Semen quality parameters and embryo lethality in mice deficient for Trp53 protein

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Received: 16 March 2011; accepted: 20 September 2011

SUMMARY
Trp53 is a protein which is able to control semen parameters in mice, but the extent of that control depends on the genetic background of the mouse strain. Males from C57BL/6Kw, 129/Sv, C57BL×129 -p53+/+ (wild type controls) and C57BL×129-p53-/- (mutants) strains were used in the study, and histology and light microscopy were applied to evaluate the influence of genetic background and Trp53 (p53) genotype on testes morphology and semen quality in male mice. We showed that sperm head morphology, maturity and tail membrane integrity were controlled only by the genetic background of C57BL/6Kw and 129/Sv males, while testes weight and sperm concentration depended on both the genetic background and p53 genotype. Cell accumulation in seminiferous tubules may be responsible for heavier testes of p53-deficient males. In addition, to examine the effect of sex and p53 genotype on embryo lethality, pairs of control (C57BL×129-p53+/+) and heterozygous (C57BL×129-p53+/-) mice were examined. Before day 7 post coitum (dpc), female and male embryos were equally resorbed in both crosses types. After 7 dpc, preferential female embryo lethality in the heterozygote pairs was responsible for the skewed sex ratio in their progeny. Also, mutant female and male newborns were

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**Key words:** genetic background, p53, semen quality, embryonic lethality

**INTRODUCTION**

Tumor suppressor gene *Trp53* (*p53*) codes for small (53 kDa) but important protein controlling cell cycle and genomic integrity [9]. The activation of the *p53* gene leads to cell cycle arrest or induction of apoptosis. The first physiological wave of germ cell apoptosis is observed in the testes of 2-4 week old male rodents [20, 27]. This process is partially dependent on p53 and affects spermatogonia and spermatocytes [2, 20, 27]. In adult individuals, the expression of the *p53* and p53 protein were shown in primary spermatocytes [2, 20, 24], but apoptosis was observed mainly in spermatogonia [1, 20]. p53-dependent apoptosis can be activated in the gonads of adult male mice with X-ray irradiation [2, 7] as well as with an elevated abdominal temperature [16, 28, 29].

The role of the p53 protein during normal spermatogenesis was also documented in *p53* knock-out male mice. Several strains with mutated p53 were examined, and sterile males were found exclusively in the 129 strain [21], while only slightly affected spermatogenesis was described in other strains [2, 18, 22]. It was also shown that *p53* mutant male mice produced fewer litters [29], while mutant females exhibited increased pre- and postnatal lethality [4, 5]. In addition, sex differences in response to mutated *p53* were observed in neural tube development, especially in females [3, 5, 10]. Thus, the genetic background, associated also with sex chromosomes’ composition, plays an important role in p53-dependent control of fertility parameters.

To elucidate the significance of p53 and genetic background for quality of mouse male gametes, we have examined the morphology of testes and some sperm parameters (alive, motile and spermatozoa number, sperm concentration, percentage of immature sperms, sperm maturity, percentage of sperms with abnormal tail membrane integrity) in C57BL/6Kw and 129/Sv as well as control *p53*+/+ and mutant *p53*—/males with the C57BL×129 genetic background. In addition, we investigated embryo lethality as well as sex ratio of the embryos and newborns in relation to *p53* genotype. In this experiment, we compared the data obtained from control animal crosses (C57BL×129- *p53*+/+) with those from the crosses between mice heterozygous for p53 (C57BL×129- *p53*+/—).
MATERIALS AND METHODS

Animals

Trp53 knock-out mice (C57BL×129-p53-/-) and control mice (C57BL×129-p53+/-, C57BL×129) with the mixed (outbred) C57BL and 129 genetic background were obtained from the Swiss Federal Institute of Technology, Zurich. We crossed these animals in our breeding department to derive C57BL×129 inbred strain, recombinant for C57BL and 129 parental strains’ genomes. Mice heterozygous for p53 (i.e. C57BL×129-p53+/-) were born (F1 generation) and paired. Animals of three possible genotypes were obtained: C57BL×129-p53+/-, C57BL×129-p53+/-, and C57BL×129-p53-/-). Only sisters and brothers heterozygous for p53 were mated in the consecutive generations. At the stage of F16-F18, a total of 25 microsatellite sequences polymorphic for 129/Sv and C57BL/6Kw were analyzed. 23 sequences (92%) revealed to be homozygous, so the inbreeding status of the newly derived C57BL×129 strain was acceptable and mice could be used for our study. Moreover, sixteen microsatellite sequences (64%) were inherited after 129/Sv strain in our C57BL×129 mice (data not shown; Mouse Genome Informatics ID accession number: J:156851).

Five to eight adult (3-6 months old) males from each of the following strains were used in the study: two parental inbred strains, i.e. C57BL/6Kw and 129/Sv, as well as a newly derived C57BL×129 inbred strain, represented by C57BL×129-p53+/- (control) and C57BL×129-p53-/- (mutant) animals. The comparison of 129/Sv, C57BL/Kw and C57BL×129-p53+/- males enabled us to evaluate the impact of the genetic background on testis morphology and sperm quality parameters. The C57BL×129-p53+/- and C57BL×129-p53-/- males were used to evaluate the influence of p53 on testis morphology and sperm quality parameters.

Cages with pairs of control (C57BL×129-p53+/-; n=6 cages) and heterozygous (C57BL×129-p53+/-; n=6 cages) animals were set to compare the number of embryos and newborns as well as their sex ratio. In addition, we genotyped 326 newborns from 55 litters delivered by heterozygotes to see if p53 genotype and sex affected their lethality in our C57BL×129 strain. All of the animals used in the study were bred in the Department of Genetics and Evolution under 12h:12h light:dark conditions with pelleted food and water ad libitum. Mice were killed by cervical dislocation and weighted. The experiments were
performed under the license of the Commission of Bioethics at the Jagiellonian University.

**Sperm quality parameters**

Both vas deferens of the males from the four studied strains were dissected and their content was gently squeezed out into 100 μl of M2 medium (Sigma-Aldrich, USA). The sperm was allowed to disperse (5 min) and sperm suspension was analyzed under light microscope (Olympus, type CH2, 400× magnification, unless indicated otherwise). Fresh semen suspension was used to determine the percentage of motile spermatozoa (light microscope, room temperature). In motile spermatozoa, the number of sperm without droplets on the tails (mature spermatozoa) and the number of spermatozoa possessing distal and proximal droplets (immature spermatozoa) were counted. Ten μl of the sperm suspension was diluted 1:10 with PBS (Biomed, Poland) and sperm count per 250 small squares was evaluated in a haemocytometer. Sperm suspension was diluted 1:10 with sterile water (Sigma-Aldrich, USA) and incubated for 5 min at 37°C to analyze sperm tail membrane integrity (hypoosmotic test, HOS test). Twenty μl of sperm suspension was mixed with 0.2% eosin Y (1:1, v/v; Sigma-Aldrich, USA) and incubated for 10 min at 37°C. Red and green sperm heads observed on smears were classified as dead or alive sperm, respectively. Smears prepared from sperm suspension (10 μl) were fixed in a mixture of ethanol and acetic acid (3:1, v/v). The smears were stained with eosin (Sigma-Aldrich, USA), and the morphology of 200 sperm heads (fig. 1) for each smear was evaluated under a 1000× magnification [6].

**Morphological analysis of testes**

The testes of four adult males from each of the four analyzed strains were dissected, weighed and fixed in a Bouin’s mixture, embedded in paraffin, and cut with a Reichert-Jung (Germany) paraffin microtome into 7 μm sections. Prepared slides were stained according to haematoxylin-eosin protocol and watched under a light microscope (Olympus, type CH2). One hundred seminiferous tubules’ sections per each testis were used to calculate the proportion of abnormal tubules.
Figure 1. Testis morphology of the control C57BL×129-p53+/+ (A, B, C) and mutant C57BL×129-p53-/- (D) males. A/ normal tubules in the testis of the control male; B/ shrank and atrophic tubule (asterik) in control male testis; C/ tubules with strongly degenerated germinal epithelium (asteriks), possessing mainly Sertoli cells, are adjacent to morphologically normal tubules (arrowheads) in control males; D/ abnormal tubules filled with the germ cells and lacking lumen (asteriks) characteristic for the testes of mutant males are adjacent to normal tubules (arrowheads).

Embryo lethality
Mutant mice (C57BL×129-p53-/-) of both sexes exhibited decreased vitality. When the mutants were paired, either no vaginal plug was produced or sterile copulation occurred during one week of co-housing. Therefore, six breeding cages were designated for heterozygous pairs of animals (C57BL×129-p53+/-), and six for control pairs of mice (C57BL×129-p53+/+). Females were checked for vaginal plug twice a day and killed seven days post coitum (dpc). Embryos were counted and dissected to extract DNA according to the standard phenol/chlorophorm protocol. To analyze the sex of the embryos, a pair of DYzEms12 primers was used (forward 5’CTACTAATGAAGGTGGCATCCC3’ and reverse 5’CTTACCCCCCTACACAAAAGC3’ primer). Obtained PCR products
of 317 bp were specific for the Y chromosome [13]. Thermal conditions were as follows: 4 min of 94°C, and then 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. We monitored the litter size and sex ratio of the first litters from each of the six pairs of C57BL×129 mice heterozygous for p53, and compared with the corresponding data obtained from each of the six pairs of C57BL×129 control mice. Also, the Trp53 genotyping data of 326 animals from 55 litters delivered by heterozygous pairs were collected, but only the first litter of each of breeding pair was analyzed.

Statistical analysis
All data were expressed as means±SD. The percentage data were arcsin transformed before statistical analysis. Data of sperm quality parameters were pooled for each strain. The parameters were compared by one-way ANOVA followed by Tukey’s post-hoc test (p<0.05). Student’s t test was used to determine the differences in mean embryo count on 7 dpc and mean litter size between the heterozygous (C57BL×129-p53+/-) and control (C57BL×129-p53+/+) pairs of mice. Chi square test was employed (p<0.05) to determine whether the sex and p53 genotype ratio differed from the theoretical Mendelian inheritance mode (1:1 and 1:2:1, respectively).

RESULTS
Sperm quality parameters
Body weight and sperm motility did not differ among the four analyzed groups of males (tab. 1). Testes weight as well as percentages of alive sperm (tab. 1) and sperm with normal tail membrane integrity (tab. 2) were higher in the 129/Sv males than in the C57BL/6Kw males. In contrast, the abnormal sperm count, sperm concentration (tab. 1) and percentage of sperm without residual droplets (tab. 2) were higher in the C57BL/6Kw males than in the 129/Sv males.

The percentage of abnormal sperm, matured sperm count (i.e. without cytoplasmic droplet) and percentage of sperm with normal membrane integrity did not differ between the control and mutant males (tabs. 1 and 2). Testes weight and sperm concentration were significantly higher in mutant than in control males. Males with p53 mutation possessed a decreased level of alive sperm when compared with the control males (tab. 1).
Table 1. Body and testes weights as well as sperm quality parameters (mean±SD) in the four examined mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Body weight (g)</th>
<th>Testes weight (g)</th>
<th>Motile sperm (%)</th>
<th>Alive sperm (%)</th>
<th>Abnormal sperm (%)</th>
<th>Sperm concentration (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv</td>
<td>31.8±2.7a</td>
<td>0.21±0.01b</td>
<td>40.0±5.9a</td>
<td>51.9±7.4ac</td>
<td>13.6±2.3b</td>
<td>58.7±9.3b</td>
</tr>
<tr>
<td>C57BL/6Kw</td>
<td>30.0±2.5a</td>
<td>0.16±0.03b</td>
<td>45.0±2.7a</td>
<td>43.4±1.3a</td>
<td>30.2±2.4d</td>
<td>80.0±6.8d</td>
</tr>
<tr>
<td>C57BL×129-p53+/+</td>
<td>30.4±1.1a</td>
<td>0.22±0.01b</td>
<td>47.8±8.7a</td>
<td>57.5±6.4b</td>
<td>7.6±1.8a</td>
<td>80.5±6.2b</td>
</tr>
<tr>
<td>C57BL×129-p53/-</td>
<td>30.9±2.5a</td>
<td>0.27±0.03c</td>
<td>37.4±6.2a</td>
<td>44.8±8.5a</td>
<td>6.7±1.9a</td>
<td>135.3±5.3c</td>
</tr>
</tbody>
</table>

Different superscripts depict significant differences among strains (ANOVA, p<0.05)

Table 2. Sperm maturity and sperm tail membrane integrity (mean±SD) in four examined mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sperm maturity (%)</th>
<th>Normal membrane integrity (swollen tails; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No droplet</td>
<td>Proximal droplet</td>
</tr>
<tr>
<td>129/Sv</td>
<td>44.0±7.6a</td>
<td>0.9±0.5a</td>
</tr>
<tr>
<td>C57BL/6Kw</td>
<td>58.8±6.5b</td>
<td>1.2±0.9a</td>
</tr>
<tr>
<td>C57BL×129-p53+/+</td>
<td>40.0±6.2a</td>
<td>0.7±0.4a</td>
</tr>
<tr>
<td>C57BL×129-p53/-</td>
<td>44.5±8.3a</td>
<td>0.3±0.4a</td>
</tr>
</tbody>
</table>

Sperm maturity was measured by cytoplasmic droplet location on the sperm tail. Tail membrane integrity was evaluated by means of hypoosmotic test. Different superscripts depict significant differences among strains (ANOVA, p<0.05)

Testis morphology

Seminiferous tubules of all analyzed male testes contained normal germ cells at various stages including mature spermatids. The morphological structure of the testes of the C57BL/6Kw and 129/Sv males were normal (data not shown). The total frequency of the abnormal tubules in the testes of the C57BL×129 control males (C57BL×129-p53+/+) was 10.6%. Strongly damaged seminiferous tubules (shrank and atrophic; fig. 1B) and tubules with degenerated germinal epithelium (fig. 1C) adjoined normal tubules. Gonads of the C57BL×129 mutant males (C57BL×129-p53+/-) had 7.2% abnormal tubules. Degenerated tubules, with mainly Sertoli cells, were sporadic in the testes of mutant males, and the majority of abnormal tubules were entirely filled with the germ cells (fig. 1D).
Embryo lethality
The control mice produced significantly more embryos than the heterozygous mice (tab. 3). The female-male ratio of the embryos on 7 dpc did not differ from 1:1 either in the heterozygous (28/25) or control (36/31) breeding pairs. The number of newborns in the heterozygous pairs corresponded with that of control breeding pairs (tab. 3). The sex ratio at delivery was 11 females/24 males and 24 females/19 males in the heterozygous and control pairs of mice, respectively (tab. 3). A significantly skewed sex ratio was observed in the progeny born to 55 heterozygous breeding pairs (124 females/202 males; tab. 4). Heterozygous (C57BL×129-p53+/-) and mutant mice (C57BL×129-p53-/-) were underrepresented in newborns of both sexes (tab. 4).

Table 3. Sex ratio and number (mean±SD) of embryos on 7 day post coitum (dpc) and newborn mice in the heterozygous (C57BL×129-p53+/-) and control (C57BL×129-p53+++) breeding pairs

<table>
<thead>
<tr>
<th>Genotype of breeding pairs</th>
<th>Embryos on 7 dpc</th>
<th>Newborns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number per breeding female</td>
<td>Female-male ratio</td>
</tr>
<tr>
<td>Heterozygotes C57BL×129-p53+/-</td>
<td>8.8±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28 / 25</td>
</tr>
<tr>
<td>Controls C57BL×129-p53+++</td>
<td>11.2±1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36 / 31</td>
</tr>
</tbody>
</table>

In each experiment six breeding pairs of mice were analyzed. The mutant pairs of mice were not analyzed due to sterile copulations occurrence or lack of the vaginal plug production. Different superscripts depict significant difference between heterozygotes and controls (p<0.05, Student’s t test); * p<0.05 (Chi square test)

Table 4. Distribution of sex and genotype in the progeny of the heterozygous breeding pairs of mice

<table>
<thead>
<tr>
<th>Total number</th>
<th>Control C57BL×129-p53+/-</th>
<th>Heterozygous C57BL×129-p53+/-</th>
<th>Mutant C57BL×129-p53-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>124&lt;sup&gt;*&lt;/sup&gt;</td>
<td>42 (34%)</td>
<td>65 (52%)</td>
</tr>
<tr>
<td>Males</td>
<td>202</td>
<td>68 (34%)</td>
<td>103 (51%)</td>
</tr>
</tbody>
</table>

*p=0.001 (Chi square test); the genotypes’ ratio in both female and male progeny was significantly different from 1:2:1 Mendelian inheritance.
DISCUSSION

Germ cells undergo mitotic and meiotic cell divisions, and to produce functional gametes, efficient control of DNA integrity and cell cycle regulation is needed. As a suppressor gene, \textit{Trp53} controls genome integrity and decides the fate of cells with mutated DNA [11, 17]. Apoptosis is a normal and physiological process that plays an essential role in controlling germ cell number and restricting abnormal cell proliferation during spermatogenesis [16, 19, 29]. If the mutation of \textit{Trp53} occurs, apoptotic pathways mediated by other proteins should be activated to retain male fertility. Indeed, germ cell type, inducing factors and even genotype can activate p53-dependent or p53-independent apoptosis in male mice gonads [14, 18, 23].

The genome of the C57BL×129 strain consists of the recombined genes derived from two parental inbred strains, 129/Sv and C57BL/6Kw, differing much in testis weight and all measured semen quality parameters except for sperm motility. However, in the C57BL×129-p53++ control males, genes controlling testes weight and sperm quality parameters seemed to be preferentially inherited after 129/Sv strain since the only parameter inherited after C57BL/6J strain was sperm concentration. Therefore, genes from the 129/Sv genetic background must be more abundant in the C57BL×129 strain, which was confirmed by microsatellite sequence analysis.

Our previous studies revealed that sperm morphology is under the strong control of the Y chromosome genes, but also autosomal genes influence this parameter to some extent [8, 26]. In the presented mouse model, the lowest percentage of abnormal sperm was noted in C57BL×129 males, and the Y chromosome of these males derived from the 129/Sv strain, characterized by low percentage of sperm head abnormality. Therefore, this parameter correlates with the inheritance pattern of the Y chromosome, and it is also positively influenced by autosomal genes’ composition of C57BL×129 males. As in the case of the C57BL/6 [12] and C57BL×C3H males [4], the percentage of abnormal sperm morphology did not correlate with \textit{p53} genotype of the C57BL×129 male mice.

We also showed that the mature sperm count (i.e. sperm without cytoplasmic droplet) and the percentage of sperm with unaffected tail membrane integrity were not regulated in the p53-dependent manner, since these parameters did not differ between the control and mutant males. However, the C57BL×129 males deficient for Trp53 protein had significantly heavier testes when compared with the control males. Testes
weight can be regulated by the genetic background of the strain, but also depends on the p53 status and treatment such as abdominal temperature [25, 28]. Testes size may affect sperm production, so that more sperm can be produced in bigger testes of p53-/- males, especially in view of a lack of p53-dependent apoptosis. Therefore, higher testes weight of our mutants could also result from germ cell accumulation due to the first apoptotic wave disruption. Yin et al. [29] showed that under physiological conditions C57BL/6-p53-/- mutant males produced more haploid cells in the testes. The results of testis morphology appear to support this hypothesis, since mutant tubules were completely filled with germ cells. Our mutant males also produced significantly more spermatozoa which was accompanied by decreased alive sperm percentage. Thus, a lack of functional p53 protein reduced germ cell elimination, but some of the cells that escaped apoptosis died possibly due to delayed Fas system activation [15, 30].

It was previously found that the testis morphology of males with the C57BL×129 mixed (heterozygous) genetic background appeared normal [21]. In the present study, the morphological profile of the control C57BL×129 males’ gonads was characterized by the presence of some degenerative tubules, resembling tubules with Sertoli cell-only (SCO) syndrome. It is known that the morphological structure of the p53-/- males’ gonads depends on the genetic background. While multinucleate giant cells were observed in the seminiferous tubules of the 129/Sv-p53-/- males [21], normal testes’ structure was found in the C57BL/6-p53-/- male mice [28, 30]. Thus, the observed phenotype in the testes of our C57BL×129 males may be a result of their highly homozygous genetic background (F16-F18). Such degenerated tubules were not found in the mutants. This suggests that empty tubules found in the testis of our C57BL×129 controls resulted from p53-dependent apoptosis, especially since the mutants’ tubules were filled with the sperm. These cells were probably not eliminated due to a lack of p53.

Breeding experiments revealed that no progeny could be obtained when mutants were crossed. However, mutant females mated with heterozygous males produced up to two litters, and 33% of reciprocal crosses revealed to be fertile. Therefore, decreased vitality and fertility of the mutant animals of both sexes were responsible for the extremely limited pregnancies. When the heterozygous animals were mated, the females possessed less embryos on 7 dpc than females from the control pairs, but female and male embryos were equally eliminated before day 7 of pregnancy in both types of breeding pairs. Embryos’ resorption was also
evident after 7 dpc in both heterozygous and control breeding pairs. However, in the heterozygotes, preferential female embryo elimination after 7 dpc was responsible for the skewed sex ratio at delivery, a phenomenon confirmed by our large scale genotyping experiment. This suggests that after 7 dpc, female embryos of p53+/- and/or p53-/- genotype were eliminated preferentially in heterozygous pairs.

Theoretically, 1:2:1 Mendelian inheritance ratio of control, heterozygous and mutant mice of each sex should be born in the crosses between heterozygous animals. However, genotyping of the heterozygotes’ offspring revealed that particularly mutant animals of both sexes were underrepresented probably due to preferential resorption of the mutant embryos after 7 dpc. Similar results were described in the CE/J×129/SvJ strain [5, 10], where autosomal genes were proposed to regulate the p53-dependent neural tube defect occurrence. Chen et al. [3] demonstrated that this developmental abnormality is also related with the number of X chromosomes, and not Y chromosome genes. Our experiment also showed that the skewed sex ratio in the newborn mice is only partly the effect of mutant females’ preferential elimination, since control and heterozygous females were also less frequent than males with respective genotypes. Thus, sex chromosomes’ composition can strongly modulate p53 impact on cell fate during the embryo development.

Summarizing, most of the examined parameters of semen quality depends on the genetic background of the mouse strain. Due to deregulated apoptosis in the testes, germ cell accumulation, heavier gonads and elevated sperm count were found in p53-/- males. The mutation of p53 affects also sex-dependent embryo lethality. Not only were born less females than males, but also the mutants of both sexes were underrepresented in the progeny of heterozygous pairs of mice. Therefore, while the genetic background is responsible for male mice gametes quality, the interplay between genes on sex chromosomes and p53 affects embryo lethality.

ACKNOWLEDGEMENTS
This work was supported by WRBW/BiNoZ/IZ/1/2009 and K/ZDS/0001718 from the Institute of Zoology of the Jagiellonian University. We thank Professor Allan Bradley (Wellcome Trust Sanger Institute, UK) and Professor Romeo Ricci (ETH Zurich, Switzerland) for providing us with the Trp53-/- mice.
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