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Ovarian morphometrics in *TP53*-deficient mice¹

Brenda M. Alexander², Edward A. Van Kirk, Leann M.A. Naughton, and William J. Murdoch

Department of Animal Science, University of Wyoming, Laramie

Abstract

The objective of these investigations was to characterize ovarian responses to hormonal stimulation in *TP53*-deficient mice. *TP53*-deficient (KO) and wild type (WT) mice were induced to ovulate with pregnant mare serum gonadotropin followed by human chorionic gonadotropin. Effect of estradiol on ovarian morphology was determined in induced and control mice implanted with estradiol containing or placebo pellets. Blood was collected and mice were killed 7 d following implantation. Preserved ovaries were serially sectioned and stained. Numbers of follicles (all classifications) decreased with ovulation induction, but did not differ between WT and KO mice. Numbers of corpora lutea (CL) were less in ovulation-induced KO mice treated with estradiol compared to WT mice. Area of individual CL and serum concentrations of progesterone were greater in ovulation-induced KO mice given estradiol compared to WT mice. Ovulation-induced KO mice had more, larger hemorrhagic follicles than similarly treated WT mice, but hemorrhagic follicles were not influenced by estradiol. Proliferation of ovarian surface epithelial cells did not differ between KO and WT mice induced to ovulate and given estradiol. Ovaries from *TP53* gene knockout mice (n = 4) induced to ovulate and given a 21 d estradiol implant three times over 58 d were observed for precursor lesions. There was no indication of precursor lesions in any *TP53* KO or WT mouse. *TP53* status did not influence recruitment of follicles, but *TP53* deficiency hindered the ability of hCG to cause ovulation.

Keywords

P53 Knockout; ovulation; mice

Introduction

In the ovary, cell death is necessary to maintain cyclicity and ensure specie-typical ovulation rate. Only a small fraction of follicles mature and ovulate with most follicles becoming atretic. Apoptosis initiates follicular atresia in most vertebrate species with *TP53* as an atretogenic factor (Hussein, 2005).

The role of *TP53* in tumorigenesis has been well defined. Mutations of the *TP53* tumor-suppressor gene are the most frequently observed genetic lesion in all human cancers (Donehower et al., 1992). These lesions are present in the majority of early stage serous carcinomas (Leitao et al., 2004), the most common variant of ovarian cancer.

The *TP53* protein responds to a variety of cellular stresses by arresting the cell cycle in the G1 phase allowing time for DNA repair or initiation of apoptosis (Levine, 1997). Expression

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²Correspondence: Dept. of Animal Science, 1000 E. University Ave. Dept. 3684, Laramie, WY 82071, phone (307) 766-2224, fax (307) 766-2355, email balex@uwyo.edu.

of *TP53* is increased in epithelial cells associated with the ovulatory site. In perimeter cells, increased expression of *TP53* was coincident with the expression of the base-excision repair enzyme polymerase β (Murdoch, 2003) implicating *TP53* for cell survival in the ovary.

Estrogen is luteotrophic in the mouse (Bachelot and Binart, 2005) but may also play an important role in ovarian carcinogenesis. Ovarian surface epithelial cells (normal and transformed) express estrogen receptors and affect tumor progression by increasing cell proliferation and motility (Cunat et al., 2004).

Female mice deficient in the *TP53* gene have reduced fertility with normal vaginal cell cyclicity (Embree-Ku and Boekelheide, 2002). However, the ovarian response of *TP53* knockout mice to hormonal stimulation has not been characterized and was the objective of the current investigation. Ovarian morphology of *TP53* knockout mice was characterized and compared to wild type mice in naturally cycling mice and following gonadotropin stimulation in the presence or absence of exogenous estradiol.

Materials and Methods

Experiments were approved by the University of Wyoming Animal Care and Use Committee. *TP53* gene deficient and wild type mice (6 – 8 wks) were obtained from Taconic Farms Inc. (Germantown, NY). Mice had free access to water and standard rodent chow (PMI Nutrition International LLC; Brentwood, MO) and were exposed to 12L: 12D photoperiod. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

To delineate the roles of ovulation and estrogen exposure on ovarian morphology and epithelial carcinomatosis, *TP53* gene-knockout (KO; n = 12) and wild type (WT; n = 10) mice were induced to ovulate using pregnant mare serum gonadotropin (PMSG; 10 iu) followed by human chorionic gonadotropin (hCG; 10 iu) 48 h later given intraperitoneally. Control mice (n = 10 WT; n = 12 KO) were injected with equal volumes of phosphate buffered saline (PBS). Effect of exogenous estradiol on ovarian morphology in *TP53* KO and WT mice was determined in induced and control mice implanted with estradiol 17 β (E-121) or placebo (C-111) implants (1.5 mg; Innovative Research of America; Sarasota, FL) 24 h following hCG in a 2 x 2 x 2 factorial design. On d 7 following implantation, blood was collected from the retro-orbital plexus by capillary tube and mice were killed by cervical dislocation (JAVMA, 2001). Serum was harvested 4 h after collection and stored at -4°C .

Serum concentration of progesterone (McDonnell et al., 2003) was determined in a single radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) with intra-assay coefficient of variation of < 1%. The progesterone assay is sensitive to 0.0156 ng. Dilutions of serum paralleled the standard curve with recovery of spiked serum > 98%.

Serum concentration of estradiol was determined by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Anti-estradiol antibody and radiolabeled estradiol was diluted 1:2 in PBS containing 0.1% gelatin with 1:400 rabbit sera added to the primary antibody. Serum (50 μl) was brought to 400 μl with PBS and extracted one time in 5 ml diethyl ether (Fisher Scientific Int., Hampton, NH). The ether layer was decanted and dried with 200 μl of PBS 0.1% gelatin added following drying. Extraction recovery of H^3 -Estradiol (New England Nuclear, Boston, MA) was 68.3% with 1.6% coefficient of variation. Reported values are corrected for extraction efficiency. Dilutions of mouse serum paralleled the standards curve. Intra-assay coefficient of variation was less than 5 %.

Reproductive tracts were collected and preserved by overnight emersion in Histochoice fixative (Amersco; Solon, OH) and stored at room temperature in 70% ethanol. Ovaries (one

per mouse) were dissected, dehydrated, cleared, infiltrated with paraffin wax, and serially-sectioned at 8 μ m throughout the ovary. Sections were mounted on gelatin-chromium subbed slides and stained in hematoxylin and eosin using standard procedures. Digital micrographs of ovarian structures on approximately every tenth section were taken using an Olympus SZX12 (40x; Olympus, Hamburg, Germany) microscope and Spot RT Slider Camera (Diagnostic Instruments, Sterling Heights, MI).

To ensure that epithelial cells had similar stimulation from endogenous hormones, only sections containing a preovulatory tertiary follicle were analyzed for epithelial cell proliferation. Digital images of epithelial cells surrounding the ovarian interstitium were captured at 200x from KO and WT mice induced to ovulate and given exogenous estradiol using an Olympus Vannox (Olympus, Hamburg, Germany) microscope and Spot RT Slider Camera. Images were analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

A second group of *TP53* KO mice ($n = 4$) were induced to ovulate as indicated above and give a 1.5 mg sustained release, 21 d estradiol 17 β implant (E-121; Innovative Research of America; Sarasota, FL). Ovulation induction followed by estradiol implant was repeated 2 more times over a 58 d period with ovaries collected 7 d following the third estradiol implant. Ovaries were collected and processed as stated above but were only observed for precursor lesions.

Numbers and size of antral and total follicles (containing an oocyte), corpora luteal structures (CL), and hemorrhagic follicles were quantified per section evaluated. Averages per animal were analyzed for effect of *TP53* gene (animal), ovulation-induction (gonadotropin), exogenous estradiol, and their interactions in a 2 x 2 x 2 factorial design using GLM methods of SAS (Ver 9.1; SAS Institute, Cary NC). Proportion of total ovarian area occupied by simple (squamous and cuboidal), columnar, and proliferative epithelial cells was compared between KO and WT mice using GLM procedures of SAS (Ver 9.1; SAS Institute, Cary NC). Least squared means and associated standard errors are reported.

Results

TP53 gene-knockout mice had morphologically normal ovaries (Fig. 1) and responded to gonadotropin stimulation. Treatment with gonadotropins decreased ($P = 0.04$) the number of preantral follicles while increasing ($P < 0.001$) the number of luteal structures per section. Although total ($P \geq 0.20$) and number of large tertiary ($P \geq 0.23$) follicles did not differ by *TP53* status of the animal or any animal by treatment interaction ($P \geq 0.23$), numbers of CL structures were less ($P = 0.02$) in ovulation-induced KO mice treated with estradiol than similarly treated WT mice (Fig. 2). An animal by gonadotropin by implant interaction ($P < 0.001$) revealed KO mice treated with exogenous estradiol had a greater ($P = 0.02$) average sized CL than similarly treated WT mice. Average area of individual CL was less ($P < 0.001$) in KO mice not induced to ovulate receiving a placebo implant when compared to similarly treated WT mice (Fig. 3). Within animal type, exogenous estradiol increased ($P < 0.01$) the mean area of CL in ovulation-induced mice compared to mice receiving the placebo implant (Fig. 3).

Serum concentrations of estradiol were increased ($P < 0.001$) in mice receiving estradiol containing implants (2966.3 ± 324.0 pg/mL) compared to placebo implanted (< 0.195 pg) mice. There was a trend ($P = 0.07$) for an animal by implant interaction. *TP53*-deficient mice with estradiol-containing implants had greater ($P < 0.001$) serum concentrations of estradiol than estradiol-implanted WT mice (4213.8 ± 314.3 vs. 1718.6 ± 566.7 pg/mL).

Serum concentrations of placebo-implanted KO and WT mice were below detectable levels at a 50 μ l sample volume.

Serum concentrations of progesterone differed ($P = 0.03$) with an animal by gonadotropin by implant interaction. Mice deficient in the *TP53* gene induced to ovulate and given exogenous estradiol had increased ($P = 0.04$) serum concentrations of progesterone when compared to similarly treated WT mice (Fig. 4). Differences were not noted ($P > 0.05$) among KO and WT mice induced to ovulate and give a placebo implant or vehicle treated mice given estradiol alone (Fig. 4).

The presence of large hemorrhagic follicles was most apparent in *TP53*-deficient mice treated with gonadotropins (Fig. 5). Numbers of hemorrhagic follicles were greater ($P = 0.004$) in KO compared to WT mice (1.3 vs. 0.4 [± 0.2], respectively). Hemorrhagic follicles comprised the greatest ($P = 0.02$) percentage of the ovarian area in KO mice treated with gonadotropins. The percentage of ovarian area occupied by hemorrhagic follicles did not differ ($P \geq 0.16$) among PBS treated KO and WT mice (Fig. 6). Exogenous estradiol did not interact ($P \geq 0.16$) with gonadotropin treatment to influence the occurrence or area of hemorrhagic follicles.

Linear circumference of the ovarian surface, as a percentage of the total ovarian circumference, occupied by simple ($P = 0.98$), pseudostratified ($P = 0.76$) and proliferative ($P = 0.64$) ovarian surface epithelial cells did not differ between KO and WT mice induced to ovulate and given exogenous estradiol (Fig. 7).

A follicle containing multiple oocytes was observed in a KO mouse receiving vehicle treatment and a placebo implant. Inclusion cysts or precursor lesions were not observed in KO mice induced to ovulate a single time or in KO mice induced to ovulate multiple times over a 58 d period.

Discussion

TP53-deficient mice are reported to have decreased fertility and litter size with normal vaginal cyclicity (Embree-Ku and Boekelheide, 2002). In the present study, recruitment and development of preovulatory follicles was not dependent on a functional *TP53* gene since numbers of preovulatory follicles were similar in KO and WT mice. Numbers of luteal structures were reduced in *TP53*-deficient mice induced to ovulate and given estradiol and likely reflect a reduction in ovulation rate in *TP53*-deficient mice. Estradiol is luteotrophic in the mouse (Bachelot and Binart, 2005) and luteal structures present 7 d following hCG likely reflect ovulatory events in response to gonadotropin treatment in mice treated with estradiol.

Since numbers of preovulatory follicles were similar, the reduction in ovulation rate and fertility in *TP53*-deficient mice cannot be accounted for by a reduction in follicle recruitment or development. The role of *TP53* in apoptosis and/or degradation of the basement membrane may hinder ovulation and predispose *TP53*-deficient mice treated with gonadotropins to develop large hemorrhagic follicles. Tumor necrosis factor α interacts with *TP53* and has an obligatory role in ovulation (Murdoch and Martinchick, 2004; Claycombe and Meydani, 2001). Alternatively, attenuation of *TP53*-mediated apoptosis in granulosa cells from *TP53*-deficient mice may hinder the ability of antral follicles not destined to ovulate to undergo atresia (Amsterdam *et al.*, 2003) in these animals. Nuclear accumulation of *TP53* protein has been documented in atretic follicles from the rat ovary (Choi *et al.*, 2004).

Mutations of the *TP53* gene occur in the majority of all ovarian cancers. Although *TP53* KO mice rapidly develop tumors (primarily lymphomas and sarcomas; Donehower et al., 1992) and have proven useful in the study of many cancers, *TP53*-deficient mice are not known to develop ovarian cancer. The limited life-span of *TP53* KO mice may not allow sufficient time for ovarian cancer to develop. However, it is possible that oxidative DNA damage that occurs during ovulation (Murdoch and Martinchick, 2004) would go unrepaired in *TP53* KO mice induced to ovulate and exogenous estradiol would increase proliferation and motility (Cunat et al., 2004) of DNA-damaged ovarian surface epithelial cells.

Ovarian surface epithelial cells proliferate in response to gonadotropin stimulation (Burdette et al., 2006). Proliferation of ovarian surface epithelial cells was not more extensive in *TP53* KO mice induced to ovulate and given exogenous estradiol when compared to similarly treated WT mice. The unexpected lack of epithelial cell proliferation in KO mice may be due to the reduced number of ovulatory events in these animals, and a consequent reduction in the potential for unrepaired oxidative DNA damage (Murdoch and Martinchick, 2004). Ovulation did, however, occur in the KO mice and the relative reduction in ovulation rate may not completely account for the lack of proliferation of ovarian surface epithelial in KO mice.

Increased serum concentrations of progesterone in the KO mice may have actively inhibited proliferation of ovarian surface epithelial cells. McDonnell et al. (2003) showed a decrease in the number of colonies formed when human epithelial derived ovarian cancer cells (SKOV-3) were treated with progesterone *in vitro*. *TP53*-deficient mice induced to ovulate and given exogenous estradiol had larger individual CL structures than did similarly treated WT mice which may account for the increased serum concentrations of progesterone. However since serum concentrations of estradiol were also increased, clearance of steroid hormones may be reduced in KO mice. Matoba et al. (2006) reported reduced oxygen consumption in liver mitochondria from *TP53*-deficient mice when compared to WT mice. Since no differences in serum estradiol were detected among ovulation-induced KO and WT mice given the placebo implant, it is unlikely that the presence of potentially estrogenic hemorrhagic follicles in the KO mice account for the increase in estradiol in these animals. The greater mean area of CL structures in vehicle treated placebo-implanted WT mice is likely a reflection of a reduced basal ovulation rate in *TP53* KO mice.

Ovarian morphology from *TP53*-deficient mice induced to ovulate and given estradiol indicate a role for *TP53* in ovulation and/or follicle atresia. However, the lack of a functional *TP53* gene did not cause proliferation of ovarian surface epithelial cells or predispose ovulation-induced mice treated with exogenous estradiol (one or multiple times) to develop ovarian cancer or precursor lesions before the KO mice would be expected to succumb to other more aggressive tumors.

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Figure 1.

Representative micrographs from *TP53*-deficient (KO) and wild type (WT) mice. Mice were treated with phosphate buffered saline (PBS) and given a placebo implant. Bar = 250 μ m.

Figure 2.

Average number of corpora luteal structures (CL) per section present on *TP53*-deficient (KO) and wild type (WT) mice ovaries 7 d following vehicle (VEH) treatment or ovulation induction with gonadotropins (SO). Mice were implanted with sustained-release estradiol (E) or placebo (P) pellets. *Difference between KO and WT mice ($P = 0.02$).

Figure 3.

Average area of corpora luteal structures (CL) present on *TP53*-deficient (KO) and wild type (WT) mice ovaries 7 d following vehicle (VEH) treatment or ovulation induction with gonadotropins (SO). Mice were implanted with sustained-release estradiol (E) or placebo (P) pellets. *Difference between KO and WT mice ($P \leq 0.02$).

Figure 4.

Serum concentrations of progesterone (ng/mL) in *TP53*-deficient (KO) and wild type (WT) mice 7 d following vehicle (VEH) treatment or ovulation induction with gonadotropins (SO). Mice were implanted with sustained-release estradiol (E) or placebo (P) pellets.

*Difference between KO and WT mice ($P = 0.04$).

Figure 5. Representative sections (40x) of *TP53*-deficient (KO) and wild type (WT) mice induced to ovulate with gonadotropins and receiving a placebo implant. Hemorrhagic follicles are denoted by arrows. Bar = 250 μ m.

Figure 6. Area (mm²) of hemorrhagic follicles as a percentage of total ovarian area in *TP53*-deficient (KO) and wild type (WT) mice 7 d following vehicle (VEH) treatment or ovulation induction with gonadotropins (SO). *Difference between KO and WT mice ($P < 0.001$).

Figure 7. Percent (%) of total surface area occupied by simple, pseudo stratified (Pseudo), and stratified ovarian surface epithelial cells in *TP53*-deficient (KO) and wild type (WT) mice. Cell type did not differ by *TP53* status of the mice (SEM $\leq$ 5.0).