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Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome

(tumor-suppressor gene/spermatogenesis/pachytene stage)

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ABSTRACT **Transgenic mice which carry hybrid p53 promoter–chloramphenicol acetyltransferase (CAT) transgenes were found to express CAT enzymatic activity predominantly in the testes. Endogenous levels of p53 mRNA and protein were lower than in the nontransgenic control mice. The various p53 promoter–CAT transgenic mice exhibited in their testes multinucleated giant cells, a degenerative syndrome resulting presumably from the inability of the tetraploid primary spermatocytes to complete meiotic division. The giant-cell degenerative syndrome was also observed in some genetic strains of homozygous p53 null mice. In view of the hypothesis that p53 plays a role in DNA repair mechanisms, it is tempting to speculate that the physiological function of p53 that is specifically expressed in the meiotic pachytene phase of spermatogenesis is to allow adequate time for the DNA reshuffling and repair events which occur at this phase to be properly completed. Primary spermatocytes which have reduced p53 levels are probably impaired with respect to DNA repair, thus leading to the development of genetically defective giant cells that do not mature.**

While inactivation of the p53 tumor-suppressor gene is considered to be a critical event in malignant transformation (1–3), the function of the wild-type p53 protein in the cycle of normal cells (4–7) has yet to be discovered. *In vitro* experimental models addressed at elucidating the normal function of the p53 suppressor gene have shown that the wild-type p53 protein is involved in apoptosis (8, 9) and cell differentiation (10–12). Support for the hypothesis that p53 plays a role in development is provided by observations that p53 is expressed in normal tissues of adult mice (13, 14) and in embryonic tissues (15) and that, at midgestation, p53 expression is confined to differentiation regions (16). In experiments with transgenic mice generated by microinjection of constructs consisting of murine or human p53 promoter sequences upstream to the chloramphenicol acetyltransferase (CAT) reporter gene, we measured high levels of CAT activity in the testes, leading us to suggest that p53 is associated with spermatogenesis (17). We observed by *in situ* analysis that both CAT and endogenous p53 expression exhibited the wave-like and cyclical patterns that are typical of spermatogenesis (17). Further, the expression of the p53 protein was confined to the tetraphoid (4*N*) primary spermatocytes at the pachytene phase of meiosis (18), just before they developed into haploid spermatids.

The conclusion that p53 plays a role in normal development and differentiation is being challenged by the observation that p53 null mice seem to develop normally (19). Apparently intact offspring, exhibiting normal immunological and developmental patterns, were reported to be obtained from mice with targeted deletion of the p53 gene (p53 knockout mice). In adult life, however, these animals developed a very high incidence of various types of tumors (19). Because (*i*) p53 null mice develop normally without expression of p53 and (*ii*) p53 is expressed in a specific manner in various developmental pathways, two alternative possibilities must be considered: either the ultimate role of p53 is associated solely with tumor suppression or p53 function(s) associated with normal development and differentiation is overruled by the existence of alternative pathways of the normal genome.

Here we show that a partial impairment of p53 expression may be associated with the development of a degenerative syndrome in the spermatogenic pathway. The well-characterized spermatogenic process (20) offers a convenient system for the examination of the possible function of p53 in cell differentiation *in vivo*.

MATERIALS AND METHODS

Histological Analysis. For histological analysis, fresh organs were fixed in Bouin's solution and embedded in paraffin. Sections (5–8 μm) were prepared and stained with hematoxylin/eosin/light green (modified trichrome staining).

Transgenic Mice. p53 promoter–CAT transgenic mice that carry the mouse-specific transgene (e.g., strain 16) were established as described (17), by microinjection of the 0.7-kb *Eco*RI–*Hin*dIII fragment mouse genomic DNA that contains the murine p53 promoter (21) linked upstream to the CAT gene. p53 promoter–CAT transgenic mice that carry the human-specific transgene (strains 28, 48, and 54) were established by microinjection of the 3.8-kb *Eco*RI fragment of human genomic DNA that contains the entire human p53 promoter sequence (22) linked upstream to the CAT gene. Control transgenic mice were established by microinjection of p2.4CAT, which contains the human p53 promoter sequence, which has a deleted p1 (the principal p53 promoter, Δp1); it was generated by deletion of an *Xba* I–*Eco*RI 5′ fragment (17).

Fluorescence-Activated Cell Sorting (FACS) Analysis. Testicular cell suspensions were prepared by mashing the testes on a fine steel mesh or with a hand homogenizer. Cells were washed in phosphate-buffered saline (PBS) and fixed for 1 hr at 4°C in 70% methanol (Bio-Lab, St. Paul). Fixed cells were incubated for 30 min on ice with anti-p53 monoclonal antibody PAb-421 (23) at 15–35 μg of protein per 10^6 cells. After washing in PBS with 1% bovine serum albumin and 0.02% sodium azide (PBS/BSA/azide), the cells were incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse second antibody (Jackson Im-

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Abbreviations: CAT, chloramphenicol acetyltransferase; FACS, fluorescence-activated cell sorting.

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munoResearch) diluted 1:10 (200 μl per 10^6 cells) in PBS/BSA/azide. Subsequently, after washings with PBS/BSA/azide, cells were suspended in 1 ml of PBS/BSA/azide plus RNase H (0.2 mg/ml) and incubated for 30 min at room temperature. When rabbit anti-mouse p53 polyclonal antibodies were used, 5-(4,6-dichlorotriazin-2-yl)aminofluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was the second antibody. Propidium iodide (PI, 50 μg/ml; Sigma) was added and cells were filtered through a 40-mm nylon mesh. Cells were analyzed in a FACScan flow cytometer (Beckton Dickinson) with an excitation beam of 488 nm, after appropriate compensation of the FITC and PI channels. Data were gated on an FL-2 (PI fluorescence) scale to eliminate the unstained cells and cell debris. Forward and side scatter were recorded along with FITC and PI fluorescence intensity (18).

RESULTS

Giant-Cell Degenerative Syndrome in the p53 Promoter–CAT Transgenic Mice. In earlier experiments we consistently noticed irregularities in the structure of the seminiferous tubules of the various p53 promoter–CAT transgenic mice (17, 18). An analysis of frozen sections which were used in our *in situ* hybridization studies revealed lesions that were manifested as holes in the seminiferous cortex. To evaluate possible histological modifications in this organ, it was important to prepare better-quality sections. Histological staining of paraffin-embedded sections indicated that p53 promoter–CAT transgenic mice, established by microinjection of human- or mouse-derived transgene, exhibited a high incidence of giant cells, spreading from the cortex into the lumen of the seminiferous tubules. These animals were diagnosed as suffering from giant-cell degenerative syndrome (24). Giant cells represent abnormal progenies of the 4*N* primary spermatocytes. These are multinucleated cells that cannot complete their meiotic division but nevertheless retain their normal capacity to progress to the seminiferous lumen. Indeed we found giant cells near the spermatogonia stem cells, probably at the site where they were initially formed, along the cortex, and in the seminiferous lumen, where they were secreted like normal mature spermatozoa.

Mice of strain 28, generated by microinjection of the human p53 promoter–CAT transgene, exhibited a rather high frequency of giant cells mapping to various regions of the seminiferous tubules (Fig. 1*B*). The giant cells were multinucleated (Fig. 1*C*) and varied in size and relative frequency per individual tubule. These cells were apparently very fragile and lysed easily, leaving behind holes and lesions in the tissue. This may explain why they were undetectable in the frozen sections, the preparation of which involved harsh steps, used in our initial experiments (17). Control mice of strain 91 (Fig. 1*A*), which were generated by microinjection of a 1.4-kb fragment (*Xba* I–*Eco*RI) that is deleted of the principal p1 promoter region (22), exhibited a normal testicular morphology and no giant cells were detected (Fig. 1*B*).

The giant-cell degenerative syndrome can be manifested at different degrees of severity, ranging from a sporadic appearance of a single giant cell, to large numbers of giant cells per individual seminiferous tubule, to total depletion of the seminiferous tubules. We observed that the severity of the syndrome was correlated with the reduction in the p53 mRNA levels that we reported previously (17). Strain 48, harboring the largest number (about eight) of integrated copies of the human p53 transgene and having the lowest level of endogenous expression of p53 mRNA (17), exhibited the most severe pathology. In this strain there was a high incidence of seminiferous tubules containing high numbers of giant cells. Strain 28, which has a lower copy number (about four) of the integrated transgene and a smaller reduction in the endogenous p53 mRNA level, had a relatively lower incidence of giant cell-positive seminiferous tubules and fewer of these cells within each positive tubule. Strain 54, with the lowest copy number (less than two) of the human transgene and a relatively slight reduction in p53 mRNA, exhibited only a mild pathology. Strain 16, generated by microinjection of mouse p53 promoter sequence, exhibited a mild pathology with severity higher than strain 54 but lower than strain 28. In addition to the appearance of giant cells, most sections of positive p53 promoter–CAT transgenic mice exhibited a reduced thickness of the seminiferous cortex, symptomatic of the giant-cell degenerative syndrome. Control mice of strains 91 and 92 that were maintained under the same conditions exhibited no giant cells in their seminiferous tubules.

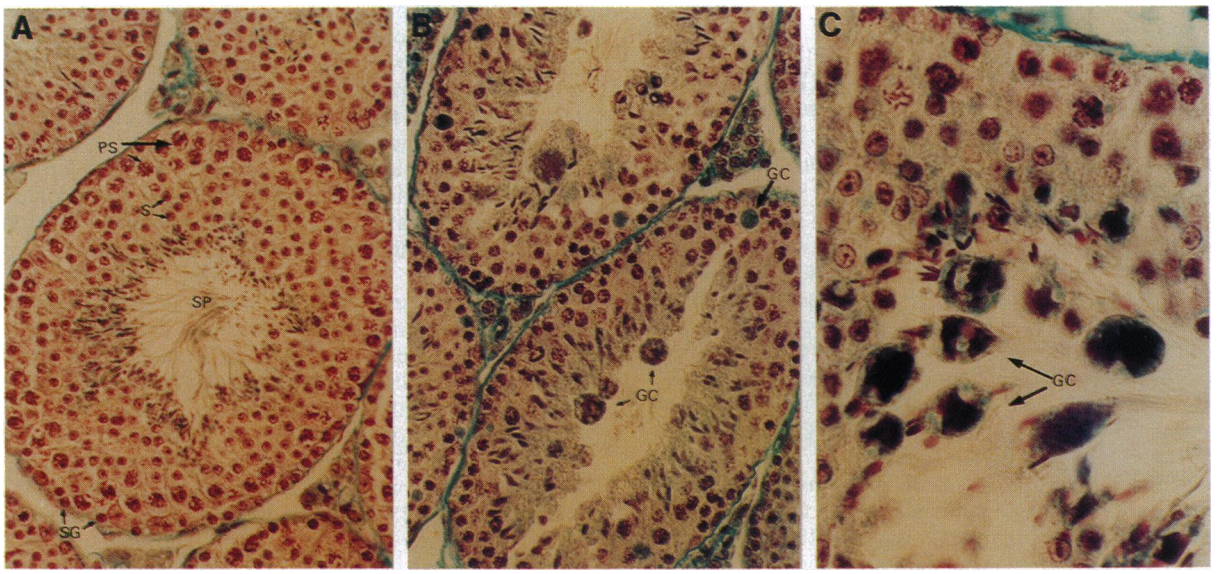

FIG. 1. Detection of giant cells in paraffin-embedded sections of testes of various p53 promoter–CAT transgenic mouse strains. (*A*) Strain 91 represents a normal seminiferous tubule. SG, diploid spermatogonia stem cell; PS, 4*N* primary spermatocytes; S, haploid spermatids; SP, mature sperm cells. (×10.) (*B*) Strain 28 represents the giant-cell degenerative syndrome. GC, giant cells. (×20.) (*C*) Giant cells of strain 28 at a higher magnification. (×40.)

Reduced Levels of Endogenous p53 Protein in Testes of p53 Promoter–CAT Transgenic Mice. That the giant cells represented 4*N* primary spermatocytes that could not complete their meiotic division and that the severity of the syndrome seemed to correlate with the reduced levels of p53 mRNA suggested an association with the deregulation of p53 protein expression. The next experiment was therefore aimed at evaluating the endogenous p53 protein levels in these mice. Previously we found that p53 expression was confined to the pachytene stage of meiosis (18). FACS analysis of testicular cells with a variety of anti-p53 antibodies showed that, in agreement with our previous observation in normal mice, p53 protein is confined to the low-DNA-density, 4*N* cells of the pachytene phase (18). However, when p53 protein levels in the tetraploid population (4*N*) were compared, we found that both the number of positive p53-stained cells (presented as distribution on the *y* axis in Fig. 2) and the intensity of staining (presented as the height on the *z* axis) were significantly reduced in adult and young p53 promoter–CAT mice relative to the corresponding negative transgenic mice. Fig. 2*A* shows a typical reduction in the expression of p53 protein in a strain 48 mouse, carrying the human-derived transgene, relative to a p53-negative transgene mouse of the same age. To further evaluate p53 expression in the p53 promoter–CAT transgenic mice, we measured the amounts of p53 protein expressed in the first round of spermatogenesis. Since we had found that p53 expression was confined to the pachytene phase, which peaks around days 16–19 of the first spermatogenic round (18), we next compared the p53 protein levels in an 18-day-old p53 promoter–CAT mouse of strain 28 with those in a negative transgenic mouse derived from the same strain and age. Again, both the number of p53-positive cells and the intensity of p53 expression per cell (see above) in the p53 promoter–CAT mouse were reduced relative to the negative control (Fig. 2*B*). The patterns of the various spermatogenic populations of the adult and the young mice differ with respect to DNA content and density; nevertheless, the p53 signal in both cases was confined to the 4*N*, low-DNA-density, pachytene-stage cells.

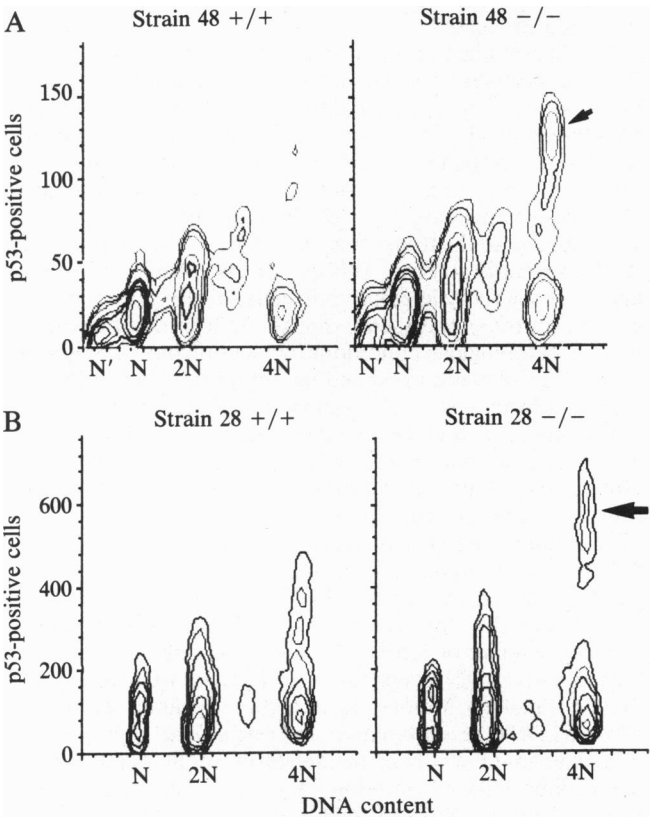

FIG. 2. p53 protein expression in the various p53 promoter–CAT transgenic mouse strains and negative transgenic mice. (*A*) Comparison of p53 protein levels in the various spermatogenic populations of adult (6 month) homozygous p53 promoter–CAT transgenic mice of strain 48 (+/+) with negative (−/−) derived transgenic mice of the same age. Negative transgenic mice were generated by backbreeding with normal SJL mice. (*B*) Comparison of p53 protein levels expressed by 18-day-old homozygous mice of strain 28 (+/+) and negative (−/−) transgenic mice of the same age. DNA content (*x* axis) is represented: N, haploid cells; 2N, diploid cells; 4N, tetraploid cells. N′ represents high-DNA-density cells which represent mature haploid spermatozoa. The number of p53 protein-positive cells, measured as binding to PAb-421 anti-p53 monoclonal antibody, is indicated on the *y* axis and the amount of p53 protein per cell is on the *z* axis (shown by contours). Arrows point to the p53-positive populations.

Reduced Levels of Endogenous p53 Protein in a Cell Line Stably Transfected with the p53 Promoter–CAT Gene. To examine the assumption that reduction of p53 expression in spermatocytes of p53 promoter–CAT transgenic mice was due to the presence of multiple copies of the transgene, we compared p53 protein levels in NIH 3T3 mouse fibroblasts and in a stable NIH 3T3 clone which was generated by transfection of the p0.7-CAT plasmid (D. Ronen, unpublished work) used for the preparation of our transgenic mice. The cell lines were grown to logarithmic phase. Single-cell suspensions were incubated with polyclonal rabbit anti-mouse p53 antibodies and then incubated with a dichlorotriazinylaminofluorescein-conjugated goat anti-rabbit antibody and analyzed as described above. Throughout the cell cycle, NIH 3T3 cells exhibited positive staining for p53, but NIH 3T3-CAT cells exhibited a significant decrease in p53 (Fig. 3). These results suggest that the exogenous p53 promoter sequences may interfere with the activity of the endogenous p53 promoter sequences.

Appearance of Giant Cells in Testicular Sections of Homozygous p53 Null Mice. The above experiments suggested that the reduction in p53 protein expression accounted for the development of the giant-cell degenerative syndrome. To verify this conclusion, we analyzed testicular sections of p53 null mice of C57BL/6 and 129 backgrounds from the Donehower colony (19). Sections from all homozygous p53 null mice of the 129 genetic background [129(−/−)] exhibited, at variable frequencies, multinucleated giant cells. The positioning of the giant cells within the seminiferous section was similar to that observed in the p53 promoter–CAT mice. Fig. 4 depicts an example of a multinucleated giant cell in the

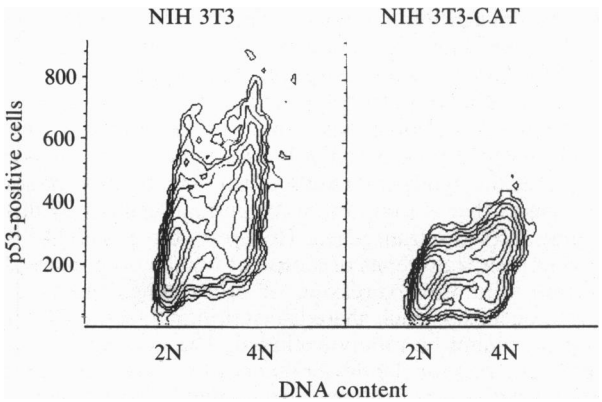

FIG. 3. p53 protein expression in NIH 3T3 and NIH 3T3-CAT cell lines. Single-cell suspensions were used for analysis of p53 protein expression. DNA content (*x* axis) is represented: 2N, diploid cells; 4N, tetraploid cells. The number of p53 protein-positive cells, measured as binding to rabbit anti-mouse p53 polyclonal antibodies, is indicated on the *y* axis and the amount of p53 protein per cell is on the *z* axis (contours).

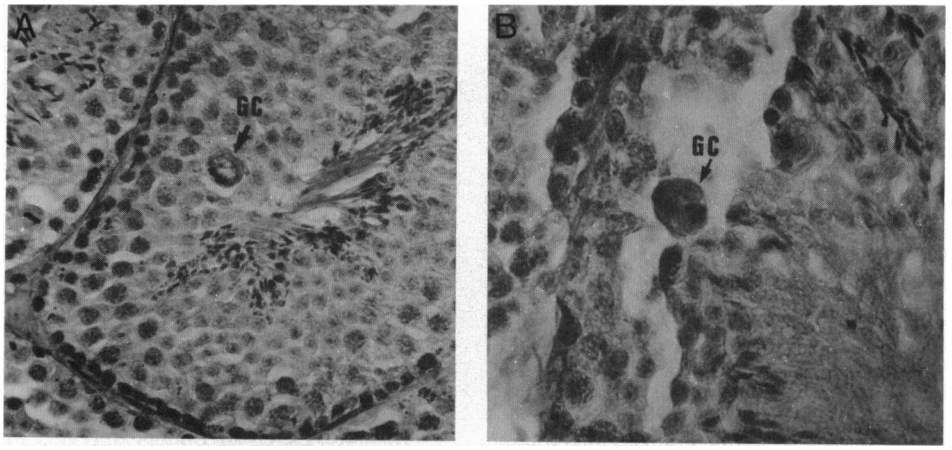

FIG. 4. Detection of giant cells in testicular sections of homozygous p53 null mice. Testicular sections were prepared as described above and screened for giant cells (GC). Homozygous p53 null mice (−/−) of strain 129, established by Donehower *et al.* (19), are presented. Sections are shown (×10) (*A*) and (×40) (*B*).

testes of a homozygous p53 null mouse of strain 129(−/−). No giant cells were detected in heterozygous mice [(129(+/−)]. Homozygous p53 null mice of C57BL/6 × 129 mixed genetic background exhibited apparently normal structure of the seminiferous tubules. In agreement with previous observations (19), testicular sections of p53 null mice of the 129 background exhibited a high incidence of seminomas and undifferentiated teratocarcinomas. Homozygous mice of 129 genetic background are infertile, whereas other strains of p53 null mice are fertile.

DISCUSSION

Transgenic mice carrying a high multiplicity of p53 promoter–CAT transgenes exhibited the giant-cell degenerative syndrome (24). In brief, this syndrome is defined as an abnormality resulting from the inability of primary 4*N* spermatocytes to undergo meiotic divisions to generate haploid sperm cells. Instead these cells undergo presumably additional DNA replications, giving rise to multinucleated giant cells (24).

We detected reduced levels of the endogenous p53 protein in the p53 promoter–CAT mice. This reduction could be attributable to competition of the molar excess concentrations of human or mouse transgene p53 promoter sequences with the endogenous p53 promoter sequences for binding of the same transcriptional transactivator(s). Such a conclusion could agree with the so-called "squelching" mechanism (25–28), where an excess of specific binding motifs was found to reduce specific promoter activities. As the giant-cell degenerative syndrome was found in several independently established p53 promoter–CAT transgenic strains, it is unlikely that the syndrome resulted from inactivation of some other genes that are important in spermatogenesis by direct integration of the transgenes. However, the possibility that p53 promoter transgenes of mouse or human origin may also interfere with the expression of other genes involved in spermatogenesis which share transcriptional factors with the p53 gene cannot be totally excluded. The notion that a lack of p53 accounts for the development of the giant-cell degenerative syndrome is further substantiated by detection of the syndrome in at least one genetic strain of homozygous p53 null animals. The existence of p53 null mouse strains with a normal testicular morphology, which exhibit no giant cells, can be explained by the assumption that in such mice, selection for total independence from p53 activities has occurred. The observation that knockout of a number of vital structural genes does not entail obvious aberrations in their phenotypes or patterns of development (29–32) supports the notion that functional redundancy may represent a general principle in regulatory networks controlling complex developmental processes.

Previous studies have shown that p53 plays a role in mitosis (4–7); our present results suggest that it also plays a role in meiosis. If p53 is arresting DNA replication (33–35), then a p53-deficient cell, which is programmed to undergo meiosis, will fail to complete this process. Subsequently, these cells may develop to multinucleated giant cells, representing an abortive intermediate product. It is unlikely that giant-cell development in the transgenic mice is the result merely of the CAT enzymatic activity. This enzyme is specific for bacteria and has no activity in eukaryotic cells; overexpression of the enzyme in other transgenic mouse models is not known to induce pathological conditions or significantly modify the expression of the corresponding endogenous gene (36–38). That a physiological aberration was induced in the p53 promoter–CAT transgenic mice can be accounted for by assuming that minor modulations, which exert no detectable effects on the function of structural proteins, may modify the function of a growth control gene such as p53, whose expression is tightly regulated.

It has been suggested that wild-type p53 plays a role in DNA repair mechanisms (39–42). In that respect p53 is regarded as the "genome guardian" which arrests cells to permit the repair of genetic damage occurring spontaneously during normal DNA replication or induced following γ irradiation (39–42). Meiosis is a highly dynamic event that involves DNA recombination and reshuffling; therefore it is bound to involve a high incidence of spontaneous genetic aberrations (43). Generation of normal functional mature spermatozoa is therefore highly dependent on an efficient DNA repair mechanism(s) (44). It should be noted that γ irradiation of spermatogenic cells is followed by intensive DNA repair (45). This repair occurs specifically in cells at the pachytene phase (45), which are the cells to which p53 protein expression is specifically confined (18). Pachytene-stage primary spermatocytes that are deficient in p53 do not undergo normal DNA repair. Such developing cells are genetically unfit to mature and therefore meiosis is aborted. It is possible that in the absence of adequate levels of wild-type p53 protein, these cells undergo additional rounds of DNA replication, possibly accompanied by uncontrolled gene amplifications (46), giving rise to polynucleated giant cells that most likely carry aberrant genetic material. A different mechanism which may account for the generation of giant cells could be connected with the inability of the spermatocytes to

complete the process of detachment of the cytoplasmic bridges. Such an arrest may cause the cytoplasmic bridges to become wider and cause clusters of cells to round up and generate giant cells.

The observation that only a fraction of cells develop into giant cells, combined with the fact that some homozygous p53 null mice appear normal, suggests that development into giant cells depends on whether a certain threshold of genetic aberrations which disqualify the cells from completing normal meiosis is passed or, alternatively, that not all of the DNA repair mechanisms are affected by the p53 protein. DNA repair is a multifacet system which involves several alternative pathways (47, 48), not all of which are essentially p53-dependent. It is possible that in p53 null mice, which display an apparently normal embryonic development and normal fertility in adult life, selection toward usage of DNA repair pathways which are independent of p53 has occurred.

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