with these data, 48 h after in vivo γ-irradiation at

![Figure 4](https://academic.oup.com/humrep/article-abstract/24/3/670/642969/0)  
Study of the involvement of the p53 signaling pathway in the radiation-induced death of human germ cells.  
Pieces of human gonads were cultured as controls or were irradiated at a dose of 1.5 Gy. For A, pieces of testis and ovary were fixed 3 h after being exposed or not to γ-rays and p53 was detected by immunohistochemistry. For B, pieces of ovaries were cultured for 24 h in a medium with DMSO or supplemented with pifithrin α, an inhibitor of the p53 pathway. Ovaries were fixed and germ cell apoptosis was measured by immunohistological staining for cleaved caspase-3. In (A), p53 immunostained germ cells are indicated with black arrowheads and unstained germ cells with white arrowheads. Means ± SEM of four values for B are shown. In B, different letters represent values differing significantly in ANOVA comparisons, with P<0.05. Bars represent 10 μm (A).

![Figure 5](https://academic.oup.com/humrep/article-abstract/24/3/670/642969/1)  
Effect of γ-irradiation on the apoptosis, proliferation and number of mouse female fetal germ cells in vitro.  
Murine ovaries from 12.5 dpc fetuses were dissected out and cultured for 24 or 48 h. One ovary was cultured as a control and the other was irradiated at a dose of 1.5 Gy. Germ cell apoptosis was followed by electron microscopy (A, bar represents 1 μm) and the percentage of apoptotic germ cells was measured by immunohistological staining for cleaved caspase-3 (B). Germ cell proliferation was evaluated by immunostaining for BrdU incorporation (C). For B, C and D (left panel), data are expressed as percentages of the value for the control ovary (defined as 100%). The number of germ cell per ovary was determined (D). On the right panel for B, C and D, the photograph shows ovary cultured for 48 h. Stained germ cells are indicated with black arrowheads and unstained germ cells with white arrowheads. In D, giant germ cells are indicated with black arrows. Means ± SEM of 4 or 5 values are shown in B, C and D. *P<0.05, **P<0.01 and ***P<0.001 in paired Student’s t-test. For B, C and D, bars represent 10 μm.
For B and C, data are expressed as percentages of the value for a control (Fig. 5D, right panel). These cells contained MVH protein, a protein characteristic of germ cells (data not shown).

**Figure 6** Study of the involvement of p53 and p63 in radiation-induced germ cell apoptosis in murine fetal ovary in vivo

We investigated the involvement of p53 in the radiation-induced death of murine germ cells by adding pifithrin α in vitro. Addition of pifithrin α had no significant effect on germ cell number or on apoptosis in irradiated ovaries (Fig. 6A and B). We then studied the effect of p53 knockout in vivo on germ cells surviving IR: 12.5 dpc fetuses were irradiated in utero and gonads were removed at 18.5 dpc. First, no effect of the knockout of p53 on germ cell number was observed in control gonads in which the number of germ cells was 11 200 ± 2307 for the testis and 3879 ± 1035 for the ovary. There was no significant difference between p53+/+ and p53−/− ovaries after irradiation, whereas in testis there was a significant increase of germ cell number in the p53−/− samples (Fig. 6C and D).

p63 is closely related to p53 and was recently reported to be involved in female germ cell apoptosis during postnatal life. Therefore, we investigated whether p63 invalidation prevented radiation-induced death of fetal germ cells. Similarly to the knockout of p53, the knockout of p63 had no effect in control gonads. For irradiated ovaries, knockout of p63 had no effect on the germ cell number (553 ± 164 germ cells in irradiated p63+/+ ovary and 531 ± 85 in irradiated p63−/− ovary), whereas in irradiated testis, there were significantly more germ cells in p63−/− than p63+/+ testis (304 ± 68 germ cells in p63−/− versus 1083 ± 148 germ cells in p63+/+ testis) (Fig. 6C and D).

**Discussion**

The results of our study suggest that female fetal germ cells undergo much less apoptosis than male fetal germ cells after irradiation. Also, the radiation-induced death of the oogonia is p53-independent both in human and in mice.

The importance of the oogonial proliferation for the establishment of a stock of female germ cells has long been known. Previous work has proven that in utero irradiation of rats and mice during the oogonia stage severely depletes the follicle stock after birth; however, details of the sensitivity of human oogonia were unknown. In rat and mouse, a dose of 1.5 Gy at the oogonia stage severely depletes the follicle stock after birth; and above trigger oogonia apoptosis in human and mouse ovary. Electron microscopy confirmed that the radiation-induced death of female fetal germ cells presented the typical features of apoptosis, and caspases-3 and -9 were activated during this process. As a result, the germ cell number decreased, consistent with apoptosis induction.

Surprisingly, low doses (below 0.5 Gy) had no detectable effect on human oogonia apoptosis. This result was unexpected as IRs induce 12.5 dpc, there was a significant decrease in the number of germ cells in the ovaries (1908 ± 299 versus 7335 ± 549 in control 14.5 dpc ovaries). Interestingly, 48 h after in vitro or in vivo γ-irradiation, we observed some huge cells with a large nucleus in the ovaries (Fig. 5D, right panel). These cells contained MVH protein, a protein characteristic of germ cells (data not shown).

**Involvement of p53 and p63 in radiation-induced germ cell apoptosis in murine fetal ovary in vivo**

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similar DNA damage in both male and female germ cells with regard to DSBs and, as we demonstrated previously, human male germ cells at the equivalent stage are highly radiation sensitive. Indeed, doses as low as 0.1 Gy increased apoptosis rates in human fetal testes (Lambrot et al., 2007). Furthermore, irradiation of the testis in organ culture with a dose of 1.5 Gy increased the percentage of cleaved caspase-3-positive germ cells by about 4-fold after 24 h (Lambrot et al., 2007), whereas similar treatment of the ovary increased this measure by only about 2-fold; after long-term culture (1 or 2 weeks later), only 7% of the male germ cells but more than 40% of the female germ cells survived. Interestingly, the percentages of BrdU-positive germ cells were similar in the human fetal ovary and testis after irradiation. These findings demonstrate that proliferating fetal germ cells respond differently to a genotoxic stress according to their sex, with male germ cells being more susceptible to apoptosis. Surprisingly, only the 0.2 Gy dose affected BrdU incorporation and induced a moderate increase in oogonia proliferation. We propose that low doses of radiation may induce an adaptive response, as has been previously reported in some adult stem cells (Wang and Cai, 2000; Liu et al., 2007).

We showed previously that IR induces p53 phosphorylation in the human fetal testis, and that the p53 inhibitor, pifithrin α, decreases radiation-induced apoptosis (Lambrot et al., 2007). Here, we detected no p53 accumulation in irradiated human fetal ovaries and pifithrin α did not prevent female germ cell apoptosis. Moreover, we detected some giant germ cells in irradiated mouse fetal ovaries. Such giant cells have previously been observed in p53 knockout testes after irradiation where they were considered to represent eliminated damaged spermatogonia associated with the absence of p53 (Beumer et al., 1998). These data fit with our results in vivo from mice lacking p53 indicating that human and mouse fetal germ cells share similar apoptotic pathways. Indeed, irradiation induced a similar germ cell loss in fetal ovaries of wild type and p53-deficient mice, whereas p53 knockout reduced radiation-induced germ cell loss in the fetal testis. An observation consistent with our findings has previously been reported: p53 promotes spontaneous apoptosis of mouse fetal germ cells in the testis but not in the ovary (Matsui et al., 2000). It was recently proposed that p53 is the guardian of the genome of somatic cells and that p63, the most ancient member of the p53 family, may replace p53 as the guardian of the genome in the female germ line (Suh et al., 2006; Livera et al., 2008). Therefore, we examined the radiation sensitivity of the ovaries of mice with p63 knockout: the absence of p63 had no effect on ovarian germ cell survival during fetal life. However, p63 knockout increased germ cell survival in the fetal testis after irradiation. In the human ovary, we and others previously reported that p63 was absent in the oogonia (Kurita et al., 2005; Livera et al., 2008). It seems therefore that, in mice and humans, both p53 and p63 are involved in the response to genotoxic stress in male germ cells but not in female germ cells. This might explain why mammalian female germ cells are less radiosensitive than male germ cells during fetal life.

Fetal mitotic germ cells are believed to be bipotential. Indeed, elegant germ cell sex reversal experiments have demonstrated that early germ cells are undifferentiated in both sexes and present a remarkable plasticity (McLaren, 1997; Bowles et al., 2006): in mice, at 12.5 dpc, these germ cells may follow either the male or the female pathway independently of their genetic sex. It may therefore be logical to consider them as equivalent. In this regard, our demonstration that a genotoxic stress prior to the differentiation of the germ cells along the male or the female pathway affects differently male or female germ cells is particularly intriguing and suggests that these cells are not equal. It has been proven that it is the somatic environment that dictates the male or the female differentiation of these early germ cells. We therefore propose that it could equally be the environment that controls the response of these germ cells to an apoptotic stimulus. Alternatively, apoptotic pathways in undifferentiated male and female germ cells may differ fundamentally, independent of their environment.

Exposure of proliferating somatic cells to IRs usually induces cell cycle arrest in either S, G1 or G2 phase to allow the repair of the DNA lesions or apoptosis (Houtgraaf et al., 2006). Adult stem cells generally respond similarly with both a decrease in cell proliferation and an increase in apoptosis; both these phenomena depend upon p53 activation (Lee and Bernstein, 1993; Kato et al., 2007). Interestingly, we detected no germ cell cycle arrest in either mouse or human fetal ovary following irradiation, either at any dose or at any time studied. Similarly, there was no change in BrdU incorporation in the human fetal testis following irradiation. It seems therefore that fetal germ cells have an unusual response to genotoxic insult: there is no slowing of their cell cycle to try to repair the DNA damage but rather direct entry into a cell death program. This could be a particularly drastic mechanism to prevent the transmission of putative repair errors into the germ line and to the genome of the progeny. The high radiation sensitivity of the fetal testicular germ cells is consistent with this notion: it avoids the transmission of mutations to the spermatogonia that will divide many times throughout adult life and may generate oncogenic lesions and propagate damaged genomes. This situation bears similarities with embryonic stem cells that have also been reported to be hypersensitive to DNA damage (de Waard et al., 2003) and do not undergo cell cycle arrest after DNA damage (Aladjem et al., 1998). The low radiation sensitivity of the germ cells in the fetal ovary, following the same line of argument, appears to be a serious risk. Possibly, the oogonia are ‘differentiation-committed’ cells that can only divide a limited number of times before initiating meiosis and this would result in a smaller risk of malignant transformation.

Lastly, though a similar amount of DNA damage is observed in male and female germ cells, DNA repair occurs faster in oogonia when compared with male fetal germ cells. γH2AX foci disappeared rapidly in female as early as 3 h after irradiation. This indicates that DNA damage sites have been repaired as it occurred too rapidly for the cells to have been lost by apoptosis. We hypothesize that this fast DNA repair in females may be due to the expression of some elements of the meiotic DNA repair machinery long before meiosis.

In summary, this work demonstrates major differences between proliferating fetal male and female germ cells. It provides a solid basis for future investigations of the apoptotic pathways in fetal germ cells. Indeed, it would be valuable to address two major questions: (i) Do progenitors of the female gametes repair their DNA faithfully after an assault by damaging agents? (ii) Are these insulted cells able to form mature gametes?

Author’s role

M.-J.G. helped conceive the study, designed and carried out organ culture experiments. C.D. carried out some of the IHC experiments. M.B. carried out electronic microscopy studies. H.C., V.R.-F., R.L. and R.F. designed and carried out the methodology for human fetal
gonad culture model. G.L. and R.H. helped conceive, design and coordinate the study, carried out some gonadal culture experiments. All authors helped to draft the manuscript, read and approved the final manuscript.

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