Mitochondrial DNA Integrity Is Maintained by APE1 in Carcinogen-Induced Colorectal Cancer

Joan Ballista-Hernández1, Margaly Martínez-Ferrer2, Roman Vélez3, Consuelo Climent3, María M. Sánchez-Vázquez2, Ceidy Torres4, Adlin Rodríguez-Muñoz5, Sylvette Ayala-Peña1, and Carlos A. Torres-Ramos5

Abstract
Changes in mitochondrial DNA (mtDNA) integrity have been reported in many cancers; however, the contribution of mtDNA integrity to tumorigenesis is not well understood. We used a transgenic mouse model that is haploinsufficient for the apurinic/apyrimidinic endonuclease 1 (Apex1+/−) gene, which encodes the base excision repair (BER) enzyme APE1, to determine its role in protecting mtDNA from the effects of azoxymethane (AOM), a carcinogen used to induce colorectal cancer. Repair kinetics of AOM-induced mtDNA damage was evaluated using qPCR after a single AOM dose and a significant induction in mtDNA lesions in colonic crypts from both wild-type (WT) and Apex1+/− animals were observed. However, Apex1+/− mice had slower repair kinetics in addition to decreased mtDNA abundance. Tumors were also induced using multiple AOM doses, and both WT and Apex1+/− animals exhibited significant loss in mtDNA abundance. Surprisingly, no major differences in mtDNA lesions were observed in tumors from WT and Apex1+/− animals, whereas a significant increase in nuclear DNA lesions was detected in tumors from Apex1+/− mice. Finally, tumors from Apex1+/− mice displayed an increased proliferative index and histologic abnormalities. Taken together, these results demonstrate that APE1 is important for preventing changes in mtDNA integrity during AOM-induced colorectal cancer.

Implications: AOM, a colorectal cancer carcinogen, generates damage to the mitochondrial genome, and the BER enzyme APE1 is required to maintain its integrity. Mol Cancer Res; 15(7): 831–41. ©2017 AACR.

Introduction
Colorectal cancer is a global health problem causing significant morbidity and mortality. The Surveillance, Epidemiology, and End Results Program (seer.cancer.gov) estimates that in 2016, there will be in the United States 134,490 new colorectal cancer cases and 49,190 colorectal cancer–related deaths. Thus, a great deal of effort has been invested in animal models of colorectal cancer to help identify molecular targets for pharmacologic interventions.

Studies using mouse models of colorectal cancer have helped elucidate molecular mechanisms underlying tumor initiation and promotion (1). A commonly used mouse model is based in exposure to the carcinogen azoxymethane (AOM; ref. 2). AOM induces mutagenic lesions within target cells by alkylating DNA primarily at positions N7 of guanine (N7MeG) and N3 of adenine (N3MeA) and to a lesser extent, at position O6 of guanine (O6MeG; ref. 3). O6MeG is primarily repaired by the suicide enzyme O6-methylguanine-DNA methyltransferase (MGMT; ref. 4), whereas the above-mentioned N-alkylated bases are repaired by the base excision repair (BER) pathway. BER is a multistep process that repairs damage induced by reactive oxygen species and alkylating agents, which involves the coordinate actions of glycosylases, endonucleases, and other end-trimming enzymes, DNA polymerases, and DNA ligases (5).

Within BER, the apurinic/apyrimidinic endonuclease 1 (APE1) is the major AP endonuclease. APE1 is a multifunctional enzyme involved in DNA repair, transcriptional regulation, and redox signaling. APE1 is normally localized in the cytoplasm, and upon NADPH-dependent ROS generation, it mobilizes to the cell nucleus (6). In addition, an N-terminal truncation of the full-length APE1 translocates to the mitochondria in response to oxidative stress (7).

Mice carrying a homozygous-null mutation in the Apex1 gene (which encodes APE1) are embryonically lethal, indicating that APE1 is necessary for normal embryonic development (8). In contrast, Apex1 heterozygous mice (Apex1+/−) are viable and have a normal lifespan, although these mice present increased markers of oxidative stress. In addition, Apex1+/− mice show enhanced mutagenesis in liver, spleen, and germ cells (9, 10). Also, Apex1+/− mice exhibit an age-dependent accumulation of mitochondrial DNA (mtDNA) lesions in germ cells as compared with WT mice, suggesting that APE1 may play a role in preventing mtDNA damage during aging (10).

MtDNA mutations, deletions, and copy-number alterations are associated with cancer (11). Among colorectal cancer, loss of...
mtDNA has been identified in the form of missense mutations, chain terminations, and frame shift mutations (12). In addition, changes in mtDNA copy number have been reported in colorectal cancer (13, 14). However, how these mtDNA changes contribute to carcinogenesis is not totally understood. Our study seeks to determine the contribution of APE1 in preventing nuclear and mtDNA alterations that could drive the tumorigenic process. We hypothesize that the effects of AOM treatment are more pronounced in the Apex1+/− mice than in WT mice due to decreased nuclear and mtDNA repair capacity. To test this hypothesis, we measured changes in mtDNA abundance, and mtDNA and nDNA damage in colonic crypts after AOM treatment. We also determined the characteristics of AOM-induced tumors. By focusing in APE1, we show that BER is important for the repair of AOM-induced mtDNA and nDNA damage and for the subsequent development of specific tumor characteristics.

**Materials and Methods**

**Animals**

Six-month-old WT and Apex1+/− in the C57BL/6J background were used in this study (8). The original breeding pair in our mouse colony was kindly provided by Dr. Christi Walter (University of Texas Health Science Center at San Antonio). Mice were housed 2 per cage with a 12-hour light-dark cycle and *ad libitum* access to food and water. Genotyping analysis was performed collecting 5 mm of tissue from the tail from each mouse at 21 days of birth. DNA was extracted from tail samples following the protocol from Manual Archive Pure DNA purification (Archive-Pure Trademark of 5 PRIME). PCR was performed using a set of primers that recognize a Neomycin resistance transgene present only in the Apex1 knockout mice, as previously described (8, 9). The University of Puerto Rico Medical Sciences Campus Institutional Animal Care and Use Committee approved the studies.

**AOM treatment**

WT and Apex1+/− mice were randomized into two groups: AOM (Sigma-Aldrich Chemical) treated or saline treated. To measure AOM-induced DNA damage and repair kinetics, mice were injected intraperitoneally with a single dose of AOM (10 mg/kg of body weight) and sacrificed 24, 48, and 72 hours after treatment. Colonic crypts were isolated followed by the isolation of genomic DNA as described below.

To study AOM-induced tumor formation, mice were treated with 10 mg/kg AOM, once a week during 4 weeks as previously reported (15). Six months after the first AOM injection, colon tissues were removed, cut longitudinally, and examined for tumor incidence (percentage of animals with tumors), multiplicity (average number of tumors per mouse), and tumor volume. Tumor volume (expressed in mm3) was determined by measuring the length, width, and height of each tumor with a caliper and then obtaining the product of these dimensions.

**Isolation of colonic crypts**

After euthanization of mice by cervical dislocation, the abdomen was opened followed by colon removal. The colon was cut along its length and rinsed with ice-cold PBS. The colon was then incubated in a 15 mL tube containing 1X (PBS), 1.5 mmol/L ethylenediaminetetraacetic acid, and 0.5 mmol/L of dithiothreitol for 90 minutes at room temperature. The incubation buffer was replaced with 1 mL 1X PBS, and the sample was vortexed for 30 seconds to obtain the crypts. The crypt suspension was transferred to a fresh tube, washed 2 times with 1 mL 1X PBS, and pelleted by centrifugation at 1,000 rpm for 5 minutes. The supernatant was removed and the crypt containing pellets were stored at −80°C until further processing.

**DNA isolation and quantification of genomic DNA from colonic crypts**

DNA isolation was performed using a high molecular weight genomic DNA purification kit as described by the manufacturer (Qiagen). DNA was quantitated using the PicoGreen dsDNA quantitation assay (Molecular Probes/Life Technologies) using a microplate reader (Wallac 1420 VICTOR) with a 535 nm excitation filter and a 485 nm emission filter. A standard curve with a Lambda DNA was constructed in order to determine the correct DNA concentration of the samples. Before performing the DNA damage analysis, DNA samples (100 ng) were visualized by 1% agarose gel electrophoresis, stained with ethidium bromide to ensure that no degradation occurred during the isolation procedure.

**Detection of DNA damage and mtDNA abundance using the qPCR assay**

Levels of DNA lesions (nDNA and mtDNA) and mtDNA abundance in AOM-treated and saline-treated WT and Apex1+/− mice were measured by qPCR as described previously (16). The rationale of using the qPCR assay for the quantification of lesions is based on the ability of various DNA lesions to act as blocks to the action of the PCR polymerase, resulting in decreased amplification of the template. The qPCR assay detects alkylation lesions as well as oxidative DNA damage such as AP sites, strand breaks, and thymine glycol, all of which block the movement of the thermostable polymerase along the DNA template. The PCR amplification was performed using the Master Amp XL Polymerase (Epicentre). Prior to qPCR analysis, we performed a cycle test to determine the optimal number of amplification cycles and a template test to determine the optimal initial DNA concentration (data not shown).

The mouse mitochondrial (10 kb) and nuclear (6.9 kb) fragments were amplified using an initial denaturation for 45 seconds at 94°C, followed by 22 cycles (mtDNA amplicon) and 28 cycles (nDNA amplicon) of denaturation for 15 seconds at 94°C, and annealing/extention at 64°C and 66°C for 12 minutes, respectively. A final extension at 72°C was performed for 10 minutes at the completion of the profile. The primer nucleotide sequences used for the amplification of the 10 kb mtDNA fragment were the following: 5′-CCA GTC CAT GCA GGA GCA TC-3′ (5733 sense; relative to sequence NC_005089) and 5′-CGA GAA GAG GGG (15733 antisense). The primer nucleotide sequences used for the amplification of a 6.9 kb mouse hypoxanthine phosphoribosyltransferase (17) gene fragment were the following: 5′-CCA GCA GGG GCA CCA CGC TGG TG-3′ (16246 antisense) and 5′-TGG GAG GCA GGG ATC TGA AGC-3′ (16246 antisense). We normalized the amplification of the 10 kb mtDNA fragment with the levels of miDNA abundance (see below).

Steady-state levels of mtDNA abundance were measured by qPCR amplification of a small, 116 bp mtDNA fragment. Because the probability of finding a lesion in a small mtDNA fragment is nearly null, changes in the amplification of the small mtDNA fragment provides an accurate measure of the abundance of mtDNA molecules. The PCR amplification profile for the 116 bp
A decrease in the amplification of the 116 bp mtDNA fragment, the 10 kb mtDNA fragment, and the 6.9 kb nDNA fragment were 15, 10, and 7.5 ng, respectively. We amplified a DNA containing 50% of the initial template concentration as a control for amplification; therefore, under optimal amplification conditions, a 50% reduction in the amount of template amplification is expected. We considered qPCR reactions to be within the linear range of DNA amplification if the resulting amplicon was within 40% to 60% amplification. The 10 kb and 6.9 kb PCR products were resolved on 1% agarose gels, whereas the 116 bp PCR products were resolved on 6% polyacrylamide gels. DNA was visualized under UV-light using ethidium bromide and quenched of endogenous peroxidase with 3% v/v H2O2. Primary antibodies diluted in 10% FBS were incubated overnight. Sections were stained for monoclonal mouse anti-β-catenin (1:50; BD Bioscience), monoclonal anti-Ki-67 (1:1,000; Vector Laboratories), monoclonal anti-cytochrome oxidase subunit 1 (COX1; 1:500; Invitrogen), and polyclonal anti-Noxl1 (1:200; Bioss). A biotinylated secondary antibody and an avidin-biotin complex with horseradish peroxidase were used followed by the addition of the chromogen 3,3′-diaminobenzidine (DAB). Finally, slides were counterstained with hematoxylin and observed under a light microscope.

We used quantitative criteria to evaluate the Ki-67–positive staining by visualizing the slides by brown nuclear staining and assessed as the percentage of Ki-67–positive cells per tissue section. The percentage of positive cells was calculated as follows: % positive cells = positive cells staining/ total cells × 100. The strength staining intensity scores for Ki-67 were semi-qualitatively calculated based on a scale of 3 for high intensity, 2 for medium intensity, and 1 for low intensity of Ki-67–positive cell staining. Four different random tissue fields per section were selected from each mouse and counted for Ki-67–positive cells staining and intensity.

The criteria used to evaluate β-catenin, COX1, and NADPH oxidase 1 (NOX1) staining in the colon and tumor sections were made following a semi-quantitative scoring system as previously described (20). For β-catenin analysis, we estimated the proportion of positive cells in the nucleus. For COX1 analysis, we estimated membrane staining. For NOX1 analysis, we estimated the cytoplasmic staining. The semi-quantitative score was made following parameters in a 1 to 4 scale of positive staining: 1 = <25% staining, 2 = 25%–50% staining, 3 = 50%–75% staining, 4 = >75% staining. Four different random tissue fields per section were selected from each mouse and counted for each marker-positive cells staining and intensity. All the immunohistochemical scoring was performed as blinded studies.

**RNA isolation and real-time reverse transcriptase-polymerase chain reaction**

RNA was isolated from mouse colonic crypts using the RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer’s instructions. The RNA was spectrophotometrically quantified and qualified at 260 nm and 280 nm on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). RNA (75 ng) was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer’s instructions. Thermal cycling was performed using a Step One Plus Real-time PCR System (Applied Biosystems). The real-time PCR was performed by using iQ SYBR Green Supermix (Bio-Rad) in a total volume of 10 μL, at 95°C for 15 seconds and 60°C for 1 minute. PCR efficiency was examined by serially diluting the template cDNA, and the melting curve data were collected to check the PCR specificity. An internal loading control of GAPDH expression was used for Apex1 1 gene. Results were quantified using the ΔΔCt method. No PCR product was detected in control samples in which reverse transcriptase was omitted. The PCR primers (IDT) were as follows: glyceraldehyde-hydrophosphate dehydrogenase (GAPDH): antisense, 5′-AATGG-GAGTGGCTCTGTCAGTC-3′ and sense, 5′-CTGCAGCCCGGTCTGGAACAC-3′; Apex1: sense, 5′-TTGGTCCTCTGCTGCTCCGA-3′ and antisense, 5′-GCTCTCTCCGCCCTGTCCTG-3′.

**Statistical analysis**

To determine the statistical significance of the effect of AOM treatment in the number of nDNA and mtDNA lesions and...
mtDNA abundance and for histologic analyses, we made comparisons using one-way ANOVA and Tukey’s honestly significant difference test for post hoc analyses. The independent variables were the treatment groups, and the dependent variables were the lesion number. For tumor incidence, size, and multiplicity, we performed the Person’s $\chi^2$ test with Yates’ continuity correction test. All statistical testing was performed at a pre-set $\alpha$ of 0.05.

**Results**

**Effect of AOM on the levels of mtDNA damage and mtDNA abundance in colonic crypts from WT and \textit{Apex1}^{+/-} mice**

AOM is an alkylating agent widely used for the induction of colorectal cancer in rodents (2, 21). It is well known that this agent induces mutations in nuclear genes; however, little is known about the effects of AOM on mtDNA and how this may lead to colorectal cancer. To define the role of the BER enzyme APE1 on the repair of mtDNA and nDNA damage in WT and \textit{Apex1}^{+/-} mice, we studied the formation and repair of mtDNA and nDNA lesions induced by AOM using qPCR. Mice were administered intraperitoneally with a single dose of AOM (10 mg/kg of body weight), and DNA was isolated from colonic crypts 24, 48, and 72 hours after treatment. In WT mice, we observe a significant 3.3-fold increase in mtDNA lesions 48 hours after AOM treatment; however, the number of lesions was reduced to control levels 72 hours after treatment (Fig. 1A). Similarly, in \textit{Apex1}^{+/-} mice, there was a 2.4-fold increase in mtDNA lesions 48 hours after treatment, but, contrary to WT mice, mtDNA lesions persisted after 72 hours of treatment (Fig. 1B).

Besides qualitative changes in mtDNA (i.e., DNA lesions and mutations), quantitative changes such as mtDNA copy number have been proposed as key determinant factors in
mitochondrial-related pathogenesis (22). We evaluated whether AOM treatment could exert changes in mtDNA abundance in the colonic crypts using QPCR. We observed that WT mice show no significant changes in mtDNA abundance 24, 48, and 72 hours after AOM treatment (Fig. 1C). However, we found a statistically significant 16% decrease in the abundance of mtDNA molecules in Ape1-/- mice 72 hours after AOM treatment (Fig. 1D).

Repair kinetics of AOM-induced nuclear DNA lesions in Ape1-/- mice colonic crypts

We sought to analyze the levels of AOM-induced damage to the nuclear genome in colonic crypts from WT and Ape1-/- mice. We found that WT mice treated with AOM exhibited a significant 27-fold increase in the number of lesions 24 hours after treatment. The lesion number was significantly reduced to levels similar to WT control 48 hours after treatment (Fig. 1E). Similarly, in Ape1-/- mice, lesion number peaked 24 hours after treatment (a 12.8-fold increase over saline-treated mice). In contrast to WT animals, nDNA lesions remained elevated in Ape1-/- mice 48 hours after treatment and returned to control levels 72 hours after treatment (Fig. 1F).

APE1 mRNA levels after AOM treatment

To determine whether AOM treatment results in changes in APE1 mRNA levels, we isolated RNA from colonic crypts and performed reverse transcriptase-PCR (RT-PCR) analysis. We chose to analyze samples 48 hours after AOM treatment because at this time point, lesions in both nDNA and mtDNA were still present (except in nDNA from WT mice). We observe that in WT animals, there is a statistically significant 5.1-fold increase in APE1 mRNA levels 48 hours after AOM treatment. In contrast, in colonic crypts from Ape1-/- mice, there was only a 2.2-fold increase.

Effect of AOM treatment in tumor burden

To analyze the contribution of mtDNA damage in the induction of colorectal cancer, we exposed mice to 10 mg/kg AOM doses (once per week) as described previously (15). Six months after the first AOM treatment, the number of mice-bearing tumors (tumor incidence), tumor number, and tumor size were determined. We observed that 65% of Ape1-/- mice exhibited tumors, all in the distal part of the colon compared with 55% of WT mice (Table 1). Although the percentage of Ape1-/- mice exhibiting tumors is higher than in WT mice, this difference was not statistically significant. Similarly, tumor multiplicity and size were not statistically significant between WT and Ape1-/- mice.

Immunohistochemical characteristics of AOM-induced tumors

Although normal colon tissue from Ape1-/- mice show no significant histopathologic changes, tumors from both WT and Ape1-/- AOM-treated animals show statistically higher values in all the parameters examined (mean number of nuclear/cytoplasmic ratio, epithelial stratification, nuclear dispolarity, goblet depletion, and structural abnormalities) when compared with normal colon (Table 2). Interestingly, a statistically significant increase in epithelial stratification was observed in tumors from Ape1-/- animals compared with tumors from WT mice. This increase in epithelial stratification describes the occurring changes in the thickness of the abnormal epithelium characteristics of dysplasia.

We examined the expression of the proliferation marker Ki-67 in AOM-induced tumors from WT and Ape1-/- mice. Colon from saline-treated WT mice shows 11.7% of Ki-67-positive cells, whereas tumors from WT mice show 25.3% (a 2.2-fold increase; Fig. 2A–C). Colon from saline-treated Ape1-/- mice show 14.5% of Ki-67–positive cells, whereas tumors from Ape1-/- mice show 34.3% (a 2.4-fold increase). A comparison in the number of Ki-67–positive cells between tumors from WT and Ape1-/- mice reveals a statistically significant 1.3-fold increase in the Ape1-/- animals compared with WT. Moreover, determination of the intensity of Ki-67 staining showed that tumors from Ape1-/- mice exhibit a statistically significant 1.6-fold increase compared with WT tumor (Fig. 2A–D).

We analyzed the expression of β-catenin in AOM-induced tumors and observed a 13.1-fold increase in the nuclear staining of this marker in tumors obtained from WT mice compared with colon from saline-treated animals (Fig. 2E and G). Similarly, AOM-induced tumors obtained from Ape1-/- mice exhibit a 12.4-fold increase in β-catenin nuclear staining compared with colon from saline-treated Ape1-/- mice (Fig. 2E and G). No significant differences were observed between β-catenin expressions in AOM-induced tumors from WT and Ape1-/- mice.

Reduced expression in colonic crypts of the mitochondrial-encoded COX1 has been associated to increased colorectal cancer

Table 1. Incidence, multiplicity, and size of AOM-induced tumors in WT and Ape1-/- mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence (%)</th>
<th>Multiplicity</th>
<th>Size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>55</td>
<td>1.36 ± 0.28</td>
<td>24.98 ± 11.03</td>
</tr>
<tr>
<td>Ape1-/-</td>
<td>65</td>
<td>1.54 ± 0.21</td>
<td>21.99 ± 5.32</td>
</tr>
</tbody>
</table>

NOTE: No statistically significant differences were observed between genotypes as determined by Person's χ² with Yates’ continuity correction (n = 18 for WT mice and n = 17 for Ape1-/- mice).

Table 2. Histopathologic analysis of AOM-induced tumors in WT and Ape1-/- mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nuclear/cytoplasmic ratio</th>
<th>Epithelial stratification</th>
<th>Nuclear dispolarity</th>
<th>Goblet depletion</th>
<th>Structural abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT control tissue</td>
<td>0 ± 0.10²</td>
<td>0 ± 0.15</td>
<td>0 ± 0.16</td>
<td>0.17 ± 0.10</td>
<td>0 ± 0.10</td>
</tr>
<tr>
<td>WT tumor</td>
<td>1.8 ± 0.10²</td>
<td>1.6 ± 0.13²</td>
<td>1.6 ± 0.16³</td>
<td>2.0 ± 0.10⁴</td>
<td>1.8 ± 0.10³</td>
</tr>
<tr>
<td>Ape1-/- control tissue</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Ape1-/- tumor</td>
<td>2.0 ± 0.09³</td>
<td>2.0 ± 0.12²</td>
<td>1.8 ± 0.16³</td>
<td>2.0 ± 0.09⁴</td>
<td>2.0 ± 0.09³</td>
</tr>
</tbody>
</table>

NOTE: The grade of histologic abnormality was scored using the following five parameters: (a) nuclear/cytoplasmic ratio(<25%: 0, 25%–50%: 1, >50%: 2); (b) epithelial stratification (none: 0, mild: 1, severe: 2); (c) nuclear dispolarity (none: 0, mild: 1, severe: 2); (d) goblet depletion (null to mild: 0, moderate: 1, severe: 2); (e) structural abnormality (none: 0, mild: 1, severe: 2). Values represented mean ± SEM. One-way ANOVA.

*P < 0.05 compared with WT control tissue group.

**P < 0.05 compared with Ape1-/- tumor group.
Risk (23). AOM-induced tumors from WT show a 39% reduction in COX1 staining when compared with colon from saline-treated WT mice (Fig. 3A and C). Similarly, there is 29% reduction in COX1 staining in Apex1+/− mice tumors when compared with colon from saline-treated Apex1+/− mice (Fig. 3B and C). Interestingly, a comparison between AOM-induced tumors from WT and Apex1+/− mice reveals that tumors from the Apex1+/− mice exhibit a 17% decrease in COX1 staining. Colon from saline-treated Apex1+/− mice shows a 28% reduction in COX1 staining when compared with colon from saline-treated control WT animals.

Damage to mtDNA, nDNA, and mtDNA abundance in tumors from AOM-treated mice

We examined tumor tissues to determine relative abundance of mtDNA molecules, mtDNA damage, and nDNA damage. We observed that tumors from WT and Apex1+/− show a 56% and 44% reduction in mtDNA abundance, respectively, as compared with colon from their respective saline-treated controls (Fig. 4A). No statistically significant differences were observed in mtDNA abundance between AOM-induced tumors from WT and Apex1+/− mice.

When we measured the frequency of mtDNA lesions, we found no significant changes in the levels of mtDNA lesions in tumors from WT mice as compared with colonic tissue from saline-treated WT mice (Fig. 4B). Similarly, no increase in the frequency of mtDNA lesions is observed in tumors from Apex1+/− mice, compared with colonic tissue from saline-treated Apex1+/− mice (Fig. 4B). However, tumors from Apex1+/− mice show a statistically significant 2.1-fold increase in mtDNA damage compared with tumors from WT mice.

We also measured nDNA damage and found no differences in AOM-induced tumors from WT mice as compared with colonic tissue from saline-treated WT animals. In contrast, we observed a significant 47.5-fold increase in the number of nDNA lesions in tumors from Apex1+/− mice as compared with colonic tissue from saline-treated Apex1+/− mice (Fig. 4C). Taken together, these results indicate that AOM-induced tumors show mtDNA depletion and that tumors from Apex1+/− mice exhibit increased burden of nDNA damage.

Expression of NOX1 in tumors from WT and Apex1+/− mice

To examine if damage to the nDNA (Fig. 4C) might be caused by other endogenous sources of ROS besides mitochondria, we turned our attention to NADPH oxidases. NADPH oxidases have been proposed as an important contributor of ROS in cancer (24). To test the hypothesis that nDNA damage was due to the action of NADPH oxidases, we performed IHC analysis to detect the expression of NOX1, which has a perinuclear location and has been suggested to play a role in ROS production in colon cancer (25). We observe abundant expression of NOX1 in normal colonic tissue from both WT and Apex1+/− mice. Moreover, the expression of NOX1 remains elevated in AOM-induced tumors in both WT and Apex1+/− mice (Fig. 5) with approximately 50% to 75% of positive cell staining. However, no statistically significant differences are observed in NOX1 expression between tumors from WT and Apex1+/− mice.
Discussion

Our results show that treatment with a single dose of AOM leads to increased mtDNA damage in both WT and Apex1+/− mice, demonstrating that AOM also targets mitochondria (Fig. 1A and B). WT mice exhibit efficient repair of AOM-induced mtDNA lesions, whereas Apex1+/− mice show delayed repair kinetics. These results indicate that mice haploinsufficient for APE1 (and therefore, their BER capacity is low) have reduced repair kinetics of alkylation damage (or an intermediate BER lesion such as abasic sites) in the mitochondrial genome. In addition to deficient mtDNA damage, we observe that colonic crypts from Apex1+/− mice show decreased mtDNA abundance 72 hours after AOM treatment (Fig. 1D). No changes in mtDNA abundance were observed in colonic crypts from WT animals (Fig. 1C), which is in agreement with previous results showing that treatment of BER-competent mouse embryonic fibroblasts with the alkylating agent methyl methanesulfonate did not lead to mtDNA depletion (26). These results indicate that deficient mtDNA repair may lead to decreased mtDNA abundance. Persistent mtDNA damage and/or decreased mtDNA abundance may result in deficient mitochondrial bioenergetics and energy metabolism, thus contributing to carcinogenesis (27). This idea is supported by experiments performed in animals carrying simultaneous heterozygous mutations in Tfam (Tfam+/−) and Apc (ApcΔmin+/−), which showed increased tumorigenesis indicating that the mtDNA deletion phenotype observed in Tfam+/− mice modifies the tumorigenic phenotype of ApcΔmin+/− mice (28).

In our study, both WT and Apex1+/− mice exhibit faster kinetics of nDNA damage compared with mtDNA since the peak of nDNA lesions occurred 24 hours after AOM treatment (Fig. 1E and F) versus 48 hours in the mtDNA (Fig. 1A and B). This faster DNA repair kinetics in the nucleus is consistent with studies measuring O6-MeG, an alkylating lesion which is completely repaired 48 hours after AOM treatment, but it is not detected by the qPCR assay employed in our study (29). We speculate that this slower mtDNA damage kinetics could be due to a slower AOM distribution inside the mitochondria as compared with the nucleus. In addition to the fast kinetics of AOM-induced nDNA damage, there was fast and efficient repair kinetics in colonic crypts from WT mice (Fig. 1E). This could be due to AOM-induced upregulation of the Apex1 gene in the distal colon as part of a DNA damage response (30). Indeed, our RT-PCR analyses of APE1 mRNA expression show that there is a strong AOM-dependent induction in APE1 mRNA levels in colonic crypts from WT mice (Fig. 1G). This DNA damage response to AOM is blunt in colonic crypts from Apex1+/− mice (Fig. 1H). In addition, efficient DNA repair in the nucleus can result from the overlapping DNA repair pathways (BER, nucleotide excision repair and recombination) that can act on the repair of abasic sites, an intermediate during the repair of AOM-induced lesions (31–33). Nevertheless, APE1 haploinsufficiency results in delayed repair of both AOM-induced nDNA and mtDNA lesions. The partial repair activity observed in the Apex1+/− mice could be attributed to the functional Apex1 allele in these animals. Altogether, these results suggest
that the APE1 haploinsufficiency sensitizes both mtDNA and nDNA to alkylating agents.

To analyze how deficient BER may affect the development of colorectal cancer, we induced colorectal cancer with multiple AOM doses as previously reported (15). No major differences between AOM-induced tumors from WT and Apex1+/− mice were observed. Although tumor incidence was higher in the Apex1+/− mice (65% vs. 55%), this difference was not statistically significant (Table 1). An important consideration is the genetic background of the animals that were used in this study. We conducted our studies in the C57BL/6 genetic background, which is not as sensitive as the A/J or SWR/J backgrounds (15). Thus, it is possible that a more permissive genetic background is needed in order to detect statistically significant differences in tumor formation between WT and Apex1+/− mice.

When we analyzed the histologic characteristics of colon and tumor tissues in WT and Apex1+/− mice, we observed that tumors of Apex1+/− mice present a higher structural abnormality score and that these tumors were less differentiated than WT tumor tissues (Table 2). Moreover, we observed that
AOM-induced tumors from Apc<sup>fl/fl</sup> mice presented increased number and staining intensity of Ki-67-positive cells as compared with tumors from WT mice (Fig. 2A–C). This observation indicates that AOM-induced tumors in Apc<sup>fl/fl</sup> mice are poorly differentiated and have a higher risk of becoming metastatic, particularly to liver tissue (34–36). To answer this question, further studies are needed in which tumors are allowed to progress into the metastatic stage.

The role of Wnt/β-catenin signaling pathway in colorectal cancer is well documented (37). Our studies show that AOM-induced tumors from both WT and Apc<sup>fl/fl</sup> mice exhibit increased levels of β-catenin staining, confirming that the nuclear DNA paradigm for colorectal cancer is prominent in the AOM-induced colorectal cancer model. However, it is interesting to note that even though the β-catenin levels are similar between tumors from WT and Apc<sup>fl/fl</sup> mice, tumors from Apc<sup>fl/fl</sup> mice exhibit increased expression of the cell proliferation marker, Ki-67. These results indicate that, besides the well-established effects mediated by the Wnt/β-catenin, other factors could be influencing the expression of cell proliferation markers such as Ki-67.

Studies have shown that mitochondrial dysfunction is present in cancer cells and contribute to the process of carcinogenesis (38). Mitochondrial dysfunction could result from mutations in genes encoding proteins that participate in the electron transport chain. For example, mutations in the mitochondria-encoded COX1 result in reduced cytochrome c oxidase activity, decreased mitochondrial respiration, and increased apoptotic resistance, processes that contribute to colorectal cancer (23, 33–36). We analyzed the immunohistochemical expression of COX1 in AOM-induced tumors from WT and Apc<sup>fl/fl</sup> mice and observed that there is a significant loss of COX1 expression when compared with their respective control colon tissue (Fig. 3). Furthermore, the loss of COX1 expression in AOM-induced tumors from Apc<sup>fl/fl</sup> mice is more pronounced than in WT mice. Even control saline-treated tissue from Apc<sup>fl/fl</sup> mice shows a significant reduction in COX1 when compared with control saline tissue from WT animals. These results indicate that APE1 is required for the prevention of mtDNA mutations in COX1 and that reduced APE1 levels lead to increase susceptibility to AOM-induced mutations in the mtDNA. The significance of these observations is that COX1 mutations have been shown to result in increased ROS generation and increased tumor growth potential in a prostate cancer model (41). This supports the hypothesis that mtDNA mutations contribute to colorectal cancer by generating ROS that could generate a positive cell growth signal and/or that can influence further damage to the mitochondrial and the nuclear genome, thus favoring tumorigenesis (42). In support of this notion, Apc<sup>min</sup> mice carrying a transgene which targets catalase to mitochondria results in decreased intestinal polyposis (28). Although the results presented in this study point to a positive correlation between mtDNA damage/repair and tumorigenesis, further studies are needed to determine if mtDNA mutations cause colorectal cancer.

APE1-dependent Repair of AOM-Induced mtDNA Damage
damage only in tumors from Ape1−/− mice, these results suggest that reduced levels of APE1, which results in deficient BER, sensitize nDNA to the effects of ROS within the tumor microenvironment. Studies performed in an inflammation-induced colorectal cancer model show that another BER enzyme, alkyladenine glycosylase (AAG), prevents DNA damage and tumorigenesis (49). Two other DNA glycosylases with overlapping DNA damage specificity with AAG, ALKBH2, and ALKBH3 also protect against inflammation-induced colorectal cancer (50). Thus, our studies in APE1 haploinsufficient mice support the notion that colorectal cancer involves an inflammatory microenvironment that promotes DNA damage.

In summary, we show that diminished DNA repair capacity due to APE1 haploinsufficiency in Ape1−/− mice leads to increased nDNA and mtDNA lesions in colonic crypts after acute AOM treatment. In addition, we demonstrate that AOM-treated and Ape1−/− mice show a time-dependent decrease in levels of mtDNA abundance. Moreover, these changes in mtDNA abundance become more profound in AOM-induced tumors in both WT and Ape1−/− mice. AOM-induced tumors from Ape1−/− mice display increased markers of dysplasia and cell proliferation such as epithelial stratification and Ki-67 staining compared with tumors from WT animals. Finally, we observe that levels of nDNA damage in AOM-induced tumors from Ape1−/− mice are higher compared with tumors from WT mice. A possible source of nDNA damage in these tumors could be the NADPH oxidase isoform, NOX1. Thus, Ape1 haploinsufficiency leads to increased sensitivity of nDNA to the deleterious effects of ROS produced within the tumor microenvironment. We conclude that AOM-induced colorectal cancer involves both nDNA and mtDNA damage and highlight the importance of BER in maintaining mtDNA integrity during carcinogenesis.

References
Mitochondrial DNA Integrity Is Maintained by APE1 in Carcinogen-Induced Colorectal Cancer


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