Cell Death and Differentiation (1998) 5, 669–677

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The role of the tumor suppressor p53 in spermatogenesis

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Received: 22.12.97; revised: 3.3.98; accepted: 20.3.98
Edited by E. Yonish-Rouach

Abstract

The p53 protein appeared to be involved in both spermatogonial cell proliferation and radiation response. During normal spermatogenesis in the mouse, spermatogonia do not express p53, as analyzed by immunohistochemistry. However, after a dose of 4 Gy of X-rays, a distinct p53 staining was present in spermatogonia, suggesting that, in contrast to other reports, p53 does have a role in spermatogonia. To determine the possible role of p53 in spermatogonia, histological analysis was performed in testes of both p53 knock out C57BL/6 and FvB mice. The results indicate that p53 is an important factor in normal spermatogonial cell production as well as in the regulation of apoptosis after DNA damage. First, p53 knock out mouse testes contained about 50% higher numbers of A_1 spermatogonia, indicating that the production of differentiating type spermatogonia by the undifferentiated spermatogonia is enhanced in these mice. Second, 10 days after a dose of 5 Gy of X-rays, in the p53 knock out testes, increased numbers of giant sized spermatogonial stem cells were found, indicating disturbance of the apoptotic process in these cells. Third, in the p53 knock out testis, the differentiating A_2-B spermatogonia are more radioresistant compared to their wild-type controls, indicating that p53 is partly indispensable in the removal of lethally irradiated differentiating type spermatogonia. In accordance with our immunohistochemical data, Western analysis showed that levels of p53 are increased in total adult testis lysates after irradiation. These data show that p53 is important in the regulation of cell production during normal spermatogenesis either by regulation of cell proliferation or, more likely, by regulating the apoptotic process in spermatogonia. Furthermore, after irradiation, p53 is important in the removal of lethally damaged spermatogonia.

Keywords: p53; spermatogenesis; mouse; apoptosis; giant cell; spermatogonial stem cell; irradiation

Abbreviations: CDKi, cyclin dependent kinase inhibitor; HLH, helix–loop–helix; PBS, phosphate buffed saline; DAB, diaminobenzidine; PAS, periodic acid Schiff; TdT, terminal deoxynucleotidyl transferase

Introduction

The tumor suppressor p53, also referred to as the guardian of the cell cycle (Lane, 1992), has important functions in cell growth and differentiation. During the cell cycle checkpoints at the G_1/S and G_2/M borders are under the control of p53 (Agarwal *et al*, 1995; Pellegata *et al*, 1996; Ko and Prives, 1996). After DNA damage, for example inflicted by ionizing irradiation, p53 is often upregulated in mammalian cell lines, resulting in the initiation of apoptosis, repair pathways (Chernova *et al*, 1995), a G_1/S (Kuerbitz *et al*, 1992; Zölzer *et al*, 1995) or G_2/M cell cycle arrest (Guillouf *et al*, 1995). P53, as a transcription factor, is able to upregulate a series of proteins, among which Bax (Miyashita and Reed, 1995), Gadd45 (Kastan *et al*, 1992) and the Cyclin Dependent Kinase inhibitor (CDKi) $p21^{Cip1/WAF1}$ (El-Deiry *et al*, 1993). In turn, p53 has a helix–loop–helix (HLH) binding site in its promoter (Ronen *et al*, 1991). So, p53 may be regulated by basic HLH containing transactivators (Reisman and Rotter, 1993) a family of proteins involved in cell differentiation programs of keratinocytes (Rehberger *et al*, 1997) and myelocytic cell lines (Rotter and Ronen, 1997).

Spermatogenesis is a well characterized process in which spermatogonia proliferate and differentiate into terminally differentiated spermatozoa. Undifferentiated spermatogonia laying at the basal membrane of the seminiferous tubule, can be subdivided according to their topographical arrangement into A single (A_s), paired (A_{pr}) or A aligned (A_{al}) and are at the beginning of the spermatogenic lineage (Oakberg, 1971; de Rooij, 1973). These cells are proliferating during part of the cycle of the seminiferous epithelium (stages X–II) and then become quiescent (stages III–VII) until most of them differentiate into differentiating type A_1 spermatogonia in stage VIII. After a series of five subsequent divisions (A_2-B), differentiating spermatogonia divide into spermatocytes that move toward a more adluminal position of the seminiferous tubules. After the two meiotic divisions the spermatocytes become round spermatids, which develop into elongating spermatids without further divisions.

During spermatogenesis, the p53 mRNA and protein are present in primary spermatocytes (Sjöblom and Lähdetie, 1996; Almon *et al*, 1993; Schwartz *et al*, 1993). From these results it was suggested that p53 has a role during the prophase of meiosis. Possibly, this is accomplished via the induction of $p21^{Cip1/WAF1}$ which is also expressed during the prophase of meiosis (Beumer *et al*, 1997; West and Lähdetie,

1997). After irradiation, p53 expression was found to be enhanced in spermatocytes (Sjöblom and Lähdetie, 1996).

We now have studied the presence and the role of the p53 protein in the mouse testis before and after X-irradiation by way of immunohistochemistry, Western blot analysis and by studying spermatogenesis in p53 knock out mice. Besides expression of p53 in spermatocytes, a distinct induction of p53 was found in spermatogonia after irradiation. Furthermore, in p53 knock out mice, increased numbers of spermatogonia were found as well as profound effects on radiation induced spermatogonial apoptosis.

Results

Immunohistochemical localization of p53 in the normal and X-irradiated testis

In the normal adult mouse testis, pachytene spermatocytes in stages XI till VIII were lightly stained for p53 (Figure 1A, Sc). Spermatogonia, Leydig cells and Sertoli cells did not stain for p53 (Figure 1A). However, after irradiation, virtually all spermatogonia in all epithelial stages, and preleptotene spermatocytes in stage VII stained heavily for p53 from 1.5 till at least 24 h post irradiation (Figure 1B). Sertoli cells also stained after irradiation (Figure 1B). After irradiation, no increase in p53 staining was observed in pachytene spermatocytes (Figure 1B, Sc). Spermatids were never stained for p53 (Figure 1A, St; 1B). In irradiated p53 knock out mice (6 h post irradiation), in the testis no immunohistochemical staining for p53 was detected (Figure 1C).

Spermatogonial apoptosis in the p53 knock out testis

To investigate whether germ cells were able to undergo apoptosis in the p53 testis, TUNEL analysis was performed on p53 knock out testis. As in the wild-type testis, in the p53 knock out testis too, morphologically apoptotic cells that were also TUNEL stained were found (Figure 1D, open arrow). Some of these apoptotic cells were laying at the basal membrane of the seminiferous tubule.

Levels or p53 change after X-irradiation

Western blot analysis was performed on whole testis lysates of normal and X-irradiated mouse testes (1.5, 3, 6, 9, 12, 18,

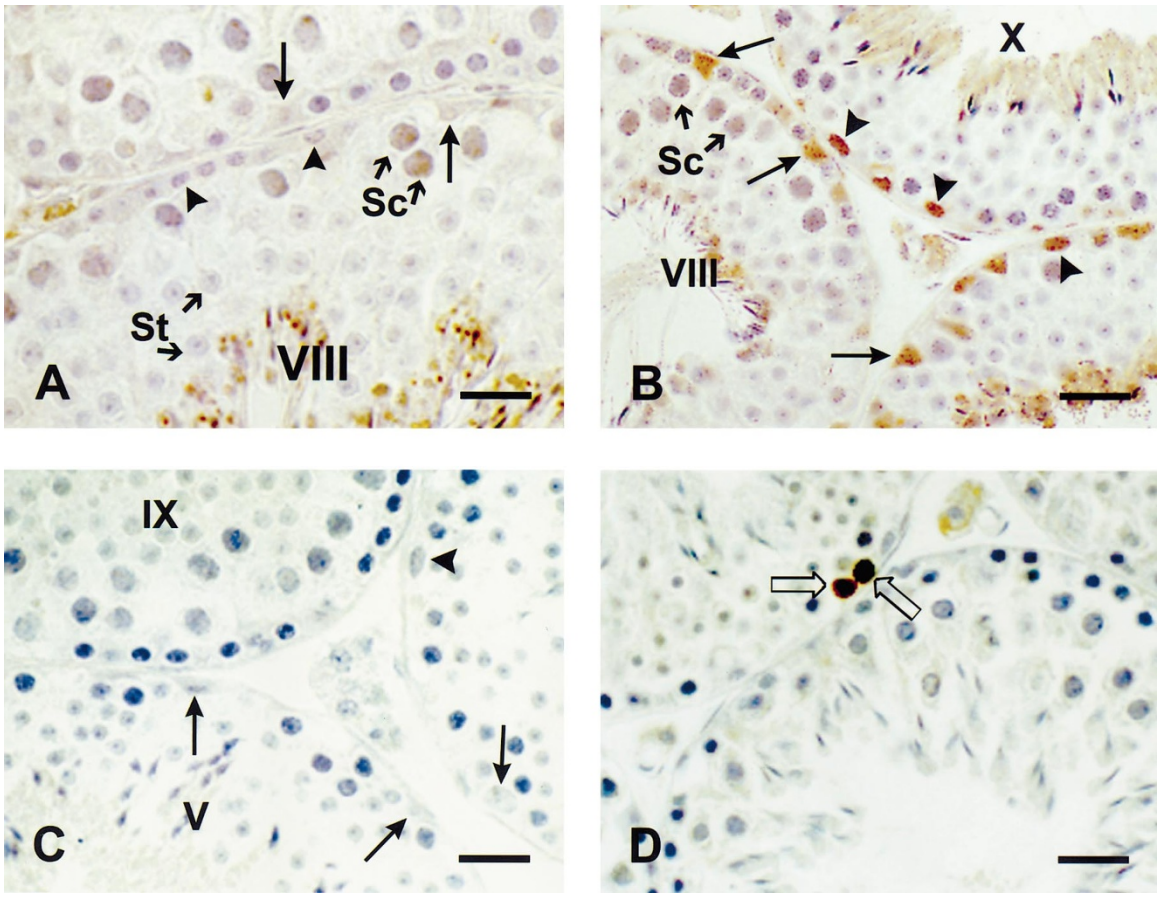

Figure 1 Immunohistochemical p53 staining in mouse testis before and after X-irradiation. (**A**) In the non-irradiated adult mouse testis a light p53 staining is seen spermatocytes (Sc). No p53 staining can be observed in Sertoli cells (arrow) or spermatogonia (arrowhead). (**B**) Levels of p53 staining were high in spermatogonia and Sertoli cells at 6 hours post irradiation. No staining was present in spermatids (St). (**C**) In 5 Gy irradiated C57Bl/6 p53 knock out testis, 6 h post irradiation, no p53 staining is present. (**D**) Morphologically apoptotic, TUNEL stained, cells are present in non-irradiated p53 knock out testis (open arrow). Stages of the seminiferous epithelium are indicated with Roman numerals. Magnification: bar represents 20 μm

24 h post irradiation). In three independent irradiation experiments, p53 was detected in the non-irradiated testis (Figure 2, time point T=0). Within 3 h post irradiation an increase of p53 was found in total testis lysates. In the three experiments 4.4–6.1-fold increases in p53 levels were found 3 h post irradiation. At 6 h post irradiation levels of p53 slightly decreased, where after levels increased at 12 h post irradiation. Twenty-four hours post-irradiation p53 levels had declined to almost control levels (Figure 2).

Irradiation affects spermatogenesis differently in p53 knock out and wild-type mice

In sections of testes of irradiated mice, a severe effect of 5 Gy of X-rays was revealed. Spermatogonia were sparse 10 days post irradiation and there was a severe depletion of spermatocytes up to pachytene phase in epithelial stage VIII, indicating that many spermatogonia were killed by the irradiation.

In C57BL/6 p53 knock out mice, as well as in the heterozygote and in wild-type mice, the effect of a dose of 5 Gy of X-rays on spermatogonial stem cells was evaluated by determination of the percentage of tubular cross sections showing one or more spermatogonia (repopulation index), 10 days post irradiation. In previous studies it was found that the extent of the repopulation was a good measure of the number of the number of surviving spermatogonial stem cells (van der Meer *et al*, 1992). Although the repopulation index tended to be higher in the p53 knock out mice, no significant differences were found (Table 1).

However, many abnormally large spermatogonia were seen in the irradiated knock out mice (Figure 3B, arrows). These spermatogonia were mostly single cells, but occasionally pairs were seen. A sevenfold increase was found in the percentage of tubular cross-sections showing one or more giant spermatogonia, laying at the basal membrane, in irradiated knock out compared to wild-type mice (Table 1, Figure 3A and B). No significant increase in the number of giant spermatogonia was seen in the heterozygotes.

Also the effects of irradiation on A_2-B spermatogonia were evaluated. Ten days after irradiation, surviving A_2-B spermatogonia will have developed into pachytene spermatocytes in epithelial stages I–VI. This because virtually all types of spermatogenic cells are subject to the principle of 'go or die', i.e. they develop further or go into apoptosis immediately, being unable to temporarily arrest development. Hence, we have determined the percentages of seminiferous tubular cross sections in stages I–VI which contained one or more pachytene spermatocytes. It was found that the testes of the irradiated p53 knock out mice contained significantly more ($P<0.01$) pachytene spermatocytes (Table 1), indicating higher numbers of surviving A_2-B spermatogonia in these mice compared to wild-type mice. Also the number of pachytene spermatocytes in heterozygotes tended to be higher but the differences were not statistically significant (Table 1).

p53 knock out and wild type testes have different spermatogonial cell numbers

In epithelial stage VIII undifferentiated spermatogonia (A_s, A_{pr}, and A_{al}) are present, as well as the first generation of the differentiating type spermatogonia, the A_1 spermatogonia. The A_1 spermatogonia were formed by the undifferentiated spermatogonia during the preceding epithelial cycle. Furthermore, there are preleptotene spermatocytes that in turn were formed by the A_1 spermatogonia via six subsequent divisions during the preceding cycle. Hence, in epithelial stage VIII 3 subsequent generations of cells can be counted and the efficiency of the cell production in these generations during the epithelial cycle evaluated.

Cell counts were performed in sections of testes of wild-type and p53 knock out C57BL/6 mice (Figure 4). In sections it was not possible to distinguish between the different types of A spermatogonia present in stage VIII but a significant 40% increase in the total numbers of A

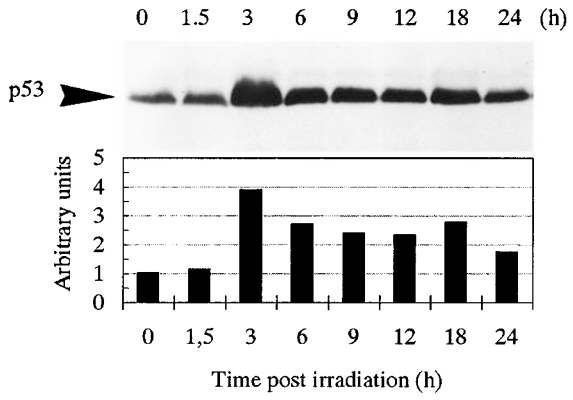

Figure 2 Western blot analysis of p53 in lysates of whole testis before and after irradiation. Total protein extracts from non- or 4 Gy irradiated adult testes were separated onto a 13% SDS–PAGE gel, transferred to PVDF membrane, and probed for p53. Densitometrical analysis is given as a bar diagram. Indicated time points are post-irradiation. Time point T=0 represents a non-irradiated testis lysate which was used as a control reference.

Table 1 The effect of a dose fo 5 Gy of X-irradiation on germ cells in p53 wild-type heterozygote and knock out C57Bl/6 mice, 10 days post irradiation. Mean±S.E.M.

	p53 +/+ ($n=4$)	p53 +/– ($n=6$)	p53 –/– ($n=4$)
% tubular cross-sections showing			
A spermatogonia	26.7±2.0	24.7±3.0	32.2±5.0
Giant spermatogonia	3.1±1.0	6.0±1.0	23.5±0.0**
Pachytene spermatocytes	7.2±5.3	26.4±11.1	46.3±2.9*

*Significantly different from wild-type $P<0.01$, **$P<0.005$

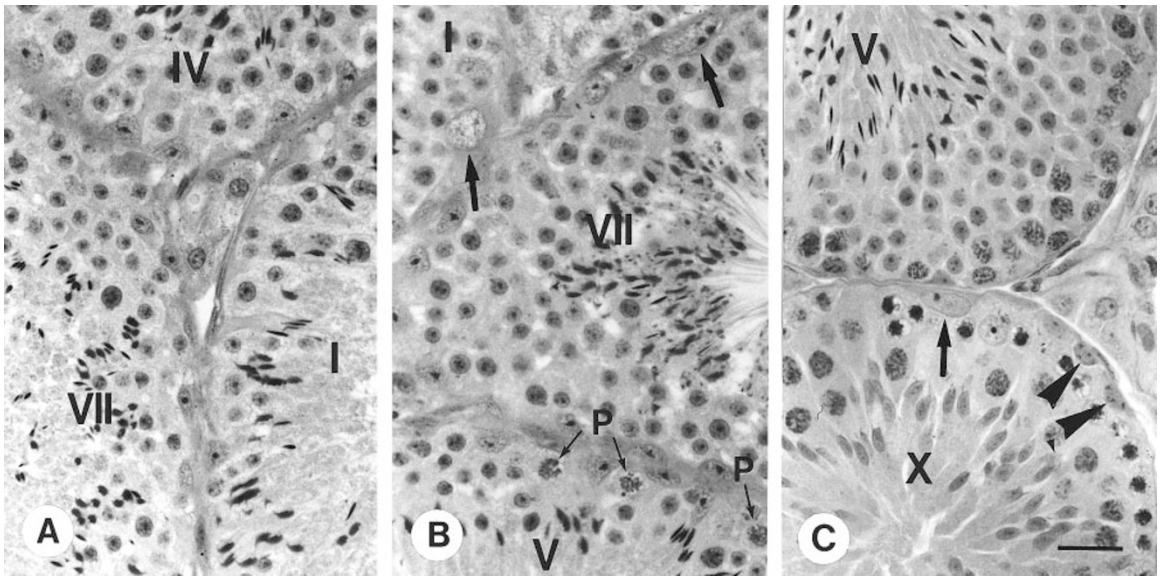

Figure 3 Histology of p53 knock out and wild type testes 10 days after a of 5 Gy of X-rays. (**A**) In wild-type testes very few pachytene spermatocytes were found in epithelial stages I-VI, indicating a virtually complete killing of their predecessors, the A_2-B spermatogonia. Also giant spermatogonia, stem cells that still duplicate their DNA but are unable to divide were rare in wild-type testes. (**B**) In contrast, in p53 knock out testes, pachytene spermatocytes and giant spermatogonia (arrows) were much more numerous. (**C**) Occasionally, in unirradiated p53 knock out testes a giant spermatogonium was seen. Spermatogonia are indicated by arrow heads. Magnification: bar represents 25 μm

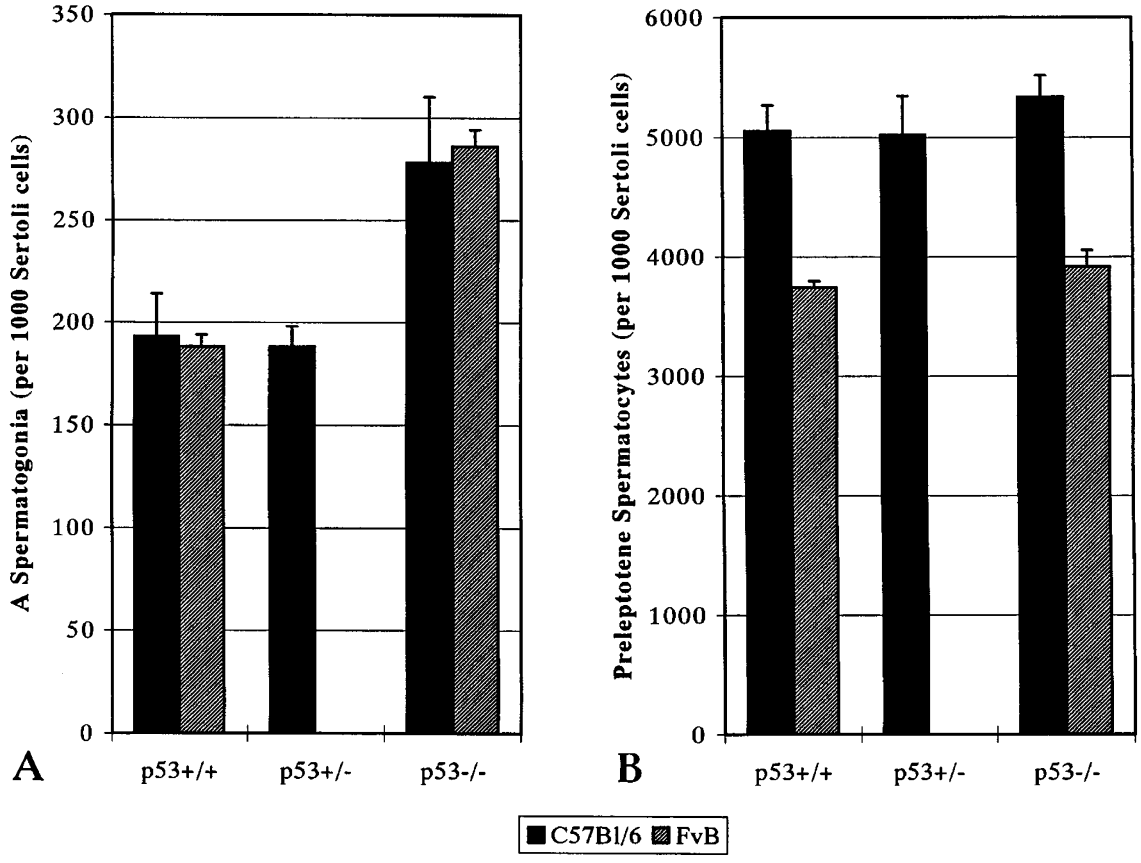

Figure 4 The number of the total population of A spermatogonia in stage VIII of the cycle of the seminiferous epithelium is higher in the absence of p53 (**A**). In both C57Bl/6 and FvB knock out mouse testes a significant increase ($P < 0.01$) of A spermatogonia was found. In contrast no difference could be found in the numbers of preleptotene spermatocytes in the p53 knock out compared to the wild-type (**B**). Numbers of spermatogonia and preleptotene spermatocytes are expressed per 1000 Sertoli cells

spermatogonia was found. No significant difference was found between the numbers of preleptotene spermatocytes in p53 knock out and wild type mice (Figure 4B).

The origin of the increase in the total numbers of A spermatogonia was studied in more detail in FvB p53 knock out mice, using whole mounts of seminiferous tubules. Also in this strain of mice, a significant increase (51%, $P<0.0005$) in the total number of A spermatogonia in stage VIII was found in p53 knock out mice compared to wild-type mice. As in C57BL/6 mice, the higher numbers of A spermatogonia did not lead to significantly higher numbers of preleptotene spermatocytes (Figure 4). In addition, in contrast to sections in whole mounts of seminiferous tubules the topographical arrangement of the A spermatogonia on the basal membrane remains intact, enabling the distinction between A_s, A_{pr}, A_{al} and A_1 spermatogonia in epithelial stage VIII. The detailed counts revealed no significant differences between p53 knock out and wild-type mice with respect to the numbers of spermatogonial stem cells (A_s), A_{pr} and A_{al} spermatogonia, or total numbers of these undifferentiated A spermatogonia (Table 2). However, there was a statistical significant difference ($P<0.0005$) in the numbers of A_1, spermatogonia between wild-type and p53 knock out mice (Table 2).

During the cell counts, in p53 knock out mice occasionally giant spermatogonia were found (Figure 3B). No such cells could be observed in the wild-type mice. Giant cells, derived from primary spermatocytes, laying at more adluminal positions of the seminiferous epithelium were not found in both p53 knock out C57BL/6 and FvB mouse testes.

Discussion

The present results, for the first time, show important and partly indispensable functions of the p53 protein in spermatogonial cell production and in the response of the various types of spermatogonia, including stem cells, to irradiation.

In the normal mouse testis, immunohistochemical staining for p53 was not observed in spermatogonia. However, after a dose of 4 Gy of X-rays, p53 staining was clearly present in many spermatogonia from 1.5–24 h post irradiation. These results indicate that p53 has a function in spermatogonia in their response to DNA damage. Because most spermatogonia go into apoptosis at 9–18 h after irradiation (Beumer *et al*, 1997), in these cells p53 might induce a cell cycle arrest, repair and/or apoptosis. However, since $p21^{(Cip1/WAF1)}$ is not expressed in spermatogonia before or after irradiation (Beumer *et al*, 1997) p53 does not exert its role via $p21^{(Cip1/WAF1)}$. A comparable situation was seen in fibroblasts in which transcriptional activation by p53 was essential for apoptosis induction but not of the p21 gene (Donatella Attardi *et al*, 1996). Another possibility is that p53 induces apoptosis by upregulation of Bax, which is another regulator of apoptosis (Miyashita and Reed, 1995). An indication for this can be seen on the fact that Bax deficiency results in an accumulation of spermatogonia caused by decreased spermatogonial apoptosis which also takes place in the normal mouse (Knudson *et al*, 1995). Clearly, the role of Bax in male germ cell apoptosis needs further study.

Table 2 Numbers of the various types of A spermatogonia in epithelial stage VIII, in p53 +/+ and p53 –/– FvB mice, as counted in whole mounts of seminiferous tubules. Numbers are expressed per 1000 Sertoli cells, mean±S.E.M.

		p53 +/+ ($n=3$)	**p53 –/–** ($n=5$)
Spermatogonia	A_1	153.4±3.1	242±7.5*
	A_s	15.2±1.2	15.2±2.2
	A_{pr}	18.2±2.9	21.6±1.7
	A_{xl}	2.2±0.9	6.6±1.8
	A_{undiff}	35.0±3.6	43.2±3.5
	A total	188.2±5.5	285.2±8.2*

*Significantly different from wild-type $P<0.005$

P53 expression in spermatogonia was not observed in the X-irradiated rat testis (Sjöblom and Lähdetie, 1996). However, Sjöblom and Lähdetie used Bouin's solution to fix the testes. In our hands, testes fixed in Bouin's solution were not suitable to immunohistochemically detect p53, while our protocol in which we fix in formalin and post fix in a diluted (0.9% picric acid) Bouin's solution does give rise to detectable p53 staining, as described.

Possible functions of p53 were also studied in p53 knock out mice. First, it was investigated whether the absence of p53 would render spermatogonial stem cells more radioresistant. In previous studies (van der Meer *et al*, 1992) it was shown that the percentage of seminiferous tubular cross sections showing A spermatogonia at 10 days after irradiation is representative for stem cell survival. After a dose of 5 Gy of X-rays, no statistically significant difference between p53 knock out and wild-type mice was found using this parameter, indicating that p53 is not an important factor in the radiosensitivity of spermatogonial stem cells. These results are consistent with previous results of Hendry *et al*. (1996) who did not find a difference in the D_0 value for stem cell killing by X-rays between p53 knock out and wild-type mice.

Second, the occurrence of giant spermatogonia was studied in testis sections of irradiated mice. We have previously shown that after irradiation some spermatogonial stem cells become unusually large. These giant spermatogonia are most frequent at 8 days after irradiation and have disappeared by about day 15 (van Beek *et al*, 1984, 1986). Apparently, these cells are still capable of DNA synthesis, can no longer divide and take a long time before they undergo apoptosis (van Beek *et al*, 1986). At 10 days after irradiation, in p53 knock out mice, a sevenfold increase was seen in the number of tubular cross sections showing one or more giant spermatogonia compared to the wild-type mice. This suggests that after irradiation many spermatogonial stem cells in p53 knock out mice have problems entering the apoptotic pathway. In addition, also in whole mounts of seminiferous tubules of unirradiated p53 knock out mice, occasionally a single giant spermatogonium was observed, while these cells were completely absent in unirradiated wild-type mice. This suggest that spermatogonial apoptosis in the normal mouse is not restricted to

differentiating spermatogonia as described before (Huckins, 1978) but that there is occasional apoptosis of stem cells too. Giant cells, originating from primary spermatocytes as previously found in some p53 knock out mice by Rotter *et al*. (1993) were not observed in testes of p53 knock out C57BL/6 and FvB strains. This may be due to strain differences, since Rotter *et al*. (1993) observed these giant cells in a 129 background, while their p53 knock out mice with a mixed genetic background C57BL/6 $\times$ 129 exhibited a normal structure of the seminiferous tubules. Taken together, the present results strongly suggest that p53 has an important role in spermatogonial stem cell apoptosis both after irradiation and in the normal situation.

Third, the cell killing effect of irradiation on differentiating type A_2-B spermatogonia was investigated by studying the presence of their descendants at 10 days post irradiation, i.e. pachytene spermatocytes in epithelial stages I–VI. In general, in all mice numbers of pachytene spermatocytes in these stages were low compared to the unirradiated controls. This indicates that in p53 knock out mice most lethally damaged A_2-B spermatogonia do degenerate, but apparently not via the p53 apoptotic pathway. Nevertheless, many more pachytene spermatocytes were found in p53 knock out than in wild-type mice. Apparently, in p53 knock out mice relatively many A_2-B spermatogonia that would otherwise be lethally damaged and killed by the irradiation are capable to carry out one or more divisions, even start meiotic prophase and reach at least pachytene stage. Obviously in p53 knock out mice these cells are more radioresistant. This indicates that p53 is also important in the apoptotic pathway of differentiating type spermatogonia. Indeed, after a dose of 5 Gy of X-rays, 16 h post irradiation no apoptotic spermatogonia were observed (Odorisio *et al*, 1998), while in wild-type mice spermatogonial apoptosis is at its peak at this interval post irradiation (Beumer *et al*, 1997).

Fourth, cell counts in both FvB and C57BL/6 p53 knock out mice revealed that the lack of p53 causes a 40–50% increase in the total number of A spermatogonia in epithelial stage VIII. The total number of A spermatogonia in epithelial stage VIII represents the sum of the undifferentiated A spermatogonia and the number of A_1 spermatogonia that was formed during the preceding cycle of the seminiferous epithelium. To study this in further detail, cell counts in whole mounts of seminiferous epithelium were carried out, which revealed no significant difference in the numbers of undifferentiated spermatogonia between knock out and wild-type mice. However, a highly significant increase of 58% ($P < 0.0005$) was seen in the numbers of A_1 spermatogonia. This either means increased proliferative activity of the undifferentiated spermatogonia in p53 knock out mice, suggesting a role of p53 in the regulation of the normal cell cycle of these cells, or a decreased apoptosis of undifferentiated spermatogonia in p53 knock out mice. Apoptosis of differentiating type A spermatogonia in the normal testis has been described extensively by a number of groups (Rodriguez *et al*, 1997; Furuchi *et al*, 1996; Allan *et al*, 1992) and could be useful in removal of aberrant cells and/or regulation of cell density (De Rooij and Lok, 1987; De Rooij and Janssen, 1987). So far, no degeneration/apoptosis of undifferentiated spermatogonia in the normal testis has been observed. Nevertheless, the present results suggest that this type of spermatogonia are also vulnerable to apoptosis during the normal epithelial cycle.

Despite the increased numbers of A_1 spermatogonia in p53 knock out mice, no statistically significant increased numbers of preleptotene spermatocytes were found. Hence, despite the absence of p53, more differentiating spermatogonia degenerate during their series of divisions into spermatocytes than in wild-type mice. Apparently, the density constraints of differentiating spermatogonia are rather strict and apoptosis occurs anyway via a secondary pathway with a similar efficiency. In addition to the presence of TUNEL positive cells at the basement membrane in the p53 knock out testis, these results also indicate that differentiating type of spermatogonia are able to undergo apoptosis in the absence of p53. These results are compatible with the results of Perez *et al*. (1997), showing that female germ cell death is independent of p53.

Immunohistochemical staining showed that p53 is expressed at low levels in spermatocytes in the normal mouse testis in stages XI–VIII of the seminiferous epithelium. This is in support of the levels of p53 mRNA expression in the normal mouse testis (Almon *et al*, 1993) and p53 protein expression in the normal rat testis (Sjöblom and Lähdetie, 1996; Schwartz *et al*, 1993). The expression of p53 in spermatocytes indicates that p53 could be important during the meiotic prophase. The reported p53 upregulation in spermatocytes after X-irradiation in the rat by Sjöblom and Lähdetie (1996) was not observed in mouse spermatocytes using immunohistochemical analysis. However, immunohistochemistry is not well suitable for making quantitative comparisons.

Sertoli cells do not stain for p53 in the normal mouse testis, however after a dose of 4 Gy of X-rays p53 staining can be seen in Sertoli cells. Because Sertoli cells are terminally differentiated and do not go into apoptosis after a dose of 4 Gy of X-rays, a possible function of p53 might be the induction of DNA repair pathways.

Levels of p53 in normal and irradiated testes were determined using Western blotting analysis of whole testis lysates. Although in these experiments both cytoplasmic and nuclear p53 was determined, these experiments clearly show that p53 is present in the normal mouse testis and is upregulated after X-irradiation, in a way compatible to our immunohistochemical data. The presence of high levels of p53 at 3 h post irradiation shows that p53 expression rapidly responds to DNA damage. This was also found by Kuerbitz and coworkers (1992) in various human cell lines. Six hours post irradiation p53 levels decline, after which a second increase in p53 levels may be present at 18 h post irradiation. This pattern may be due to differences in the timed p53 expression in spermatogonia and Sertoli cells. The observed p53 expression pattern may be testis or dose specific, since single or double p53 waves post X-irradiation are reported dependent on tissue (Midgley *et al*, 1995) and dose of X-rays (Wang *et al*, 1996).

In conclusion, the present data show, for the first time, that p53 has an important role in all subsequent types of spermatogonia. First, while p53 is not important in determining the radiosensitivity of spermatogonial stem cells, without p53 lethally damaged stem cells take much longer to undergo apoptosis. Second, cell production by the undifferentiated spermatogonia is much more efficient in p53 knock out mice than in wild-type mice, indicating either enhanced proliferative activity or less apoptosis of these cells. Third, differentiating type spermatogonia strongly express p53 after irradiation and are more radioresistant in the sense that after a dose of 4 Gy considerably more of these cells survived and were able to reach at least pachytene stage. When these severely damaged cells would be able to become spermatozoa, this would mean that during a restricted period of time after irradiation, upon fertilization the risk of inducing genetically compromised offspring is high. In this, it is reassuring that severely damaged stem cells do die in spite of a lack of p53.

Materials and Methods

Animals, irradiation and fixation

The testes of adult male FvB/NAU mice (Central Laboratory Animal Institute, Utrecht, The Netherlands) of at least 7 weeks of age were locally irradiated (Philips, 200 kV, 20 mA, 0.5 mm Cu^{2+} filter). A single dose of 4 Gy was given. Groups of four mice were sacrificed by cervical dislocation at 1.5, 3, 6, 9, 12, 18 and 24 h after a dose of 4 Gy. Control mice were sham-irradiated.

The p53 knock out C57BL/6 mice originally were generated by Jacks and coworkers (1994) and were raised at the Leiden University via breeding of heterozygotes. Part of the different genotypes (p53+/+, p53+/−, p53−/−) received a dose of 5 Gy of total body X-irradiation using a Andrex SMART 225 machine operating at 200 kV and 4 mA, resulting in an HVL of 1.0 mm Cu^{2+} and a dose rate of 0.6 Gy/min. FvB P53 knock out mice were generated by Donehower and coworkers (1992) and were reared at the Netherlands Cancer Institute at Amsterdam.

For histology and immunohistochemistry, testes were fixed in 10% neutral buffered formalin for 4 h and post fixed in a diluted Bouin solution (71% picric acid (0.9%), 24% formaldehyde (37%), 5% acetic acid) for 16 h, at 4°C. Tissues were washed in 70% EtOH prior to embedding in paraffin for immunohistochemistry (Stemcowax, Adamas Instruments, The Netherlands) or Technovit 7100, for histological examination (Kulzer & Co. GmbH, Wehrheim, Germany).

For protein isolation, testes were frozen in liquid nitrogen and stored at −80°C.

Immunohistochemistry

To avoid slide-to-slide differences, 5 μm paraffin sections, of testes at different intervals after irradiation were mounted together on a silane coated glass slide. Series of testes of animals from at least three different irradiation experiments were used. Also testes of irradiated p53 knock out mice were used. Unmasking of the epitope was established by boiling the sections three times for 10 min in 0.01 M sodium citrate, using a microwave oven (H2500, Bio-Rad). Endogenous peroxidase was blocked by incubation in 0.35% H_2O_2 in PBS for 10 min. The slides were washed in phosphate buffered saline (PBS) and then incubated in 10% normal horse serum to block nonspecific binding sites of the antibodies. Subsequently, the slides were incubated with a rabbit polyclonal p53 antibody (Novocastra Laboratories Ltd., NCL-p53-CM5p), diluted 1 : 200 in PBS including 5% normal horse serum in a humidified chamber overnight at 4°C. After extensive washing steps in PBS, slides were incubated for 60 min with a secondary biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., USA) diluted 1 : 200 in PBS including 5% normal horse serum, in a humidified chamber. The horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol (Vectastain Elite, Vector Laboratories). To visualize bound antibodies, sections were washed in PBS and covered with 0.3 μg/μl 3, 3′-diaminobenzidine (DAB, Sigma) in PBS, to which 0.03% H_2O_2 was added. Sections were counterstained with Mayer's haematoxylin.

Negative control sections were treated as described above, except that the primary antibody was omitted during the procedure and replaced by normal rabbit serum. Adjacent sections were used for a periodic acid Schiff (PAS) staining to identify the stages of the cycle of the seminiferous epithelium of the tubules.

TUNEL analysis

Apoptotic cells were detected at single cell level, using the TUNEL method (In Situ Cell Death Detection Kit, Boehringer Mannheim). Procedures were carried out according to the manufacturer's protocol. In short, sections were deparaffinated and rehydrated, after which sections were treated with 10 μg/ml proteinase K in 10 mM Tris/HCl (pH 7.5). Endogenous peroxidase was blocked in 0.35% H_2O_2 for 30 min. Single and double strand breaks were 3′ labeled with fluorescein labeled nucleotides, using terminal deoxynucleotidyl transferase (TdT). Incorporated fluorescein was detected by anti-fluorescein antibody Fab fragments, conjugated with horseradish peroxidase. Bound antibodies were covered with 0.3 μg/μl 3,3′-diaminobenzidine tetrachloride (DAB, Sigma) in PBS, to which 0.03% H_2O_2 was added.

Protein gel electrophoresis and Western blotting

Total protein lysates were prepared by mincing the testes in a membrane disrupter (micro-dismembrator II, B. Braun Biotech) after which the cells were lysed in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1 % SDS) for 30 min on ice. Lysates were sonicated on ice and cleared by centrifugation. Protein levels were measured using BCA analysis (Pierce). SDS–PAGE was performed as described by Laemmli (1970). Fifty μg of protein was denaturated by boiling for 5 min in Laemmli SDS sample buffer and separated on a 13% PAGE gel. Proteins were blotted onto a PVDF membrane (MilliPore). After blotting the gel was Coomassie stained for transfer efficiency.

Western blots were blocked using Blotto-A, containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline (10 mM Tris-HCl, pH 8.0; 150 mM NaCl), including 0.05% Tween-20 (TBT). The antibody against p53 (Pab 240 1 : 100, Mouse monoclonal SC-99, Santa Cruz Biotechnology Inc., USA) was diluted in Blotto-A. Incubation with Rabbit anti Mouse (RAMPO, DAKO), conjugated to horse radish peroxidase, 1 : 10 000 in Blotto-A, was performed after at least three rinses in TBT for 5 min each.

Chemiluminescence (ECL, Amersham) was used for analyzing levels of protein according to the manufacturer's protocol. Blots were exposed to X-ray film (RX, Fuji, Japan). Intensity of p53 protein signals were measured using a densitometer (GS-700, Bio-Rad).

Histological examination

For histological examination, testes were embedded in Technovit 7100. Five μm sections were stained by the Periodic-acid Schiff reaction (PAS) and counterstained with Mayer's haematoxylin. The stages of the cycle of the seminiferous epithelium were classified according to Oakberg (1956). In testis sections of p53 knock out and wild type C57BL/6 mice the numbers of both A-spermatogonia and preleptotene spermatocytes were counted in stage VIII of the seminiferous epithelium. In each animal, cell counts were carried out until at least 250 Sertoli cells were counted.

For the determination of the percentages of tubular cross-sections containing A spermatogonia (repopulation index) and/or giant spermatogonia, in each mouse 200 tubular cross-sections were studied. The percentage of tubular cross sections in epithelial stages I–VI containing pachytene spermatocytes at 10 days after a dose of 5 Gy was determined by studying 100 tubular cross-sections in these stages in each animal.

Whole mount seminiferous tubules were prepared from five wild-type and five p53 knock out FvB mice, as described by Clermond and Bustos-Obregon (1968). Whole tubules were fixed in Bouin's solution and stained with Harris' haematoxylin. With the help of an ocular grid covering an area of 111×111 μm, using a $100\times$ objective, spermatogonia with their geometrical center and Sertoli cells with their typical nucleoli, within the frame of the grid were counted. In stage VIII A_s, A_{pr}, A_{al} spermatogonia and (pre)leptotene spermatocytes were counted as described before (de Rooij, 1973). In the second half of stage VIII/early IX the A_1 spermatogonia synchronously prepare for a division in stage IX. These cells are then large as they are in G_2 phase or early prophase. In contrast, the undifferentiated spermatogonia which will start proliferation later on in the epithelial cycle are small and can easily be distinguished from the A_1 spermatogonia. According to their typographical arrangement the undifferentiated spermatogonia were subdivided into clones consisting of 1 (A_s), 2 (A_{pr}) or more ($_{A\alpha l}$) cells. Undifferentiated A spermatogonia lying less than 25 μm from each other were considered to belong to the same clone. Paired *t* test analysis was used to verify significance of the obtained data.

Acknowledgements

The authors thank Mr. R. Scriwanek and Mr. A. van Rijn for assistance with photography and Mrs. van Duijn-Goedhart for assistance regarding the C57BL/6 p53 knock out mice. This work was supported by the J.A. Cohen Institute for Radiopathology and Radiation Protection Leiden, The Netherlands.

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