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## Dynamics of testicular germ cell apoptosis in normal mice and transgenic mice overexpressing rat androgen-binding protein

D Antony Jeyaraj\*, Gail Grossman and Peter Petrusz

Address: Department of Cell and Developmental Biology and Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC 27599, USA

Email: D Antony Jeyaraj\* - antojeya@med.unc.edu; Gail Grossman - gail\_grossman@med.unc.edu; Peter Petrusz - petrusz@med.unc.edu

\* Corresponding author

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### Abstract

The number and type of testicular germ cells undergoing apoptosis in different age groups of mice (from 7 to 360 days of age) was determined and compared in age-matched wild type (WT) control and in a transgenic (TG) mice homozygous to rat androgen binding protein (ABP) using flow cytometry. Flow cytometric quantification revealed that the total number of germ cells undergoing apoptosis did not differ significantly in WT and TG mice up to Day 14. From Day 21 to Day 60, the number of germ cells undergoing apoptosis was consistently higher in TG than in WT mice. Starting from Day 90, the number of germ cells undergoing apoptosis in TG mice was lower than controls until Day 360. In 21–60 days old TG mice, spermatogonia, S-Phase cells, and primary spermatocytes are the cell types undergoing apoptosis at significantly greater numbers than those in WT mice. However, starting from day 60, the total number of spermatids undergoing apoptosis was significantly lower in TG mice than in age-matched WT controls. TdT-mediated dUTP-biotin nick end labeling (TUNEL) in testicular sections from TG mice of 21 and 30 days of age confirmed the presence of increased numbers of apoptotic germ cells compared to their age matched controls.

These data indicate that the continuous presence of greater than physiological concentrations of ABP in the mouse testis has a biphasic effect on the frequency of apoptosis in germ cells. The initial pre-pubertal increase in testicular germ cell apoptosis may result from direct or indirect actions of ABP and is likely to determine the subsequent life-death balance of germ cell populations in TG mice, whereas the subsequent reduction may result from maturation depletion. A wave of apoptosis during the pre-pubertal period is required for normal spermatogenesis to develop, and our data indicate that this apoptotic wave may be regulated by ABP and/or androgens.

### Background

Normal spermatogenesis represents a precisely regulated balance between continuous cell proliferation and concomitant programmed cell death, apoptosis [1–4]. More than 50% of the germ cells die during their normal developmental process [5,6]. Apoptosis in the testis of rodents is detected as early as the 14<sup>th</sup> embryonic day [7]. After birth, an early apoptotic wave of germ cells during a criti-

cal pre-pubertal period (around 3–4 weeks) [8] may be required for determining the subsequent normal pattern of spermatogenesis [3].

Androgen binding protein (ABP) is produced by Sertoli cells of the testis, secreted into the lumen of seminiferous tubules, and transported with tubular fluid to the epididymis, where it is internalized and degraded by the

epithelial cells of the initial segment and the caput; it binds testosterone (T), dihydrotestosterone and estradiol with high affinity [9–11]. The functions of ABP have been the subject of many reviews [e.g., [10,12–15]]. It is thought to regulate spermatogenesis and sperm maturation by maintaining high androgen levels in the testis and epididymis [16,17] and has been proposed as a biochemical marker for Sertoli cell function and formation of blood-testis barrier [12,18]. More recently, cell surface receptors for ABP and its liver-derived homolog sex hormone-binding globulin (SHBG) were identified in several androgen target tissues, suggesting that these extracellular steroid-binding proteins may have a much broader, hormone-like function [19–21].

In order to study ABP's functions *in vivo*, Reventos *et al.* [22] developed transgenic (TG) mice overexpressing rat ABP. These TG mice showed progressive structural and functional abnormalities in their testes, leading eventually to infertility [23–25]. In our recent paper, we described an altered pattern of germ cell proliferation throughout postnatal life in the ABP-TG mice, resulting in a progressive reduction in total germ cell numbers with increasing age [26]. However, it was not clear whether the reduction in germ cell numbers was the result of a reduced rate of proliferation, an increased rate of apoptosis, or a combination of the two.

The present study was performed to provide quantitative data on germ cell apoptosis in control and in ABP-TG mice aged from 7 to 360 days. Apoptotic cells were identified by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method and detected either by flow cytometry [27] or by light microscopy in histological sections [28]. The results indicate that transgenic ABP alters the rate of testicular germ cell apoptosis throughout life, but it is most likely to cause its germ-cell depleting effect by promoting germ cell apoptosis during the critical pre-pubertal period ("puberty" in laboratory rodents occurs at 50–60 days of life and is defined as the first appearance of spermatozoa in the tail of the epididymis [29,30]).

## Materials and Methods

### Animals

Mice were housed in an animal facility under controlled conditions and given food and water *ad libitum*. The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved the protocols used in this study. Transgenic mice overexpressing rat ABP in their testes were developed by Reventos *et al.* [22] and were propagated as described previously [25,26]. Control (wild-type, WT) and homozygous male mice of 7, 10, 14, 21, 30, 60, 90, 120, 180, 240 and 360 days of age were used in the present

study. A minimum of 5 animals per group were analyzed. Mice were sacrificed by overexposure to ether.

### Chemicals

TdT enzyme and TdT buffer were purchased from Promega (Madison, WI, USA). All other chemicals, unless stated otherwise, were purchased from Sigma Chemical Company, St. Louis, MO, USA.

### Preparation of germ cell suspension

The method described by O'Brien [31], as modified by Jeyaraj *et al.* [26], was used to isolate germ cells from the seminiferous tubules. Briefly, the decapsulated testes were incubated in PBS containing 0.5 mg/ml collagenase (type IV) for 15 minutes at 32°C in a shaking water bath. After washing twice with PBS containing 1.0 µg/ml DNase, 1.0 µg/ml trypsin in PBS was added and incubated for 15 minutes at 32°C. Equal amount of soybean trypsin inhibitor was added and the suspension was mixed by gentle pipetting with a plastic transfer pipette for 3–5 minutes and was filtered through 80 µm nylon mesh (50 µm for 7–30 days old). The filtrate was washed twice with PBS, fixed in 70% ice-cold ethanol, and stored at 4°C.

### TUNEL labeling of germ cells for flow cytometry analysis

The procedure followed in the present study for TUNEL labeling was described by Krishnamurthy *et al.* [27]. Approximately 1–2 million/ml ethanol-fixed testicular germ cells were washed twice in PBS, treated with 300 µl of 0.5% pepsin solution (dissolved in 0.9% saline, pH 2.0) for 5 minutes at 37°C. After washing twice with 1.0 ml PBS, the cells were resuspended in 50 µl of TdT buffer containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCL (pH 6.6), 2.5 mM CoCl<sub>2</sub>, 0.25 mg/ml bovine serum albumin, 5.0 units of terminal deoxynucleotidyl transferase and 0.5 nM biotinylated deoxyuridine triphosphate (b-UTP) and incubated at 37°C for 30 minutes. After washing in PBS, the cells were resuspended in 100 µl of a reagent containing 4x-concentrated saline-sodium citrate buffer, 2.5 µg/ml fluoresceinated avidin, 0.1% Triton X-100, and 5% dry milk, incubated for 30 minutes at room temperature in the dark, and washed twice in 1.0 ml of PBS. The cells were resuspended in 1.0 ml of PBS containing 5 µg/ml propidium iodide and 0.1% RNase. Control cells were processed as described but without TdT. The intensity of the green fluorescence of avidin-FITC-stained cells and the red fluorescence of PI stained cells was measured in the FACScan flow cytometer (Becton-Dickinson Immunocytometry, San Jose, CA, USA). A marker was set in the TUNEL dot plot at the cut-off between background signals and positive staining, as determined from the control samples. Cells were considered TUNEL-positive if their fluorescence intensity exceeded that set level. The percentage of positive cells was quantified by using the

"Summit" software (Cytomation Inc, Fort Collins, CO, USA).

#### ***In situ end labeling and peroxidase staining on testicular tissue sections***

*In situ* apoptosis detection kit (Apoptag peroxidase kit) was purchased from Intergen Company, NY, USA and their protocol was followed. Testicular tissue was fixed in Bouin's fixative, dehydrated in graded concentrations of ethanol and embedded in paraffin. 8  $\mu$ m sections were deparaffinized, re-hydrated and washed in PBS. Twenty  $\mu$ g/ml proteinase-K was placed on the section and kept at room temperature for 15 minutes. After washing twice in distilled water, the sections were covered with equilibration buffer for 10–15 seconds followed by the addition of TdT enzyme for 15–20 minutes. The reaction was stopped by placing the sections in stop/wash buffer. The sections were washed thrice with PBS and anti-digoxigenin-peroxidase conjugate was added on the section and incubated in a humidified chamber for 30 minutes at room temperature. After washing the sections 4 times in PBS, diaminobenzidine tetra-hydrochloride (DAB) was used to develop the color (dark brown). Control slides were processed in an identical manner except that TdT was omitted. The sections were counterstained with toluidine blue, dehydrated with ethanol and xylene, coverslipped, and images viewed and recorded using a Nikon Eclipse 600 microscope equipped with Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

#### ***Induction of testicular germ cell apoptosis***

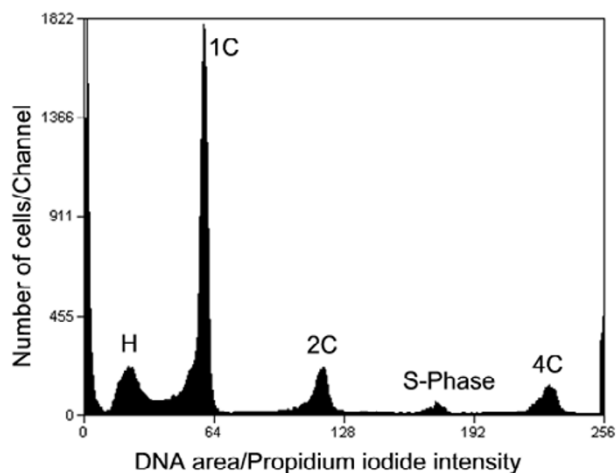
To confirm the reliability of the above methods, apoptosis was induced by treatment with methoxy acetic acid (MAA), which is known to cause apoptosis in testicular germ cells [27,32]. Two adult male control mice (90 days old) were given a single intraperitoneal injection of 650 mg/kg of MAA. After 24 hours, the mice were decapitated, testes removed, isolated germ cells processed for flow cytometric estimation, and tissue sections for TUNEL as described above.

#### ***Statistical Analysis***

Changes were evaluated using unpaired "t" test and the software GraphPad InStat (San Diego, California, USA).  $P = 0.05$  or less was considered significant. Quantitative results throughout this paper will be stated as the mean  $\pm$  standard deviation.

#### **Results**

Figure 1 is the representative flow cytometry histogram of propidium iodide-stained testicular germ cells isolated from adult mouse (90 days). Five populations of cells were detected depending upon their DNA content. They are 1) elongating and elongated spermatids (H); 2) round spermatids (1C); 3) spermatogonia and secondary sper-



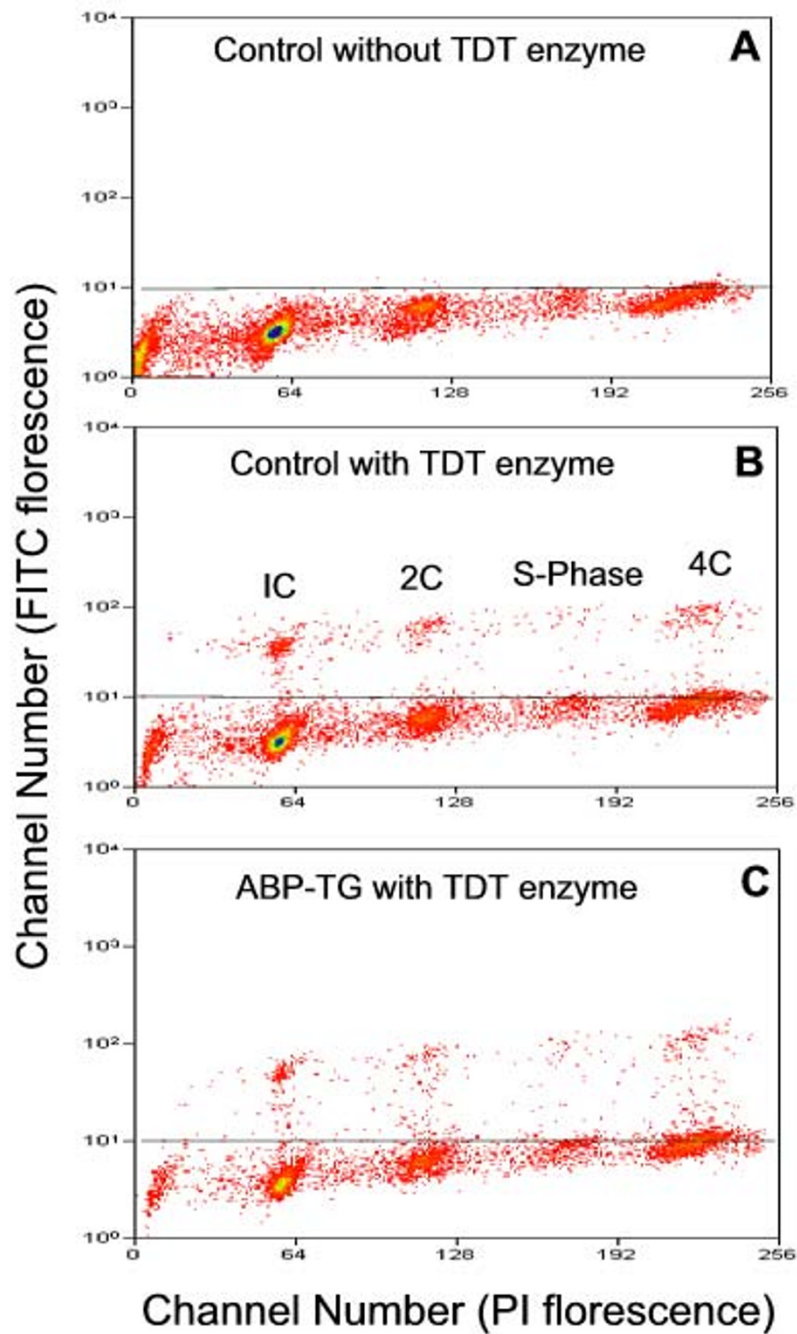
**Figure 1**

Flow cytometry histogram of propidium-iodide-stained testicular germ cells isolated from an adult mouse (90 days of age). Five populations of cells were detected depending upon their DNA content. They are 1) elongating and elongated spermatids (H); 2) round spermatids (1C); 3) Spermatogonia and Secondary Spermatocytes (2C); 4) Spermatogonia Synthesizing DNA (S-phase) and 5) Primary Spermatocytes (4C).

matocytes (2C); 4) spermatogonia synthesizing DNA (S-phase) and 5) primary spermatocytes (4C). The 2C population includes the somatic cells of Sertoli cells, peritubular myoid cells, Leydig cells and others, representing about 3% of total testicular cells [33].

Figure 2 represents the flow cytometry dot plot of germ cells from 90 days old control and ABP-TB mice processed for TUNEL to quantify flow cytometrically the number of germ cells undergoing apoptosis. Panel A is a negative control representing germ cells from control mice, processed for TUNEL without the enzyme (TdT) which catalyzes the template-independent addition of labeled or unlabeled nucleotide triphosphates to the 3'-OH ends of double-stranded or single-stranded DNA. Note the lack of fluorescent signal from germ cells above the marker line. Panel B & C are the dot plots of germ cells from control and ABP-TG mice processed for TUNEL with TdT enzyme.

Figure 3 is representative of the flow cytometry dot plot of (A) control and (B) MAA-treated germ cells isolated from 90 days old mice and processed for TUNEL. Flow cytometry analysis of germ cells from control mice revealed that four germ cell populations (1C, 2C, 4C and S-Phase) had TUNEL-positive cells. Analysis of germ cells from MAA treated mice showed an increase in the percentage of



**Figure 2**

Flow cytometry dot plot of germ cells from 90 days old control and ABP-TB mice processed for TUNEL to quantify flow cytometrically the number of germ cells undergoing apoptosis. Panel A is a negative control; germ cells from control mice, processed for TUNEL without the enzyme (TdT). Panel B & C are the dot plots of germ cells from control and ABP-TG mice processed for TUNEL with TdT enzyme.

TUNEL-positive cells in all types of germ cells with the frequency being highest in the 4C cell population (Fig 3B).

#### **Total number of germ cells undergoing apoptosis in control and ABP-TG mice**

In control mice, the total number of germ cells undergoing apoptosis was  $0.47 \pm 0.07$  million on day 7 (Figure 4 & Table 1). It gradually increased to  $24.77 \pm 1.88$  million on day 21 followed by a sharp increase on day 30 ( $141.74 \pm 30.13$  million) and a plateau thereafter until Day 120. From Day 180 onwards, a gradual increase was observed until Day 360. The general pattern of germ cells undergoing apoptosis in ABP-TG mice was similar; however, the total number of germ cells undergoing apoptosis in ABP-TG mice was consistently higher than in controls on Days 21, 30 and 60, respectively (the increase was statistically significant on Days 21 and 30). Starting from Day 90, the number of germ cells undergoing apoptosis in TG mice was consistently lower than in controls until Day 360 (Figure 4 & Table 1).

#### **Spermatogonia and secondary spermatocytes (2C)**

The total number of spermatogonial cells undergoing apoptosis in control and ABP-TG mice remained unchanged until Day 14. In ABP-TG mice, between Days 21 and 60, the number of spermatogonial cells undergoing apoptosis was significantly increased. After 60 days, even though a decrease was observed in the total number of spermatogonia undergoing apoptosis, the decrease was statistically significant only on Days 90 and 240 (Figure 5A & Table 2).

#### **Spermatogonia synthesizing DNA (S-phase)**

Between Days 21 and 60, the number of S-phase cells undergoing apoptosis was significantly higher in ABP-TG than in control mice. Starting from 90 days, a consistent reduction in the number of apoptotic S-phase cells was seen in ABP-TG mice; the decrease was statistically significant on Days 90, 240 and 360 (Figure 5B & Table 2).

#### **Primary spermatocytes (4C)**

In the testes of ABP-TG mice, the total number of 4C cell population undergoing apoptosis was increased significantly (as compared to controls) on Days 21 and 30. The number of primary spermatocytes undergoing apoptosis in ABP-TG mice relative to controls was decreased after 60 days; the decrease was statistically significant on days 90, 240 and 360 (Figure 5C & Table 2).

#### **Spermatids (1C)**

Although a greater number of spermatids underwent apoptosis in ABP-TG mice than in controls on Days 21, 30 and 60, none of these changes were statistically significant. After 60 days of age, the number of spermatids undergoing apoptosis was significantly lower in ABP-TG

mice than controls in all age groups studied (Figure 5D & Table 2).

#### **Percentage of germ cells undergoing apoptosis**

On Days 21, 30 and 60, the percentage of germ cells undergoing apoptosis was higher in ABP-TG mice than controls (see Table 1).

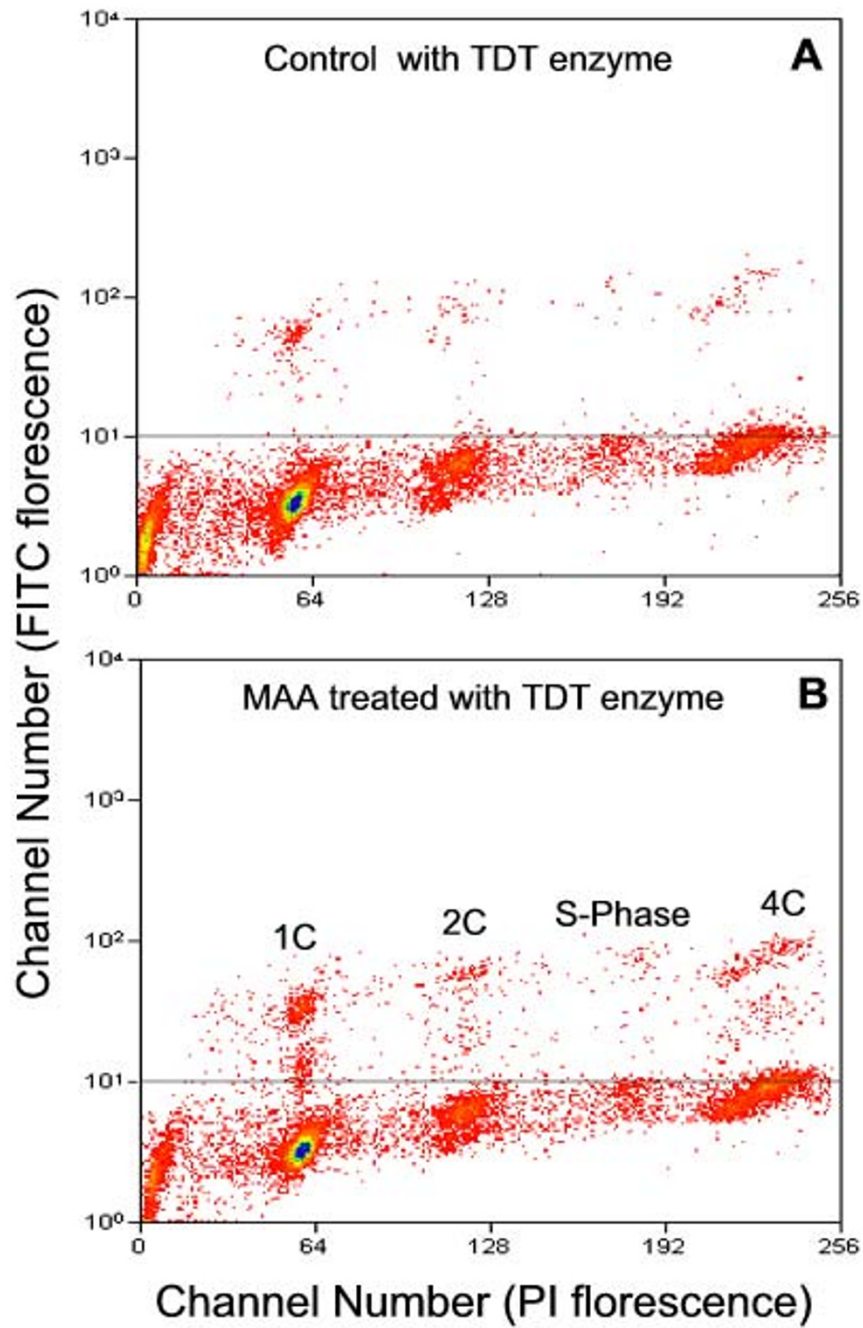
#### **TUNEL on testicular sections**

To further validate the results obtained in flow cytometric analysis, TUNEL assay was done on testicular paraffin sections from mice of different age groups (Figures 6 & 7). Microscopic observation of the sections revealed the presence of greater numbers TUNEL positive germ cells in the testicular sections of ABP-TG mice at the ages of 21 (Fig 6F) and 30 (Fig 6H) days compared to their age-matched controls than in any other age group. Figure 7E is the representative photomicrograph of a testicular section from an MAA-treated 90 days old WT mouse processed for TUNEL. Germ cells undergoing apoptosis are considerably more frequent in the testis of the MAA-treated mouse (Fig 7E) than the untreated control (Fig 7A). The increase seen in the frequency of TUNEL-positive germ cells in MAA-treated mice corroborates the results obtained from flow cytometry analysis. Figure 7F is a negative control.

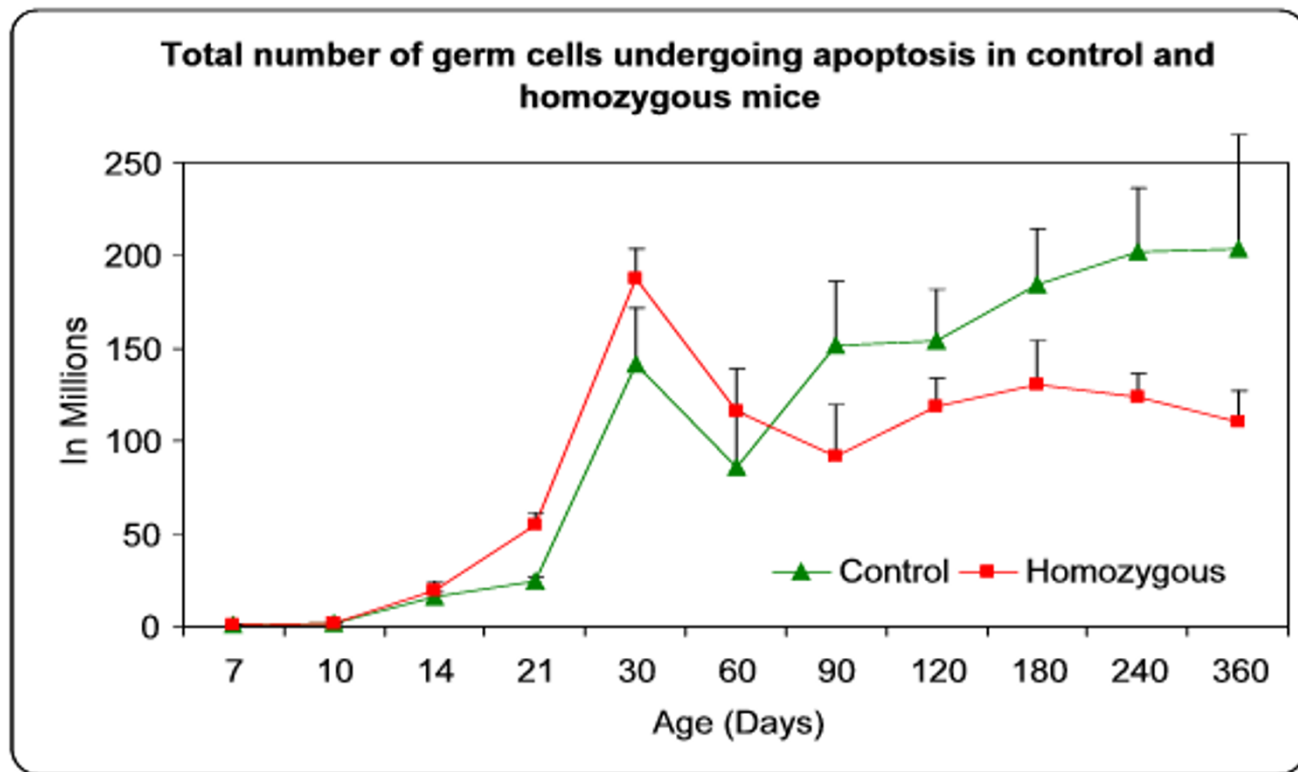
#### **Discussion**

It is well established that TG mice overexpressing rat ABP [22] develop a testicular impairment [23–25] leading gradually to a reduced number of germ cells and infertility [26]. Various theories were proposed to explain the possible mechanisms behind this testicular impairment seen in the ABP-TG mice overexpressing rat ABP. The first theory invokes the indirect involvement of ABP. The increased concentration of ABP present in the testes of TG mice (see Joseph *et al.* [25]) will chronically reduce the concentration of intra-testicular free T available for interaction with androgen receptor (AR) and other molecular targets. This is supported by the *in vitro* study of Roberts and Zirkin [34] demonstrating that ABP can actually inhibit T's action at the level of the AR. Our earlier findings including a reduced number of elongated spermatids in the ABP-TG mice [26] also support the notion that higher than physiological concentrations of ABP affect spermatogenesis indirectly, through sequestration of androgens; the transformation of round spermatids to elongated spermatids is known to be the main androgen-dependent step in spermatogenesis [35–37].

The second theory suggests the possible direct involvement of ABP in spermatogenesis. Specific cell surface receptors for ABP have been detected in several cell types including germ cells [38–41]. Gerard *et al.* [41] reported that ABP was bound and internalized by germ cells and it also modified protein synthesis in germ cells in a manner



**Figure 3**  
Flow cytometry dot plot of (A) control and (B) MAA treated germ cells isolated from the 90 days old mice, processed for TUNEL.

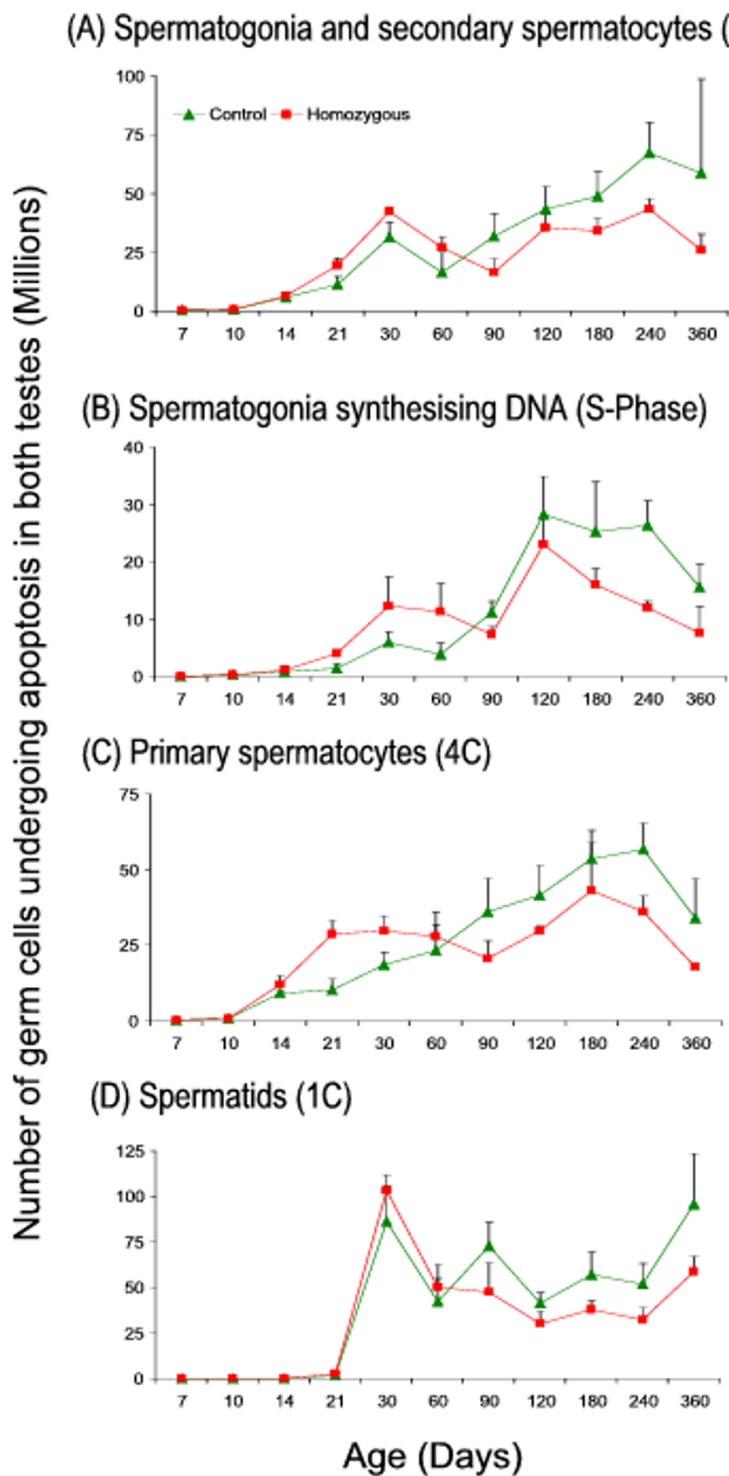


**Figure 4**  
Total numbers of germ cells undergoing apoptosis in control and ABP-TG mice. All the values are mean ± SD (n = 5).

**Table 1: Total number (×10<sup>6</sup>) and percentage of germ cells undergoing apoptosis in age-matched control (WT) and in ABP transgenic (TG) mice.**

Animal age	Total number of germ cells Undergoing apoptosis (l × 10 <sup>6</sup> )		Percentage of germ cells undergoing apoptosis	
	WT	TG	WT	TG
7 days	0.47 ± 0.074	0.56 ± 0.29	0.72 ± 0.18	0.79 ± 0.19
10 days	1.89 ± 0.32	1.75 ± 0.33	0.77 ± 0.13	0.79 ± 0.19
14 days	15.79 ± 2.36	19.70 ± 4.39	4.18 ± 0.93	4.64 ± 0.87
21 days	24.77 ± 1.88	54.97 ± 6.85 <sup>c</sup>	5.45 ± 1.68	11.47 ± 2.54 <sup>b</sup>
30 days	141.74 ± 30.13	187.62 ± 16.09 <sup>a</sup>	8.26 ± 1.55	10.86 ± 0.99 <sup>a</sup>
60 days	86.10 ± 28.62	116.10 ± 22.82	3.12 ± 1.28	4.93 ± 1.03 <sup>a</sup>
90 days	151.61 ± 34.21	91.85 ± 27.78 <sup>a</sup>	4.17 ± 0.86	3.70 ± 0.79
120 days	154.41 ± 27.10	118.30 ± 15.49	4.59 ± 0.50	4.38 ± 0.53
180 days	184.49 ± 30.51	130.64 ± 23.59 <sup>a</sup>	6.25 ± 1.61	6.40 ± 1.12
240 days	202.26 ± 34.17	123.72 ± 12.78 <sup>b</sup>	6.39 ± 0.99	6.25 ± 1.14
360 days	203.97 ± 61.04	110.18 ± 16.77 <sup>a</sup>	6.36 ± 3.20	6.41 ± 2.54

Unpaired 't' test was used. Values are Mean ± SD of 5 estimations; a p < 0.05; b p < 0.01; c p < 0.001 Statistical comparisons are between wild type control and TG mice at each individual age.



**Figure 5**  
 Changes in total number of spermatogonia and secondary spermatocytes (A), S-Phase cells (B), primary spermatocytes (C) and spermatids (D) undergoing apoptosis in control and ABP-TG mice. The values are mean  $\pm$  SD (n = 5).



**Table 2: Number ( $\times 10^6$ ) of germ cells undergoing apoptosis in age-matched control (WT) and in transgenic (TG) mice homozygous to rat ABP.**

Days	Spermatogonia and Secondary spermatocytes (2C)		Spermatogonia synthesizing DNA (S-Phase)		Primary spermatocytes (4C)		Spermatids (1C)	
	WT	TG	WT	TG	WT	TG	WT	TG
<b>7 days</b>	0.18 $\pm$ 0.04	0.23 $\pm$ 0.09	0.065 $\pm$ 0.026	0.065 $\pm$ 0.046	0.23 $\pm$ 0.061	0.26 $\pm$ 0.15		
<b>10 days</b>	0.77 $\pm$ 0.16	0.73 $\pm$ 0.17	0.270 $\pm$ 0.156	0.266 $\pm$ 0.166	0.84 $\pm$ 0.072	0.75 $\pm$ 0.178		
<b>14 days</b>	5.88 $\pm$ 2.12	6.49 $\pm$ 1.52	0.832 $\pm$ 0.41	1.172 $\pm$ 0.61	9.08 $\pm$ 0.70	12.04 $\pm$ 2.81		
<b>21 days</b>	11.21 $\pm$ 3.38	19.48 $\pm$ 3.16 <sup>a</sup>	1.39 $\pm$ 0.64	4.03 $\pm$ 0.42 <sup>c</sup>	10.31 $\pm$ 3.61	28.87 $\pm$ 4.31 <sup>c</sup>	1.88 $\pm$ 1.58	2.58 $\pm$ 0.56
<b>30 days</b>	31.24 $\pm$ 6.76	42.32 $\pm$ 1.95 <sup>b</sup>	5.91 $\pm$ 1.79	12.28 $\pm$ 5.12 <sup>a</sup>	18.48 $\pm$ 4.21	29.78 $\pm$ 5.08 <sup>b</sup>	86.10 $\pm$ 19.51	103.42 $\pm$ 8.65
<b>60 days</b>	16.43 $\pm$ 8.61	26.94 $\pm$ 4.75 <sup>a</sup>	3.92 $\pm$ 1.85	11.35 $\pm$ 4.85 <sup>a</sup>	23.38 $\pm$ 8.38	28.08 $\pm$ 8.04	42.36 $\pm$ 12.64	50.01 $\pm$ 12.54
<b>90 days</b>	31.64 $\pm$ 9.54	16.44 $\pm$ 5.82 <sup>a</sup>	11.16 $\pm$ 1.95	7.34 $\pm$ 1.29 <sup>a</sup>	36.14 $\pm$ 11.0	20.69 $\pm$ 5.78 <sup>a</sup>	72.66 $\pm$ 13.00	47.49 $\pm$ 16.16 <sup>a</sup>
<b>120 days</b>	43.47 $\pm$ 9.30	35.64 $\pm$ 5.78	28.33 $\pm$ 6.51	23.08 $\pm$ 4.67	41.46 $\pm$ 9.94	29.72 $\pm$ 1.67	41.16 $\pm$ 5.75	29.84 $\pm$ 7.02 <sup>a</sup>
<b>180 days</b>	48.72 $\pm$ 10.71	34.30 $\pm$ 5.18	25.31 $\pm$ 8.59	15.95 $\pm$ 2.76	53.70 $\pm$ 9.37	43.01 $\pm$ 15.71	57.05 $\pm$ 12.14	37.70 $\pm$ 4.88 <sup>a</sup>
<b>240 days</b>	67.24 $\pm$ 13.04	43.44 $\pm$ 4.51 <sup>a</sup>	26.29 $\pm$ 4.34	12.03 $\pm$ 1.11 <sup>a</sup>	56.68 $\pm$ 8.59	36.16 $\pm$ 5.15 <sup>c</sup>	52.03 $\pm$ 11.06	32.08 $\pm$ 6.66 <sup>c</sup>
<b>360 days</b>	58.62 $\pm$ 40.10	26.10 $\pm$ 6.56	15.57 $\pm$ 3.99	7.61 $\pm$ 4.61 <sup>a</sup>	34.03 $\pm$ 13.0	17.70 $\pm$ 1.24 <sup>c</sup>	95.74 $\pm$ 27.93	58.76 $\pm$ 8.11 <sup>a</sup>

Unpaired 't' test was used; Values are Mean  $\pm$  SD of 5 estimations; <sup>a</sup> p < 0.05; <sup>b</sup> p < 0.01; <sup>c</sup> p < 0.001 Statistical comparisons are between wild type control and TG mice at each individual age.

dependent on the presence or absence of sex steroids. The presence of increased numbers of ABP-containing germ cells in the testes of ABP-TG mice [26] and immunocytochemical observations of intense ABP immunoreactivity found in and around all types of germ cells in ABP-TG mice [25] support a possible direct involvement of ABP in the testicular impairment seen in the ABP-TG mice. The late stages of spermatogenesis (spermiogenesis) were extensively studied for the possible direct effects of ABP [16,17,42–45]. In these studies, however, ABP (at least at physiological concentrations) appeared to be supportive, rather than detrimental, to spermatogenesis. Thus, available evidence does not seem to indicate a direct inhibitory or "damaging" role of ABP in spermatogenesis.

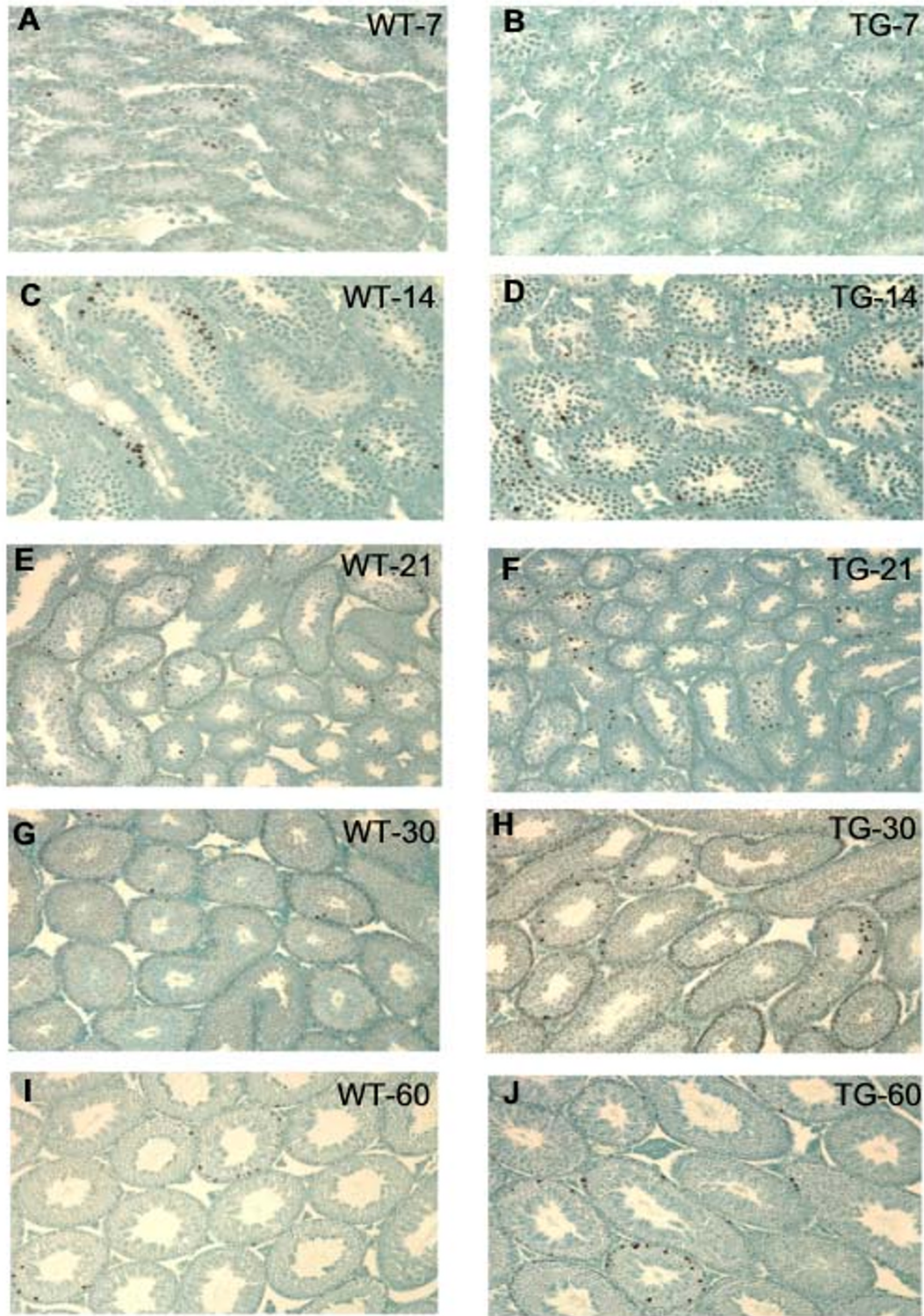
Although optimal and highly regulated rates of "spontaneous" germ cell apoptosis are essential for normal spermatogenesis [1,2,46–48], there is evidence to link the increased or decreased apoptosis of germ cells with abnormal spermatogenesis and male infertility [3,49,50].

In the ABP-TG mice, an overall reduction in the number of spermatids was reported [26] and suggested as one of the reasons for the reduced fertility seen in these animals. Our finding of an increased rate of germ cell apoptosis in 21 and 30 days old ABP-TG mice as compared to controls is consistent with the possibility that the reduced fertility (reduced germ cell number) seen in the ABP-TG mice during adulthood may be due to increased apoptosis of germ cells during the first wave of spermatogenesis in the pubertal period. In a recent paper, Selva *et al.* [51] described meiotic arrest and an "increased occurrence of

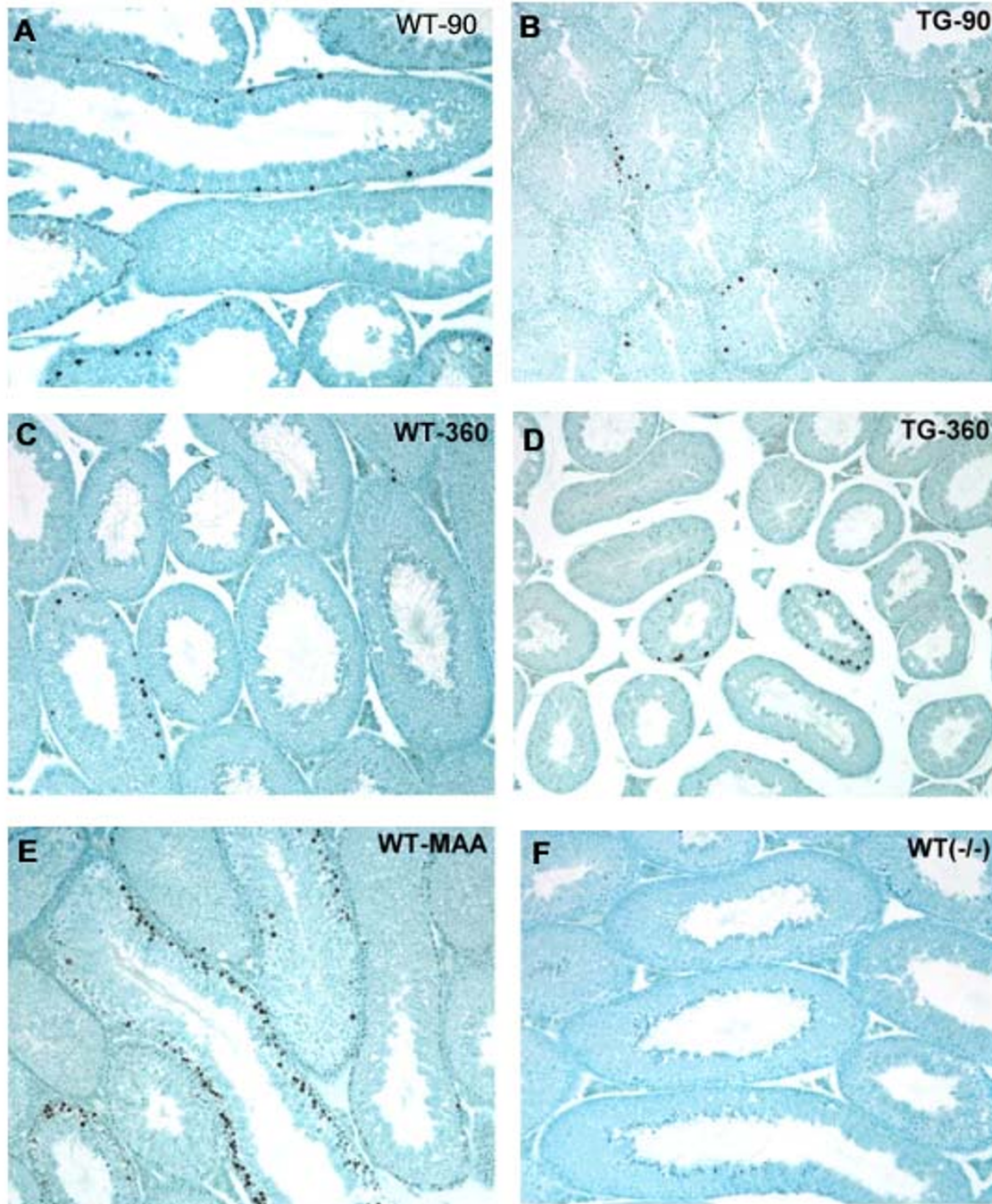
apoptosis" of testicular germ cells (primarily pachytene spermatocytes and cells in metaphase) in 3-, 6-, and 12-month old ABP-TG mice and suggested that these events were the cause of the fertility impairment seen in these animals. However, in the study of Selva *et al.* [51], apoptosis was not analyzed by flow cytometry but was detected by TUNEL reaction in histological sections only from a small number of homozygous animals (3 each of 3- and 6-month old and 1 of 12-month old) and no statistical analysis of the results was provided.

The present study is the first to describe the detailed quantitative pattern of testicular germ cell apoptosis throughout life in normal as well as in ABP-TG mice. Flow cytometry detects TUNEL-positive cells in four populations of germ cells in adult mice (Figure 2). The greater numbers of TUNEL-positive cells detected in the four populations of germ cells (highest in 4C, primary spermatocytes) from MAA-treated mice than controls confirm the reliability of the methods we used in this study. Selective targeting of spermatocytes by MAA has been reported earlier [52–55].

In 21 days old control mice 5.45  $\pm$  1.68% and in ABP-TG mice 11.47  $\pm$  2.57% of all germ cells are apoptotic. Analysis within each population reveals that, in control mice, 2.47  $\pm$  0.65% of 2C, 0.32  $\pm$  0.23% of S-phase, 2.26  $\pm$  0.76% of 4C, and 0.44  $\pm$  0.40% of 1C cells were apoptotic. In ABP-TG mice, the percentage of 2C (4.45  $\pm$  0.70%) and 4C (5.82  $\pm$  2.27%) cells undergoing apoptosis was significantly higher than in controls.



**Figure 6**  
TUNEL-positive germ cells in testicular sections of age-matched control (WT) and ABP-TG mice at the ages of 7 (A&B), 14 (C&D), 21 (E&F), 30 (G&H) and 60 (I&J) days.



**Figure 7**

TUNEL-positive germ cells in testicular sections of age-matched control (WT) and ABP-TG mice at the ages of 90 (A&B) and 360 (C&D) days. Photomicrograph in E is from a 90 days old control mouse treated with methoxy acetic acid (MAA). Compare number of apoptotic germs cells in Fig. 7E with that in untreated WT control (Fig. 7A) and the negative control (Fig. 7F).

Though the total number of germ cells undergoing apoptosis in control mice increased gradually from Day 7 to 360 (the total number of germ cells also increased in general, see Jeyaraj *et al.* [26]), the percentage of germ cells undergoing apoptosis was at its peak on Day 30 (Table 1). These findings are consistent with the morphological analysis of Russel *et al.* [8] reporting higher incidence of degenerative cells in the seminiferous tubules of 20 and 32 days old normal animals and lower levels found in adults.

In ABP-TG mice, the total number of germ cells undergoing apoptosis also increased from Day 7 and peaked on Days 21 and 30. After a decline by Day 60, the number of germ cells undergoing apoptosis leveled off in ABP-TG mice. From Day 90 onwards, in terms of total number of germ cells undergoing apoptosis, there is a clear and consistent reduction in ABP-TG mice compared to controls. However, since the total number of germ cells in ABP-TG mice of these age groups (day 90 to day 360) is also reduced [26], the actual proportion (or percentage) of germ cells undergoing apoptosis remains similar in the two groups.

The presence of increased numbers of apoptotic germ cells in ABP-TG mice compared to WT controls on Days 21, 30 and 60 suggests that this age group is more vulnerable than adult animals to increased levels of intra-testicular ABP. This suggestion is supported by many of our observations: (1) the percentage of germ cells undergoing apoptosis at these age groups (days 21, 30 and 60) was higher in ABP-TG mice than in WT controls. (2) The absolute numbers of germ cells undergoing apoptosis in 21 and 30 days old ABP-TG mice were significantly higher than in WT controls, albeit the total number of germ cells present in the testes of 21 and 30 days of ABP-TG mice was similar to that in WT-controls [26]. (3) On day 60, even though the total number of germ cells present in the ABP-TG mice was significantly lower than in age-matched WT controls [26], the number of germ cells undergoing apoptosis was nevertheless higher. Thus, at Days 21, 30, and 60, the changes observed must be directly related to the increased levels of testicular ABP present in these TG animals.

Our data indicate that the presence of greater than physiological concentration of ABP in the mouse testis has a biphasic effect on the frequency of apoptosis in germ cells. The initial increase seen in germ cell apoptosis (especially at 21 and 30 days) is likely to result from a direct or indirect action of ABP and may affect the subsequent life-death balance of germ cell populations in TG mice. The subsequent reduction is most likely due to maturation depletion (reduced germ cell numbers). Taken together, the presence of increased levels of intra-testicular ABP in the TG mice after 7 days of age [25,26] and a wave of germ

cell apoptosis seen in normal mice at 21 and 30 days of age in this study and by Rodriguez *et al.* [3] suggest that the heightened apoptotic wave found in our TG mice during the pre-pubertal period may be regulated by ABP. However, at this point it is not clear whether ABP regulates this apoptotic wave by direct action on germ cells or indirectly, by reducing the intra-testicular concentrations of free (bioavailable) T. The known biochemical properties and actions of ABP make the second mechanism more likely (see Jeyaraj *et al.* [26]). This would also indicate that the pre-pubertal wave of apoptosis is regulated, at least in part, by T. Indeed, it has been reported that experimental reduction in the levels of intra-testicular T leads to the activation of caspase-3 [56] and thereby to an increase in germ cell apoptosis [57,58]. The roles of other apoptosis-regulating proteins, such as the Fas/Fas ligand system [47,59,60], the Bcl-2 family of proteins [61,62], and several "inhibitor of apoptosis" proteins (IAP) are also likely to play a role in germ cell death and survival [63-65].

## Conclusions

In conclusion, our data suggest that the presence of high concentrations of ABP in the mouse seminiferous tubules results in a dramatically increased wave of germ cell apoptosis during the pre-pubertal period. Since the most likely mechanism of this effect of ABP is androgen sequestration, androgens are likely to play an important role in regulating the wave of germ cell apoptosis in mice. The altered rate of apoptosis at this time may in part be responsible for the subsequent progressive spermatogenic defect seen in the ABP-TG mice. An analysis of androgen levels and ABP- and androgen-regulated gene expression within the seminiferous tubules may reveal the precise molecular mechanisms of these complex regulatory processes.

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