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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], Christi A. Walter[a,b,h,i,*]

[a] South Texas Veteran's Health Care System, 7400 Merton Minter Blvd., San Antonio, TX 78229, USA
[b] Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA
[c] Duke Cancer Institute, Duke University, 20 Duke Medicine Circle, Durham, NC 27710, USA
[d] Cell & Tissue Imaging Center, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA
[e] Department of Radiation Oncology, Houston Methodist Research Institute, 6550 Fannin Street, Houston, TX 77030, USA
[f] Graduate Center for Toxicology, 1095 V.A. Drive, Lexington, KY 40536, USA
[g] Department of Pathology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA
[h] Cancer Therapy & Research Center, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA
[i] Barshop 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

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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 endonuclease 1 (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 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.

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]. Abasic 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]. Abasic 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).

* 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.
E-mail address: walter@uthscsa.edu (C.A. Walter).
[1] Co-first authorship.

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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-κB, HIF-1α 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 subcloned 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 (*mPgk-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, Palo Alto, 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 μg/ml) 48 h after transfection. Puromycin resistant clones were collected, expanded and tested for the presence of the *Apex1* expression vector by Southern

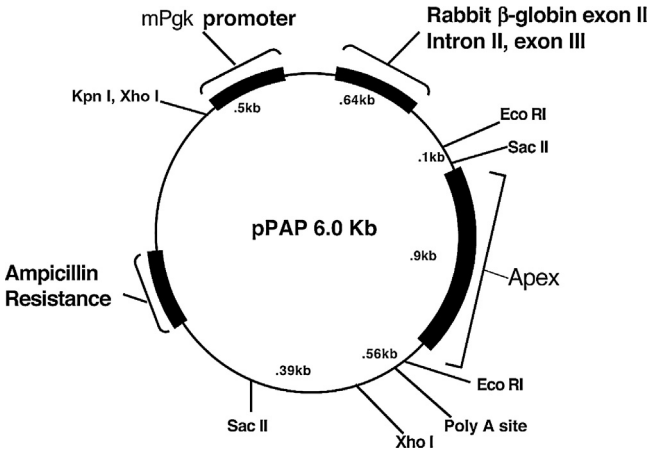

Fig. 1. m*Apex1* expression vector. The murine *Apex1* (m*Apex1*) cDNA was subcloned into pBluescript(r) II SK+ (Stratagene). The murine phosphoglycerol kinase (Pgk-1) promoter directs expression of *Apex1*. Rabbit β-globin exons II and III and intron II were added to potentially enhance expression of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

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% CO_2 and10 μg/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 KH_2PO_4, 0.138 M NaCl, 8.10 mM Na_2HPO_4-$7H_2O$), 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 H_2O. Ten micrograms of DNA were digested with EcoR I restriction endonuclease. Complete digestion was confirmed by subjecting a small aliquot to electrophoresis and visualized using ethidium bromide (EtBr) and UV light. Afterward, 10ug 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 Na_2HPO_4 pH 7.2, 7% SDS for 30 min at 65 °C. Murine *Apex1* cDNA was radioactively labeled with α [^{32}P] 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 Na_2HPO_4 pH 7.2, 7% SDS. After hybridization the membrane was washed twice in 20 mM Na_2HPO_4, pH 7.2, 5% SDS, twice in 20 mM Na_2HPO_4, 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