Absence of p53 and FasL Has Sexually Dimorphic Effects on Both Development and Reproduction

MICHELLE EMBREE-KU AND KIM BOEKELHEIDE[1]

Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island

Reproduction and development are highly dependent on apoptosis to balance the proliferation that necessarily occurs during these processes. How the absence of two apoptotic factors in mice would affect reproduction and development was examined. Given previous reports of increased neural tube defects in p53–/– female fetuses, decreased fertility in *gld* female mice, and altered spermatogenesis in both p53 and *gld* male mice, the possibility that these phenotypes might be enhanced by the elimination of a second apoptotic factor was investigated. The reproductive vigor and the health of offspring were monitored during the production of the new double-deficient strain (FasL–/–p53–/–) for any changes from the reported phenotypes. Thus, any unusual phenotypes that could lead to new models for studying mechanisms of health and disease would be identified. Double-deficient male offspring appeared healthy and occurred at expected frequencies. Additionally, spermatogenesis and male fertility were unaffected by the gene deficiencies. On the other hand, FasL+/+p53–/– and FasL–/–p53–/– female mice were susceptible to increased malformations and post-natal death. These abnormalities were consistent with previous reports of neural tube defects in p53–/– female mice. Fertility rates were also significantly decreased in p53–/– female mice that lived to be adults, an observation not previously reported. Finally, the absence of both FasL and p53 led to dystocia in pregnant female mice, suggesting that the two genes play complementary roles in parturition. Therefore, although male mouse development and reproduction remained unaffected by p53 and FasL deficiencies, female mouse development was adversely affected by the absence of p53, and no live litters were born to female mice with the combined absence of both FasL and p53. In this report, we suggest a potential mechanism involving corpora luteal regression to explain this defect in parturition in FasL–/–p53–/– female mice.

[Exp Biol Med Vol. 227(7):545–553, 2002]

Key words: apoptosis; reproduction; development; p53; FasL

This work was supported, in part, by NIEHS grant RO1-ES05033 and by the Burroughs Wellcome Fund.

[1] To whom requests for reprints should be addressed at Department of Pathology and Laboratory Medicine, Box G-B5, 171 Meeting Street, Brown University, Providence, RI 02912. E-mail: Kim_Boekelheide@Brown.edu

Received February 14, 2002.
Accepted April 9, 2002.

1535-3702/02/2277-0545$15.00

Apoptosis is as important to reproductive health as is cellular proliferation. It plays a role in everything from spermatogenesis to remodeling of tissues during embryogenesis. Disruption of normal apoptotic mechanisms can effect changes in development of the reproductive organs, regression of the endometrium during the estrous cycle, limitation of spermatocyte numbers, regression of Graafian follicles, and remodeling of hormone-responsive tissues such as prostate and breast. The importance of apoptotic factors in reproduction is most evident in transgenic and mutant mice, which are rendered sterile or super-fertile due to alterations in apoptotic genes. Examples of such mice are bax knock outs, in which spermatogenesis is disrupted (1) and oogenesis is prolonged (2), bcl-2 knock outs, in which the number of oocytes and primordial follicles are decreased in the post-natal mouse (3), and bcl-2 transgenic mice, in which not only are spermatogenesis and oogenesis altered (4–7), but vaginal opening is also prevented (8). Here, we examine the role of p53 and the Fas system, two other important apoptotic factors, in murine reproduction and development.

Fas (CD95/Apo-1) is a cell surface protein in the tumor necrosis factor receptor family and is activated by its ligand, FasL. When engaged, Fas initiates a cascade of enzymatic activation that results in apoptosis. Mice defective in Fas (*lpr* and lpr^{cg} mice) or FasL (*gld* mice) develop obvious lymphoproliferative and autoimmune disease (9), and they have provided a model for studying the role of Fas in immune system function. As a result, the role of the Fas system in immune cells is particularly well defined. p53, on the other hand, is an intracellular protein that plays several roles in cell cycle checkpoints, apoptosis induction, and DNA repair; which role it plays depends on the cell type, the state of the cell, and the initiating signal (10, 11). The role of p53 in tumorigenesis is particularly well defined, and, as expected, p53 knock-out mice rapidly develop tumors. However, given the number of reproductive and developmental processes that depend on apoptosis, it is surprising that fertility and viability in *lpr*, *gld*, and p53 knock-out mice are relatively unaffected.

Evidence that p53 and Fas systems are important in

reproductive processes comes from expression patterns of these molecules in the reproductive tracts of both males and females. Immunohistochemical localization in female human and rat shows p53 expression in granulosa cells during both luteolysis and atresia (12–15). Additionally, there are several reports of immunohistochemical localization of Fas to granulosa cells of ovarian follicles (15–18) and oocytes (19). Similarly to p53, expression of Fas and FasL has been detected in the male reproductive tract. Both Fas and p53 are expressed in spermatocytes, and Fas is expressed in spermatids (20–24). Consistent with a model in which Sertoli cells limit the number of germ cells that they support via Fas/FasL interactions, FasL expression has been found in Sertoli cells (20, 21, 25, 26).

Additional evidence of the importance of Fas ligand and p53 in reproduction at the functional level comes from reports of altered fertility in mice lacking these genes. *Gld* mice have been reported to have reduced litter sizes and increased resorption rates (27), presumably due to the loss of FasL as a factor important in maintaining the immune-privilege status of the placenta. However, this hypothesis has been disputed (28). Male mice lacking p53 have been reported to be sterile when the gene knock out is expressed on a 129 background (22), and alterations in spermatogenesis have been reported (22, 24, 29). Likewise, increased testis weights and spermatid head counts have been reported for *gld* male mice (30). Overall, though, the reported alterations in reproductive function are qualitatively mild.

On the other hand, although reproductive success remains relatively unaffected by the elimination of FasL or p53, previous reports indicate that up to 80% of p53-deficient female mice die due to exencephaly during development (31, 32). Because most mutant mouse models for neural tube defects are affected to a great extent by the genetic background on which the mutation is expressed (33), modifying factors could play a role in determining the extent of the expression of the defect. We sought to determine whether the Fas system might be one of these modifying factors and whether the absence of FasL would exacerbate the p53-null exencephalic phenotype.

Mice with a targeted disruption of the p53 tumor suppressor gene were crossed with *gld* mice (deficient in Fas ligand) with the goal of making mice deficient in both p53 and the Fas ligand. p53 knock-out mice are transgenic for a neomycin resistance gene insertion, which results in the loss of exons 2–6 of the p53 gene (34), and the *gld* mice are the result of a spontaneous mutation in the FasL gene (35). The resulting thymidine to cytidine missense mutation changes Phe^{273} to Leu^{273} in the C-terminus (35), rendering the Fas ligand unable to successfully interact with the Fas receptor (36, 37). The fact that animals with either the *gld* mutation or the p53 mutation are at least partially fertile and viable suggests either a redundancy in the apoptotic systems or a lack of requirement for these particular systems during development and reproduction. By eliminating both of these apoptotic factors in mice, we have addressed the question of whether these two particular systems provide redundant pathways in reproductive physiology and normal development.

Materials and Methods

Animals. Mice on a mixed background were bred and maintained in four separate in-house colonies. Each colony was derived from crosses of F1 generation mice resulting from the cross of an initial stock of 5 p53–/– male mice (C57BL/6J-*Trp53*^{tm1Tyj}) and 15 *gld* female mice (B6Smn.C3H-*Tnfsf6*^{gld}) (The Jackson Laboratory, Bar Harbor, ME). Animals were housed in humidity- (30–70%) and temperature- (74 ± 2°F) controlled rooms and maintained on an alternating 12-hr light–dark cycle. Animals had access to Purina Rodent Chow 5001 and water *ad libitum*. All procedures involving animals were performed in accordance with the guidelines of Brown University's Institutional Animal Care and Use Committee in compliance with guidelines set by the National Institute of Health. Tail tips (0.4 cm) for genotyping were collected from 21- to 28-day-old mice while mice were anesthetized by inhalation of Metophane (Schering-Plough, Union, NJ). Animals were individually identified at the time the tail tips were collected with either a unique combination of clipped toes and ear punches or a microchip implanted subcutaneously (BioMedic Data Systems, CO).

Genotyping. Template DNA (25 ng) was used for a 50-μl multiplex PCR reaction containing 200 μ*M* dNTPs (Gibco, Gaithersburg, MD), 1× PCR buffer (Perkin-Elmer, Branchburg, NJ), 6 m*M* $MgCl_2$ (Perkin-Elmer), 0.05 U/μl of AmpliTaq Gold (Perkin-Elmer), 0.4 μ*M* of each primer set: FasL primers (5′ ataggtcttaagaagactctcattcaag 3′ and 5′ tgatcaattttgaggaatctaaggcc 3′), neomycin primers (5′ aggtgagatgacaggagatc 3′ and 5′ cttgggtggagaggctattc 3′), p53 primers (5′ gcgtcttagagacagttgact 3′ and 5′ ggataggtcggcggttcatgc 3′), and *Zfy* primers (5′ aagataagcttacataatcacatgga 3′ and 5′ cctatgaaatcctttgctgcacatgt 3′). The reaction was incubated in a Perkin-Elmer 2400 Thermocycler as follows: 95°C for 10 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, ending with 72°C for 5 min. To identify the presence or absence of the FasL mutation in *gld* mice, a restriction digest was performed using 15 μl of the PCR product, 1 μl of *Stu*I (Gibco), and 0.5 μl of 1 *M* NaCl. The samples were incubated for 3 hr at 37°C followed by 10 min at 65°C. The resulting product was separated on a 2.5% agarose gel, and the presence or absence of bands at 112, 136, 280, 458, and 618 bp indicated the presence of a wild-type FasL allele, a *gld* mutant FasL allele, a p53 knock-out allele, a p53 wild-type allele, or a Y chromosome, respectively. The resulting gel was photographed under UV-transillumination.

Litter Observations. Matings designed to obtain information regarding post-partum pup health were conducted. Female mice were placed in cages with male breeder mice after 1600 and co-housed for 18 days. After 18 days, males were removed and females were observed for signs of parturition twice daily, once in the morning and

once in the afternoon. Dams that had completed delivery of a litter by the morning check were considered to be at lactational day 1. Dams observed with litters during the afternoon check, that did not have a completed delivery during the morning check, were considered to be at post-natal day 0. The pups were observed for any malformations and for the presence of milk in their stomachs. If a pup was found dead on post-natal day 0, a determination of whether the dead pup was born dead or alive was made; the lungs were removed and placed in water. If the lungs floated, the pup was presumed to have been born alive, otherwise the pup was presumed to have been born dead. The number and sex of the pups was recorded on days 0, 1, 4, 7, 14, 21, and 28. If dams showed signs of parturition (vaginal blood or a visible lowering of the litter in the abdomen) without showing signs of having delivered a litter (i.e., no loss of palpable litter from abdomen) within 48 hr, they were considered to be in dystocia and were killed by CO_2 asphyxiation. The uterine contents were examined. Alternatively, if a dam showed signs of pregnancy (i.e., a palpable litter), but a litter was never observed, the dam was killed by CO_2 asphyxiation, and a positive pregnancy was verified by examining the uterus for implant sites. Fertility rates were determined from the ratio of the number females with confirmed pregnancy to the number females co-housed with a male.

Caesarean Sections. Several matings were designed to obtain information regarding developmental status of fetuses *in utero*. Female mice were placed in cages with male breeder mice 1–2 hr before the start of the dark cycle. Each morning, 1–2 hr after the end of the dark cycle, female mice were observed for the presence of a vaginal plug. The morning (0600) of the day that the plug was observed was considered to be gestational day 0. On the scheduled day of gestation (days 8–19), female mice were killed by CO_2 asphyxiation, and caesarean sections were performed to determine whether there was a delay in neural tube development in double-deficient female mice. Yolk sacs were collected from each fetus for genotyping, and all fetuses were examined externally and internally, with the aid of a dissecting microscope, for the presence of malformations and to determine sex. Information regarding implant placement, number of early resorptions, late resorptions, and the number of corpora lutea on each ovary was recorded. The pregnancy rate was determined by the ratio of the number of gravid female mice to the number females co-housed with a male. Pre-implantation loss for each pregnant female mouse was determined by the ratio of the number of corpora lutea minus the number of implants divided by the number of corpora lutea. Post-implantation loss was determined by the ratio of the number of dead pups plus the number of resorptions divided by the number of implants.

Estrous Cycle Determination. To determine the cyclicity of vaginal epithelium, female mice (4–38 animals per genotype) were examined daily. Vaginal lavage was used to obtain cells for microscopic examination, and samples were classified as being consistent with metestrus, diestrus, proestrus, or estrus. An animal was considered to be cycling normally if she became pregnant (verified by caesarean section) or if there was a change in cell types over a 4-day period.

Spermatid Head Counts. Male mice were killed by CO_2 asphyxiation, and testes were removed and stored at −80°C until analysis. For the assessment of spermatogenesis at post-natal day 37 and week 12, both testes from each animal were homogenized separately. For evaluation of spermatogenesis in 8-week-old adult double-deficient versus wild-type mice, only the right testis was homogenized for counts. Sperm heads were counted on a hemacytometer using previously described methods (38). When available, the counts from the two testes of each animal were averaged for statistical analysis.

Histology. To examine the histology of adult testes and to measure seminiferous tubule diameters, formalin-fixed testes were embedded in glycol methacrylate using a Historesin embedding kit (Reichert-Jung, Heidelberg, Germany). Sections (2 μm) were stained with periodic acid-Schiff (PAS) reagent and hematoxylin.

Seminiferous Tubule Diameters. Seminiferous tubule diameters were measured using a reticule in the eyepiece of the microscope, which was calibrated using a micrometer. Each tubule was measured at the smallest width of the cross-section. Approximately 100 tubule cross-sections were measured for each animal.

Statistics. The following parameters were evaluated for statistical significance using the χ^2 test: predicted genotypic frequencies (based on Mendelian genetics) versus actual genotypic frequencies, post-partum pup death relative to genotype, post-partum pup death relative to sex, pregnancy rates, and fertility indices, estrous cycle incidence, post-partum malformations relative to genotype, and neural tube defects found *in utero* relative to genotype.

The following statistical evaluations were performed using nonparametric ANOVA followed by a Fisher's protected least-significant differences test: weaning rates, litter size, time to copulation, ovulation rate, number of implants per litter, pre-implantation loss and post-implantation loss, spermatid head counts, and seminiferous tubule diameters. Significance was calculated at both $P < 0.01$ and $P < 0.05$.

The histology, seminiferous tubule diameters, and spermatid head counts were analyzed by an evaluator who had no knowledge of the group identification of the samples. The data were decoded, summarized, and analyzed statistically.

Results

Establishment of Mouse Colonies. Five male mice homozygous for a targeted disruption of p53 on the C57Bl/6 background were crossed with 15 female *gld* mice (deficient in FasL) on the same background. F1 generation mice, heterozygous at both loci (FasL and p53), were mated with non-siblings, and the resulting 266 offspring were genotyped using DNA extracted from tail tips. A modified

multiplex PCR method using primers for p53, the targeted *neo* insert, and FasL was used to determine genotypes.

On the basis of genotypes, the mice were split into four distinct colonies and matings were designed to produce FasL+/+p53+/+ (wild-type), FasL−/−p53+/+ (*gld*), FasL+/+p53−/− (p53 knock-out), or FasL−/−p53−/− (double-deficient) mice. Because wild-type and *gld* mice were both viable and fertile, the colonies were maintained by breeding homozygous mice, and genotyping was not performed after the F3 generation. However, because FasL+/+p53−/− female mice were not as abundant as other genotypes, presumably due to neural tube defects (32, 33), breeding to maintain the p53 knock-out colony was performed using FasL+/+p53+/− female mice, and all resulting offspring were genotyped. Likewise, FasL−/−p53−/− female mice were scarce; therefore, FasL−/−p53+/− female mice were bred to maintain the double-deficient colony, and offspring were genotyped.

Genotype Frequencies in F2 Generation of Mice. During the production of the F2 generation of mice, 313 pups were born alive. Male mice were more abundant at birth with a ratio of male to female offspring of 1.13 (166 males:147 females). By weaning, the ratio had increased slightly to 1.16 (143 males:123 females). Male mice were produced with expected genotypic frequencies (Table I). However, the frequencies observed in the F2 female offspring were significantly different from those predicted by Mendelian genetics ($P < 0.01$, χ^2 test). Notably, the number of FasL+/+p53−/−, FasL+/−p53−/−, and FasL−/−p53−/− female mice was lower than expected (35%, 80%, and 100% decreases from expected values, respectively).

The loss of female double-deficient mice was intriguing because of the apparent gene dosage effect of FasL. Hence, the possibility of using the mouse model to understand the mechanisms that lead to neural tube defects was of interest. However, given the relatively small sample size (266 offspring), the mathematical probability that a pup would be a double-deficient female (3.13%), and the reduction of p53 null female mice [up to 80% of expected (32)], it was possible that the absence of FasL−/−p53−/− knock-out female mice in the F2 generation was simply due to the loss of p53.

Therefore, subsequent to the F2 generation, matings were designed to produce litters with the genotypes of interest within each of the four mouse colonies.

Post-natal Malformations. Any pups with malformations were removed from the litter, euthanized, and necropsied. A sample of tail was used to genotype the pup. Spina bifida, exencephaly, and kinked tails were all associated with p53−/−, regardless of the status of FasL (data not shown). Additionally, aphakia, micro-ophthalmia, malocclusion incisors, syndactyly, hydrocephaly, edema, and aortic arch malformations were observed. However, these latter observations either were single events that could be attributed to background incidence (39–41) or were not associated with any one genotype. So, although the p53−/− genotype increased the incidence of neural tube defects, the presence or absence of FasL was not specifically associated with any malformation.

The post-natal malformations suggested that neural tube defects played a role in the loss of female mice with the FasL+/+p53−/− or FasL−/−p53−/− genotypes and that the presence or absence of FasL had no effect on the expression of the defect. However, the extent and timing of the loss had to be considered by examination of fetuses *in utero* as well.

***In Utero* Malformations.** To examine the role of *in utero* malformations in the loss of female mice, mice from each of the four colonies were mated according to the mating scheme described in the section "Establishment of Mouse Colonies," and caesarean sections were performed. Dams were killed during gestation (GD 8–19), and fetuses were removed, necropsied, and genotyped.

No malformations were observed in male fetuses (FasL+/+p53+/+, 0/23; FasL−/−p53+/+, 0/15; FasL+/+p53+/−, 0/15; FasL+/+p53−/−, 0/6; FasL−/−p53+/−, 0/21; and FasL−/−p53−/−, 0/28). Additionally, female fetuses of several genotypes did not have any malformations (FasL+/+p53+/+, 0/23; FasL−/−p53+/+, 0/16; FasL+/+p53+/−, 0/10; and FasL−/−p53+/−, 0/25). However, there was an increase in the incidence of neural tube defects in female fetuses with genotypes of FasL+/+p53−/− (3/9) and FasL−/−p53−/− (4/16). There was no significant difference between these incidences in p53 knock-out and double-deficient female mice.

Distribution of Sex and Genotype During Gestation and at Weaning. To further examine the timing and extent of the loss of female mice, sex, and genotype distributions were determined during gestation (from caesarean sections) and at weaning (from litter observations). Wild-type and *gld* mice produced litters with sex ratios that were not significantly different from the expected ratio of unity. On the other hand, sex ratios in litters containing p53+/− and p53−/− mice were significantly increased at

Table I. F2 Generation Genotypes at Weaning

	FasL−/− p53−/−	FasL−/− p53+/−	FasL−/− p53+/+	FasL+/− p53−/−	FasL+/− p53+/−	FasL+/− p53+/+	FasL+/+ p53−/−	FasL+/+ p53+/−	FasL+/+ p53+/+
No. of male mice	9	14	11	18	39	21	8	11	12
% of male mice	6.29%	9.79%	7.69%	12.59%	27.27%	4.69%	5.59%	7.69%	8.39%
No. of female mice	0	22	10	3	46	21	5	11	5
% of female mice[a]	0.00%	17.89%	8.13%	2.44%	37.40%	17.07%	4.07%	8.94%	4.07%
Expected percent	6.25%	12.50%	6.25%	12.50%	25.00%	12.50%	6.25%	12.50%	6.25%

[a] Distribution of genotypes compared to the expected distribution (based on Mendelian genetics) is significantly different (χ^2, $P < 0.01$).

birth (Table II). This increase was even more pronounced by the time of weaning, indicating that not only was loss of female mice occurring *in utero*, but it was also occurring during lactation.

By genotyping fetuses and pups, it was evident that only FasL+/+p53−/− and FasL−/−p53−/− female fetuses and pups were affected, and that p53+/− female animals were equally viable as male animals (Table III). Consistent with the increased sex ratios at weaning in litters containing p53−/− and p53+/− mice, the percent of FasL+/+p53−/− and FasL−/−p53−/− mice decreased between the time of gestation and weaning from 14% to 3% and from 13% to 6%, respectively.

Male Mouse Fertility. Fertility rates, the mean time to copulation, and mean number of live fetuses in the four genotypes were similar among the four mouse colonies, suggesting that neither the male nor the female mice used in this mating scheme had reduced fertility (Table IV).

Analysis of testes from wild-type, FasL−/−p53+/+, FasL+/+p53−/−, and FasL−/−p53−/− male mice during the first wave of spermatogenesis indicated that testis weights were not significantly different between genotypes (data not shown). Seminiferous tubule diameters were measured on post-natal day 22 as a reflection of germ cell content (data not shown). However, there were no significant increases in any of the four genotypes analyzed. Spermatid head counts measured on post-natal day 37 also revealed no statistically significant increases in any of the genotypes (data not shown).

Finally, examination of 8-week-old wild-type and double-deficient male mice indicated that spermatogenesis was normal in the double-deficient animal (Table V). Additionally, male fertility rates and litters sired by the double-deficient mice were of similar size as those of wild-type mice (Table V), and testis histopathology did not reveal any obvious defects in spermatogenesis (data not shown).

Female Mouse Reproductive Parameters. There were no differences in time to copulation, ovulation rates (corpora lutea), pregnancy rates, or number of implants per litter in FasL−/−p53+/+, FasL+/+p53+/−, or FasL−/−p53+/− female mice when compared to wild-type animals (Table IV). Despite previous reports of reduced litter sizes in *gld* mice (27), FasL−/− mice did not have any reductions in pregnancy rate, number of implants, pre-implantation loss, or post-implantation loss (Table IV).

On the other hand, several matings with FasL+/+p53−/− and FasL−/−p53−/− female mice, when mice with these rare genotypes became available, showed a significant decrease in fertility rates, litter size, and survival to weaning (Table VI). Interestingly, although the fertility rates were similar to those of FasL+/+p53−/− mice, no pups were born alive to FasL−/−p53−/− female mice. There were two FasL−/−p53−/− female mice that did become pregnant, but they were both killed due to dystocia. Necropsies revealed that although the number of pups per litter was normal, all of the pups were dead and were larger than normal. To determine whether the reduction in fertility rates was due to an alteration in estrous cycle, vaginal smears were evaluated for signs of normal cyclicity. No differences in estrous cycling were detected between the genotypes (data not shown).

Discussion

As with any new transgenic mouse model, exciting insights into gene interactions can be gained from observing the genetic frequencies and phenotypes of the offspring. For this reason, when the p53 knock-out mice were crossed with Fas ligand mutant mice, the mouse colonies were tested for reproductive parameters, such as pregnancy, ovulation rate, and resorption rates. Additionally, mice were monitored during litter deliveries to determine fertility rates and litter sizes at birth and at weaning. Thus, the effects of eliminating two apoptotic factors—FasL and p53—on reproductive performance and mouse development were evaluated with the goal of determining whether these two apoptotic systems play complementary roles in reproduction and development.

Analysis of reproductive function in the different mouse colonies showed that the absence of p53 in female mice reduced fertility rates (Table VI). Additionally, the absence of FasL in addition to p53 in female mice led to dystocia in pregnant females, suggesting that the Fas system plays a complementary role to p53 in parturition. On the other hand, male mouse development and fertility remained unaffected by the absence of p53 and FasL.

Although the preliminary data from the F2 generation of mice suggested that the absence of FasL enhanced the loss of p53−/− female mice, further analysis showed that the presence or absence of FasL did not affect fetal viability. The extent and timing of the loss due to neural tube defects

Table II. Sex Ratios at Birth and at Weaning

Colony	Sire genotype	Dam genotype	No. of litters	Male:female ratio	
				At birth	At weaning
Wild-type	FasL+/+p53+/+	FasL+/+p53+/+	17	0.73	0.79
gld	FasL−/−p53+/+	FasL−/−p53+/+	17	0.97	1.19
p53-Deficient	FasL+/+p53−/−	FasL+/+p53+/−	23	1.22[a]	1.32[a]
Double-Deficient	FasL−/−p53−/−	FasL−/−p53+/−	27	1.57[a,b]	1.87[a,b]

[a] Significantly different from wild-type ratio ($P < 0.01$, χ^2 test).
[b] Significantly different from the expected ratio of 1, based on Mendelian genetics ($P < 0.01$, χ^2 test).

Table III. Sex and Genotype Distribution of p53+/– and p53–/– Mice

Colony	Sire genotype × dam genotype	No. of litters	Sex and genotype distribution in litters								
			During gestation					At weaning			
			Males		Females		No. of litters	Males		Females	
			p53 +/–	p53 –/–	p53 +/–	p53 –/–		p53 +/–	p53 –/–	p53 +/–	p53 –/–
p53-Deficient	FasL+/+p53–/– FasL+/+p53+/–	7	42%	17%	28%	14%	23	35%[a]	23%[a]	40%[a]	3%[a]
Double-deficient	FasL–/–p53–/– FasL–/–p53+/–	13	27%	27%	33%	13%	27	35%[a]	30%[a]	29%[a]	6%[a]

[a] Significantly different from expected ratios based on Mendelian genetics ($P < 0.01$, χ^2 test).

Table IV. Reproductive Data from Caesarean Sections

Colony		Genotype of sire and dam	Mean days to copulation (*N*)[b]	Pregnancy rate (*N*)[a]	Number of implants per litter ± SEM (N)[b]	Number of resorptions and dead per litter ± SEM (N)[b]	Number of corpora lutea per litter ± SEM (N)[b]	Pre-implantation loss ± SEM[b]	Post-implantation loss ± SEM[b]
Wild-type	Sire	FasL+/+p53+/+	5.76	86%	8.07 ± 0.7	1.47 ± 0.7	10.17 ± 0.7	0.19 ± 0.06	0.18 ± 0.08
	Dam	FasL+/+p53+/+	(21)	(21)	(18)	(15)	(18)	(18)	(15)
gld	Sire	FasL–/–p53+/+	4.33	83%	7.00 ± 1.1	0.60 ± 0.4	8.40 ± 0.5	0.26 ± 0.1	0.09 ± 0.07
	Dam	FasL–/–p53+/+	(6)	(6)	(5)	(5)	(5)	(5)	(5)
p53-deficient	Sire	FasL+/+p53–/–	4.36	73%	7.89 ± 1.4	1.57 ± 0.7	10.13 ± 0.4	0.26 ± 0.1	0.24 ± 0.08
	Dam	FasL+/+p53+/–	(11)	(11)	(8)	(7)	(8)	(8)	(7)
Double-deficient	Sire	FasL–/–p53–/–	4.67	64%	8.00 ± 0.9	2.00 ± 0.6	10.08 ± 0.5	0.22 ± 0.08	0.24 ± 0.08
	Dam	FasL–/–p53+/–	(22)	(22)	(14)	(14)	(12)	(13)	(13)

[a] No significant differences were found ($P < 0.05$, χ^2 test).
[b] No significant differences were found ($P < 0.05$, ANOVA test).

Table V. Male Reproductive Parameters of Double FasL–/–p53–/– Compared to FasL+/+p53+/+ Mice

	FasL+/+p53+/+	FasL–/–p53–/–
Testis weights[a]	99.55 ± 1.43 mg ($n = 5$)	98.31 ± 2.8 mg ($n = 5$)
Spermatid head counts[a]	12.3 ± 1.9 million/testis ($n = 5$)	9.8 ± 1.5 million/testis ($n = 5$)
Tubule diameters[a]	192 ± 9 μm ($n = 5$)	187 ± 4 μm ($n = 5$)
Fertility rate	78% ($n = 24$ male mice)	77% ($n = 17$ male mice)
Live-birth litter size[a]	6.36 ± 0.28 pups ($n = 50$ litters)	6.06 ± 0.45 pups ($n = 25$ litters)

[a] Mean ± SEM.

was similar for FasL+/+p53–/– mice and FasL–/–p53–/– mice.

The observation that the absence of p53 in female mice reduced fertility rates (Table VI) has not been reported previously. In fact, p53–/– female mice have been reported to have normal fertility (42). One explanation for the reduced fertility in p53–/– female mice is a change in ovulatory function. There is some evidence that p53 plays a role in ovulation. Immunohistochemical localization of p53 in human and rat shows that p53 is expressed in granulosa cells during both luteolysis and atresia (12–15). Additionally, *in vivo* gonadotropin priming inhibited apoptosis with concomitant suppression of p53 (43). In light of these data, it is plausible that the absence of p53 could inhibit the normal apoptosis that occurs in the ovary during the estrous cycle or following pregnancy. As such, the absence of p53 could inhibit the normal apoptosis that occurs in the ovary during the estrous cycle or at the termination of pregnancy. If luteolysis was inhibited by the absence of p53, a disruption in estrous cycle could occur. However, vaginal cell cycling was grossly normal in FasL+/+p53–/– and FasL–/–p53–/– female mice, suggesting that ovarian hormonal control of endometrial cellular stages was not affected by the lack of p53. Necropsies of p53–/– female mice did not reveal any

Table VI. Pregnancy Outcome for p53–/–Female Mice

Colony	Sire genotype	Dam genotype	No. of matings	Fertility rate	Mean number of pups born ± SEM	Mean number of pups weaned ± SEM
Wild-type	FasL+/+p53+/+	FasL+/+p53+/+	25	72%	6.06 ± 0.5	4.28 ± 0.7
p53-Deficient	FasL+/+p53–/–	FasL+/+p53–/–	10	20%[a]	3.50 ± 0.5	1.50 ± 1.5
Double-deficient	FasL–/–p53–/–	FasL–/–p53–/–	7	29%[a]	0.00 ± 0.0[b]	0.00 ± 0.0

[a] Statistically significantly different from wild type ($P < 0.01$, χ^2).
[b] Statistically significantly different from wild type ($P < 0.01$, ANOVA, Fisher's PLSD).

gross abnormalities in reproductive tracts (data not shown); therefore, the reason for reduced fertility in p53–/– female mice remains to be determined.

Another possible explanation for reduced female fertility is that p53 status influences reproductive function via its role as a transcription factor. In fact, p53 has been shown to be a negative regulator of estrogen receptor signaling pathways (44), implying that any estrogen responsive tissue, such as ovarian follicles, uterine stroma, uterine myometrium, and uterine endometrium, might be over-stimulated in the absence of p53. This, in fact, could help to explain why Li-Fraumeni patients (with a p53 mutation) are susceptible to developing mammary tumors. Although this mechanism has not previously been investigated, it is a likely possibility in light of the data. Additionally, parturition, which is initiated by estrogen/progesterone ratio changes, might be inhibited by an alteration in estrogen activity. Whether the reduction in fertility in p53–/– female mice is due to a direct effect of p53 on the biology of female reproductive tract, due to a behavioral modification, or is an indirect effect of an alteration in another organ system requires further investigation.

The roles for Fas/FasL interactions in the female reproductive tract have not been as well defined as those for p53. There are several reports of immunohistochemical localization of Fas to granulosa cells of ovarian follicles (15–18) and oocytes (19). However, the significance of this Fas expression has not been explored. A previous report suggested that Fas/FasL interactions are responsible for preventing activated leukocytes from attacking placenta and fetal tissue during pregnancy (27). *Gld* mice were reported to have smaller litters and increased resorption rates. However, this hypothesis has been disputed (28), and the data presented here suggest that there is no effect of the loss of FasL, alone, on reproductive function.

On the other hand, the absence of FasL in addition to p53 in female mice prevented normal delivery; the two FasL–/–p53–/– female mice that did become pregnant were killed because of dystocia. Necropsies revealed litters with an average number of pups. However, in both cases, all of the pups were dead and were larger than normal term weight, suggesting that the combination of the FasL and p53 deficiencies had a deleterious effect on parturition. This would suggest that the Fas system plays a complementary role to p53 in parturition and that it is able to compensate, at least in part, for the absence of p53. Given that both p53 and Fas are expressed in granulosa cells and p53 is expressed during luteolysis, one possibility is that both apoptotic systems are involved in luteal regression at the end of pregnancy. In the absence of luteal regression, progesterone levels would remain high and prevent uterine contractions.

The decreased frequency of FasL+/+p53–/– females in the F2 generation of mice (4.07% instead of the expected 6.25%) was consistent with previous reports (31, 32). Depending on the mouse strain, up to 85% decreases in the number of p53 null female mouse offspring have been observed (32). The sexually dimorphic expression of neural tube defects is well documented. As in humans, female mice are more susceptible to neural tube defects than male mice (45, 46). The reason for this sexual dimorphism, however, remains elusive.

Interestingly, the absence of one of the two FasL alleles in addition to the p53 null genotype led to an enhanced reduction in the number of female offspring that survived to weaning, and the absence of both FasL alleles in conjunction with p53 deficiency effectively eliminated all of the F2 female offspring, suggesting a gene-dosage effect of the FasL (Table I). Based on these preliminary data, we explored the possibility that the Fas ligand deficiency enhanced the predisposition for female p53 knock-out mice to develop neural tube defects.

Analysis of fetuses from caesarean sections indicated that there was an increase in neural tube defects in p53–/– female conceptuses, regardless of the status of FasL. Analysis of genotypes during gestation, at birth, and at weaning verified that loss of p53–/– female mice occurred not only during gestation, but also post-partum, and that there was no difference between FasL+/+p53–/– and FasL–/–p53–/– mice (Tables II and III). This variability in the timing of the loss has been reported previously for p53–/– mice (32), and it is probably due to variable expressivity; pups with more severe forms of exencephaly may die *in utero*, while those with mild forms of delayed neural tube closure might live several days post-partum. Therefore, although the preliminary data from the F2 generation of mice suggested that the absence of FasL enhanced the loss of p53–/– female mice, further analysis showed that the presence or absence of FasL did not affect fetal viability. The extent and timing of the loss due to neural tube defects were similar for FasL+/+p53–/– and FasL–/–p53–/– mice.

A report of a double-deficient Fas–/–p53–/– mouse, made by crossing *lpr* mice with p53 knock-out mice, also

indicated a reduction in double-deficient offspring (47). However, at this time it is unclear whether the reduction in offspring was due to the loss of p53–/– females with neural tube defects or whether a reproductive deficiency was responsible for the difficulty in breeding double-mutant mice.

In summary, the loss of p53 was detrimental to female mice, not only because of the increased incidence of neural tube defects but also because it reduced reproductive function. On the other hand, despite reports to the contrary, the loss of FasL, alone, had no effect on reproductive function in female mice. Male development and reproductive function remained unaffected by the double deficiency. Finally, although neither gene deficiency alone had an effect on parturition, the combined deficiency of p53 and FasL prevented successful deliveries in mice, suggesting that these two apoptotic systems play complementary roles in parturition.

1. Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science **270:**96–98, 1995.
2. Perez GI, Robles R, Knudson CM, Flaws JA, Korsmeyer SJ, Tilly JL. Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. Nat Genet **21:**200–203, 1999.
3. Ratts VS, Flaws JA, Kolp R, Sorenson CM, Tilly JL. Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. Endocrinology **136:**3665–3668, 1995.
4. Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J **16:**2262–2270, 1997.
5. Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y. Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing bcl-2 in spermatogonia. Development **122:**1703–1709, 1996.
6. Flaws JA, Hirshfield AN, Hewitt JA, Babus JK, Furth PA. Effect of bcl-2 on the primordial follicle endowment in the mouse ovary. Biol Reprod **64:**1153–1159, 2001.
7. Hsu SY, Lai RJ, Finegold M, Hsueh AJ. Targeted over-expression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. Endocrinology **137:**4837–4843, 1996.
8. Rodriguez I, Araki K, Khatib K, Martinou JC, Vassalli P. Mouse vaginal opening is an apoptosis-dependent process which can be prevented by the over expression of Bcl-2. Dev Biol **184:**115–121, 1997.
9. Cohen PL, Eisenberg RA. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu Rev Immunol **9:**243–269, 1991.
10. Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR. The p53 network. J Biol Chem **273:**1–4, 1998.
11. Bellamy CO. p53 and apoptosis. Br Med Bull **53:**522–538, 1997.
12. Makrigiannakis A, Amin K, Coukos G, Tilly JL, Coutifaris C. Regulated expression and potential roles of p53 and Wilms' tumor suppressor gene (WT1) during follicular development in the human ovary. J Clin Endocrinol Metab **85:**449–459, 2000.
13. Tilly KI, Banerjee S, Banerjee PP, Tilly JL. Expression of the p53 and Wilms' tumor suppressor genes in the rat ovary: gonadotropin repression in vivo immunohistochemical localization of nuclear p53 protein to apoptotic granulosa cells of atretic follicles. Endocrinology **136:**1394–1402, 1995.
14. Keren-Tal I, Suh BS, Dantes A, Lindner S, Oren M, Amsterdam A. Involvement of p53 expression in c2AMP-mediated apoptosis in immortalized granulosa cells. Exp Cell Res **218:**283–295, 1995.
15. Kim JM, Yoon YD, Tsang BK. Involvement of the Fas/Fas ligand system in p53-mediated granulosa cell apoptosis during follicular development and atresia. Endocrinology **140:**2307–2317, 1999.
16. Matsubara H, Ikuta K, Ozaki Y, Suzuki Y, Suzuki N, Sato T, Suzumori K. Gonadotropins and cytokines affect luteal function through control of apoptosis in human luteinized cells. J Clin Endocrinol Metab **85:**1620–1626, 2000.
17. Hakuno N, Koji T, Yano T, Kobayashi N, Tsutsumi O, Taketani Y, Nakane PK. Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. Endocrinology **137:**1938–1948, 1996.
18. Quirk SM, Cowan RG, Joshi SG, Henrikson KP. Fas antigen-mediated apoptosis in human granulosa/luteal cells. Biol Reprod **52:**279–287, 1995.
19. Jose de los Santos M, Anderson DJ, Racowsky C, Hill JA. Presence of Fas-Fas ligand system and bcl-2 gene products in cells and fluids from gonadotropin-stimulated human ovaries. Biol Reprod **63:**1811–1816, 2000.
20. Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology **138:**2081–2088, 1997.
21. Xu JP, Li X, Mori E, Guo MW, Matsuda I, Takaichi H, Amano T, Mori T. Expression of Fas-Fas ligand in murine testis Am J Reprod Immunol **42:**381–388, 1999.
22. Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, Donehower LA, Levine AJ. Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. Proc Natl Acad Sci U S A **90:**9075, 1993.
23. Sjoblom T, Lahdetie J. Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p53 in meiotic recombination and repair. Oncogene **12:**2499–2505, 1996.
24. Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH, de Rooij DG. The role of the tumor suppressor p53 in spermatogenesis. Cell Death Differ **5:**669–677, 1998.
25. Koji T, Hishikawa Y, Ando H, Nakanishi Y, Kobayashi N. Expression of Fas and Fas ligand in normal and ischemia-reperfusion testes: involvement of the Fas system in the induction of germ cell apoptosis in the damaged mouse testis. Biol Reprod **64:**946–954, 2001.
26. Wang R-A, Nakane PK, Koji T. Autonomous cell death of mouse male germ cells during fetal and postnatal period. Biol Reprod **58:**1250–1256, 1998.
27. Hunt JS, Vassmer D, Ferguson TA, Miller L. Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between mother and the conceptus. J Immunol **158:**4122–4128, 1997.
28. Chaouat G, Clark DA. FAS/FAS ligand interaction at the placental interface is not required for the success of allogeneic pregnancy in anti-paternal MHC pre-immunized mice. Am J Reprod Immunol **45:**108–115, 2001.
29. Yin Y, Stahl BC, DeWolf WC, Morgentaler A. p53-mediated germ cell quality control in spermatogenesis. Dev Biol **204:**165–171, 1998.
30. Richburg JH, Nanez A, Williams LR, Embree ME, Boekelheide K. Sensitivity of testicular germ cells to toxicant-induced apoptosis in gld mice that express a nonfunctional form of Fas ligand. Endocrinology **141:**787–793, 2000.
31. Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibits exencephaly. Nat Genet **10:**175–180, 1995.
32. Armstrong JF, Kaufman MH, Harrison DJ, Clarke AR. High-frequency developmental abnormalities in p53-deficient mice. Curr Biol **5:**931–936, 1995.
33. Copp AJ. Genetic models of mammalian neural tube defects. CIBA Found Symp **181:**118–143, 1994.
34. Jacks T, Remington L, Williams BO. Tumor spectrum analysis in p53-mutant mice. Curr Biol **4:**1–4, 1994.

35. Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, Nagata S. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell **76:**969–976, 1994.
36. Schneider P, Bodmer J-L, Holler N, Mattmann C, Scuderi P, Terskikh A, Peitsch MC, Tscopp J. Characterization of Fas (Apo-1, CD95)-Fas ligand interaction. J Biol Chem **272:**18827–18833, 1997.
37. Cheng LE-C, Chan FK-M, Cado D, Winoto A. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. EMBO J **16:**1865–1875, 1997.
38. Blazak WF, Treinen KA, Juniewicz PE. Application of testicular sperm head counts in the assessment of male reproductive toxicity. In: Chapin RE, Heindel J, Eds. Methods in Toxicology. New York: Academic Press, Vol **3A:**pp86–105, 1993.
39. Kalter H. Sporadic congenital malformations of newborn inbred mice. Teratology **1:**193–200, 1968.
40. Dagg CP. Teratogenesis. In: Green EL, Ed. Biology of the Laboratory Mouse. New York: McGraw-Hill, pp309–328, 1966.
41. Robinson ML, Holmgren A, Dewey MJ. Genetic control of ocular morphogenesis: defective lens development associated with ocular anomalies in C57BL/6 mice. Exp Eye Res **56:**7–16, 1993.
42. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr., Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature **356:**215–221, 1992.
43. Tilly KI, Banerjee S, Banerjee PP, Tilly JL. Expression of the p53 and Wilms' tumor suppressor genes in the rat ovary: gonadotropin repression *in vivo* and immunohistochemical localization of nuclear p53 protein to apoptotic granulosa cells of atretic follicles. Endocrinology **136:**1394–1402, 1995.
44. Yu CL, Driggers P, Barrera-Hernandez G, Nunez SB, Segars JH, Cheng S. The tumor suppressor p53 is a negative regulator of estrogen receptor signaling. Biochem Biophys Res Commun **239:**617–620, 1997.
45. Seller MJ. Neural tube defects and sex ratios. Am J Med Genet **26:**699–707, 1987.
46. Seller MJ, Perkins-Cole KJ. Sex difference in mouse embryonic development at neurulation. J Reprod Fertil **79:**159–161, 1987.
47. Yin Y, Stahl BC, DeWolf WC, Morgentaler A. P53 and Fas are sequential mechanisms of testicular germ cell apoptosis. J Androl **23:**64–70, 2002.