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Age-Related Instability in Spermatogenic Cell Nuclear and Mitochondrial DNA Obtained from *Apex1* Heterozygous Mice

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Abstract

The prevalence of spontaneous mutations increases with age in the male germline; consequently, older men have an increased risk of siring children with genetic disease due to *de novo* mutations. The *lacI* transgenic mouse can be used to study paternal age effects, and in this system, the prevalence of *de novo* mutations increases in the male germline at old ages. Mutagenesis is linked with DNA repair capacity, and base excision repair, which can ameliorate spontaneous DNA damage, decreases in nuclear extracts of spermatogenic cells from old mice. Mice heterozygous for a null allele of the *Apex1* gene, which encodes apurinic/apyrimidinic endonuclease I (APEN), an essential base excision repair enzyme, display an accelerated increase in spontaneous germline mutagenesis early in life. Here, the consequences of lifelong reduction of APEN on genetic instability in the male germline were examined, for the first time, at middle and old ages. Mutation frequency increased earlier in spermatogenic cells from *Apex1*$^{+/-}$ mice (by 6 months of age). Nuclear DNA damage increased with age in the spermatogenic lineage for both wild-type and *Apex1*$^{+/-}$ mice. By old age, mutation frequencies were similar for wild-type and APEN-deficient mice. Mitochondrial genome repair also depends on APEN, and novel analysis of mitochondrial DNA damage revealed an increase in the *Apex*1$^{+/-}$ spermatogenic cells by middle age. Thus, *Apex1* heterozygosity results in accelerated damage to mitochondrial DNA and spontaneous mutagenesis, consistent with an essential role for APEN in maintaining nuclear and mitochondrial DNA integrity in spermatogenic cells throughout life.

Keywords

paternal age; mutagenesis; base excision repair; AP endonuclease; *lacI*; transgenic mice

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INTRODUCTION

In male mammals, germ cells undergo DNA replication throughout reproductive life, which extends from puberty to old age. This process occurs in proliferating spermatogonia and as cells prepare for meiosis. The effects of radiation and chemical mutagens on genomic integrity throughout spermatogenesis have been examined in mice using several methods (Singer et al., 2006), including the heritable translocation test (Adler, 2000), the morphological specific locus test (Russell et al., 1998; Russell, 2004), and the *lacI* mutation frequency assay (Xu et al., 2008). *LacI* transgenic animals have also been used to characterize increased spontaneous mutagenesis in the spermatogenic cells of old mice, as a model system for the mechanisms underlying the paternal age effect in humans (Walter et al., 1998). This correlation between an increased incidence of *de novo* genetic disorders and greater paternal age arises from the development and accumulation of spontaneous mutations, as the total number of spermatogonial cell divisions increases throughout male reproductive life (Crow, 2000; Glaser and Jabs, 2004).

Potentially mutagenic DNA damage, whether spontaneous or induced, is normally ameliorated by DNA repair pathways. For example, the highly conserved base excision repair (BER) pathway corrects DNA damage caused by alkylation, deamination, and oxidative stress (Robertson et al., 2009). Four proteins – apurinic/apyrimidinic endonuclease 1 (APEN/REF-1), DNA polymerase beta (POLB), X-ray cross-complementing 1 (XRCC1), DNA ligase IIIα (LIG3) - comprise the core of the BER short-patch subpathway, which functions to remove and then replace a damaged DNA base (Xu et al., 2008; Robertson et al., 2009). Repair is initiated by specific DNA glycosylases that preferentially recognize and excise aberrant purines or pyrimidines, leaving an apurinic/apyrimidinic (abasic) site (Hitomi et al., 2007; Hegde et al., 2008); subsequent strand incision by APEN prepares the site for the completion of short-patch BER. Unrepaired abasic sites, whether generated by hydrolysis, oxidative stress, or damaged base removal, can lead to DNA strand breaks, mutagenesis, and apoptosis (Fishel and Kelley, 2007).

BER capacity varies among different tissues and changes throughout life (Intano et al., 2001; Intano et al., 2002; Larsen et al., 2007). In young male mice, expression levels for BER pathway proteins, as well as the levels of uracil-DNA glycosylase-initiated (UDG) BER activity, are higher in extracts prepared from mixed germ cells (MGC), as compared to extracts from somatic tissues, such as brain, liver, and small intestine (Hirose et al., 1989; Alcivar et al., 1992; Walter et al., 1994; Intano et al., 2001). Age-related declines in UDG-BER activity and in the levels and activities of individual BER pathway proteins have been reported for both somatic and spermatogenic cell populations (Xu et al., 2008). Consistent with the crucial role of this pathway in maintaining genome integrity, these spatial and temporal differences in BER activity are correlated with changes in spontaneous mutant frequencies. For example, spontaneous mutant frequencies are higher in spermatogenic cells isolated from old mice (28 months), than in those isolated from young (60 days) and middle-aged (15 months) animals (Walter et al., 1998). The non-linear increase in mutation frequency observed in the male germline, apparent in pachytene spermatocytes, round spermatids, and spermatozoa, contrasts with the gradual, linear, age-related increase in mutations reported for somatic cells, such as splenocytes and lymphocytes (Wei et al., 1993; Lee et al., 1994; Walter et al., 1998).

Most studies of spatial and temporal changes in BER activity have focused on repair of the nuclear genome. Some BER proteins, however, are translocated to the mitochondrial matrix and are involved in repairing mitochondrial DNA. Because mitochondrial DNA (mtDNA) is located physically near the site of reactive oxygen species production and is not associated with histones, it is subject to substantial oxidative damage (Hamilton et al., 2001). In

particular, abasic sites are generated frequently, and the demonstrable repair of these lesions, rather than degradation of damaged mtDNA, indicates that abasic endonuclease activity is present in mitochondria (Tomkinson et al., 1988; Tell et al., 2001). mtDNA repair has not been examined in mammalian spermatogenic cells deficient in APEN, thus it is not known if this process is impaired.

An alternative approach to examine the relationships between BER activity and spontaneous mutagenesis is to manipulate levels of individual BER pathway components through use of targeted alleles and transgenes in mice. Recently, we showed that BER activity is reduced in MGC extracts obtained from *Polb*$^{+/-}$ mice, whereas spontaneous mutation frequency is elevated in the spermatogenic cells of these animals as compared to wild-type littermates (Allen et al., 2008). Similarly, *Apex1*-deficient mice exhibit elevated spontaneous mutant frequencies at 9 months of age in MGCs, the liver, and the spleen (Huamani et al., 2004). In the present study, we use the *lacI* transgene to characterize mutagenesis in the germline of *Apex1*$^{+/-}$ mice, with a focus on changes from young adulthood to old age. Moreover, we characterize apoptosis in different spermatogenic cell types and directly examine both nuclear and mitochondrial DNA damage levels in the context of APEN-deficiency throughout life.

RESULTS

Reduction of APEN protein levels and activity in the germline of Apex1$^{+/-}$ mice

Although *Apex1*$^{-/-}$ embryos die *in utero*, *Apex1*$^{+/-}$ mice survive to adulthood and are fertile, albeit with characteristics associated with increased oxidative stress (Xanthoudakis et al., 1996; Ludwig et al., 1998; Meira et al., 2001). To confirm that heterozygosity for the targeted null mutation in the *Apex1* gene corresponded to reduced APEN protein levels in spermatogenic cells, we performed immunoblotting on mixed germ cell (MGC) nuclear extracts from *Apex1*$^{+/-}$ and wild-type control (*Apex1*$^{+/+}$) mice between 5 and 7 months of age (young adults). In *Apex1*$^{+/-}$ mice, APEN protein levels were significantly lower than in wild-type controls for nuclear extracts prepared from MGCs (Figure 1A). In addition to APEN, the levels of other BER proteins were examined in these cells; Figure 1A shows the values for POLB, XRCC1, and LIG3 in MGCs isolated from young adult *Apex1*$^{+/-}$ mice, as normalized to values obtained for age-matched controls. To confirm that this reduction in APEN protein levels in the germline persisted into old age, we compared levels in MGC nuclear extracts isolated from *Apex1*$^{+/-}$ and $^{+/+}$ mice at 28 months of age (Figure 1B). For the spermatogenic cell population, levels of APEN protein are reduced in *Apex1*$^{+/-}$ mice at both young and old adult stages.

The 5′-endonuclease activity of APEN can be measured in vitro, using a duplex oligonucleotide substrate that includes a synthetic abasic site (Wilson et al., 1995). We compared APEN-mediated incision of a G:D oligomer by MGC extracts prepared from *Apex1*$^{+/-}$ and $^{+/+}$ mice at 28 months (old adults). Figure 2 shows that, at two different concentrations, the percentage incision of the G:D 18-mer was reduced for MGC protein extracts prepared from *Apex1*$^{+/-}$ mice, as compared to wild-type controls. Thus, *Apex1* heterozygosity results in reduced APEN protein levels and in endonuclease activity in spermatogenic cells, in both young and old mice.

Age-related increases in nuclear and mitochondrial DNA damage in the germline of Apex1$^{+/-}$ and wild-type mice

The normal development and functions of male germ cells are dependent on the integrity of both the nuclear and mitochondrial genomes (Agarwal et al., 2008; Aitken et al., 2009). Activity of the BER pathway, which has a major role in maintaining germline genomic

stability, is diminished in MGC populations isolated from old mice (Intano et al., 2002). To characterize the relationship between *Apex1* gene dosage and DNA damage, we determined the steady-state levels of nuclear DNA damage using quantitative PCR (QPCR). As the DNA polymerase amplifies only undamaged templates in this assay, amplification is inversely proportional to the level of DNA damage; this technique allows detection of abasic sites, strand breaks, and other consequences of oxidative DNA damage (Yakes and Van Houten, 1997; Ayala-Torres et al., 2000). We analyzed nuclear DNA obtained from MGCs isolated from 6-, 16-, and 28-month old wildtype and *Apex1*$^{+/-}$ mice, by amplifying a 6.9-kb fragment of the murine hypoxanthine phosphoribosyltransferase (*Hprt*) gene. Figure 3A shows an age-dependent reduction in amplification of this nuclear DNA fragment in MGC from both wild-type and *Apex1*-deficient mice. Specifically, amplification was reduced by 30% and 34% in the 16- and 28-month old wild-type mice, respectively; in *Apex1*$^{+/-}$ mice, amplification of the nuclear DNA fragment was reduced by 35% at 16 months and 48% at 28 months, as compared to MGCs isolated at 6 months of age (Figure 3A, B). At 16 months, the frequency of DNA lesions per 10 kb in the nuclear DNA fragment was 0.50 for wild-type and 0.63 for *Apex1*$^{+/-}$. The lesion frequencies at 28 months of age (0.59 lesions/10kb/strand for wild-type, and 0.93 lesions/10 kb/strand for *Apex1*$^{+/-}$) were not significantly different from the numbers obtained for the MGC of middle-aged mice (Table 1).

The mitochondrial genome is particularly susceptible to oxidative damage and dependent on the BER pathway for its repair (Maynard et al., 2009). APEN protein is translocated to the mitochondria, consistent with a role in repairing mitochondrial DNA (mtDNA) damage (Chattopadhyay et al., 2006). To assess this potential function specifically in the male germline, we used the QPCR technique to compare mtDNA damage in MGCs isolated from wild-type and *Apex1*$^{+/-}$ mice. Although no mtDNA damage was detected in the MGC from 6-month-old wild-type mice, we observed an 11% decrease in the amplification of the mtDNA fragment in the young *Apex*$^{1+/-}$ mice (Figure 4A, B). Figure 4B shows a 30% decrease in the amplification of the mtDNA fragment in 16-month old *Apex1*$^{+/-}$ mice as compared to age-matched wild-type mice. This damage corresponds to lesion frequencies of 0.36 and 0.16 lesions/10 kb/strand in *Apex1*$^{+/-}$ and wild-type MGC mtDNA, respectively (Table 1). In *Apex1*$^{+/-}$ mice, the level of mtDNA damage remained unchanged between 16 and 28 months of age; in contrast, 28-month old wild-type mice exhibited a further reduction in mtDNA fragment amplification, as compared to 16-month old animals. At 28 months of age, the mtDNA lesion frequency was 0.31 in MGCs isolated from *Apex1*$^{+/-}$ mice, and 0.49 in those from wild-type mice; this difference is not statistically significant (Table 1). To exclude the possibility that the decreased amplification of a 10-kb mtDNA fragment we observed resulted from the loss of mtDNA copy number, we amplified a small mtDNA fragment (92 bp), the amplification of which is independent of the presence of lesions and provides an accurate determination of the steady-state levels of mtDNA molecules (Ayala-Torres et al., 2000). No significant differences in the amounts of mtDNA steady-state levels were observed for wild-type and *Apex1*$^{+/-}$ mice at any age (Figure 4A, lower panel), indicating that the age-dependent decrease in amplification of the 10-kb mtDNA fragment results from damage to the mitochondrial genome. Our data are consistent with an accelerated accumulation of mtDNA damage in MGC from APEN-deficient mice.

Accelerated age-related increases in germline spontaneous mutation frequency in Apex1$^{+/-}$ mice

Our previous studies, utilizing *lacI* mutagenesis assays in *Apex1*-deficient mice, indicated that although mutation frequency values for mixed spermatogenic cells were not significantly different from wild-type at 3 months, by 9 months of age spontaneous mutation frequency values in the germ cells of *Apex1*$^{+/-}$ mice were increased compared to controls (Huamani et al., 2004). To identify ages at which APEN deficiency affected mutagenesis in

the male germline, we measured spontaneous mutantion frequency in MGCs isolated from *Apex1*$^{+/-}$ and wild-type mice at 6, 16, and 26 months. For each age and genotype, genomic DNA from 5 different MGC samples was used for *lacI* mutagenesis assays, and a minimum of 1.4×10^6 plaque-forming units were scored for mutant (blue) plaques (Table 2). Figure 5 shows that at 6 months (young), there was an increased spontaneous mutation frequency for spermatogenic cells isolated from APEN-deficient mice ($1.309 \times 10^{-5} \pm 0.267$) as compared to wild-type controls ($0.480 \times 10^{-5} \pm 0.181$). By middle age (16 months), there was no significant difference in the mutation frequency for spermatogenic cells isolated from *Apex1*$^{+/-}$ ($1.327 \times 10^{-5} \pm 0.265$) and wild-type ($0.744 \times 10^{-5} \pm 0.206$) mice. Moreover, average mutation frequency values for both genotypes at 16 months were not significantly different from those obtained for young adult mice (Figure 5, Table 2). At 26 months, spontaneous mutation frequency was increased approximately two-fold in mixed spermatogenic cells isolated from both *Apex1*$^{+/-}$ ($2.565 \times 10^{-5} \pm 0.296$) and wild-type ($2.488 \times 10^{-5} \pm 0.330$) mice, as compared to mutation frequency values obtained from young and middle-aged mice (Figure 5, Table 2). Taken together, our previous data (Huamani et al., 2004) and the present results indicate that sustained APEN deficiency leads to an accelerated increase in spontaneous mutation frequency in the male germline, which is apparent as early as 6 months of age and persists through at least 9 months of age. By middle age (16 months), however, differences in mutation frequency for MGC from *Apex1*$^{+/-}$ and wild-type were not significant, and in old mice (26 months), spermatogenic cells from both genotypes exhibited the elevated MF.

In addition to frequency, we determined the mutation spectrum for MGCs isolated from young and old mice of *Apex1*$^{+/-}$ and $^{+/+}$ genotypes. Table 3 shows that there were no significant differences in the percentages of transitions, transversions, or deletions between *Apex1*$^{+/-}$ and wild-type mice at young or old ages. However, we observed a significant decrease in the percentage of transversions in the MGC of old mice for both genotypes (40% and 11.5% for *Apex1*$^{+/-}$ mice; 40% and 23.3% for wild-type mice; Table 3). The distribution of mutations along the *lacI* gene was significantly different ($P = 0.001$) for *Apex1*$^{+/-}$ MGCs at both ages and for old wild-type MGCs. We used the distributions of mutation position to empirically identify three hotspots, which account for 39.5% of the 81 mutants examined (Table 4). In addition, there was a significant association ($P = 0.0016$) of mutation type with hotspot. Transitions predominated at positions 1206 (11/11, or 100%), 92, and 93 (10/11, or 90.9%); whereas transversions were more common at positions 39 and 42 (6/10, or 60%).

To determine if increased mutant frequencies in the MGCs of *Apex1*$^{+/-}$ mice were accompanied by changes in apoptotic rates in these spermatogenic populations, we characterized the proportions of TUNEL-positive cells in the testis of APEN-deficient and wild-type mice at three different ages (6, 16, and 26 months). First, the percentages of seminiferous tubules that contained at least one TUNEL-positive (apoptotic) cell were determined; Figure 6A shows that no significant differences were observed for *Apex1*$^{+/-}$ and wild-type mice at any age examined. Next, to further refine our analyses of apoptotic rates in subpopulations of spermatogenic cell types, we determined the numbers of TUNEL-positive spermatogonia and pachytene spermatocytes in cross-sections of seminiferous tubules of APEN-deficient and wild-type mice at 6, 16, and 26 months (Figure 6B). Again, *Apex1* gene dosage had no significant effect on the numbers of apoptotic spermatogenic cells, although a decrease in the number of TUNEL-positive spermatogonia in *Apex1*$^{+/-}$ mice was observed between 16 and 26 months (Figure 6B).

DISCUSSION

Mature, functional gametes are produced throughout the adult lifespan in male mammals, and several mechanisms have evolved to protect the genomic integrity of the germline, which potentially undergoes hundreds of chromosomal replications during that period (Crow, 2000; Crow, 2006). Nevertheless, with increasing age, there is a corresponding increase in levels of spontaneous mutagenesis in the male germline, as manifested by the paternal age effect in humans. At least 20 sporadic, autosomal dominant disorders have been identified as exhibiting an increased incidence in the offspring of older fathers (Glaser and Jabs, 2004). For example, Apert, Crouzon, Pfeiffer, and progeria syndromes, as well as multiple endocrine neoplasia type 2A and medullary thyroid carcinoma, show strong paternal-age effects (Risch et al., 1987; Glaser and Jabs, 2004). In several of these disorders, the mutation has been identified as a paternally-derived base substitution, such as the G-to-A transition in the fibroblast growth factor receptor 3 (*FGFR3*) gene for achondroplasia (Rousseau et al., 1994; Shiang et al., 1994), and the C-to-G transversions in the *FGFR2* gene for Apert syndrome (Moloney et al., 1996).

Replication error can account for a linear increase in the number of paternal germ cell mutations with age ("copy-error hypothesis"), but the increased incidence of sporadic genetic disorders in children of aged fathers reflects an exponential trend (Crow, 2000; Crow, 2006). Through analysis of sperm DNA from men of different ages, it was shown that the increase in *FGFR3* G1138A mutation frequency is not sufficient to account for the increased incidence of sporadic achondroplasia with advanced paternal age (Tiemann-Boege et al., 2002). Similarly, mutations in *FGFR2* may alter ligand binding and specificity for the receptor protein, thus favoring clonal expansion of spermatogonia harboring the gain-of-function mutation (Goriely et al., 2005); one hypothesis is that the Apert syndrome mutation (C755G) may alter the division pattern of adult, self-renewing Ap spermatogonial cells (Qin et al., 2007). Diminished capacities for recognizing and repairing DNA damage, as well as decreased apoptosis in spermatogonial populations (Barnes et al., 1999; Kimura et al., 2003), are also likely to contribute to the paternal age effect. In humans, the values for an index of chromatin defects in sperm double between 20 and 60 years of age, and increase five-fold between ages 20 and 80 (Wyrobek et al., 2006). Targeted mutations in mice can be used to directly examine the mutagenic consequences of reduced DNA repair capacity during the aging process, and in the present study, we assessed spontaneous mutagenesis and apoptosis in the spermatogenic cells of mice deficient for the *Apex1* gene, which encodes APEN, a key component of the BER pathway.

Deletion of *Apex1,* through conditional mutagenesis in mouse embryonic fibroblast lines, results in apoptosis and an increased number of abasic sites (Mitra et al., 2007). *Apex1*$^{-/-}$ blastocysts are more sensitive to ionizing radiation, likely due to the APEN incision deficiency (Ludwig et al., 1998). Reduction in APEN levels, through antisense manipulations in human HeLa cells, increases sensitivity to a variety of DNA damaging agents and diminishes the ability of the cells to adapt to changes in oxygen tension (Walker et al., 1994). Consistent with the results of these experimental manipulations of *Apex1* gene expression, we have demonstrated that both APEN protein and abasic endonuclease activity are decreased in MGCs isolated from *Apex1*$^{+/-}$ mice. Because these levels remain diminished into old age for *Apex1*$^{+/-}$ mice, we could examine the consequences of APEN deficiency for maintenance of genomic integrity throughout reproductive life.

Unrepaired abasic sites can interfere with DNA replication and can potentially increase mutagenesis. Both APEN and POLB act in a coordinated manner in the BER pathway to remove altered bases, and thus preserve genomic integrity (Bennett et al., 1997). Our previous results, using mice that harbor targeted mutations in either the *Polb* or *Apex1* gene,

indicate that deficiencies in the corresponding BER pathway proteins results in increased spontaneous mutagenesis in the male germline. Although mutant frequencies were also elevated for somatic tissues in *Apex1*$^{+/-}$ mice (Huamani et al., 2004), there was no such increase in the somatic tissues (brain, liver) in *Polb*$^{+/-}$ mice (Allen et al., 2008). In the present study, we assessed mutation frequency throughout reproductive life, and demonstrated that the differences in spontaneous mutagenesis in the spermatogenic cells of APEN-deficient mice can be detected as early as 6 months of age; however, by middle age (16 months), the difference between mutation frequency in wild-type and *Apex1*$^{+/-}$ MGCs is not significant. By 26 months (old age), mutation frequency has increased to similar levels for MGCs isolated from both wild-type and *Apex1*$^{+/-}$ mice, and spontaneous mutagenesis is significantly higher than at 6 months and 16 months in both genotypes. Although both APEN protein levels and incision activity are reduced in older *Apex1*$^{+/-}$ mice, these differences may be overshadowed by other age-related declines in DNA damage detection and repair, which could contribute to increased mutagenesis in the germline of both wild-type and *Apex1*$^{+/-}$ mice. For example, our *lacI* mutation spectrum data (Table 3) indicate that while the numbers of transversions in MGCs isolated from old mice of both genotypes decreases, the numbers of deletions (both 1 bp and >1 bp) increases, when compared to the mutation spectra of MGCs isolated from young mice. In a previous mutation frequency analysis of spermatogonial cells in wild-type mice, the highest proportions of mutations at hotspots were observed at young and middle ages (Walter et al., 2004); we did not compare middle-aged animals in the present study, but observed mutation hotspots in the *lacI* gene for both young and old MGCs (Table 4).

In addition to the *lacI* mutagenesis studies, we used QPCR amplification to assess steady state nuclear DNA damage levels in MGCs throughout reproductive life, and observed an age-dependent increase in DNA damage for mice of both genotypes. With this method, which detects consequences of oxidative DNA damage in the *Hprt* gene, reductions in the percentages of undamaged templates in MGC nuclear DNA occurred in middle age (16 months) for both wildtype and *Apex1*$^{+/-}$ mice. Taken together, the present results and our previous studies are consistent with crucial roles for the BER pathway components POLB and APEN in the maintenance of genomic integrity in the male germline throughout reproductive life and with increased spontaneous mutagenesis as a consequence of diminished repair capacity in spermatogenic cells. We propose that a threshold level of APEN and BER activity is required to maintain nuclear genomic integrity in spermatogenic cells. Age-related declines in other DNA damage detection and repair pathways may account for the similar mutation frequencies and nuclear DNA damage levels we observed in MGCs isolated from old mice of both genotypes.

Another possible consequence of unrepaired DNA damage is activation of apoptotic pathways, which remove cells that potentially harbor deleterious mutations. Apoptosis is an important component of normal spermatogenesis, and serves to remove excess germ cells that cannot be supported by the Sertoli cells or to eliminate spermatogonia and spermatocytes that have rearrangements or unrepaired DNA damage produced during mitosis and meiosis (Baum et al., 2005; Xu et al., 2010). In humans, the proportion of sperm harboring DNA double-strand breaks increases with age, whereas the proportion of apoptotic sperm in semen samples from older men decreases (Singh et al., 2003). In spermatozoa isolated from old (21 months) rats, age-related alterations in chromatin packaging are correlated with increased susceptibility to DNA breaks and modifications following oxidative challenge with agents such as hydrogen peroxide (Zubkova et al., 2005; Zubkova and Robaire, 2006). We compared apoptotic rates among spermatogenic cells in the seminiferous tubules of young, middle-aged, and old mice, of both *Apex1*$^{+/-}$ and $^{+/+}$ genotypes, and determined numbers of TUNEL-positive spermatogonia and pachytene spermatocytes specifically. No significant differences, however, were observed in apoptotic

rates for testes isolated from *Apex1*$^{+/-}$ and wild-type mice at any of the three ages examined. Thus, increased DNA damage and mutation frequency in the mitochondrial and nuclear genomes associated with APEN deficiency were not accompanied by increased levels of apoptosis in the male germline.

Because of its proximity to respiratory chain enzymes and its lack of protective histones, mitochondrial DNA is especially vulnerable to oxidative damage, and the accumulation of mtDNA damage occurs concomitantly with declining mitochondrial function in aging somatic and germline cells. Normal mitochondrial function is, of course, essential for sperm motility (Hermo et al., 2010), and deletions in the mitochondrial genome contribute to reduced fertility in men through impaired motility and decreased sperm numbers (Kao et al., 1995; Kao et al., 1998). In many cell types, persistent, unrepaired damage in the mitochondrial genome leads to aberrant function, and ultimately, arrested proliferation and apoptosis (Yakes and Van Houten, 1997). There is, however, a repair mechanism available within mitochondria to ameliorate some types of DNA damage, and several components of the BER pathway have been identified in these organelles (Hegde et al., 2008): Apurinic/apyrimidinic endonuclease activity can be purified from the mitochondria of mouse plasmocytoma cells (Tomkinson et al., 1988). More recently, APEN protein was identified immunohistochemically in the mitochondria of thyroid follicular cells (Tell et al., 2001), and purified to homogeneity from the mitochondria of bovine liver (Chattopadhyay et al., 2006). To determine if reduced levels of APEN protein would affect mitochondrial DNA repair capacity, we compared levels of mtDNA damage in *Apex1*$^{+/-}$ and wildtype MGCs isolated through the life, using QPCR. Consistent with the age-related declines in nuclear genomic integrity in the spermatogenic lineage (Walter et al., 1998), we observed little or no mtDNA damage in MGCs from young (6 months) and middle-aged (16 months) wild-type mice, and a 30% decrease in amplification of the mtDNA fragment in MGCs from old (26 months) wild-type mice. Reduction in APEN levels accelerated the onset of detectable mtDNA damage in the spermatogenic lineage, as amplification of the mtDNA fragment was significantly reduced in MGCs isolated from both young and middle-aged *Apex1*$^{+/-}$ mice. In other words, in wild-type but not in *Apex1*$^{+/-}$ mice, there was an increase in mtDNA damage in 28-month-old mice as compared to 16-month-old animals. Thus, although APEN deficiency had no detectable effect on nuclear DNA damage in the QPCR assay, both the *lacI* mutagenesis and mtDNA damage assays revealed significant differences in genomic integrity in the germline of *Apex1*$^{+/-}$ mice.

Experimental manipulation of APEN expression alters DNA repair capacity and levels of DNA damage in both the nuclear and mitochondrial genomes of somatic and germ cells. Over-expression of mitochondria-targeted, truncated APEN in human umbilical vein endothelial cells increases both mtDNA repair capacity and cell survival following hydrogen peroxide-induced oxidative stress (Li et al., 2008). Although spermatogenic cells isolated from old (28 months) mice exhibit diminished BER activity, addition of purified APEN to MGCs nuclear extracts restores activity of this pathway to levels typical of those obtained from young (3 months) animals (Intano et al., 2002). In the present study, we used a targeted null allele of *Apex1* to reduce levels of APEN in the male germline, and compared the effects of this genetic manipulation on mitochondrial and nuclear DNA damage in young, middle-aged, and old mice. We have demonstrated that APEN is a key component of the BER pathway that is required to maintain mitochondrial and nuclear genomic integrity in spermatogenic cells throughout reproductive life in mice; however, the mechanisms and genetic interactions that contribute to age-related changes in the abundance, activity, and subcellular localization of this protein remain to be investigated.

MATERIALS and METHODS

Animals

Apex1 heterozygous ($^{+/-}$) mice (Ludwig et al., 1998) were crossed into C57BL/6J for more than 10 generations. Then, they were crossed with homozygous *lacI* transgenic mice (Stratagene) in the C57BL/6J strain. Genotyping for *Apex1* and *lacI* was performed using primers described previously (Zhou et al., 2001; Huamani et al., 2004; Allen et al., 2008). Animals were allowed to attain predetermined ages (6-, 16-, or 26-months-old), without intervention. At the appropriate age, the animals were humanely terminated by cervical dislocation, after being anesthetized with isofluorane. Testes were removed surgically and used immediately for mixed germ cell preparations. Mice were maintained in an American Association for Accreditation of Laboratory Animal Care-accredited facility, on standard food and water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee prior to implementation.

Mixed germ cell (MGC) preparations

Testes were decapsulated by incising the tunica albuginea, and placed in 25ml of Krebs solution (Bellvé et al., 1977), containing 0.5mg/ml collagenase, under CO_2 for 15 minutes. Afterward they were placed in a gently shaking, 31°C water bath for 15 min. MGCs were allowed to settle to the bottom of the flask, and the supernatant was then carefully removed. The MGCs were resuspended in 5ml Krebs solution and swirled gently to wash; this washing step was performed twice. The MGCs were then resuspended in 25ml Krebs solution, containing 0.5mg/ml trypsin and 1 μg/μl DNase, in a 31°C gently shaking water bath for 12 min. Cell aggregates were disaggregated by repeated pipetting. Tissue debris was removed by filtering the germ cell solution through a 100-μm nylon cell strainer. The cells were then pelleted by spinning at 1100 × *g* for 15 minutes at 4°C. The supernatant was discarded, and MGCs were washed once by suspending the pellet in 15ml Krebs solution. The MGC suspension was spun down at 1100 × *g* for 15 min. Pellets were flash frozen in liquid nitrogen and stored in a −80°C freezer until use.

Crude protein extracts

Whole cell crude protein extracts were prepared from MGC pellets by adding 1 ml lysis buffer (0.1M NaCl, 50mM Tris-HCl pH7.2, 2mM EDTA, 1mM DTT for buffer, to which 1 μg/ml pepstatin and one Complete-Mini protease inhibitor cocktail tablet (Roche) per 10 ml were added immediately before use) to 100 μg of tissue sample. MGCs were homogenized on ice for 40–50 strokes. The homogenate was then centrifuged at 100,000 × *g* for 30 minutes at 4°C. The supernatants were collected and the protein was concentrated using Microcon YM-10 centrifugal filter devices (Millipore, Bedford, MA). Protein concentrations were determined using the Bradford assay (Bradford, 1976) with Immunoglobulin (Ig) as a standard (Bio-Rad). Protein preparations were aliquotted and stored at −80°C for future use.

Western blot analysis

Protein extracts (100 μg) from MGCs prepared from young (6-month-old), middle-aged (16-month-old) and old (26-month-old) mice were fractionated on a 12% SDS polyacrylamide gel (acrylamide:bisacrylamide, 29:1). Electroblotting was performed overnight at 4°C onto Trans-Blot nitrocellulose membrane (Bio-Rad). Resulting blots were cut into two sections based on molecular mass, to facilitate the detection of XRCC1 and APEN proteins from the same blot. Each blot section was blocked for 2 hours with 1X PBS solution containing 0.1% Tween-20 (Bio-Rad) and 5% Blotting-Grade Non-Fat Milk (Bio-Rad). Membranes were then washed with 1X PBS containing 0.1% Tween-20, 3 times for 1 min each with quick washes of 15 seconds in between.

XRCC1 was detected using a 1:2500 dilution of rabbit anti-human XRCC1 polyclonal primary antibody (Serotec, Raleigh, NC). APEN was detected using a 1:1000 dilution of rabbit anti-human APE/REF-1 (Novus Biologicals, Littleton, CO). POLB was detected using a 1:1000 dilution of mouse monoclonal POLB antibody (NeoMarkers), and LIG3 was detected using a 1:500 dilution of mouse monoclonal DNA ligase IIIα antibody (Genetex). Purified APEN (Novus Biologicals, Littleton, CO) was used as a standard. All antibodies, both primary and secondary antibodies, were added to membranes in a 1X PBS solution containing 0.05% Tween-20 and 0.5% Blotting-Grade Non-Fat Milk. Primary antibodies were incubated with membranes for 1 hour 30 minutes at room temperature. Membranes were then washed 4 times for 5 minutes with 50 ml of Wash Solution (1X PBS, 0.1% Tween-20) with quick washes of 15 seconds in between.

For detection of APEN and XRCC1, washes were followed by incubation of membrane with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce), diluted 1:250,000, for 1 hour at room temperature. For detection of POLB and LIG3, a 1:250,000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce) was used. Membranes were then washed 4 times at 5 minutes with 50 ml of Wash Solution with quick washes of 15 seconds in between. A signal was generated using enhanced chemiluminescence (Pierce). Bands were visualized and intensity of bands was quantified using a ChemiImager 4400 (Alpha Innotech, San Leandro, CA).

APEN Incision Activity

G:D coding strand (5′ GTC ACC GTC D TAC GAC TC 3′) and G:D/U complimentary strand (5′ GAG TCG TAG GAC GGT GAC 3′) oligonucleotides (The Midland Certified Reagent Company, Midland, TX) were reconstituted in double-distilled H_2O to a final concentration of 100 pmol/μl. The G:D coding strand was end-labeled by treatment with T4 polynucleotide kinase and γ-[^{32}P]-ATP, and annealed to the complimentary strand as described (Wilson et al., 1995). Incision reactions were performed with wild-type and *Apex1*$^{+/-}$ MGC protein extracts at 0.15, 0.3, and 0.6 μg with reaction buffers and mix parameters as described (Wilson et al., 1995), for 5 minutes at 37°C. Reactions were stopped by adding 4 μl formamide loading dye, and kept on ice until ready for loading onto the 20% acrylamide/urea gel. Samples, controls, and a radiolabeled 10-bp ladder were loaded onto the gel, and run for 1.5 to 2.0 hours at 138V in 1X TBE buffer. At the end of electrophoresis, the gel, on one glass plate, was covered with plastic film, exposed to the phosphoimager screen for 10 minutes, and then visualized and volume-analyzed on a BioRad Molecular Imager FX. Percent incision was calculated as product/(substrate + product), and specific activity as (% incision − % background) pmol substrate used/length of reaction time/mg of protein extract used.

DNA Damage Analysis by QPCR

DNA isolation, quantification, and damage analysis by QPCR were performed as described previously (Acevedo-Torres et al., 2009). Details of the QPCR technique for the detection of DNA lesions have been described previously (Ayala-Torres et al., 2000; Santos et al., 2006). Briefly, amplification of large mtDNA (10 kb) and nuclear DNA (6.9 kb) amplicons was used to detect DNA lesions. Lesion frequency per DNA strand was calculated assuming a Poisson distribution of DNA lesions within the target sequence. We applied the formula, DNA lesions = $-\ln(A_D/A_0)$; where A_D represents the amount of amplification of the damaged template and A_0 is the amplification product from undamaged DNA (6 months-old wild type animals). Lesion number was extrapolated to 10 kilobases. Amplification of a small mtDNA amplicon (91 bp) was used to normalize the results of amplification of the large mtDNA amplicon, and to monitor possible changes in mtDNA steady-state levels. PCR reactions were conducted under conditions that ensure exponential amplification of the

different amplicons. Primer sequences have been described in detail previously (Acevedo-Torres et al., 2009).

LacI Mutagenesis Assays

The RecoverEase™ DNA isolation kit (Stratagene) was used to prepare high molecular weight genomic DNA according to the manufacturer's recommendations. MGC pellets were homogenized by pipetting up and down in lysis buffer until homogenous. The homogenate was incubated with proteinase K for 3 hrs. Otherwise, the aforementioned protocol was followed.

The transgene was recovered by packaging the DNA with Stratagene Transpack® Packaging Extracts according to the manufacturer's specifications. Packaged DNA was incubated with SCS-8 cells, mixed with agarose that contained the chromogenic substrate X-gal (5-bromo-4-chloro-e-indolyl-β-D-galactopyranoside), and poured onto NZY agar assay trays. The trays were incubated at 37°C overnight. Putative mutants, identified as blue-appearing plaques, were cored and re-plated to confirm the mutant, as previously described (Huamani et al., 2004). Mutation frequency was calculated by the ratio of confirmed mutants to the total number of plaque-forming units.

To determine mutation spectrum, confirmed, cored, blue plaques were sequenced by the DNA Sequencing Facility, Centre for Biomedical Research at the University of Victoria, to identify *lacI* mutations and positions. Mutations were classified as transitions, transversions, 1 bp deletions, >1bp deletions, or double base pair changes, and expressed as percentages of total mutations for a given age and genotype in Table 3.

TUNEL (Terminal Deoxynucleotidyltransferase-mediated dUTP-biotin Nick End Labeling) Assay for Apoptosis

Testes isolated from 6-, 16-, and 26-month old mice were fixed in 10% formalin solution, paraffin embedded, and sectioned at 4 μm; these sections were then processed for the TUNEL assay (Gavrieli et al., 1992) and for hematoxylin and eosin staining by the San Antonio Cancer Institute Pathology Core Shared Resource. For each animal, sections from four different depths, evenly distributed across the testis, were analyzed for the presence of TUNEL-positive (apoptotic) cells. The percentage of seminiferous tubule cross-sections that contained one or more apoptotic cells was quantified using two microscopic fields at 630X magnification. For each age and genotype, between 120 and 450 tubule cross-sections were examined for apoptotic cells, in testes isolated from each of five different mice. Total tubule cross-sections examined were as follows: 6-month wild-type = 1373, 6-month $Apex1^{+/-}$ = 1239, 16-month wild-type = 1043, 16-month $Apex1^{+/-}$ = 793, 26-month wild-type = 1054, 26-month $Apex1^{+/-}$ = 1178. In addition, the percentages of apoptotic (TUNEL-positive) spermatogonia and pachytene spermatocytes were quantified in seminiferous tubule cross-sections; these cell types were distinguished using the criteria of size, position relative to the tubule lumen, and morphology (Bellvé et al., 1977). For each age and genotype, between 50 and 300 spermatogonia, and between 25 and 100 pachytene spermatocytes, were scored for TUNEL staining in the tubule cross-sections quantified above, in testes isolated from each of five different mice.

Statistical Methods

Mutation types were analyzed using the chi-squared test for independence (Agresti, 2002). Where low expected frequencies were encountered, an exact chi-square test was used. While an overall P-value may not be significant, the chi-square statistic might be large and it is well known that one or more components may be significant (Maxwell, 1971). Multiple logistic regression (Hosmer and Lemeshow, 2000) was used to investigate the interactions

(whether the effect of age was different in the two genotypes) of genotype and age on mutation type.

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Abbreviations

abasic	apurinic/apyrimidinic
APEN	apurinic/apyrimidinic endonuclease 1
BER	base excision repair
LIG3	DNA ligase IIIα
MGC	mixed germ cells
mtDNA	mitochondrial DNA
POLB	DNA polymerase β
QPCR	quantitative polymerase chain reaction
XRCC1	X-ray cross-complementing 1

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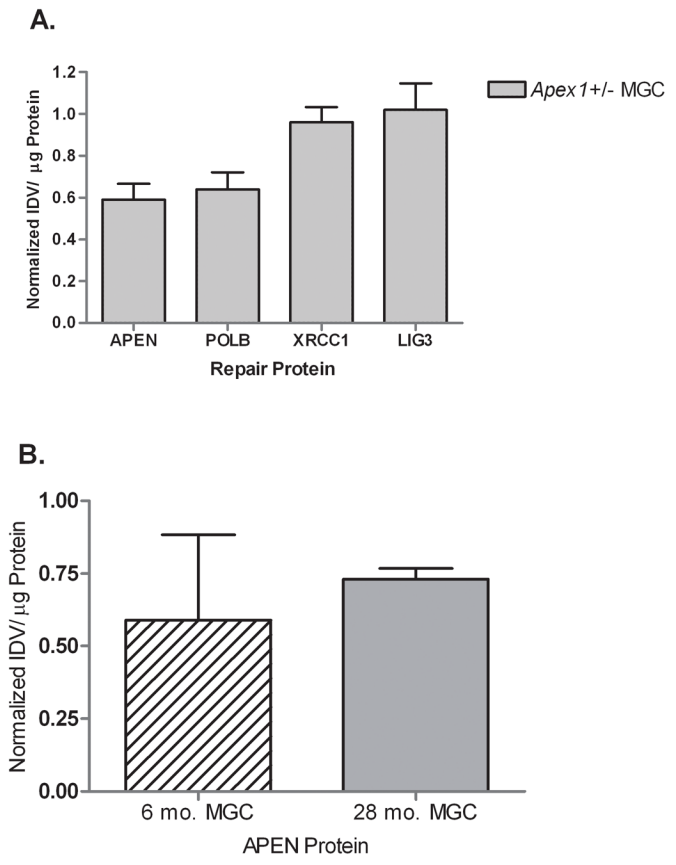

Figure 1. APEN protein levels are decreased in MGC isolated from young and old *Apex1*$^{+/-}$ mice

A. Western blot analysis of BER protein levels in MGCs isolated from young (5 to 6 months) *Apex1*$^{+/-}$ mice, normalized to values obtained from age-matched wild-type control samples. Each bar represents the mean of densitometry values from individual MGC protein preparations, isolated from 4 different *Apex1*$^{+/-}$ mice; error bars indicate standard deviation. B. Western blot comparison of APEN protein levels in MGC isolated from young (5–6 months) and old (28 month) *Apex1*$^{+/-}$ mice, normalized to values obtained from age-matched wild-type control samples. For young mice, the bar represents the mean of densitometry values from 4 individual *Apex1*$^{+/-}$ MGC protein preparations; for old mice, MGC from two *Apex1*$^{+/-}$ individuals were used. Error bars represent the upper range of values obtained.

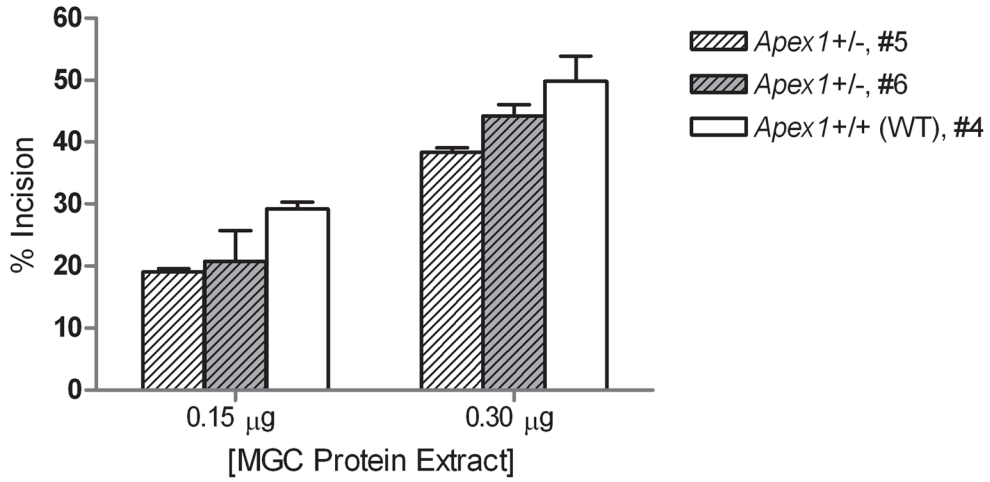

Figure 2. APEN activity is decreased in MGC isolated from old Apex1$^{+/-}$ mice

MGC extracts were prepared from one wild-type and two *Apex1*$^{+/-}$ mice at 28 months, and APEN activity assays performed in two trials, at two different concentrations of protein extract in each trial. APEN activity is plotted as percent incision of the oligonucleotide containing the synthetic AP site; error bars represent standard error of the mean. In Trial 1, the % Incision values for *Apex1*$^{+/-}$ #5 at 0.15 and 0.3 μg MGC extract were 18.49 and 37.67, for *Apex1*$^{+/-}$ #6 15.93 and 46.03, and for wild-type 25.27 and 48.74. In Trial 2, the % Incision values for *Apex1*$^{+/-}$ #5 at 0.15 and 0.3 μg MGC extract were 19.55 and 39.09, for *Apex1*$^{+/-}$ #6 25.55 and 42.42, and for wild-type 33.24 and 50.91.

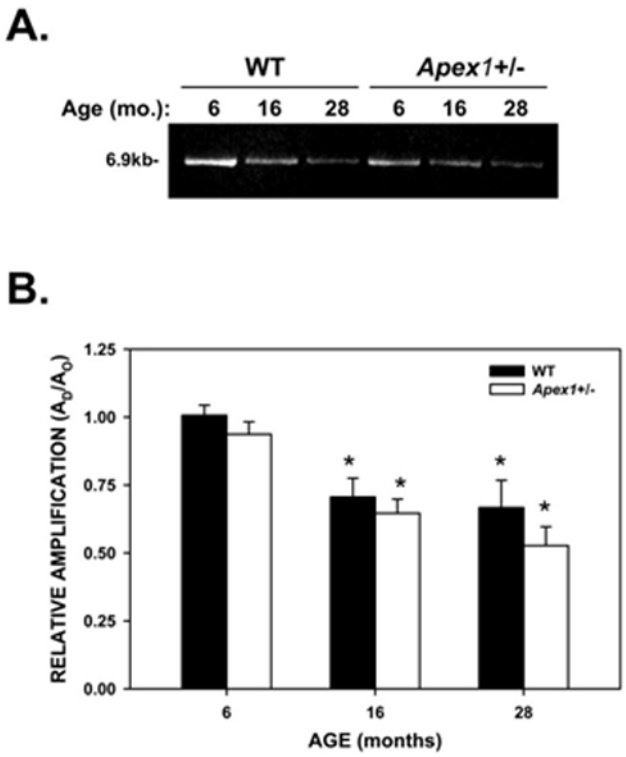

Figure 3. Age-dependent increase in nuclear DNA damage in *Apex1*$^{+/-}$ MGC

QPCR analysis of a 6.9-kb fragment was performed on nDNA isolated from the MGC of 6-, 16-, and 28-month-old wild-type and *Apex1*$^{+/-}$ mice. A. Representative agarose gel electrophoresis indicating the expected size of the PCR product. B. Relative levels of amplification of the 6.9-kb nuclear DNA fragment (n = 10 to 12 animals per group, QPCR analyses performed in triplicate). Single (*) asterisks indicate statistically significant differences ($p < 0.05$), when compared to values obtained for 6-month-old wild-type.

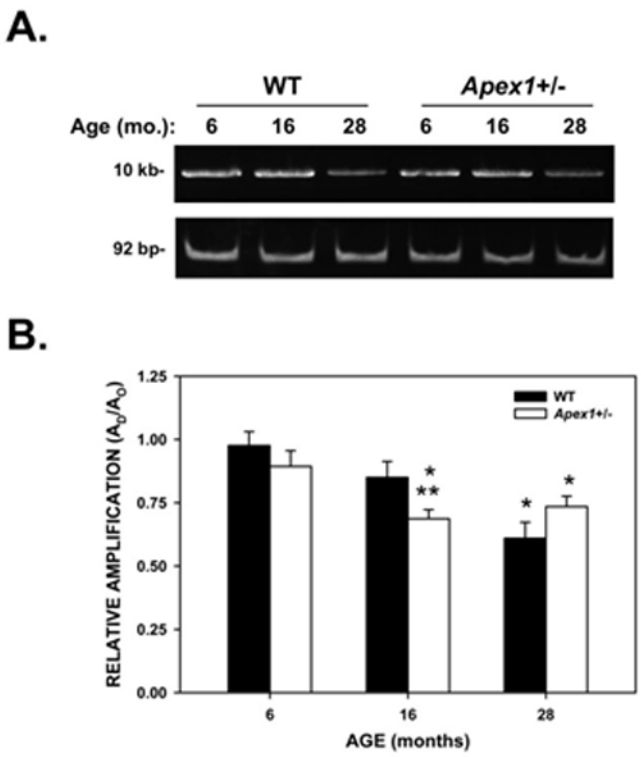

Figure 4. Age-dependent increase in mitochondrial DNA damage in both *Apex1*$^{+/-}$ and wild-type MGC

QPCR analysis of a 10.0-kb fragment was performed on mtDNA isolated from the MGC of 6-, 16-, and 28-month-old wild-type and *Apex1*$^{+/-}$ mice. A. Representative agarose gel electrophoresis indicating the expected size of the PCR product. B. Relative levels of amplification of the 10.0-kb mtDNA fragment, after normalization with a 92-bp mtDNA fragment (n = 10 to 12 animals per group, QPCR analyses performed in triplicate). p values of <0.05 were considered significant. Single (*) and double (**) asterisks indicate statistically significant differences ($p<0.05$), when compared to values obtained for 6 and 16 month old wild-types, respectively.

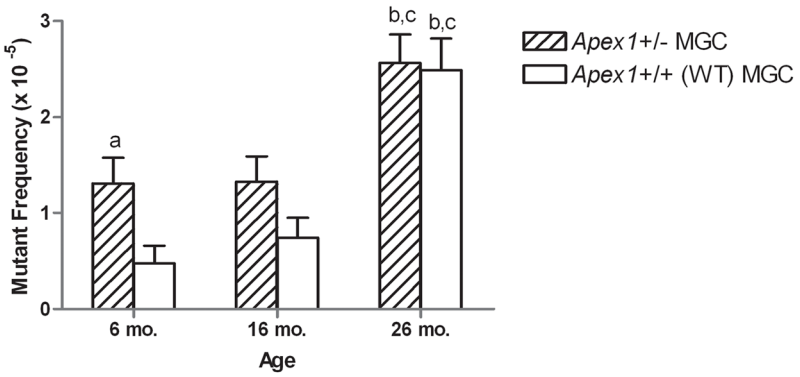

Figure 5. Increased mutation frequency in MGC isolated from old *Apex1*$^{+/-}$ and wild-type mice

Mutant frequencies in mixed germ cells isolated from *Apex1*$^{+/-}$ and $^{+/+}$ mice, at 6, 16 and 26 months of age. For each genotype and age, genomic DNA was isolated from the MGCs of 5 mice, and analyzed for mutation frequency in two separate assays. For each genotype and age, a total of 1,400,000 to 2,900,000 plaque-forming units were scored for mutant plaques. (a) Significantly different from wild-type (WT) of the same age (b) Significantly different from WT and *Apex1*$^{+/-}$ at 6 months (c) Significantly different from WT and *Apex1*$^{+/-}$ at 16 months.

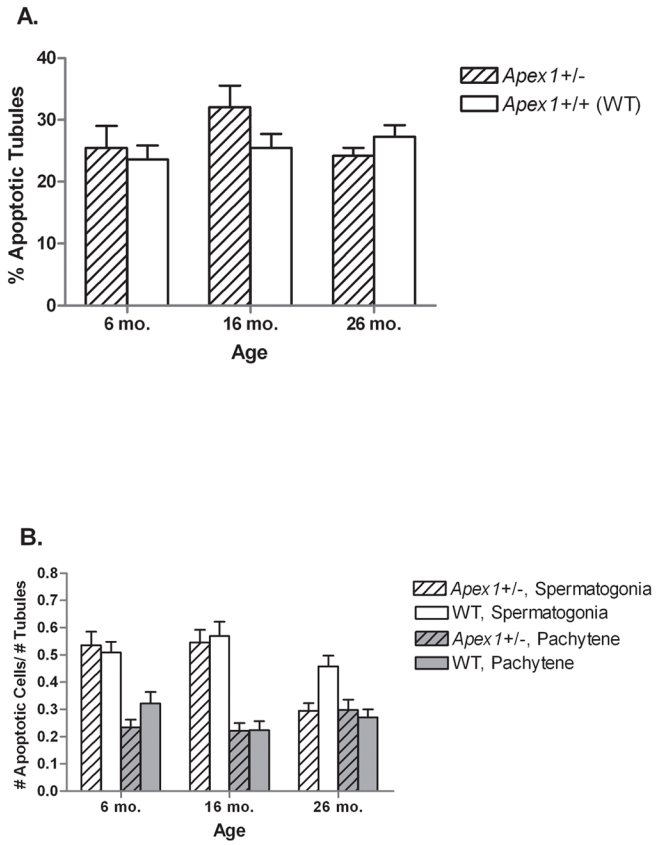

Figure 6. Apoptosis in spermatogenic cells in *Apex1*$^{+/-}$ and wild-type mice throughout reproductive life

TUNEL-positive (apoptotic) cells were counted in cross sections of seminiferous tubules at 6, 16, and 28 months of age. A. Number of seminiferous tubules that contain at least one apoptotic cell, expressed as a percentage of the total number of tubules counted, for *Apex1*$^{+/-}$ (hatched bars) and wild-type (solid bars) mice. For each age and genotype, 12 cross-sections of seminiferous tubules were examined, in the testes of each of five individuals. Each bar represents the mean for a given age and genotype, and error bars indicate standard error of the mean (SEM). B. Numbers of apoptotic spermatogonia (white bars) and pachytene spermatocytes (gray bars), expressed as a proportion of the total number of tubules counted, for *Apex1*$^{+/-}$ (hatched bars) and wild-type (solid bars) mice. For each age and genotype, 12 cross-sections of seminiferous tubules were examined, in the testes of each of five individuals. Each bar represents the mean for a given age and genotype, and error bars indicate SEM.

Table 1

Nuclear and Mitochondrial DNA Lesion Numbers

	6 months	16 months	28 months
WT, nDNA	0.007	0.5049	0.5869
***Apex1*+/−, nDNA**	0.0946	0.6333	0.9283
WT, mtDNA	0.0243	0.1622	0.4943
***Apex1*+/−, mtDNA**	0.1127	0.3623	0.3077

Lesion numbers were calculated per 10.0 kb using the Poisson equation.

Table 2

Spontaneous Mutant Frequencies in Mixed Germ Cells Isolated from *Apex1*+/− and +/+ Mice, at 6, 16, and 26 Months

Age	Genotype/ID	# PFU	# Mutants	MF (× 10^{-5})	Total PFU	Total Mutants
6 months	WT #1.1	213,433	2	0.94		
	WT #1.2	123,200	0	0		
	WT #2.1	84,800	1	1.2		
	WT #2.2	82,516	0	0		
	WT #3.1	91,233	0	0		
	WT #3.2	161,300	0	0		
	WT #4.1	274,650	1	0.36		
	WT #4.2	120,667	1	0.83		
	WT #5.1	185,900	2	1.1		
	WT #5.2	120,833	0	0		
					1,458,532	**7**
6 months	*Apex1*+/− #1.1	161,233	1	0.62		
	Apex1+/− #1.2	107,767	1	0.93		
	Apex1 +/− #2.1	191,600	3	1.6		
	Apex1+/− #2.2	257,300	11	4.3		
	Apex1+/− #3.1	111,100	3	2.7		
	Apex1+/− #3.2	199,333	2	1.0		
	Apex1+/− #4.1	93,000	0	0		
	Apex1+/− #4.2	170,450	2	1.2		
	Apex1+/− #5.1	331,100	1	0.30		
	Apex1+/− #5.2	211,033	0	0		
					1,833,916	**24**
16 months	WT #1.1	196,100	4	2.0		
	WT #1.2	139,433	0	0		
	WT #2.1	186,467	3	1.6		
	WT #2.2	129,133	1	0.77		
	WT #3.1	207,900	1	0.48		

Age	Genotype/ID	# PFU	# Mutants	MF (× 10^{-5})	Total PFU	Total Mutants
	WT #3.2	193,200	3	1.6		
	WT #4.1	171,967	0	0		
	WT #4.2	158,500	0	0		
	WT #5.1	211,333	1	0.47		
	WT #5.2	153,633	0	0		
					1,747,666	**13**
16 months	*Apex1*+/− #1.1	271,933	1	0.37		
	Apex1+/− #1.2	191,200	5	2.6		
	Apex1+/− #2.1	280,333	3	1.1		
	Apex1+/− #2.2	228,000	4	1.8		
	Apex1+/− #3.1	300,100	7	2.3		
	Apex1+/− #3.2	195,033	3	1.5		
	Apex1+/− #4.1	121,667	1	0.82		
	Apex1+/− #4.2	119,400	0	0		
	Apex1+/− #5.1	93,200	0	0		
	Apex1+/− #5.2	82,433	1	1.2		
					1,883,299	**25**
26 months	WT #1.1	307,333	8	2.6		
	WT #1.2	143,833	3	2.1		
	WT #2.1	353,867	9	2.5		
	WT #2.2	161,467	5	3.1		
	WT #3.1	338,200	4	1.2		
	WT #3.2	134,400	7	5.2		
	WT #4.1	331,133	12	3.6		
	WT #4.2	189,967	7	3.7		
	WT #5.1	181,167	1	0.55		
	WT #5.2	149,467	1	0.67		
					2,290,834	**57**
26 months	*Apex1*+/− #1.1	240,600	2	0.83		

Age	Genotype/ID	# PFU	# Mutants	MF (× 10^{-5})	Total PFU	Total Mutants
	Apex1+/− #1.2	321,750	5	1.6		
	Apex1+/− #2.1	257,967	3	1.2		
	Apex1+/− #2.2	311,750	3	0.96		
	Apex1+/− #3.1	257,200	17	6.6		
	Apex1+/− #3.2	405,950	23	5.7		
	Apex1+/− #4.1	315,650	6	1.9		
	Apex1+/− #4.2	307,900	8	2.6		
	Apex1+/− #5.1	281,633	5	1.8		
	Apex1+/− #5.2	223,233	3	1.3		
					2,923,633	**75**

For each genotype and age, mutant numbers and mutant frequencies were determined for MGC isolated from five different mice; two separate assays were performed for each MGC preparation.

Table 3

Mutation Spectra in MGC from *Apex1*+/− and +/+ Mice, at 6 and 26 Months

Genotype	Age (mo.)	Total number	Transition Number (%)	Transversion Number (%)	Del. = 1 bp Number (%)	Del. > 1bp Number (%)	Double bp Number (%)
***Apex1*+/−**	6	20	11 (55)	8 (40)	0 (0)	0 (0)	1 (5)
***Apex1*+/−**	26	26	19 (73.1)	3 (11.5)	3 (11.5)	1 (3.9)	0 (0)
Wild-type	6	5	2 (40)	2 (40)	0 (0)	1 (20)	0 (0)
Wild-type	26	30	15 (50)	7 (23.3)	4 (13.3)	4 (13.3)	0 (0)

Number and percentage of each mutation type are presented by genotype and age. The Pearson chi-square statistic was 16.5 with 12 degrees of freedom (exact P = 0.1663). Although not significant, the large chi-square statistic warrants further investigation. Analyses of Transitions vs. Other, Transversions vs. Other, and Combined Deletion = 1 bp, Deletions > 1 bp, and Double bp Change vs. Other, using multiple logistic regression analysis indicated that there were no significant interactions of genotype and age; i.e., the effect of age was similar in the two genotypes.

Table 4

Mutation Position in the *lacI* Gene, in MGC from *Apex1*+/− and +/+ Mice, at 6 and 26 Months

Genotype	Age (mo.)	Total number	39 or 42 Number (%)	92 or 93 Number (%)	1206 Number (%)	Other
***Apex1*+/−**	6	20	8 (40)	0 (0)	0 (0)	12 (60)
***Apex1*+/−**	26	26	1 (3.9)	3 (11.5)	11 (42.3)	11 (42.3)
Wild-type	6	5	0 (0)	0 (0)	0 (0)	5 (100)
Wild-type	26	30	1 (3.3)	8 (26.7)	0 (0)	21 (70)

The mutation distribution along the length of the *lacI* gene was compared to a uniform distribution (mutation equally likely at all positions) and among groups using the Kolmogorov-Smirnov test. Pairwise significance levels were Bonferroni adjusted.