

Infertility associated with incomplete spermatogenic arrest and oligozoospermia in *Egr4*-deficient mice

Warren G. Tourtellotte^{1,2}, Rakesh Nagarajan¹, Anthony Auyeung⁴, Christina Mueller⁵
and Jeffrey Milbrandt^{1,3,*}

The Department of ¹Pathology and Divisions of ²Neuropathology and ³Laboratory Medicine, and Department of ⁴Obstetrics and Gynecology, Washington University School of Medicine, St Louis, MO 63110, USA

⁵Department of Dermatology and Andrology, University of Giessen, Giessen, Germany

*Author for correspondence (e-mail: jeff@milbrandt.wustl.edu)

Accepted 11 August; published on WWW 21 October 1999

SUMMARY

Male fertility is complex and depends upon endocrine/paracrine regulatory mechanisms and morphogenetic processes occurring during testicular development, spermatogenesis (mitosis and meiosis) and spermiogenesis (spermatid maturation). *Egr4* (*NGFI-C*, *pAT133*), a member of the *Egr* family of zinc-finger transcription factors, is thought to be involved in cellular growth and differentiation, but its specific function has been previously unknown. We derived *Egr4* null mice through targeted mutagenesis and found that they were phenotypically normal with the exception that males, but not females, were infertile. *Egr4* is expressed at low levels within male germ cells during meiosis and is critical for germ cell maturation during the early-mid pachytene stage.

While most *Egr4* null male germ cells undergo apoptosis during early-mid pachytene, some are capable of maturing beyond an apparent *Egr4*-dependent developmental restriction point. Consequently, a limited degree of spermiogenesis occurs but this is accompanied by markedly abnormal spermatozoon morphology and severe oligozoospermia. *Egr4* appears to regulate critical genes involved in early stages of meiosis and has a singularly important role in male murine fertility. These data raise the possibility that *Egr4* may contribute to some forms of human idiopathic male infertility.

Key words: Infertility, Spermatogenesis, *Egr*, Transcription factor, Gene targeting

INTRODUCTION

Infertility is estimated to affect 5% of human males and, of those, a genetic basis is recognized in approximately 30%. As many as 1.5% of males (approximately 45 million) are estimated to have a genetically based impairment in fertility worldwide (McLachlan et al., 1998). These numbers, together with recent data suggesting that worldwide sperm quality is declining, mandate a greater understanding of the molecular mechanisms involved in male fertility (for review see, Irvine, 1997). For example, some infertile males have chromosomal anomalies such as the loss of a region of the Y chromosome (Yq11) where some 'azoospermia factors' are located (for review see, Krausz and McElreavey, 1999). However, only about 10-15% of azoospermic and about 5-10% of oligozoospermic men have Yq microdeletions (Gunduz et al., 1998; Krausz and McElreavey, 1999). Thus, while Y chromosome-linked azoospermia factors have received a great deal of scrutiny, they are likely to account for only a small proportion of genetically based male infertility.

In recent years, a large number of genes have been identified that directly participate in spermatogenesis. Using targeted mutagenesis in mice, different classes of autosomal genes that

code for transcription factors (e.g. A-myb, *crem* τ , c-Fos), heat-shock proteins (e.g. HSP70-2), growth factors (e.g. BMP8B), steroid receptors (e.g. AR, ER), cell-death proteins (e.g. Bcl-w, Bax) and DNA mismatch repair enzymes (e.g. Pms2, Mlh1, Msh2) have all been identified as important participants in spermatogenesis (for review see, Cooke et al., 1998; Sassone-Corsi, 1997). Presuming that these murine gene products function similarly to their human homologues, some may represent autosomal recessive fertility factors involved in human infertility. However, many genes that have been identified as critical for spermatogenesis in mice also have a variety of functions in non-germ cells. Therefore, it may be particularly interesting to study genes with essential functions confined to the testis since many infertile human males have no other identifiable phenotypic abnormalities.

The *Egr* family of zinc-finger transcription factors, whose members include *Egr1* (NGFI-A), *Egr2* (Krox20), *Egr3* and *Egr4* (NGFI-C, pAT133), are thought to regulate critical genetic programs involved in cellular growth and differentiation. Gene targeting experiments in mice have revealed specific biological processes that critically depend upon these transcription factors. For example, whereas male *Egr1* null mice are fertile, female mutants are infertile due to

impaired luteinizing hormone β (LH β) gene regulation (Lee et al., 1996). Moreover, *Egr2* null mice have severe hindbrain abnormalities (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993) and impaired peripheral nerve myelination (Topilko et al., 1994), whereas *Egr3* null mice have profound ataxia and abnormal proprioception due to dysmorphogenesis of muscle spindles (Tourtellotte and Milbrandt, 1998). The fact that *Egr2* mutations have been identified in humans with congenital myelinopathies provides some support for the concept that *Egr* transcription factors may be important in other human diseases (Warner et al., 1998).

There has been very little additional information reported regarding the function of the last *Egr* family member, *Egr4*, since it was originally isolated (Crosby et al., 1991; Holst et al., 1993). It is expressed at high levels in the forebrain in areas where one or more other *Egr* genes are simultaneously expressed. However, unlike other *Egr* genes that are widely expressed outside the nervous system, *Egr4* was initially identified only within the central nervous system (Crosby et al., 1992). Thus, it was presumed that *Egr4* function would be restricted to the central nervous system. In this study, we report that *Egr4* null mice develop without obvious phenotypic abnormalities with the exception that males, but not females, are infertile. Moreover, we have identified *Egr4* expression at very low levels in maturing male germ cells, where it appears to have an essential function during meiosis. This animal model of infertility is particularly interesting because it is restricted to males (mutant female mice are fertile and have no identifiable germ cell abnormalities) and there is incomplete germ cell maturation arrest. In male *Egr4* null mice, germ cell maturation is nearly completely blocked at the early-mid pachytene stage, leading to oligozoospermia characterized by the production of a comparatively small number of spermatozoa with abnormal morphology (teratozoospermia). These observations are reminiscent of a subset of infertile human males that are phenotypically normal except for the production of small amounts of morphologically abnormal spermatozoa. *Egr4* may function similarly in humans and, if so, its dysfunction could result in genetically based male infertility.

MATERIALS AND METHODS

Egr4 gene targeting

A 7 kb genomic DNA fragment from the 129/SvJ mouse strain containing the *Egr4* gene was subcloned into pBluescript KS⁺ (Stratagene). A 1.5 kb DNA fragment containing a PGK-neomycin transferase selection cassette, pMC1NEOpA (McMahon and Bradley, 1990) was used to disrupt the gene coding sequence at a *NotI* site 19 nucleotides 5' of the first zinc finger in the DNA-binding domain (Fig. 1A). The PGK-Neo selection cassette contains stop codons in all three reading frames, thus creating a truncation of any formed protein N-terminal to the zinc-finger DNA-binding domain. The MC1-herpes simplex virus thymidine kinase gene (HSV-TK) was inserted in the *HindIII* site at the 3' end of the homologous region in the targeting construct. Four targeted embryonic stem cell clones were isolated. Two clones injected into C57BL/6 blastocysts gave rise to chimeric offspring, which in turn were mated to C57BL/6 females. The two independent ES-cell-derived lines produced homozygous mutant male mice with similar phenotypes.

Germ cell fractionation and gene expression analysis

Purified Leydig and germ cells were obtained from wild-type adult

testes (10-20 week old) using a modification of the method by Vernon et al. (1991). Decapsulated testes were gently triturated in DMEM supplemented with 0.5% fetal calf serum (FCS) and 25 mM HEPES, and filtered through nylon mesh. The cell suspension was layered on a 45% continuous Percoll density gradient containing density marker beads (Pharmacia AB, Uppsala, Sweden) as described (Browning et al., 1981). The cells were centrifuged and fractions of enriched Leydig cells (s.g.=1.06-1.07) and germ cells (s.g. <1.05) were obtained. The Leydig cells were further purified by culturing them in DMEM for 1-3 days and their purity was established by morphology and β -hydroxysteroid dehydrogenase histochemistry (Payne et al., 1980).

Sertoli and myoid peritubal cells were isolated from 2- to 3-week-old mouse testes using a modification of the method of Lian et al. (1992). The testes were decapsulated and the seminiferous tubules incubated in three sequential digestions in DMEM/F12 medium containing 0.05% collagenase/dispase (Boehringer-Mannheim), 0.005% soybean trypsin inhibitor (Sigma) and 0.01% DNase (Sigma). The myoid peritubal cell-enriched fraction from the second digestion was filtered through a 50 μ m nylon mesh, layered on a 45% continuous Percoll density gradient and eluted from the s.g.=1.074 fraction after centrifugation. The purity of the cells was established by morphology and α -smooth muscle actin immunohistochemistry (Tung and Fritz, 1990). The Sertoli cell enriched fraction from the third digestion was filtered through a 50 μ m nylon mesh and the cells cultured for 2-4 days in DMEM/F12 medium supplemented with 7.5% FCS and cytosine arabinoside (10 μ M). Contaminating germ cells were removed by lysis in 20 mM hypotonic citrate buffer.

Total RNA from each of the purified cell fractions was normalized to the expression of a housekeeping gene (GAPDH) by northern blot analysis. The RNA samples were then subjected to RT-PCR and Southern blotting. Each of the cell fraction samples was amplified using gene-specific primers for varying cycle numbers (15-21) and blotted to ensure a linear range of amplification. The purity of the cell fractions was additionally established by using primer pairs to amplify lineage-specific markers for Leydig (side chain cleavage, P450_{SCC}), germ (heat-shock protein, HSP70-2), Sertoli (androgen-binding protein, ABP) and myoid peritubal (smooth muscle actin, α -SMA) cells.

Histological analysis and germ cell staging

For general histological analysis, animals were deeply anesthetized (87 mg/kg ketamine; 13 mg/kg xylazine, i.p.), subjected to transcardiac perfusion with 4% phosphate-buffered paraformaldehyde (pH 7.4), and the tissues were processed in paraffin using standard methods. Sections from selected testes were processed for TUNEL histochemistry to identify apoptotic germ cells (Gavrieli et al., 1992) or for immunohistochemistry using antibodies to either GCNA1 (Enders and May, 1994) or LDHC4 (Hintz and Goldberg, 1977) according to standard methods. For some experiments, *p53* mutant mice (Sah et al., 1995) were mated to *Egr4* mutant mice.

For germ cell staging, glutaraldehyde-fixed adult wild-type and *Egr4* null testes were embedded in plastic, sectioned at 1 μ m thickness and counterstained with Toluidine blue. The cross-sections of the seminiferous tubules were staged according to previously established morphological criteria for the mouse testis (Oakberg, 1956; Russell et al., 1990).

Isolation of spermatozoa from the cauda epididymis

The epididymis was cut into multiple pieces in 2.2% sodium citrate and incubated at 32°C for 15 minutes. The suspensions of spermatozoa (5 μ l) were smeared on a glass slide, air-dried and stained with Hematoxylin-Eosin.

Flow cytometry of adult germ cells

Adult wild-type and *Egr4*^{-/-} testes were decapsulated into ice-cold phosphate-buffered saline (PBS, pH 7.4). The testicular cells were isolated by smearing the seminiferous tubules between two glass

microscope slides, gently triturating and filtering through 200 μm nylon mesh. Adequate numbers of premeiotic, meiotic and postmeiotic germ cells were morphologically identified in Cytospin prepared samples. The total number of cells obtained from identically prepared testes (four wild-type and four *Egr4*^{-/-} animals) were determined using a hemocytometer to enumerate viable cells based upon their ability to exclude Trypan blue dye. The cells were transferred to Krishan's reagent to isolate the nuclei and stain them with propidium iodide (Krishan, 1990). The DNA content of 5×10^4 testicular cell nuclei was determined by flow cytometry (FACSCAN, Becton Dickinson) and the relative percentage of cells in each peak was calculated (Janca et al., 1986; Spano and Evenson, 1993). Absolute cell numbers were determined from the percentage of cells represented by each DNA content peak and the total cell counts obtained from each prepared testis.

Gene expression analysis

For northern blot analysis, total RNA from brain (25 μg) or poly(A)⁺ RNA from testis (10 μg) was transferred to nylon membrane after electrophoresis and hybridized with an *Egr4*-specific probe. In situ hybridization was performed on fresh frozen testis using a ³³P-labeled *Egr1* ribonucleotide probe. To detect the low abundance *Egr4* message in testis, increased sensitivity was obtained using a digoxigenin-labeled ribonucleotide probe (Boehringer-Mannheim) that was detected using immunohistochemistry and tyramide signal amplification (NEN Life Sciences).

Testicular gene expression profiling was performed using RT-PCR on 5% of the RNA obtained from each testis regardless of its size. Thus, the amount of RNA in each RT reaction was closely correlated with the testicular weight. This method was preferred over traditional methods that either use equivalent amounts of RNA in each sample or normalize to a reference 'housekeeping' gene, since the gene expression can be compared on the basis of an entire testis. This is particularly appropriate for heterogeneous organs that lose specific

cell constituents relative to one another. By contrast, samples normalized to the total amount of RNA (or to a reference gene), skew the representation of RNA in favor of cells that are not effected by the mutation. Since it was clear that a specific cell population was lost in testis (i.e. early-mid pachytene spermatocytes), the 'unskewed' RNA representation was preferred to more accurately determine relative gene expression in the remaining cells (i.e. in the entire testis). The gene expression comparisons were performed using RT-PCR on samples obtained by pooling RNA from the testes of four wild-type and four *Egr4*^{-/-} mice. The cDNA samples were amplified by PCR with gene-specific primers at cycle numbers corresponding to the linear phase of amplification (15-21 cycles) and Southern blotted using the amplified products as probes. The resulting bands were quantified on a phosphorimager (Molecular Dynamics) and the results expressed as changes relative to the wild-type values.

RESULTS

Targeted disruption of *Egr4*

To examine the in vivo function of *Egr4*, null mice were generated using gene targeting in embryonic stem (ES) cells. The gene targeting construct was designed to remove the entire zinc-finger DNA-binding domain and carboxyl-terminal region of the protein by inserting a PGK-neomycin transferase (NEO) cassette, which contains stop codons in all three reading frames (McMahon and Bradley, 1990) as depicted (Fig. 1A). Southern blot analysis of genomic DNA from targeted ES cells or selected F₂ progeny identified the targeted homologous recombination event (Fig. 1B). Since there are currently no antibodies available to *Egr4*, northern blot analysis was used to confirm that the wild-type transcript was absent from adult brain and testis (Fig. 1C). A low-abundance mutant transcript, containing coding sequence 5' of the zinc fingers and a portion of the Neo resistance gene, was also identified (not shown). Two independent lines were established and mice from both lines demonstrated similar phenotypes. Wild-type (+/+), heterozygous (+/-), and null (-/-) F₂ progeny were born at the expected Mendelian ratio of 1:2:1, respectively ($n=400$). *Egr4*^{-/-} mice developed normally and exhibited no identifiable behavioral abnormalities. Despite a thorough gross and microscopic examination of all major organs

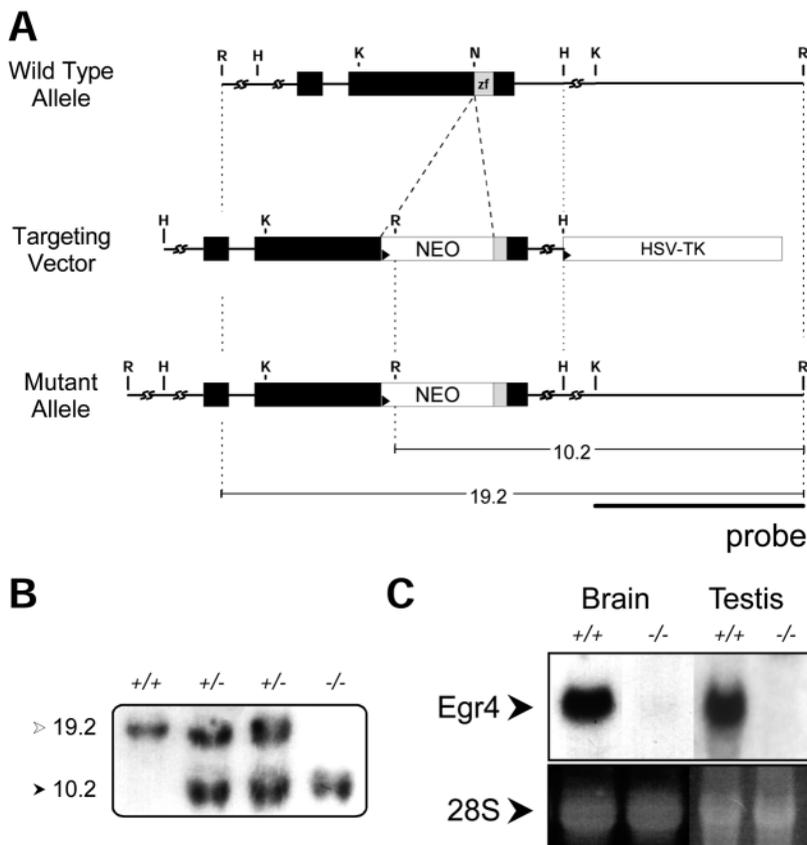


Fig. 1. Disruption of the murine gene encoding the transcription factor *Egr4*. (A) The wild-type allele, targeting vector and resulting mutant allele demonstrate the strategy used to disrupt *Egr4* in ES cells. Restriction sites are indicated: H, *Hind*III; K, *Kpn*I; R, *Eco*RI. The probe used to identify homologous recombination events is indicated. (B) Southern blot analysis of *Eco*RI-digested genomic DNA from four selected F₂ littermates identified wild-type, heterozygous and homozygous mice. Arrows indicate the DNA fragment sizes (kbp) expected in wild-type (white arrow) and mutant (black arrow) alleles. (C) Northern blot analysis of total RNA from brain (25 μg) and poly(A)⁺ RNA from testis (10 μg) demonstrated loss of the wild-type transcript in the *Egr4* mutant mice (wild type, +/+; homozygous mutant, -/-). Note that the expression of *Egr4* in testis is estimated to be 25- to 50-fold lower than in brain.

Fig. 2. *Egr4* expression in testicular germ cells. (A) In situ hybridization demonstrated low-level *Egr4* expression in primary spermatocytes (asterisk) and higher levels in secondary spermatocytes and early spermatids (arrowhead). *Egr4* expression was not detected in either spermatogonia or Sertoli cells found along the seminiferous tubule basement membrane (arrow). The primary spermatocytes (asterisk) consist largely of several layers of pachytene spermatocytes in the stage I-IV tubule shown. (B) No staining was detected using a sense *Egr4* probe. Scale, 40 μm . (C) Wild-type testes were fractionated into highly enriched populations of Leydig (L), germ (GC), Sertoli (S) and myoid peritubal (PT) cells. The purity of the cell fractions was verified using morphological as well as immuno- and enzyme-histochemical criteria. Lineage-specific gene expression using P450 side chain cleavage enzyme (P450_{SCC}) for Leydig cells, heat-shock protein 70-2 (HSP70-2) for germ cells, androgen-binding protein (ABP) for Sertoli cells and alpha-smooth muscle actin (α -SMA) for myoid peritubal cells, confirmed the purity of the individual cell fractions. *Egr4* expression was detected only in the purified germ cell fractions.

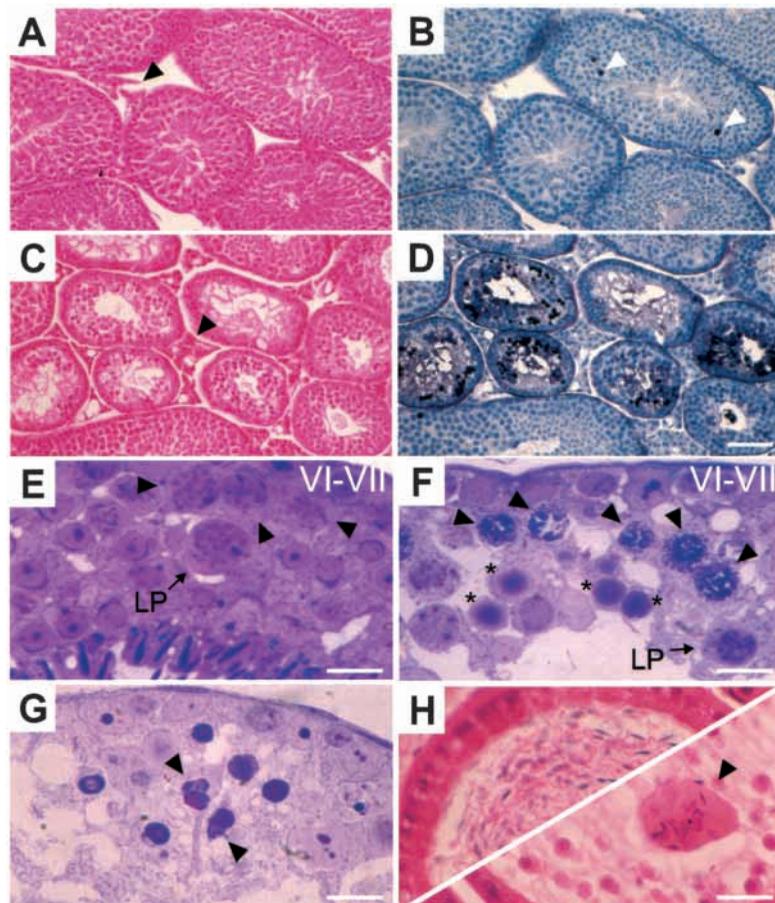
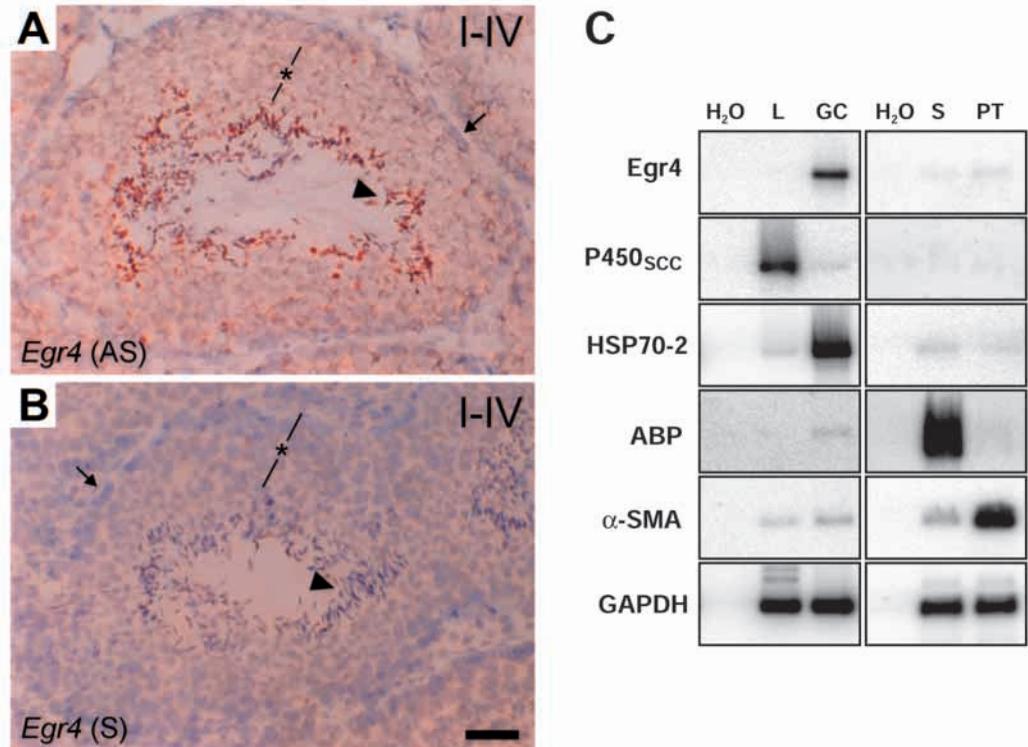


Fig. 3. Premature germ cell death and ineffective spermatogenesis in *Egr4*^{-/-} testis. (A) Wild-type testis showed normal seminiferous tubule architecture, Leydig cell morphology (arrowhead) and (B) TUNEL histochemistry identified rare apoptotic germ cells (arrowheads). (C) In *Egr4*^{-/-} testis, however, frequent pyknotic germ cells, distortion of the epithelial architecture and Leydig cell hyperplasia (arrowhead) were noted. (D) Large numbers of apoptotic germ cells were observed in some tubules in *Egr4*^{-/-} testis using TUNEL histochemistry. (A-D) Scale, 100 μm . A detailed comparison of (E) wild-type and (F) *Egr4*^{-/-} testis identified early apoptotic changes in early-mid pachytene spermatocytes (arrowheads) that were often associated with markedly degenerated cells in mutant testis (asterisks). While many early-mid pachytene spermatocytes appeared to degenerate, the maturation arrest was incomplete as some late pachytene spermatocytes could still be identified (arrow, LP). (G) Most of the seminiferous tubules examined in *Egr4*^{-/-} testis contained highly degenerated cells that could not be staged (arrowheads). (E-G) Scale, 10 μm . (H) While wild-type cauda epididymis was replete with mature spermatozoa (top), in most *Egr4*^{-/-} epididymides, only a small number of cellular aggregates contained mature spermatozoa (arrowhead). Scale, 30 μm .

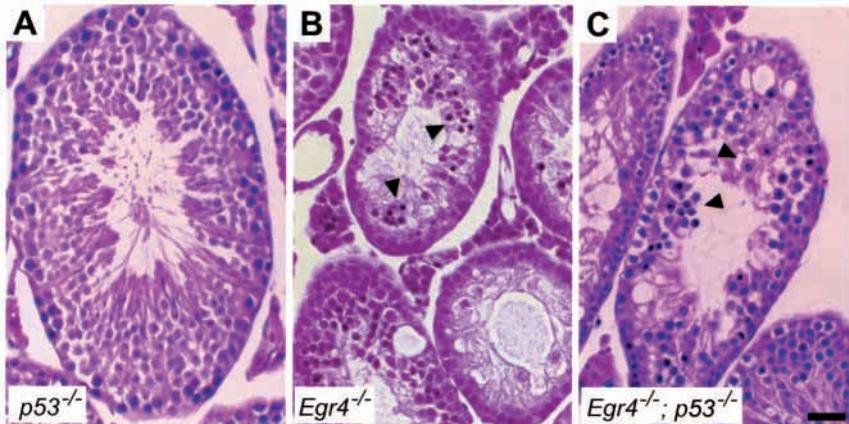


Fig. 4. *p53*-independent germ cell apoptosis in *Egr4* null testis. (A) Normal spermatogenesis was observed in *p53* null mice. (B,C) The germ cell apoptosis that occurred in *Egr4* null testis was not affected by the concomitant loss of *p53* function. Clusters of apoptotic germ cells are illustrated in (B) *Egr4* single and (C) *Egr4/p53* double null testis (arrowheads). Scale, 20 μ m.

from female and male mice, only the testes showed prominent cellular abnormalities. The *Egr4*-dependent defects were associated with male-only infertility as the cellular changes were restricted to the male germline. Female mice had normal fertility and no identifiable microscopic abnormalities in their ovaries.

***Egr4* is expressed specifically in testicular germ cells**

Early studies identified *Egr4* expression at high levels in the brain but not in other tissues (Crosby et al., 1992). Thus, a phenotype confined to male germ cells in the *Egr4* null mice was unexpected and prompted a detailed analysis of its expression in testis. With northern blot analysis, the level of *Egr4* expression was estimated to be 25- to 50-fold lower in testis than in brain (compare 48 hour exposures Fig. 1C; brain, 25 μ g total RNA to testis, 10 μ g poly(A)⁺ RNA). We used in situ hybridization to examine its expression in testis, as immunohistochemical analysis was not possible. We detected low levels of *Egr4* expression in primary spermatocytes and higher levels in secondary spermatocytes and some spermatids (Fig. 2A, *Egr4* antisense probe and Fig. 2B, *Egr4* sense probe). However, neither spermatogonia, Sertoli cells, Leydig cells nor myoid peritubal cells expressed *Egr4*. The germ cell specificity of *Egr4* expression was further corroborated by fractionating testes into highly enriched populations of Leydig, Sertoli, germ or myoid peritubal cells. The purity of the cell fractions was determined using a combination of morphological as well as enzyme and immunohistochemical markers for a specific cell population (data not shown). *Egr4* expression was analyzed in each cell type using reverse transcription (RT)-PCR and the purity of the fractions was additionally confirmed by examining genes with expression restricted to either Leydig (P450_{ScC}), Sertoli (ABP), germ (HSP70-2) or myoid peritubal (α -SMA) cells. *Egr4* expression was detected only in RNA obtained from the germ cell fraction (Fig. 2C).

Male infertility is associated with microrchidia and *p53*-independent germ cell apoptosis in early-mid pachytene primary spermatocytes

Test matings demonstrated that nearly all *Egr4* null males were infertile, whereas females showed no identifiable fertility defects. Occasional null males (2 in 21 test matings) sired very small litters (4-5 pups) but never with the frequency of their wild-type male littermates. Adult *Egr4* null testes (>8 weeks

old) were smaller and weighed 43% of wild type (wild type, 96.4 \pm 1.7 mg; *n*=63; *Egr4* null, 41.9 \pm 2.3 mg; *n*=37; *P*<0.001 Fig. 7I). Microscopic analysis of *Egr4* null testes showed Leydig cell hyperplasia (Fig. 3C, arrowhead) when compared to wild-type testes (Fig. 3A, arrowhead). Within seminiferous tubules, maturing spermatids filled the lumina in adult wild-

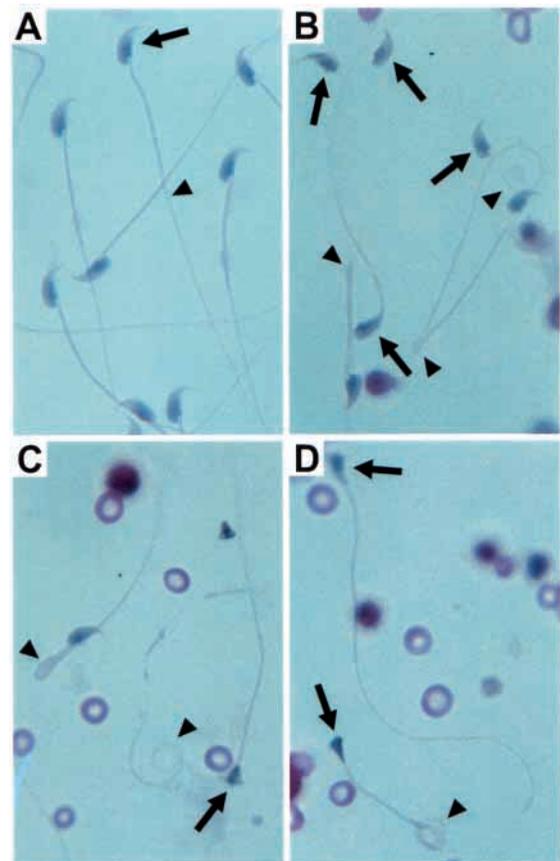


Fig. 5. Oligozoospermia and abnormal spermatozoan morphology in *Egr4*^{-/-} mice. Compared to (A) wild type, spermatozoa from (B-D) *Egr4*^{-/-} cauda epididymis showed abnormal morphology characterized by flagella that were fragmented, sharply kinked, or had tightly curled distal ends (arrowheads). Frequently, spermatozoon heads were either separated entirely or bent sharply back on the flagellum to obscure the acrosomal cap (arrows).

type testes (Fig. 3A), whereas null testes had comparatively small caliber tubules with open lumina due to markedly decreased numbers of maturing spermatocytes (Fig. 3C). Moreover, Tdt-mediated dUTP-biotin nick end labeling (TUNEL) histochemistry identified rare apoptotic germ cells in wild-type testes (Fig. 3B, arrowheads), but in *Egr4* null testes large numbers of apoptotic germ cells were concentrated in scattered tubules (Fig. 3D). Increased numbers of apoptotic cells were noted in approximately 20% of the tubule cross-sections suggesting an association with a specific stage of germ cell maturation (Oakberg, 1956). Although the spermatogenic staging analysis was complicated by the disrupted germ cell architecture, it was possible to identify germ cells undergoing early stages of apoptosis in some tubules. Where staging was possible, many of the degenerating germ cells were identified in stage VI-VII tubules in the early-mid pachytene phase of meiosis (Oakberg, 1956; Russell et al., 1990). In stage VI-VII tubules from wild-type mice, early-mid pachytene spermatocytes were identified adjacent to the outermost cell layers of the tubule (Fig. 3E, arrowheads). However, in tubules from *Egr4* null testis of comparable stage, similar spermatocytes showed marked nuclear and cytoplasmic alterations consistent with early apoptosis. Many germ cells showed marked aggregation/margination of nuclear contents and early fragmentation of the cytoplasm (Fig. 3F, arrowheads). Germ cells in early stages of apoptosis were frequently associated with highly degenerated cells of indeterminate maturational stage near the adluminal space of the seminiferous tubules (Fig. 3F, asterisks). In many tubules, however, germ cell degeneration was far advanced, precluding precise morphological characterization and staging (Fig. 3G, arrowheads). Both spermatogonia and Sertoli cells appeared morphologically intact in all of the tubules examined. These data indicate that, in adult testis, primary spermatocytes require *Egr4* to transgress the early-mid pachytene stage of meiotic prophase.

Apoptosis in the context of DNA damage (Lowe et al., 1993) or during tumor growth and progression (Symonds et al., 1994) is related to the transcriptional activity of *p53*. In spermatogonia, *p53* plays a critical role in apoptosis that occurs after double-strand breaks in DNA, but not for checkpoint monitoring of synapsis during meiosis in primary spermatocytes (Odorisio et al., 1998). In *Egr4* null testis, we observed that spermatocyte apoptosis was also *p53*-independent since spermatocyte death was not affected in the *p53* null background (Fig. 4). Moreover, the similar (low) weights between *Egr4* null and *Egr4/p53* double null testes further suggested that *p53* inactivation was not capable of rescuing early-mid pachytene spermatocytes (data not shown).

Incomplete germ cell maturation arrest is characterized by markedly decreased spermiogenesis and abnormal sperm morphology

While a large number of germ cells degenerated at the early-mid pachytene stage of meiosis, the maturation arrest was incomplete. Indeed, some late pachytene stage spermatocytes, showing no morphological abnormalities, were identified in the material prepared for staging of *Egr4* null testes (Fig. 3E; wild type, late pachytene (LP) and Fig. 3F; *Egr4* null, late pachytene (LP)). Although late pachytene spermatocytes were not analyzed quantitatively, they were encountered far less

frequently in *Egr4* null testes than in wild type. Whereas adult wild-type cauda epididymides were replete with spermatozoa (Fig. 3H, top), in most *Egr4* null mice they contained frequent immature germ cells and negligible numbers of mature spermatozoa (Fig. 3H, bottom). In some *Egr4*-deficient animals, a sufficient number of spermatozoa were present for morphological analysis. Compared to wild-type spermatozoa (Fig. 5A), those isolated from *Egr4* null epididymides were present in much lower numbers and showed a variety of structural abnormalities. Frequently, spermatozoon heads were either separated entirely or bent sharply back on the flagellum to obscure the acrosomal cap (Fig. 5B-D; arrows). In addition, flagella were often fragmented, sharply kinked, or had tightly coiled distal ends (Fig. 5B-D; arrowheads).

Ploidy analysis by flow cytometry confirms the loss of germ cells during prophase I of meiosis

Apoptosis of early-mid pachytene spermatocytes was observed in adult testis prepared for staging analysis, but was based upon the staging of a small number of cells. Most of the cells were identified at advanced stages of apoptosis and were not classifiable. To determine if apoptosis occurred as germ cells entered meiosis, we used flow cytometry to compare wild-type and *Egr4* null testicular cells based upon their DNA content. The flow cytograms showed four discrete peaks consisting of: (i, ii) a 1N (haploid) double peak representing mature spermatozoa and immature elongating and round spermatids, (iii) a 2N (diploid) peak representing G₁ phase spermatogonia, preleptotene spermatocytes, and some additional testis somatic cells, and (iv) a 4N (tetraploid) peak consisting of primary spermatocytes (Janca et al., 1986; Spano and Evenson, 1993) (Fig. 6). The cell fractions represented by each peak were normalized to the absolute number of cells isolated from each testis. When compared to wild type, there was no difference in the number of 2N cells isolated from *Egr4*^{-/-} testes. However, the 4N and 1N cell fractions showed a reduction to 30% (30±7.6%, *P*<0.003) and 22% (22±7.2%, *P*<0.002) of wild-type levels, respectively (Fig. 6, inset). Thus, *Egr4* does not appear to be required in spermatogonia and preleptotene spermatocytes (2N). However, after maturing germ cells entered the chromosomal doubling phase of meiosis (4N), 70% of the primary spermatocytes were lost to apoptosis. These data are compatible with the results obtained from the staging analysis in which apoptosis of early-mid pachytene spermatocytes was observed.

Male germ cell apoptosis in *Egr4* null mice precedes the onset of puberty and occurs during the first round of spermatogenesis

In adult testis, *Egr4* was first expressed in early-mid pachytene spermatocytes and was essential for their progression through early stages of meiosis. This most likely reflects an autonomous requirement for *Egr4* within maturing germ cells rather than endocrine influences from the hypothalamic-pituitary-gonadal axis. We examined the levels of LHβ and follicle stimulating hormone β (FSHβ) by northern blotting and immunohistochemistry in pituitary glands, as well as serum levels measured by radioimmunoassay, and found no significant differences between adult male wild-type and *Egr4*^{-/-} mice (W. G. T., unpublished data). An autonomous germ cell defect was further corroborated by examining

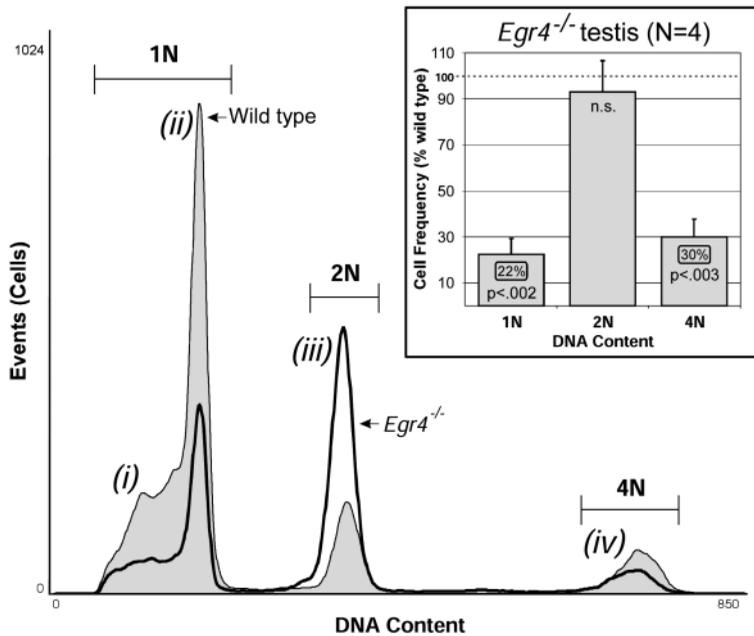


Fig. 6. Flow cytometry of cells isolated from adult wild-type and *Egr4*^{-/-} testis. The flow cytograms contained four discrete peaks: (i) a 1N (haploid) peak representing immature elongating and round spermatids (secondary spermatocytes), (ii) a 1N (haploid) peak representing mature spermatozoa, (iii) a 2N (diploid) peak representing G₁ phase spermatogonia, preleptotene spermatocytes, and some additional testis somatic cells, and (iv) a 4N (tetraploid) peak representing primary spermatocytes. Compared to wild type, the flow cytograms obtained from *Egr4*^{-/-} testes showed a markedly abnormal distribution of maturing germ cells. (inset) Absolute cell numbers were calculated from the relative number of cells represented by each DNA content peak. No difference was noted in the 2N cell population, indicating that spermatogonia and preleptotene spermatocytes were not effected in *Egr4*^{-/-} testis. However, both the 4N and 1N peaks showed a reduction to 30% (30±7.6%, *P*<0.003) and 22% (22±7.2%, *P*<0.002) relative to wild type, indicating that 70–80% of the germ cells were lost during meiosis after chromosomal doubling. These results are consistent with a loss of early-mid pachytene spermatocytes observed in the tissue prepared for staging analysis.

prepubertal testes during the first round of spermatogenesis. No histological abnormalities were identified in *Egr4*^{-/-} testes at birth (data not shown). Moreover, by P7, a stage at which the testis contained primarily proliferating spermatogonia (Janca et al., 1986), testicular weights and morphology between wild-type and *Egr4* null testes were indistinguishable (Fig. 7I). By P14 however, an age at which germ cells have entered meiosis but spermiogenesis has not yet begun (Janca et al., 1986), *Egr4* null testes were smaller and weighed 73% of wild type (wild type, 15.0±0.32 mg; *n*=8; *Egr4* null, 11.0±0.31 mg; *n*=12, *P*<0.0001) (Fig. 7I; dashed box). At this developmental stage, prior to the onset of puberty (which occurs around P20 in mice), apoptotic germ cells were noted in *Egr4* null testes (Fig. 7C,D; arrowheads) but in only rare germ cells in wild-type testes (Fig. 7B, arrowhead). The apoptotic germ cells were located near the adluminal space of the seminiferous tubules and did not appear to involve spermatogonia or Sertoli cells located along the tubular basement membrane. Early-mid pachytene spermatocyte death was correlated with the expression of GCNA1 and LDHC4, which have expression patterns restricted to specific stages of germ cell maturation. In P14 mouse testis, GCNA1 is expressed at high levels in spermatogonia and primary spermatocytes up to the mid-pachytene stage, after which it is downregulated (Enders and May, 1994). Reduced numbers of GCNA1⁺ germ cells were observed in *Egr4* null testis (Fig. 7F) compared to wild-type testis (Fig. 7E), consistent with a loss of germ cells at the early-mid pachytene stage of meiosis. Similarly, the testis-specific LDHC4 is highly expressed in mid and late pachytene primary spermatocytes in P14 mouse testis (Hintz and Goldberg, 1977). There was a marked reduction of LDHC4⁺ germ cells in *Egr4* null testis (Fig. 7H) compared to wild type (Fig. 7G), also consistent with a marked reduction of viable early-mid pachytene spermatocytes. These data demonstrate that *Egr4* function is required, prior to the onset of puberty, within early-mid pachytene spermatocytes and does not appear to depend upon prominent hypothalamic-pituitary-gonadal axis control.

Disruption of *Egr4* leads to widespread alterations in testicular gene expression

In an attempt to identify possible target genes regulated by *Egr4* within testis, we examined the expression of a variety of genes known to play a role in spermatogenesis. These studies identified alterations in the level of expression of many genes in *Egr4* null versus wild-type testes (Fig. 8A). The haploid-specific germ cell markers, heat-shock protein 70-2 (*HSP70-2*), transition protein 1 (*TPI*) and *RT7* (Higgy et al., 1994; Yelick et al., 1989; Zakeri and Wolgemuth, 1987), were expressed at levels that poorly correlated with the large loss of haploid germ cells in *Egr4* null testis. For example, their expression was either decreased slightly (*RT7*, 1.4-fold), increased (*HSP70-2*, 2.6-fold) or unaffected (*TPI*, 0.3-fold), despite an overall 4.5-fold decrease in the number of haploid germ cells produced in mutant testis (Fig. 6, inset). This may have reflected an induction of these genes in surviving haploid germ cells. The expression of several Leydig cell-specific genes, including luteinizing hormone receptor (LHR) and several steroidogenic enzymes (*P450_{SCC}*, *P450_{C17}* and *3βHSD*), showed induction ranging from 2.5-fold to 8.2-fold. While serum testosterone levels were normal in *Egr4* null male mice (W. G. T., unpublished data), the increased mRNA levels may reflect an increased fraction of Leydig cells present in the testicular lysates due to their hyperplasia (Fig. 3C, arrowhead). Two Sertoli cell-specific genes, androgen-binding protein (*ABP*) and procathepsin L (*Pro-L*) (Attramadal et al., 1981; Erickson-Lawrence et al., 1991), were induced only modestly (*ABP*, 1.7-fold and *Pro-L*, 2.0-fold).

Androgen receptors (AR) are expressed by myoid peritubal, Sertoli and some Leydig cells (Vornberger et al., 1994), while estrogen receptors (ER) are expressed primarily by Leydig and Sertoli cells (Lin et al., 1982). The expression of both receptors was increased (*AR*, 3.3-fold and *ER*, 7.2-fold) in *Egr4* null testis. Moreover, the principle androgen-to-estradiol converting enzyme, aromatase (*P450_{Arom}*) was also markedly upregulated (8.8-fold).

A variety of DNA repair enzymes, transcription factors and

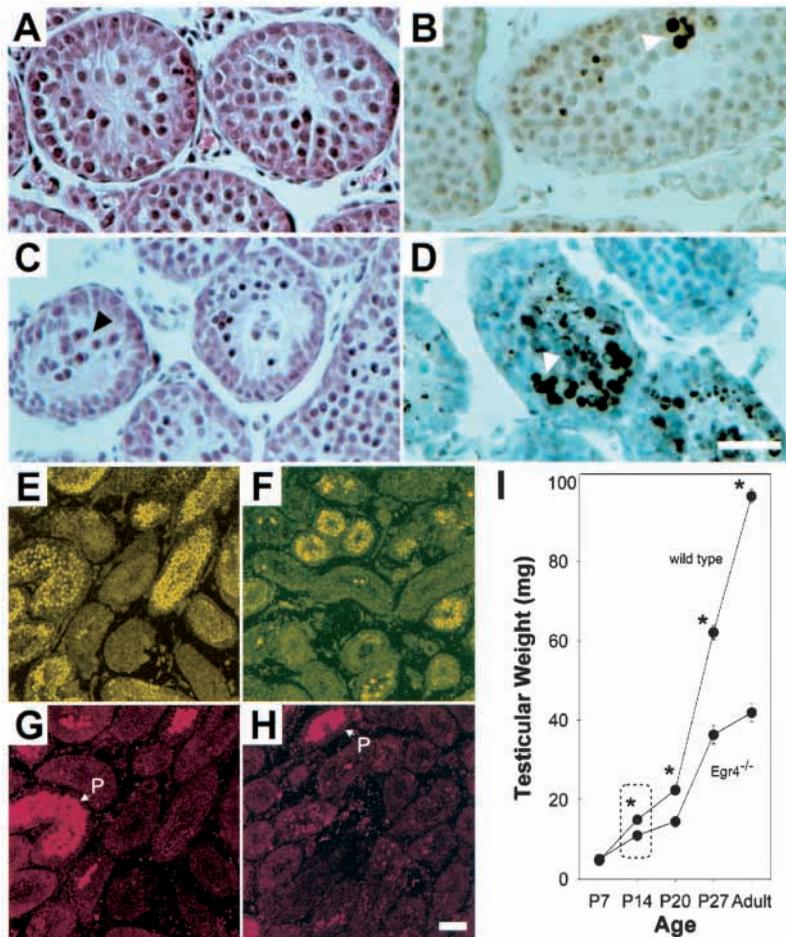


Fig. 7. Germ cell death during the first round of spermatogenesis in prepubescent *Egr4* null testis. (A) During the first round of spermatogenesis, the seminiferous tubules in wild-type P14 testis contained spermatogonia, Sertoli cells and primary spermatocytes. The spermatocytes were positioned near the central portions of the tubules and (B) TUNEL histochemistry identified rare apoptotic germ cells (arrowhead). (C) In *Egr4* null testis, large numbers of pyknotic spermatocytes were sloughed into the adluminal space of the seminiferous tubules (arrowhead) and (D) TUNEL histochemistry identified marked spermatocyte apoptosis (arrowhead). (A–D, scale, 100 μ m). (E) In wild-type P14 testis, GCNA1 labeled spermatogonia and primary spermatocytes up to the mid pachytene stage. (F) In *Egr4* null testis, there was a substantial reduction in GCNA1 labeling, consistent with the loss of a large portion of early-mid pachytene spermatocytes. (G) LDHC4 is restricted to mid and late pachytene primary spermatocytes in P14 wild-type testis. (H) Reduced numbers of LDHC4⁺ spermatocytes were observed in P14 *Egr4* null testis, consistent with the loss of early-mid pachytene spermatocytes. Although reduced in number, some mid to late pachytene spermatocytes were present, indicating that spermatocyte death was incomplete. (E–H) Scale, 50 μ m. (I) Differences in testicular weight were first identified in P14 testis after germ cells entered the first round of spermatogenesis but prior to the onset of puberty (asterisk indicates statistical difference, $P < 0.001$).

growth factors play essential roles in spermatogenesis. We examined the expression levels of several DNA repair enzymes and found that they were all markedly upregulated in *Egr4* null testis. Two DNA mismatch repair enzymes, *PMS2* (upregulated 6.1-fold) and *MLH1* (upregulated 4.2-fold), are essential for normal murine spermatogenesis (Baker et al., 1995, 1996; Edlmann et al., 1996). In addition, the mismatch repair enzyme, *Msh2* (upregulated 8.8-fold) and the recombination repair enzyme, *Rad51* (upregulated 8.4-fold) are integral components of the synaptonemal complexes formed between paired homologous chromosomes in pachytene spermatocytes (Ashley et al., 1995; de Wind et al., 1995; Haaf et al., 1995). Apoptosis in *Egr4*-deficient early-mid pachytene spermatocytes together with an induction of DNA repair constituents may indicate that DNA damage is a predetermining factor in their demise.

While *Egr4* deficiency leads to a partial spermatogenic arrest and oligozoospermia, mice deficient in the transcription factors *CREM τ* and *A-myb* have a complete spermatogenic arrest (Blendy et al., 1996; Nantel et al., 1996; Toscani et al., 1997). In *Egr4* null testis the upregulation of *CREM τ* (4.8-fold) and *A-myb* (8.0-fold) may reflect compensatory transcriptional mechanisms induced by the ineffective spermiogenesis. We also examined the levels of expression of the three other closely related Egr transcription factors and found only *Egr1* to be markedly upregulated (9.5-fold). *Egr1* induction was confirmed at the cellular level by in situ hybridization and showed high levels of expression specifically in Leydig cells

(Fig. 8B). The expression levels of several growth-related genes produced in the testis (*TGF β* , *Inhibin α* and *Activin/Inhibin β*) were not markedly altered.

DISCUSSION

Our results indicate that the zinc-finger transcription factor *Egr4* has a critical role in spermatogenesis. Both male and female *Egr4*-deficient mice appeared neurologically normal. Males were infertile due to massive germ cell death in early-mid pachytene spermatocytes. Early-mid pachytene spermatocytes most likely have an autonomous requirement for *Egr4* since it appears to be expressed first at this stage of spermatocyte maturation. Moreover, neither of the pituitary hormones, luteinizing hormone (LH) or follicle stimulating hormone (FSH) appeared to play a significant role, as pachytene spermatocytes were affected during the first round of spermatogenesis prior to the onset of puberty (Fig. 7I; dashed box). Accordingly, no significant alterations in either the expression of LH β or FSH β in the pituitary or in their levels in serum were detected in adult *Egr4*-deficient mice (W. G. T., unpublished data).

The block in spermatogenesis was incomplete, as a small percentage (20–30%) of the primary spermatocytes was capable of developing beyond the pachytene stage in *Egr4*-deficient mice (Fig. 6). Moreover, the incomplete spermatogenic arrest led to oligozoospermia characterized by abnormal spermatozoa

morphology (teratozoospermia). These results indicate that some pachytene spermatocytes are capable of transgressing an *Egr4*-dependent restriction point despite the fact that they may still harbor abnormalities, as they give rise to structurally abnormal spermatozoa. Alternatively, since *Egr4* expression was identified in secondary spermatocytes and spermatids (Fig. 2A), structural abnormalities in spermatozoa could also be due to *Egr4*-mediated gene regulation at later stages of spermiogenesis.

Inactivation of *Egr4* transcriptional activity may have accounted for the deregulation of critical target gene(s) required for normal pachytene spermatocyte maturation in mice. We attempted to identify *Egr4*-regulated genes by examining the expression of 25 genes that are highly expressed in the testis, many of which have a critical role in spermatogenesis in mice. While the gene expression profiles of wild-type and *Egr4* null testis were dramatically different, no definitive target genes were identified amongst the small set of genes surveyed (Fig. 8A). For example, *Cremt* and *A-myb*, two transcription factors that are critical for promoting germ cell maturation beyond the pachytene stage (Blendy et al., 1996; Nantel et al., 1996; Toscani et al., 1997), were markedly upregulated in *Egr4* null testis. The significance of this finding is unclear, but it may reflect compensatory transcriptional mechanisms that are modulated by ineffective pachytene spermatocyte maturation. *Egr4* null testes also expressed increased levels of several genes primarily in Leydig and Sertoli cells that participate in steroidogenesis (P450_{SCC}, P450_{C17}, 3 β HSD, P450_{Arom}) or steroid signaling (ER, AR). Normal AR and ER function is essential for spermatogenesis (Eddy et al., 1996; Reddy and Ohno, 1981) and it is possible that increased aromatization of androgens and steroid receptor gene expression reflect compensatory responses to inadequate levels of spermatogenesis. Most likely, these changes are an indirect effect of *Egr4* deficiency, since it is not expressed in either Leydig or Sertoli cells. In fact, the changes in steroid related gene expression may reflect increased numbers of Leydig cells in the testicular lysates due to Leydig cell hyperplasia, as well as intracellular gene upregulation. For example, *Egr1*, which is expressed preferentially in Leydig cells in the testis, was upregulated 9.5-fold in *Egr4* null testis. In situ hybridization studies on wild-type and *Egr4* null testis confirmed the intracellular *Egr1* upregulation in hyperplastic Leydig cells. Thus, the disruption of spermatogenesis appears to alter feedback mechanisms leading to activation of Leydig cell gene expression. Similarly, no apparent Sertoli cell proliferation in *Egr4* null testis was observed, thus the increased ER and AR expression may also reflect intra-Sertoli cell upregulation due to ineffective spermatogenesis.

The fact that some pachytene spermatocytes matured beyond an apparent *Egr4*-dependent restriction point indicates that some stochastic processes may be involved in determining the fate of germ cells in *Egr4*-deficient spermatocytes. Evidence is accumulating that meiotic checkpoints are critical for regulating spermatogenesis and presumably for maintaining the integrity of the genome. For example, the transcription

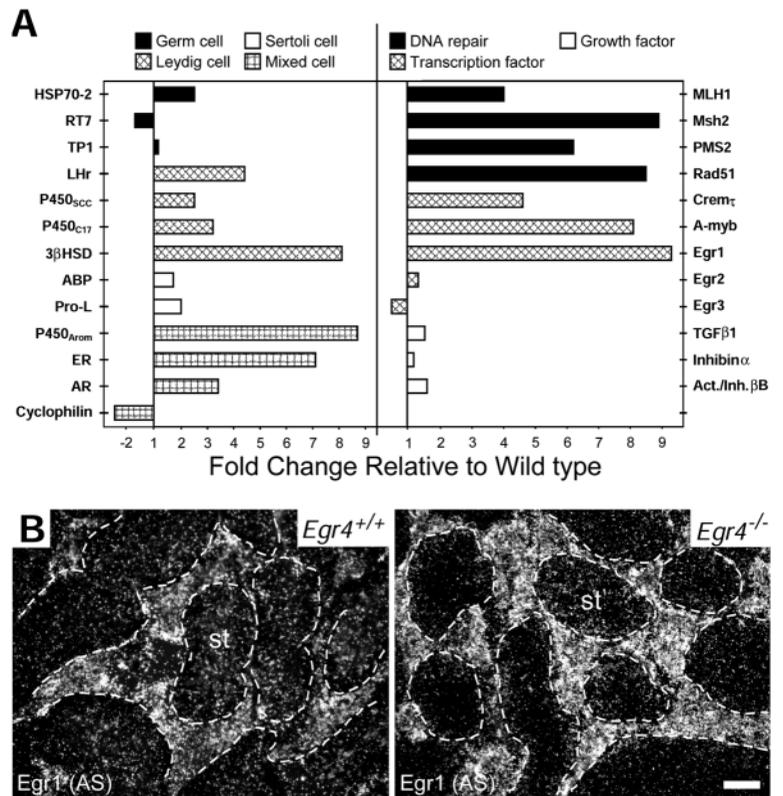


Fig. 8. Aberrant gene expression in *Egr4* null testis. (A) Gene expression analysis using semiquantitative RT-PCR (see Materials and Methods) demonstrated marked alterations in the level of expression of many genes in *Egr4* null testis (see text for discussion). (B) An antisense (AS) probe for *Egr1*, a closely related transcription factor to *Egr4*, identified high levels of expression specifically in Leydig cells. Note that *Egr1* expression is markedly increased in *Egr4* null testis relative to wild type. The seminiferous tubules (st) are outlined to identify the interstitial zones between the tubules where Leydig cells reside. Scale, 100 μ m.

factor *p53*, which is involved in apoptosis after DNA damage (Lowe et al., 1993) or during tumor growth and progression in a variety of cell types (Symonds et al., 1994), also plays a role during spermatogenesis. It is essential for apoptosis in spermatogonia, which occurs after irradiation-mediated DNA damage (Beumer et al., 1998) and possibly for monitoring the genomic integrity of germ cells due to spontaneous mutations occurring during normal spermatogenesis (Yin et al., 1998). However, *p53* is not essential for apoptosis that occurs at a recently described synaptic checkpoint after improper chromosome synapsis in meiosis in X $S_{Xr(a)O}$ mice (Odorisio et al., 1998). Similarly, we observed that massive germ cell apoptosis occurred in *Egr4*-deficient pachytene spermatocytes even in the *p53* null genetic background (Fig. 4). These results raise the possibility that the defect in *Egr4*-deficient spermatocytes may relate to chromosomal synapsis. This hypothesis is supported by the fact that pachytene spermatocytes normally express *Egr4*, the apoptosis appears confined to pachytene (a stage that involves chromosomal synapsis and homologous recombination), and the apoptosis is independent of *p53* transcriptional activity. Recent studies involving targeted mutagenesis of DNA mismatch repair enzymes further support this contention. For example, mice

deficient in the DNA mismatch repair enzyme *PMS2* have several characteristics similar to those observed in *Egr4*-deficient mice. For instance, they have male-only infertility characterized by massive pachytene spermatocyte apoptosis, incomplete spermatogenic arrest at pachytene with oligozoospermia and teratozoospermia, and abnormal chromosomal synapsis in meiosis (Baker et al., 1995). However, *PMS2*-deficient mice also have an increased frequency of tumorigenesis (presumably due to its DNA mismatch repair function in non-germ cells) (Prolla et al., 1998), which we have not observed in the *Egr4*-deficient mice to date. *PMS2*-deficient mice also have numerous defects in chromosomal structure during synapsis in meiosis, including numerous discontinuities of bivalent axes in mid-late pachytene spermatocytes. While we did not observe synaptonemal complex abnormalities in *Egr4*-deficient spermatocytes using light microscopy (data not shown), detection of subtle abnormalities such as occur in *PMS2*-deficient and X^{Sxr(a)}O mice may require electron microscopy (Baker et al., 1995; Odorisio et al., 1998). We examined the levels of expression of several DNA mismatch repair enzymes (*PMS2*, *Msh2* and *MLH1*) and a double-stranded DNA break repair enzyme (*Rad51*), and found all of them to be markedly upregulated in *Egr4* null testis. Additional experiments will be necessary to clarify whether this reflects compensatory changes in DNA repair machinery as a consequence of the misregulation of *Egr4*-dependent genes or because of increased levels of DNA damage within germ cells.

In summary, these results demonstrate that *Egr4* is critical for egress through the pachytene stage of spermatogenesis. The fact that *Egr4*-deficient mice are neurologically normal strongly suggests a redundant function in the nervous system. Presumably, other *Egr* transcription factors, many of which have similar expression patterns to *Egr4*, functionally compensate for its absence within the nervous system. Although additional work is required to elucidate specific target genes regulated by *Egr4* during spermatogenesis, it represents a potential candidate gene for some forms of genetically based male infertility. Indeed, male-only infertility disorders with autosomal recessive inheritance patterns and early meiotic arrest have been described (Cantu et al., 1981).

The authors thank L. Cabalka Tourtellotte, G. Gavrilina, T. Gorodinsky and C. Bollinger for excellent technical assistance. We thank E. Goldberg and G. Enders for providing the LDHC4 and GCNA1 antibodies, respectively. This work was supported by NIH grants 5 K08 MH1426-02 (W. G. T.) and 5 P01 CA49712-08 and a grant from The Association for the Cure of Cancer of the Prostate (CapCURE) (J. M.).

REFERENCES

- Ashley, T., Plug, A. W., Xu, J., Solari, A. J., Reddy, G., Golub, E. I. and Ward, D. C. (1995). Dynamic changes in Rad51 distribution on chromatin during meiosis in male and female vertebrates. *Chromosoma* **104**, 19-28.
- Attramadala, A., Bardin, C. W., Gunsalus, G. L., Musto, N. A. and Hansson, V. (1981). Immunocytochemical localization of androgen binding protein in rat Sertoli and epididymal cells. *Biol. Reprod.* **25**, 983-988.
- Baker, S. M., Bronner, C. E., Zhang, L., Plug, A. W., Robatzek, M., Warren, G., Elliott, E. A., Yu, J., Ashley, T., Arnheim, N. et al. (1995). Male mice defective in the DNA mismatch repair gene *PMS2* exhibit abnormal chromosome synapsis in meiosis. *Cell* **82**, 309-319.
- Baker, S. M., Plug, A. W., Prolla, T. A., Bronner, C. E., Harris, A. C., Yao, X., Christie, D. M., Monell, C., Arnheim, N., Bradley, A. et al. (1996). Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. *Nat. Genet.* **13**, 336-342.
- Beumer, T. L., Roepers-Gajadien, H. L., Gademan, I. S., van Buul, P. P., Gil-Gomez, G., Rutgers, D. H. and de Rooij, D. G. (1998). The role of the tumor suppressor p53 in spermatogenesis. *Cell. Death. Differ.* **5**, 669-777.
- Blendy, J. A., Kaestner, K. H., Weinbauer, G. F., Nieschlag, E. and Schutz, G. (1996). Severe impairment of spermatogenesis in mice lacking the CREM gene. *Nature* **380**, 162-165.
- Browning, J. Y., D'Agata, R. and Grotjan, H. E., Jr. (1981). Isolation of purified rat Leydig cells using continuous Percoll gradients. *Endocrinology* **109**, 667-669.
- Cantu, J. M., Rivas, F., Hernandez-Jauregui, P., Diaz, M., Cortes-Gallegos, V., Vaca, G., Velazquez, A. and Ibarra, B. (1981). Meiotic arrest at the first spermatocyte level: a new inherited infertility disorder. *Hum. Genet.* **59**, 380-385.
- Cooke, H. J., Hargreave, T. and Elliott, D. J. (1998). Understanding the genes involved in spermatogenesis: a progress report. *Fertil. Steril.* **69**, 989-995.
- Crosby, S. D., Puetz, J. J., Simburger, K. S., Fahrner, T. J. and Milbrandt, J. (1991). The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. *Mol. Cell. Biol.* **11**, 3835-3841.
- Crosby, S. D., Veile, R. A., Donis-Keller, H., Baraban, J. M., Bhat, R. V., Simburger, K. S. and Milbrandt, J. (1992). Neural-specific expression, genomic structure, and chromosomal localization of the gene encoding the zinc-finger transcription factor NGFI-C. *Proc. Natl. Acad. Sci. U S A* **89**, 4739-4743.
- de Wind, N., Dekker, M., Berns, A., Radman, M. and te Riele, H. (1995). Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* **82**, 321-330.
- Eddy, E. M., Washburn, T. F., Bunch, D. O., Goulding, E. H., Gladen, B. C., Lubahn, D. B. and Korach, K. S. (1996). Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinol.* **137**, 4796-4805.
- Edelmann, W., Cohen, P. E., Kane, M., Lau, K., Morrow, B., Bennett, S., Umar, A., Kunkel, T., Cattoretti, G., Chaganti, R. et al. (1996). Meiotic pachytene arrest in MLH1-deficient mice. *Cell* **85**, 1125-1134.
- Enders, G. C. and May, J. J., 2nd. (1994). Developmentally regulated expression of a mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. *Dev. Biol.* **163**, 331-340.
- Erickson-Lawrence, M., Zabludoff, S. D. and Wright, W. W. (1991). Cyclic protein-2, a secretory product of rat Sertoli cells, is the proenzyme for of cathepsin L. *Mol. Endo.* **5**, 1789-1798.
- Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119**, 493-501.
- Gunduz, G., Luleci, G. and Baykara, M. (1998). Cytogenetic study in 102 infertile men. *Urol. Int.* **61**, 32-34.
- Haaf, T., Golub, E. I., Reddy, G., Radding, C. M. and Ward, D. C. (1995). Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. *Proc. Natl. Acad. Sci. U S A* **92**, 2298-2302.
- Higgy, N. A., Pastoor, T., Renz, C., Tarnasky, H. A. and Van der Hoorn, F. A. (1994). Testis-specific RT7 protein localizes to the sperm tail and associates with itself. *Biol. Reprod.* **50**, 1357-1366.
- Hintz, M. and Goldberg, E. (1977). Immunohistochemical localization of LDH-x during spermatogenesis in mouse testes. *Dev. Biol.* **57**, 375-384.
- Holst, C., Skerka, C., Lichter, P., Bialonski, A. and Zipfel, P. F. (1993). Genomic organization, chromosomal localization and promoter function of the human zinc-finger gene pAT133. *Hum. Mol. Genet.* **2**, 367-372.
- Irvine, D. S. (1997). Declining sperm quality: a review of facts and hypotheses. *Baillieres. Clin. Obstet. Gynaecol.* **11**, 655-671.
- Janca, F. C., Jost, L. K. and Evenson, D. P. (1986). Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. *Biol. Reprod.* **34**, 613-623.
- Krausz, C. and McElreavey, K. (1999). Y chromosome and male infertility. *Front. Biosci.* **4**, E1-8.
- Krishan, A. (1990). Rapid DNA content analysis by the propidium iodide-hypotonic citrate method. *Methods. Cell. Biol.* **33**, 121-125.
- Lee, S. L., Sadovsky, Y., Swirnow, A. H., Polish, J. A., Goda, P., Gavrilina, G. and Milbrandt, J. (1996). Luteinizing hormone deficiency and female

- infertility in mice lacking the transcription factor NGFI-A (*Egr-1*). *Science* **273**, 1219-1221.
- Lian, G., Miller, K. A. and Enders, G. C.** (1992). Localization and synthesis of entactin in seminiferous tubules of the mouse. *Biol. Reprod.* **47**, 316-325.
- Lin, T., Chen, G. C., Muroso, E. P., Osterman, J. and Nankin, H. R.** (1982). Estradiol receptors of two distinct populations of Leydig cells. *Steroids* **40**, 53-63.
- Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. and Jacks, T.** (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-849.
- McLachlan, R. I., Mallidis, C., Ma, K., Bhasin, S. and de Kretser, D. M.** (1998). Genetic disorders and spermatogenesis. *Reprod. Fertil. Dev.* **10**, 97-104.
- McMahon, A. P. and Bradley, A.** (1990). The Wnt-1 (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.
- Nantel, F., Monaco, L., Foulkes, N. S., Masquillier, D., LeMeur, M., Henriksen, K., Dierich, A., Parvinen, M. and Sassone-Corsi, P.** (1996). Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature* **380**, 159-162.
- Oakberg, E. F.** (1956). A description of spermatogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99**, 391-413.
- Oodorio, T., Rodriguez, T. A., Evans, E. P., Clarke, A. R. and Burgoyne, P. S.** (1998). The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis. *Nat. Genet.* **18**, 257-261.
- Payne, A. H., Downing, J. R. and Wong, K. L.** (1980). Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. *Endocrinol.* **106**, 1424-1429.
- Prolla, T. A., Baker, S. M., Harris, A. C., Tsao, J.-L., Yao, X., Bronner, C. E., Zheng, B., Gordon, M., Reneker, J., Arnheim, N. et al.** (1998). Tumour susceptibility and spontaneous mutation in mice deficient in Mlh1, Pms1 and Pms2 DNA mismatch repair. *Nat. Genet.* **18**, 276-279.
- Reddy, J. K. and Ohno, S.** (1981). Testicular feminization of the mouse: paucity of peroxisomes in Leydig cell of the testis. *Am. J. Pathol.* **103**, 96-104.
- Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P. and Clegg, E. D.** (1990). Staging for laboratory species. In *Histological and Histopathological Evaluation of the Testis*, (ed. L. D. Russell, R. A. Ettlin, A. P. Sinha Hikim and E. D. Clegg), pp. 62-194. Clearwater: Cache River Press.
- Sah, V. P., Attardi, L. D., Mulligan, G. J., Williams, B. O., Bronson, R. T. and Jacks, T.** (1995). A subset of p53-deficient embryos exhibit exencephaly. *Nat. Genet.* **10**, 175-180.
- Sassone-Corsi, P.** (1997). Transcriptional checkpoints determining the fate of male germ cells. *Cell* **88**, 163-166.
- Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P.** (1993). Disruption of *Krox-20* results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-1214.
- Spano, M. and Evenson, D. P.** (1993). Flow cytometric analysis for reproductive biology. *Biol. Cell* **78**, 53-62.
- Swiatek, P. J. and Gridley, T.** (1993). Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene *Krox20*. *Genes. Dev.* **7**, 2071-2084.
- Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T. and Van Dyke, T.** (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell* **78**, 703-711.
- Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A. B., Seitanidou, T., Babinet, C. and Charnay, P.** (1994). *Krox-20* controls myelination in the peripheral nervous system. *Nature* **371**, 796-799.
- Toscani, A., Mettus, R. V., Coupland, R., Simpkins, H., Litvin, J., Orth, J., Hatton, K. S. and Reddy, E. P.** (1997). Arrest of spermatogenesis and defective breast development in mice lacking A-myb. *Nature* **386**, 713-717.
- Tourtellotte, W. G. and Milbrandt, J.** (1998). Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor *Egr3*. *Nat. Genet.* **20**, 87-91.
- Tung, P. S. and Fritz, I. B.** (1990). Characterization of rat testicular peritubular myoid cells in culture: alpha-smooth muscle isoactin is a specific differentiation marker. *Biol. Reprod.* **42**, 351-365.
- Vernon, R. B., Lane, T. F., Angello, J. C. and Sage, H.** (1991). Adhesion, shape, proliferation, and gene expression of mouse Leydig cells are influenced by extracellular matrix in vitro. *Biol. Reprod.* **44**, 157-170.
- Vornberger, W., Prins, G., Musto, N. A. and Suarez-Quian, C. A.** (1994). Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinol.* **134**, 2307-2316.
- Warner, L. E., Mancias, P., Butler, I. J., McDonald, C. M., Keppen, L., Koob, K. G. and Lupski, J. R.** (1998). Mutations in the early growth response 2 (*EGR2*) gene are associated with hereditary myelinopathies. *Nat. Genet.* **18**, 382-384.
- Yelick, P. C., Kwon, Y. H., Flynn, J. F., Borzorgzadeh, A., Kleene, K. C. and Hecht, N. B.** (1989). Mouse transition protein 1 is translationally regulated during the postmeiotic stages of spermatogenesis. *Mol. Reprod. Dev.* **1**, 193-200.
- Yin, Y., Stahl, B. C., DeWolf, W. C. and Morgentaler, A.** (1998). p53-mediated germ cell quality control in spermatogenesis. *Dev. Biol.* **204**, 165-171.
- Zakeri, Z. F. and Wolgemuth, D. J.** (1987). Developmental-stage-specific expression of the *hsp70* gene family during differentiation of the mammalian male germ line. *Mol. Cell. Biol.* **7**, 1971-1976.