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Increased human AP endonuclease 1 level confers protection against the paternal age effect in mice

Jamila R. Sanchez^{b,1}, Traci L. Reddick^{c,1}, Marissa Perez^b, Victoria E. Centonze^d, Sankar Mitra^e, Tadahide Izumi^f, C. Alex McMahan^g, and Christi A. Walter^{a,b,h,i,*}

^aSouth Texas Veteran's Health Care System, 7400 Merton Minter Blvd., San Antonio, TX 78229, USA

^bDepartment of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA

^cDuke Cancer Institute, Duke University, 20 Duke Medicine Circle, Durham, NC 27710, USA

^dCell & Tissue Imaging Center, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA

^eDepartment of Radiation Oncology, Houston Methodist Research Institute, 6550 Fannin Street, Houston, TX 77030, USA

^fGraduate Center for Toxicology, 1095 V.A. Drive, Lexington, KY 40536, USA

^gDepartment of Pathology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA

^hCancer Therapy & Research Center, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA

ⁱBarshop Institute for Longevity and Aging Studies, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA

Abstract

Increased paternal age is associated with a greater risk of producing children with genetic disorders originating from *de novo* germline mutations. Mice mimic the human condition by displaying an age-associated increase in spontaneous mutant frequency in spermatogenic cells. The observed increase in mutant frequency appears to be associated with a decrease in the DNA repair protein, AP endonuclease1 (APEX1) and *Apex1* heterozygous mice display an accelerated paternal age effect as young adults. In this study, we directly tested if APEX1 over-expression in cell lines and transgenic mice could prevent increases in mutagenesis. Cell lines with ectopic expression of APEX1 had increased APEX1 activity and lower spontaneous and induced mutations in the *lacI* reporter gene relative to the control. Spermatogenic cells obtained from mice

*Corresponding author at: Department of Cellular and Structural Biology Univ of TX Health Sci Ctr at San Antonio M.C. 7762 7703 Floyd Curl Dr. San Antonio, TX 78229-3900, USA. Fax: +1 210 567 3803. walter@uthscsa.edu (C.A. Walter).

¹Co-first authorship.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

transgenic for human *APEX1* displayed increased APEX1 activity, were protected from the age-dependent increase in spontaneous germline mutagenesis, and exhibited increased apoptosis in the spermatogonial cell population. These results directly indicate that increases in APEX1 level confer protection against the murine paternal age effect, thus highlighting the role of APEX1 in preserving reproductive health with increasing age and in protection against genotoxin-induced mutagenesis in somatic cells.

Keywords

Paternal Age; DNA repair; APEX1; Mutagenesis; LacI; Spermatogenesis

1. Introduction

Over the last three decades, the frequency of births to fathers over the age of 35-years-old, the age at which the number of mutations potentially transmitted to children are approximately doubled compared to a 20-year-old father and which continue to double approximately every 16 years [1], has increased by 30% [2]. The increased risk of genetic disease for children born to older fathers is known as the paternal age effect and has traditionally been associated with dominant disorders displaying Mendelian inheritance. More recently, a strong association between the paternal age effect and autism-like syndromes and schizophrenia has been demonstrated [1,3–5].

The driving force of the paternal age effect is increased mutagenesis [1,6,7]. A basic sites, known also as AP sites, are generated spontaneously in the genome at the rate of approximately 10,000–40,000 lesions/cell/day [8,9]. These sites are harmful because they inhibit DNA replication and transcription [10,11], and lead to cell death [12] and increased mutagenesis [13–16]. A basic sites are also produced by mono-functional DNA glycosylases that hydrolyze the *N*-glycosidic bond of alkylated bases, oxidized bases and incorrect bases, such as uracil, to initiate base excision repair (BER). The AP sites are processed by AP endonuclease 1 (APEX1). Reduced APEX1 activity leads to persistent AP sites in the genome, which can result in the aforementioned deleterious effects.

APEX1 incises the phosphodiester backbone at the 5'-phosphate during repair of an abasic site, leaving a 5'-deoxyribosephosphate (5'-dRP) and 3'-hydroxyl (3'-OH) group [17]. APEX1 enhances binding of DNA Polymerase β (POLB) to DNA and enhances its lyase activity [16,18], aids in the release of DNA glycosylases from damaged DNA [19], coordinates activities of proteins in long-patch BER [20], and is involved in mitochondrial DNA repair [21]. APEX1 has a redox function, which led to the gene being identified originally as *Ref-1* (redox factor-1) [22]. APEX1 can reduce and activate other transcription factors, such as c-Fos/c-Jun heterodimer, NF- κ B, HIF-1 α and p53 [22–25]. While it is clear that APEX1 performs multiple functions within the cell, its central role in BER seems most likely to have a direct effect in regulating mutagenesis [16,18–24,26].

APEX1 abundance correlates directly with BER activity [27,28] and inversely with mutant frequency [29,30]. Increased APEX1 in tumor cells is associated with resistance to chemotherapeutic drugs and ionizing radiation, suggesting that APEX1 enhances repair from

genotoxic agents and therefore survival of the tumor cells [31–35]. Izumi et al. presented evidence that APEX1 activity is rate limiting in the repair of 3' blocking damage caused by reactive oxygen species (ROS) [36]. APEX1 plays a critical role in spontaneous germline mutagenesis, such that mutant frequency was elevated in germ cells obtained from young *Apex1* heterozygous mice compared to wild-type mice of the same age [29]. In this *in vivo* model system, mice heterozygous for *Apex1* also displayed reduced BER activity [29,30]. These young *Apex1* heterozygous mice recapitulate the phenotype that is observed at old age in wild-type mice, thus making them an excellent model for studying the paternal age effect. Together, these studies indicate the importance of APEX1 in the repair of DNA damage and regulation of mutagenesis. The present study was performed to determine if increased APEX1 expression and activity could enhance protection against the mutagenic effects of DNA damage *in vitro* and minimize or abrogate the age-dependent increase in mutant frequency previously observed in germ cells obtained from old mice.

2. Materials and methods

2.1. Construction of an *Apex1* expression vector

The murine AP endonuclease cDNA (*Apex1*) was cloned by reverse transcription coupled PCR (RT-PCR) amplification using testis RNA obtained from a C57BL/6J mouse. The cDNA was sub-cloned into pBluescript®. DNA sequence analysis was performed to confirm the correct gene had been cloned, and subsequently *Apex1* cDNA was placed under the transcriptional regulation of the murine phosphoglycerol kinase (*mPkg-1*) promoter, generously provided by Dr. Micheal McBurney of the University of Ottawa, Ottawa, Ontario, Canada [37]. Rabbit β -globin exons II, III and intron II were added to potentially enhance expression of *Apex1* [38,39] and DNA sequences encoding a polyadenylation signal completed the expression vector (Fig. 1).

2.2. Cell lines and culture conditions

The *Apex1* expression vector was co-transfected with a plasmid containing a puromycin resistance gene, (pPUR, Clontech, PaloAlto, CA), into the Big Blue Rat™ (BBR) primary fetal fibroblast line, carrying a *lacI* mutation reporter, purchased from Stratagene (now Agilent) and transfected as described previously [40]. Cells were placed under puromycin selection (10 μ g/ml) 48 h after transfection. Puromycin resistant clones were collected, expanded and tested for the presence of the *Apex1* expression vector by Southern blot analysis. Clones that contained the *Apex1* expression vector were designated Pap. The BBR™ primary fetal fibroblast cell line was also transfected with pPUR alone to serve as a transfected control line and was designated Pur. Pur and Pap cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with low glucose (1000 mg/l D-glucose, L-glutamine, 110 mg/l sodium pyruvate, GIBCO), supplemented with 10% fetal bovine serum (FBS; GIBCO) at 37°C, 5% CO₂ and 10 μ g/ml of puromycin. Cells were harvested with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA·4Na, Gibco), subjected to centrifugation at 1200 rpm for 4 min at 4°C, then rinsed with Dulbecco's phosphate buffered saline, without calcium and magnesium (DPBS; 2.67 mM KCl, 1.47 mM KH₂PO₄, 0.138 M NaCl, 8.10 mM Na₂HPO₄·7H₂O), and stored at –80 °C until further use.

2.3. Southern analysis

DNA was prepared from harvested cells using lysis buffer (1% SDS, 10 mM Tris (pH 7.5), 5 mM EDTA) followed by digestion with 2 mg of proteinase K (100 mg/ml stock solution) at 55° for 1 h. Deproteinization was carried out with Tris-buffered (pH 8.0) phenol/chloroform (1 volume: 1 volume), then chloroform/isoamyl alcohol (24 volume: 1 volume), and the DNA was precipitated with 100% ethanol. DNA was then reconstituted in 500 µl double distilled H₂O. Ten micrograms of DNA were digested with EcoRI restriction endonuclease. Complete digestion was confirmed by subjecting a small aliquot to electrophoresis and visualized using ethidium bromide (EtBr) and UV light. Afterward, 10 µg of each sample was separated in a 0.8% agarose gel in (Tris-acetate EDTA buffer (TAE), 0.04 M Tris (pH 8.0), 0.018 M glacial acetic acid, 0.001 M EDTA), and transferred to a Zeta-Probe[®] genomic nylon membrane (Bio-Rad, Hercules, CA), by capillary action. DNA was fixed to the membrane by UV cross-linking (UVC 515 Ultraviolet multilinker, Ultra-Lum, Claremont, CA). The membrane was pre-hybridized in 0.25 M Na₂HPO₄ pH 7.2, 7% SDS for 30 min at 65°C. Murine *Apex1* cDNA was radioactively labeled with α [³²P] dCTP utilizing a random priming kit (Amersham Pharmacia Biotech). Unincorporated dNTP's were removed by filtration through a sephadex column (Quick-spin, Roche, Indianapolis, IN). Labeled probe was added to hybridization solution, and hybridized overnight at 65 °C in 0.25 M Na₂HPO₄ pH 7.2, 7% SDS. After hybridization the membrane was washed twice in 20 mM Na₂HPO₄, pH 7.2, 5% SDS, twice in 20 mM Na₂HPO₄, pH 7.2, 1% SDS, for 1 h each at 65 °C, and exposed on a Molecular Imager GS-363 (Bio-Rad) for quantitation. *Apex1* probe was removed by boiling in 0.1 × SSC, 0.5% SDS for 20 min. The membrane was then hybridized with radiolabeled rat *Gapdh* cDNA to normalize differences in loading and transfer. Quantification of *Gapdh* hybridizing bands was as described for *Apex1*.

2.4. Immunofluorescence microscopy

To identify the subcellular localization of APEX1 in the transfected cell lines, immunofluorescence microscopy was performed. Briefly, cells were grown on coverslips. After 48 h, medium was aspirated and cells washed with Dulbecco's phosphate buffered saline (DPBS) and fixed with 80% methanol for 10 min at -20 °C. Coverslips were rinsed with DPBS and then permeabilized with ice-cold acetone for 10 min at room temperature, then rinsed with DPBS. Nonspecific binding was blocked by incubation with 50 µl of normal goat serum added to each coverslip. After 1 h, coverslips were rinsed with borate-buffered saline (BBS, 0.1 M boric acid, 25 mM borax, 75 mM NaCl, pH 8.5) containing 0.05% (w/v) Triton X-100 (Fisher Biotech). Fifty µl of rabbit polyclonal anti-APEX1/REF-1 polyclonal antibody generated against the human APEX1 protein (NB 100-101, Novus Biological, Littleton, CO) was used as primary antibody, added to coverslips and incubated for 1 h at 37 °C, then rinsed with BBS. 50 µl of goat anti-rabbit IgG fluorescein conjugated secondary antibody (Chemicon Intl) was then added to coverslips, incubated for 1 h at 37 °C, then rinsed with BBS and followed with a rinse of a 0.05% Triton X-100 solution. Coverslips were mounted on a microscope slide with Vectashield[®] mounting medium (Vector Laboratories, Burlingame, CA).

Samples were imaged on a FV500 confocal lens mounted on an IX-81 microscope (Olympus USA). Images were acquired with a 60X PlanApo oil immersion lens, 1.4 NA.

The fluorescence excitation source was a 488-nm line of an Argon laser and the emission was collected through a 505–525 nm bandpass filter. All image acquisition parameters were kept consistent for each sample. At each field of view a stack of optical slices was collected at 0.2 micron intervals through the volume of the cells. Differential interference images were also collected to document overall cell morphology. Each optical slice was evaluated to determine the distribution of APEX1. To represent the distribution through the depth of the nucleus, the data sets were digitally resliced to create orthogonal sections (X, Z or Y, Z) using the Multiplane function available in the Fluoview (v. 5.0) software.

2.5. APEX1 activity assay

APEX1 activity assays were performed as described previously [41]. Single-strand oligonucleotides were purchased from Midland Certified Reagent Company, (Midland, TX). The lesion containing strand was an 18-mer with the sequence 5'GTCACCGTCQTACGACTC3'. A tetrahydrofuran residue was positioned at nucleotide 10, as indicated by "Q". The sequence of the complementary strand was 5'CAGTGGCAGGATGCTGAG3'. 100 pmol of the lesion containing oligonucleotide was 5' radiolabeled by incubating at 37 °C for 30 min with 3 µl of γ [32 P]-ATP and 10 U of polynucleotide kinase (Invitrogen, Grand Island, NY), then heated at 95 °C for 10 min to inactivate the kinase. 500 pmol of the complementary strand and 75 µl of double distilled H₂O was added and incubated overnight at room temperature. 250 ng of whole cell extract was incubated with the 5' [32 P]-end labeled 18 mer duplex oligonucleotide (1 pmol) in a 20 µl reaction mixture (50 mM Hepes (pH 7.5), 50 mM KCl, 10 µg/ml BSA, 10 mM MgCl₂, 10% glycerol, and 0.1% Triton X-100) for 5 min in a 37 °C water bath. Enzyme activity was stopped by placing immediately on dry ice and adding 4 µl of formamide loading dye (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF). 1 pmol of duplex oligonucleotide alone, and duplex oligonucleotide incubated with 12 pg of recombinant purified human APEX1/REF-1 protein (NB 100-100PEP, Novus Biologicals, Littleton, CO) were used as controls. Samples were heated at 85 °C for 10 min, immediately cooled on ice, and 5 µl of the reaction mixture was analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea in Tris/borate/EDTA buffer (TBE; 89 mM Tris-borate, 2 mM EDTA pH 8.0). Radioactivity of the 18-mer substrate and 9-mer products was measured on a BioRad Personal Molecular Imager FX Phosphoimager (Bio-Rad, Hercules, CA).

2.6. DNA damage inducing treatments

Cells grown to 75- to 80% confluence, were harvested, replated at 3.0×10^4 cells per flask, and treated 16 h later with 500 cGy ionizing radiation (IR) by exposure to a [137 Cs] Gammacell Irradiator (Ottawa, Canada) or with 5 mM methyl methane sulfonate (MMS, Sigma). For IR, the medium was removed and cells were rinsed with DPBS that contained calcium and magnesium. After exposure to IR, fresh DMEM with 10% FBS replaced the DPBS. For MMS exposure, medium was removed; cells were rinsed twice with DPBS (containing calcium and magnesium) to remove FBS then cells were incubated for 15 min at 37 °C using DMEM without FBS and containing the appropriate dose of MMS. After treatment, MMS was removed, cells were rinsed twice with DPBS that contained calcium and magnesium, followed by fresh DMEM with 10% FBS. Cells were harvested 80 h post-

exposure to IR or post-treatment with MMS and proceeded to Big Blue[®] mutant frequency assay (see below).

2.7. Animals

hAPEX1 transgenic mice [42] were crossed with homozygous *lacI* transgenic mice (Agilent, Cedar Creek, TX) in the C57BL/6J genetic background. Genotyping for *lacI* was performed using primers described previously for *lacI* [29,43]. For the amplification of the *lacI* transgene, primers 5'-

TAATACGACTCACTATAGGGACACCATCGAATGGTGCAAAAC-3' and 5'-

ATTTAGGTGACACTATAGGAGAACTTAATGGGCCCCG-3' were used. The following primers were used for identification of both the hAPEX1 and endogenous mApex1, Forward primer: 5'-TGGAATGTGGATGGGCTTCGAGCC and reverse primer: 5'-

ATCAAGGCGCCAACCAACATTCTT. The human PCR gene fragment is 150 bp longer than mApex1, which is 1.2 kb in length, and can be distinguished in transgenic animals.

Animals were allowed to attain predetermined ages for young adult (6-months-old), middle-age (16-months-old), or old (26- to 28-months-old), without intervention. At appropriate ages, the animals were anesthetized with isofluorane and then humanely terminated by cervical dislocation. Testes were removed and used immediately for 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.

2.8. Germ cell preparations

Testes were decapsulated by incising the tunica albuginea, and placed in 25 ml of KREBS solution [44], containing 0.5 mg/ml collagenase, under CO₂ for 15 min. Afterward they were placed in a gently shaking, 31 °C water bath for 15 min. Germ cells were allowed to settle to the bottom of the flask, and the supernatant was then carefully removed. The germ cells were resuspended in 5 ml KREBS solution and swirled gently to wash; this washing step was performed twice. The germ cells were then resuspended in 25 ml KREBS solution, containing 0.5 mg/ml trypsin and 1 mg/ml DNase, in a 31 °C gently shaking water bath for 12 min. Cell aggregates were disaggregated by repeated pipetting with a plastic Pasteur pipette. Tissue debris was removed by filtering the germ cell mixture through a 100-μm nylon cell strainer. The cells were then pelleted by spinning at 1100 × g for 15 min at 4 °C. The supernatant was discarded, and germ cells were washed once by suspending the pellet in 15 ml KREBS solution. The germ cells were pelleted then flash frozen in liquid nitrogen and stored at -80 °C until use.

2.9. Preparations of whole cell extracts

Whole cell extracts were prepared from germ cells. Cells were lysed in 1 ml of Lysis Buffer containing 0.1 M NaCl, 50 mM Tris-HCl pH 7.2, 2 mM EDTA, 1 mM DTT, 0.01 mg pepstatin and 1 cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science). Cells were homogenized by hand with a 2 ml glass homogenizer, fitted with a tight pestle, for 40 strokes. Lysed cell suspension was spun at 100,000 × g at 4 °C for 30 min using a Beckman TLA-100.4 rotor (Beckman). The supernatant, containing whole

protein extract, was collected and concentrated using YM-10 centrifugal filters (Amicon). Protein concentrations were determined using the Bradford assay (Bio Rad Laboratories) with bovine gamma globulin used as a standard [45].

2.10. APEX1 immunohistochemistry on wild-type and hAPEX1 transgenic mice sections

Paraffin sections were prepared from testes obtained from wild-type and *hAPEX1* transgenic mice that were fixed in 10% formalin. Sections were rehydrated in each of the following solutions, in this order: 100% xylene, 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol. For antigen retrieval, slides were submerged in 1 M Tris-HCl, pH 9.5, and micro waved. 3% H₂O₂ was used as a blocking reagent. A Vectastain kit (Vector Laboratories) was used (following manufacturer's instructions) to block and probe for APEX1. The kit used corresponded to the species the primary antibody was made in. Rabbit polyclonal anti-APEX1 antibody (Novus Biologicals) was prepared in 1× PBS with 0.1% immunohistochemistry grade BSA (Sigma). Color was developed, using peroxidase substrate solution (DAB) (Vector Laboratories). Hematoxylin (Sigma) was used to counter-stain. Sections were de-hydrated using the opposite order of solutions for re-hydration. A coverslip with mounting medium, xylene-based cyto seal resin (ThermoScientific) was added.

2.11. Big Blue[®] mutant frequency assays

For Big Blue[®] Rat Mutant Frequency Assays, cells were grown to 80% confluence and harvested by trypsin/EDTA as previously described [46]. Cells were pelleted by centrifugation at $1200 \times g$ for 4 min at 4 °C, then rinsed with ice cold DPBS. High molecular weight DNA was isolated utilizing RecoverEase[®] DNA isolation kit (Agilent Technologies) per the manufacturer's protocol. For Big Blue[®] Mouse Mutant Frequency Assays, GC pellets were homogenized by pipetting up and down in lysis buffer until homogenous. The homogenate was incubated with proteinase K for 3 h, otherwise the aforementioned protocol was followed.

The *lacI* gene was recovered by packaging the DNA with Transpack[®] Packaging Extracts (Agilent Technologies) 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-3-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 [29]. Mutant frequency was calculated by the ratio of confirmed mutants to the total number of plaque-forming units (PFUs). Two independent biological replicates and two technical replicates of each biological sample were performed for the cell lines. More than 3.5×10^5 PFUs were analyzed from each cell line to determine spontaneous mutant frequency and more than 4.5×10^5 PFUs were analyzed from each cell line to determine induced mutant frequencies. For the *in vivo* studies, an $n = 5$ mice and greater than 1.6×10^6 PFUs were analyzed from each transgenic and control mouse cohort for each age group.

2.12. TUNEL assay for apoptosis

Testes harvested from young, middle-age and old mice were fixed in 10% formalin solution, paraffin embedded, and sectioned at 4 μ m; these sections were then processed for TUNEL (Terminal Deoxynucleotidyltransferase-Mediated dUTP-Biotin Nick End Labeling) staining [47] and for hematoxylin and eosin staining by the Cancer Therapy and Research Center 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 for each depth and each animal. For each age and genotype, between 120 and 450 tubule cross-sections were examined for apoptotic cells. The total numbers of tubule cross-sections examined were as follows: young wild-type = 2469, young *hAPEX1* transgenic = 3898, middle-age wild-type = 1195, middle-age *hAPEX1* transgenic = 3595, old wild-type = 3311, old *hAPEX1* transgenic = 3824. The percentages of apoptotic (TUNEL-positive) spermatogonia and pachytene spermatocytes were also quantified in seminiferous tubule cross-sections; these cell types were distinguished using the criteria of size, position relative to the tubule lumen, and morphology [44].

2.13. Statistical analysis

Analysis of variance (ANOVA) was used to analyze APEX1 activity data. Comparisons among means were Bonferroni adjusted. Numbers of mutants in the Big Blue[®] assays were described by Poisson random variables. Mutant frequencies between treatments and lines were compared using the χ^2 test.

3. Results

3.1. Characterization of stably transfected cell lines

The rat fetal fibroblast cells were transfected with pPAP and stable clones were selected with puromycin. Clones successfully transfected with pPAP, the *mApex1* expression vector (Fig. 1), were then identified by the presence of a 1 kb band through Southern blot analysis (Fig. 2). Additional hybridizing bands are typical of multiple copy and multiple site integrations of the expression vector. Two clones, Pap 2a and Pap 6a, were selected for further analysis. Subcellular localization studies were performed to determine if there were differences in the distribution of APEX1 in transfected clones compared to control cells (Fig. 3). Distribution of endogenous APEX1 is largely cytoplasmic with a concentrated staining surrounding the nucleus. Pap 2a displays a largely cytoplasmic distribution of APEX1, but the distribution in the cytoplasm is not as concentrated around the nucleus as for Pur 1, the transfection control line. For clone Pap 6a immunofluorescence detection of APEX1 revealed a strong concentration of staining around the nucleus and also a broader cytoplasmic distribution.

3.2. APEX1 activity assays on transfected clonal lines

APEX1 activity assays were performed to quantify the APEX1 incision activity in the *mApex1* transfected clones, fibroblasts from *mApex1* heterozygous mice and fibroblasts

from C57BL/6 wild-type mice. A 74% reduction in APEX1 activity was observed in whole cell extracts obtained from *mApex1* heterozygous fibroblasts compared to extracts from wild-type fibroblasts (Fig. 4). The APEX1 activity was found to be similar among rat fibroblast whole cell extracts prepared from the transfected control cell line carrying only the puromycin resistance gene, Pur 1, and wild-type cells. Whole cell extracts prepared from fibroblast transfected lines, Pap 2a and Pap 6a, demonstrated significant 25% and 32% increases, respectively, in APEX1 activity compared to Pur 1. Upon northern blot analysis, Pap 2a and Pap 6a displayed low to modest over-expression of *Apex1* RNA (Fig. 5).

3.3. Mutant frequency analyses on transfected cell lines

LacI mutation assays were performed to determine if the spontaneous mutant frequencies were affected by *Apex1* over-expression (Fig. 6). Total PFU's scored were 492, 910 PFU's for Pur1, 1057, 354 PFU's for Pap 2a and 358, 359 PFU's for Pap 6a. Pur 1 displayed a spontaneous mutant frequency of 2.2×10^{-4} , while Pap 2a and Pap 6a displayed significantly lower mutant frequencies of 1.0×10^{-4} and 0.5×10^{-4} , respectively.

To test the mutagenic response of *Apex1* over-expressing cells to a monofunctional alkylating agent, cells were treated with 5 mM methyl methanesulfonate (MMS). Total PFU's scored were 451, 351 PFU's for Pur1, 820, 292 PFU's for Pap 2a and 770,749 PFU's for Pap 6a. The transfected control line Pur 1 mutant frequency increased to 3.7×10^{-4} in response to MMS treatment. Pap 2a and Pap 6a mutant frequencies increased to 3.0×10^{-4} and 2.3×10^{-4} , respectively, after MMS treatment, but were significantly lower than MMS-treated Pur 1 (Fig. 6). Despite the overall lower mutant frequency after MMS treatment for Pap2a and Pap6a, the absolute increase in mutant frequency was similar to that for Pur 1.

To test the mutagenic response of *Apex1* over-expressing cells to ionizing radiation, cells were treated with 500 cGy. Total PFU's scored were 513,537 PFU's for Pur1, 691,059 PFU's for Pap 2a and 646,650 PFU's for Pap 6a. Mutant frequencies were similar for clones Pur 1 and Pap 2a at 2.0×10^{-4} . A significantly lower mutant frequency of 0.9×10^{-4} was observed for Pap 6a, compared with Pur 1 and Pap 2a (Fig. 6). A significant difference was observed for each treatment group compared to untreated controls in all cell lines, except for Pur 1, for which no difference was observed between spontaneous mutant frequency and IR-induced mutant frequency. MMS-treated cells displayed greater mutant frequencies than IR-treated cells.

3.4. APEX1 activity assays on spermatogenic cells from hAPEX1transgenic mice

APEX1 activity assays were performed to assess the effect of APEX1 over-expression in germ cells obtained from *hAPEX1* transgenic male mice at 6-months-old and 28-months-old (old). APEX1 activity was elevated 15% in germ cells obtained from young *hAPEX1* transgenic mice, compared to controls of the same age. APEX1 activity levels were similar among *hAPEX1* transgenic and control mice at 28-months-old (Fig. 7), but only 2 mice for each age and genotype were available for this assay, due to natural end of life losses that occur at old age.

3.5. Subcellular distribution of APE1 does not change in hAPEX1 transgenic mice

To determine if over-expression of APE1 resulted in changes in subcellular distribution, immunohistochemistry was performed on testes sections obtained from wild-type and hAPEX1 transgenic mice (Fig. 8). APEX1 was detected largely in nuclei with modest cytoplasmic staining in testis sections.

3.6. Age-dependent changes in germ cell apoptosis in hAPEX1 transgenic mice

To determine if increased APEX1 activity affects the amount of germline apoptosis, TUNEL assays were performed on testes sections obtained from 6-months-old, 16-months-old and 26-months-old mice (Fig. 9). Adjacent testes cross-sections were stained with H&E. There was an approximate 1.3-fold increase in the percentage of seminiferous tubule cross sections with apoptotic cells in 26-months-old compared with 6-month-old mice, regardless of genotype ($P = 0.05$) (Fig. 9A). The percentage of seminiferous tubule cross sections with apoptotic cells in the hAPEX1 transgenic over-expressing males was similar to the control males at all age groups. In 6-month-old hAPEX1 transgenic mice, there were 31.2% seminiferous tubule cross sections with apoptotic cells, compared to 26.5% in control mice of the same age. At 16-months-old, hAPEX1 transgenic mice had 25.2% seminiferous tubule cross sections with apoptotic cells compared to 25.0% observed in control mice of the same age. hAPEX1 transgenic mice had 40.9% seminiferous tubule cross sections with apoptotic cells at 26-months-old, compared to 35.3% in control mice of the same age.

The ratio of apoptotic spermatogonia to seminiferous tubule cross section was also analyzed (Fig. 9B). The number of apoptotic spermatogonia per seminiferous tubule significantly increased by 1.6-fold in the 26-month-old wild-type males compared with that in 16-month-old mice. In the 26-month-old hAPEX1 transgenic mice, the number of apoptotic spermatogonia per tubule cross-section increased significantly by 2.3-fold, compared to transgenic 16-month-old mice. There was also a 1.5-fold significant increase in the number of apoptotic spermatogonia per seminiferous tubule cross section in the 26-month-old hAPEX1 transgenic mice, compared to control mice of the same age.

The ratio of apoptotic pachytene spermatocytes per seminiferous tubule cross section was significantly different at 26-months-old (Fig. 9C). There was a 3-fold increase in this ratio in 26-month-old hAPEX1 transgenic mice, compared to 6-month-old mice of the same genotype. Twenty-six-month-old wild-type mice displayed a significant 2.7-fold increase in the apoptotic pachytene spermatocyte ratio compared to 6-month-old wild-type mice. hAPEX1 old transgenic mice displayed a 3.5-fold increase and old control mice displayed a 2.1-fold increase in apoptotic pachytene spermatocytes compared to middle-age mice of the same genotype.

3.7. Spontaneous mutant frequencies in spermatogenic cells from hAPEX1 transgenic mice

The *lacI* mutant frequency assay was performed using spermatogenic cells from mice at various ages either carrying or not the hAPEX1 transgene to determine if APEX1 overexpression would prevent the age-associated increase in germline mutagenesis (Fig. 10). Total PFU's scored for 6 months of age were 1579,933 PFU's for control *lacI* and 1626,160

PFU's for hAPEX1 transgenic mice. Total PFU's scored for 16 months of age were 1822,300 PFU's for control *lacI* and 1840,500 PFU's for hAPEX1 transgenic mice. Total PFU's scored for 26 months of age were 2954,680 PFU's for control *lacI* and 3059,700 PFU's for hAPEX1 transgenic mice. In control *lacI* transgenic animals, there was a 1.5-fold significant increase in mutant frequency for cells from 26-month-old mice, compared to 16-month-old mice. However, in 26-month-old hAPEX1 transgenic animals, the mutant frequency remained low and statistically similar to that obtained for 6-month-old mice. While mutant frequency significantly increases to 1.2×10^{-5} with age in germ cells obtained from 26-month-old control *lacI* transgenic mice, the 26-month-old hAPEX1 transgenic mice maintain a low spontaneous mutant frequency at 0.6×10^{-5} . Spontaneous mutant frequencies were similar among hAPEX1 transgenic mice and control *lacI* transgenic mice at 6-months-old and 16-months-old.

4. Discussion

In humans, an increased prevalence of *de novo* mutations in sperm of older fathers is thought to be a major mechanism that results in an increased frequency of offspring with genetic diseases [48,49]. APEX1 appears to be a pivotal enzyme in determining if BER activity is sufficient to ameliorate DNA damage and maintain genetic integrity in both germ and somatic cells. During normal aging, the abundance of APEX1 in mouse spermatogenic cells declines, with a corresponding decrease in BER activity [28]. Mice heterozygous for *Apex1* display elevated mutant frequency in spermatogenic cells as young adults [29,30]. Aging wild-type and *Apex1* heterozygous mice display increasing levels of DNA damage in differentiating spermatogenic cells [30]. It seems reasonable to hypothesize that APEX1 over-expression could protect the male germline from the age-dependent increase in spontaneous mutant frequency.

To test directly if increased APEX1 activity can abrogate the paternal age effect, a two-prong approach was taken. First, we determined the consequences of APEX1 over-expression in cell lines because imbalances in base excision repair can be detrimental [50]. After determining that over-expression of APEX1 protects against spontaneous and induced mutagenesis in cells, we used transgenic mice with over-expression of human APEX1 to directly test whether APEX1 has a fundamental role in the murine paternal age effect of mice.

Initially, two m*Apex1* stable transfectants with low to moderate over-expression were examined *in vitro* for protection against spontaneous and induced mutagenesis. The clones over-expressing m*Apex1* displayed greater APEX1 activity and correspondingly lower spontaneous, MMS-induced and IR-induced mutant frequencies. These results support the hypothesis that an increase in APEX1 level provided better protection against mutagenesis, which is consistent with the generally acknowledged inverse correlation between levels of repair and mutagenesis.

Wild-type and over-expressing clones displayed similar and largely cytoplasmic distribution of APEX1. Cytoplasmic localization of APEX1 may result from the mitochondrial DNA repair role of the protein [51]. Another possible explanation stems from APEX1's

association with endoplasmic reticulum membranes to keep newly synthesized transcription factors in a reduced state while transporting to the nucleus [52,53]. This suggests that the subcellular location of APEX1, which can be found in multiple cellular locations, may have important consequences with regard to nuclear mutagenesis.

Although modest overexpression of APEX1 resulted in predominantly cytoplasmic staining for APEX1 *in vitro*, subcellular localization was largely nuclear in spermatogenic cells when APEX1 was overexpressed *in vivo*. Spermatogenic cells from wild-type mice also displayed mostly nuclear staining. Together, these results suggest location of APEX1 may vary by cell type, consistent with previous reports [51,53]. Importantly these results suggest that modest overexpression of APEX1 does not alter its localization within the cell, which could impact APEX1 function.

After establishing that moderate APEX1 over-expression enhanced genetic integrity in fibroblasts, hAPEX1 transgenic mice were crossed with *lacI* transgenic mice to determine *in vivo* if APEX1 over-expression could protect the male germline against the age-related increase in mutagenesis. Importantly, old hAPEX1 transgenic mice are protected from the age-dependent increase in spontaneous mutant frequency that occurs in the control mice of the same age. To test whether the transgene led to elevated APEX1 activity in germ cells and if it remains elevated at older ages, activity assays were performed. There was a significant increase in APEX1 activity observed in young hAPEX1 transgenic mice, compared to control mice. Unfortunately, the inclusion of old ages is accompanied by loss of animals due to natural end of life and only two old mice, for the hAPEX1 transgenic genotype, were available resulting in inconclusive results for APEX1 activity. The majority of old mice were used for mutant frequency assays and clearly revealed germ cells from hAPEX1 transgenic mice are protected against the age-related increase in spontaneous mutant frequency that was apparent in control mice. The changes observed in spontaneous mutant frequency are likely due to the altered APEX1 abundance.

Cell death is a possible consequence of unrepaired DNA damage and has been shown to play a role in regulating germline mutant frequency [54,55]. Apoptosis can remove cells with extensive DNA damage and thus aid in maintaining a low mutant frequency. Apoptosis was significantly elevated among spermatogonia in hAPEX1 transgenic mice at old age. Thus, it appears that there is a relationship in the germline between APEX1 expression, apoptosis and mutagenesis with increased APEX1 activity correlating with increased apoptosis among spermatogonia and a decreased mutant frequency among germ cells. This correlation suggests that spermatogonial cell apoptosis is a mechanism for preventing age-dependent increases in spontaneous mutant frequency.

The redox function of APEX1 has been shown to be a potent activator of p53 DNA binding activity and the overexpression of APEX1 enhances the ability of p53 to transactivate a number of target promoters, which are involved in apoptosis, such as *Bax* [23]. With the overexpression of APEX1 in germ cells, apoptosis could be triggered through the activation of p53 and its downstream targets. The removal of abnormal cells, harboring higher levels of DNA damage, through apoptosis is a possible explanation for the lower mutant frequency observed at old age in the hAPEX1 transgenic mouse spermatogenic cells.

In summary, our results indicate that over-expression of APEX1 *in vitro* can enhance protection against spontaneous and induced mutagenesis. *In vivo* over-expression of APEX1 can protect against the age-dependent increase in mutant frequency in spermatogenic cells likely through base excision repair. The *in vivo* data also suggest that in the male germline, cell death may be related to APEX1 expression and contribute to the regulation of mutant frequency. Delineating the mechanism/s resulting in decreased APEX1 abundance and activity in spermatogenic cells at old age, may be key to counteracting the paternal age effect.

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Fig. 1. *mApex1* expression vector. The murine *Apex1* (*mApex1*) cDNA was subcloned into pBluescript(r) II SK+ (Stratagene). The murine phosphoglycerol kinase (Pgk-1) promoter directs expression of *Apex1*. Rabbit β-globin exons II and III and intron II were added to potentially enhance expression of *Apex1*.

Fig. 2. Southern blot identification of stable *Apex1* transfectants. Puromycin resistant clones were subjected to Southern blot analysis to identify clones carrying the m*Apex1* expression vector, using radiolabeled m*Apex1* cDNA probe. Top panel: a 1kpb m*Apex1*-hybridizing band indicates the presence of the expression vector. Clone Pur 1, not transfected with m*Apex1* expression vector does not have a 1kbp hybridizing *Apex1* band and serves as a puromycin transfected control cell line. Bottom panel: *Gapdh* hybridization was used to correct for loading and transfer differences among samples. The representative negative control sample

is taken from the same gel, but was separated by clones that did not carry the *ApexI* expression vector. These lanes were removed digitally and the negative control lane placed adjacent to the first sample that displayed hybridization to the probe.

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Fig. 3.

Confocal immunofluorescence detection of APEX1 subcellular localization. Confocal fluorescence images (left) show the distribution of APEX1 (XY) in a single optical section selected from a series of images collected throughout the cell volume. YZ1 and YZ2 depict APEX1 distribution through the depth of the cells at positions XY1 and XY2 respectively. Differential interference contrast (DIC) images (right) show the cell morphology. (A) Detection of endogenous APEX1 in Pur 1 cells transfected with the puromycin resistance plasmid only. (B) APEX1 localization in Pap2a (C) APEX1 localization in clone Pap 6a.

Fig. 4.

APEX1 activity assays. Decreased activity was observed in extracts prepared from Apex1 heterozygous primary fibroblasts compared to all other tested cell lines. A modest increase in activity, $P < 0.05$, was observed in extracts prepared from clones Pap 2a and Pap 6a compared to the transfected control cell line, Pur 1. Data are presented as means $\pm$ SEM and represent 3 independent preparations of whole cell extracts with 2 repetitions of the assay for each preparation. WT = wild-type primary fibroblasts obtained from a C57BL/6J mouse. Het = primary fibroblasts obtained from Apex1 heterozygous mice. BBR = primary fibroblasts obtained from Big Blue Rat[®]. a = significantly different from Het, b = significantly different from Pur 1. Analysis of variance (ANOVA) was used to analyze APEX1 activity data.

Fig. 5.

Clones display varying levels of *Apex1* RNA over-expression. A greater abundance of *Apex1* RNA was observed in multiple clones. Pap 2a and Pap 6a represent low and moderate over-expression of *Apex1* RNA, respectively.

Fig. 6.

A greater mutant frequency was observed in all MMS treated cell lines compared to untreated and IR-treated conditions. A greater mutant frequency was observed in all IR treated cell lines compared to untreated spontaneous controls except for Pur 1, which displayed no significant difference between spontaneous and IR treatments. A minimum of 350,000 PFUs were screened for each cell line and treatment. Error bars represent standard errors. a = significantly different from spontaneous, same cell line; b = significantly different from MMS, same cell line; c = not significantly different from IR, same cell line. Cross-hatched bars = Pur 1, white bars = Pap 2a, diagonal bars = Pap 6a.

Fig. 7.

Changes in APEX1 activity with age in mixed germ cells. APEX1 activity is increased in GCs isolated from hAPEX1 transgenic mice. GC extracts were prepared from four transgenic 6-months-old, three non-transgenic 6-months-old wild-type, two transgenic 28-months-old and three non-transgenic 28-months-old wild-type mice. APEX1 activity is plotted as percent incision of the oligonucleotide containing the synthetic AP site relative to wild-type activity for each age. Black bars represent wild-type; white bars represent transgenic. Error bars represent standard error of the mean. a = significantly different from WT, same age, $P < 0.01$.

Fig. 8. hAPEX1 overexpression in mice does not alter the overall subcellular distribution pattern of the APEX1 protein. APEX1 immunohistochemistry was performed on testes sections prepared from four transgenic 6-months-old and three non-transgenic 6-months-old wild-type mice. Predominantly nuclear staining for APEX1 is detected in transgenic and wild-type mice. Upon greater magnification, APEX1 is also detected largely in nuclei (insets).

Fig. 9.

(A) The percent of seminiferous tubule cross sections containing apoptotic cells was determined for each genotype. The fraction of tubule cross sections containing apoptotic cells is increased in transgenic and wild-type animals with increasing age. a = significantly different from 6-months-old, same genotype; b = significantly different from 16-months-old, same genotype. Wild-type are black bars, hAPEX1 transgenic are white bars. (B) The number of TUNEL positive spermatogonial cells increased with increasing age in transgenic and wild-type mice. There are significantly more TUNEL positive spermatogonial cells in

the 26-months-old *hAPEXI* testes compared to the 26-months-old wild-type revealing a greater level of apoptosis in this cell type at old age. Wild-type are black bars, *hAPEXI* transgenic are white bars. (C) Both non-transgenic wild-type testes and *hAPEXI* transgenic testes demonstrated an age-dependent increased in TUNEL positive pachytene spermatocytes. Error bars represent standard errors. Wild-type are black bars, *hAPEXI* transgenic are white bars. a = significantly different from 6-months-old, same genotype; b = significantly different from 16-months-old, same genotype. c = significantly different from WT, same age.

Fig. 10.

Spontaneous mutant frequencies for GCs obtained from control *lacI* and *hAPEXI* transgenic mice. Mutant frequencies were determined for the *lacI* gene carried in mixed germ cells isolated from control *lacI* and *hAPEXI* transgenic mice, at 6, 16, and 26 months of age. The mutant frequency is significantly increased in cells obtained from 26-month-old control *lacI* transgenic mice, but remains low in cells obtained from old *hAPEXI* transgenic mice. Control *lacI* transgenic are black bars, *hAPEXI* transgenic are white bars. a = significantly different from control *lacI* transgenic at 26-months-old. b = significantly different from 16-months-old of the same genotype.