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Suppression of oxidative phosphorylation in mouse embryonic fibroblast cells deficient in apurinic/aprimidinic endonuclease

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Abstract

The mammalian apurinic/aprimidinic (AP) endonuclease 1 (APE1) is an essential DNA repair/gene regulatory protein. Decrease of APE1 in cells by inducible shRNA knockdown or by conditional gene knockout caused apoptosis. Here we succeeded in establishing a unique mouse embryonic fibroblast (MEF) line expressing APE1 at a level far lower than those achieved with shRNA knockdown. The cells, named MEF^{la} (MEF^{low}APE1), were hypersensitive to methyl methanesulfonate (MMS), and showed little activity for repairing AP-sites and MMS induced DNA damage. While these results were consistent with the essential role of APE1 in repair of AP sites, the MEF^{la} cells grew normally and the basal activation of poly(ADP-ribose) polymerases in MEF^{la} was lower than that in the wild-type MEF (MEF^{wt}), indicating the low DNA damage stress in MEF^{la} under the normal growth condition. Oxidative phosphorylation activity in MEF^{la} was lower than in MEF^{wt}, while the glycolysis rates in MEF^{la} were higher than in MEF^{wt}. In addition, we observed decreased intracellular oxidative stress in MEF^{la}. These results suggest that cells with low APE1 reversibly suppress mitochondrial respiration and thereby reduce DNA damage stress and increases the cell viability.

Keywords

DNA repair; AP endonuclease 1; Endogenous DNA damage; Oxidative phosphorylation

1. Introduction

Aerobic energy generation in eukaryotic cells takes place in mitochondria through oxidative phosphorylation (OXPHOS). While glycolysis is less efficient than OXPHOS in generating ATP, cancer cells often need to survive under hypoxic conditions due to undeveloped vasculature. This hypoxia compels cancer cells to utilize glycolysis to much larger extent

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Conflict of Interest

The authors declare no conflict of interest.

than normal cells to generate ATP [1]. A recent finding that rates of random mutations in mitochondrial DNA were lower in cancer cells than in normal cells [2] is consistent with the fact that reactive oxygen species (ROS) are generated due to leaked electrons during OXPHOS [3]. Together these studies indicate that a consequence of active mitochondrial respiration is a higher risk of DNA damage.

Endogenous DNA damage includes base damage, apurinic/aprimidinic (AP) sites and DNA single-strand breaks (SSBs) which are generated at rates more than 10,000/cell/day in normal conditions [4,5]. In addition, recent studies have demonstrated highly active cytosine methylation and demethylation that generate AP sites in the genome [6,7]. These DNA lesions are primarily repaired via the DNA base excision repair (BER) process in which AP-endonucleases play a pivotal role in generating 3'-OH termini required for the DNA repair synthesis by DNA polymerases [8,9]. Both *Escherichia coli* and yeast cells harbor two functional AP endonuclease genes, and double mutants of these genes are still viable, because the repair of AP sites can be carried out by the backup activities provided by the DNA nucleotide excision repair (NER) [10,11]. In contrast, the mammalian AP endonuclease (APE1) is essential for cellular viability [12–16], underscoring the unique requirement of APE1 for repairing endogenously generated DNA damage. However, while an earlier study reported apoptosis in cells by APE1 down-regulation with an inducible shRNA system [16], cell lines with stable APE1 down-regulation have been successfully established in multiple laboratories [17–20]. The difference may be explained by a cellular adaptation to the low APE1 levels.

A possible mechanism for the cellular adaptation is an increase of repair activities independent of APE1. In principle, AP sites and SSBs in mammalian cells can be repaired without involving APE1 by SSB repair pathway in which PNKP (polynucleotide kinase/3'-phosphatase) plays the central role [9,21–24]. The lack of APE1-deficient cell lines or a mouse model hinders further investigations for unraveling the roles of APE1 and APE1-independent BER in protecting cells from endogenous DNA damage.

In this study, we established and examined unique mouse embryonic fibroblasts (MEF) that express APE1 at a level near threshold and far lower than those achieved with shRNA knockdown. Yet the APE1 deficient cells grew normally. Further investigation led us to conclude that intracellular levels of APE1 influence mitochondrial respiration activities, i.e., oxidative phosphorylation, thus reducing the cellular oxidative stress. These results indicate unexpected resilience of cells, which may have significant implications for cancer therapeutics that target APE1.

2. Materials and methods

2.1. Cell culture

The human colon carcinoma cell line HCT116 [20,25,26] and JHU28, a human squamous cell carcinoma line from head and neck cancer [27] kindly provided by Dr. Walvekar, were grown in DMEM High glucose (Hyclone) supplemented with 10% FBS (Gemini Bio-products), 1% L-glutamine, and 1% streptomycin/penicillin (Hyclone). A human APE1 shRNA adenovirus vector was a generous gift from Dr. Crowe [19]. The APE1 shRNA in

the retrovirus vector pSIREN-RetroQ (Clontech) were introduced into HCT116 and JHU28 along with vector only controls, and then maintained in growth medium containing 1 µg/mL puromycin (Invivogen).

2.2. Isolation of mouse embryonic fibroblasts

Generation of APE1 transgenic mice and isolation of MEF from 9.5E embryos were described previously [13,15]. All animal procedures were carried out at University of Texas Medical Branch (UTMB) in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by UTMB Animal Care and Use Committee (#00-01-007). Briefly, mating pairs of mouse *Ape1* gene-heterozygous and hAPE1 transgene positive (*mApe1*^{+/-} hAPE1^{Tg}) C57BL/6 mice were used to generate embryos. The MEF line was screened by PCR [15] for the genotype of mouse *Ape1* gene-homozygous and hAPE1 transgene positive (*mApe1*^{+/-}hAPE1^{Tg}). Analyses of *mApe1* and hAPE1 transcripts were carried out with reverse-transcriptase PCR using total RNA from MEFs (RNeasy Micro Kit, Qiagen) and PCR primers specific to the mouse and human RNA. Primers (Integrated DNA Technologies) for human APE1 mRNA were 5'-GCTTCGAGCCTGGATTAAGA-3' and 5'-TTGGTCTCTTGAAGGCACAGT-3', and those for mouse *Apex* mRNA were 5'-CCATTCTTTGTGCCGTGAG-3' and 5'-GAGCACCGAAGCAGTGTTTA-3'. The 18s rRNA transcripts were used as an internal control (primers: 5'-GCAATTATTCCCCATGAACG-3' and 5'-GGGACTTAATCAACGCAAGC-3'). The MEFs were transformed with SV40 T-antigen [15], and stably transfected with pFRT/lacZeo (Invitrogen). The MEF^{wt} (*mApe1*^{+/-}; hAPE1^{Tg}), was also isolated from a sibling, and used as a control. All cells were grown in DMEM (high glucose) supplemented with 10% FBS, 1% L-glutamine and 1% streptomycin/penicillin. The wild-type (wt) human APE1 (hAPE1) and mouse *Ape1* (*mApe1*) cDNA [20] were cloned into the pcDNA5.1FRT/TO vector (Invitrogen), and isogenic MEF^{la} derivatives expressing hAPE1 or *mApe1* stably were established via Flp/FRT site-specific recombination [28]. Cells expressing hAPE1 and *mApe1* genes were named MEF^{la}/hAPE1 and MEF^{la}/*mApe1*, respectively. Control cells with the empty vector were named MEF^{la}/vec. Supporting Fig. S1A provides a flowchart for construction of the MEF lines.

2.3. Immunoblot analysis

Cells were washed in PBS twice and lysed in RIPA lysis buffer (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 0.1% SDS, 1 mM DTT), containing proteinase inhibitor cocktail (Roche). The total fractions of cell extracts were run in 10% SDS/PAGE, transferred onto PVDF membranes (Bio-Rad), which were blocked in 5% nonfat milk (Bio-Rad). The membranes were then blotted with appropriate primary and secondary antibodies listed below, and developed with chemiluminescent substrates (DURA or FEMTO, Pierce). Intensities of unsaturated (non-overexposed) signals were analyzed using ChemiDoc (Bio-Rd) and ImageJ software (NIH).

2.4. Cytotoxicity assays

Cells were plated (60 mm dishes at 2000 cells/dish), incubated for overnight and then treated with methyl methanesulfonate (MMS, Sigma) for 1 h at 37 °C. After removing MMS, cells

were grown without MMS for 7–9 days or until colonies grew 1 mm in size, at which point the colonies were stained with crystal violet (Fisher) and counted to calculate survival fractions by dividing numbers of colonies of MMS-treated dishes (S) with those of untreated dishes (S0). A colorimetric assay (WST-8, Dojindo) to measure cellular NADPH dehydrogenase activity was also used to assay MMS cytotoxicity. Cells (5000/well) were incubated for 3 h on 96 well plates, treated with MMS for 1 h, washed and incubated for 24 h before the dye-formation assay at 450 nm (Emax, Molecular Device).

2.5. AP-endonuclease activity assay

Intracellular AP-endonuclease activity was examined using an oligonucleotide substrate as previously reported [29] with following modifications. A 32-mer 5'-Cy5-labeled oligonucleotide containing a single tetrahydrofuran (THF, 5'-AGGCCAATGATCGGTAT/TET/AAGTCGCGGGATAA-3') and its reverse-complementary oligo were synthesized by Integrated DNA technologies (Coralville, IW). The duplex substrate DNA was incubated with 10 ng nuclear extracts [30] of MEFs at 37 °C for 8 min. The DNA was then subsequently separated in denaturing 20% polyacrylamide gel electrophoresis (8% urea, Tris-borate buffer) at 65 °C. The substrate and cleaved products were analyzed with GeneStorm (General Electric).

2.5.1. Analysis of basal levels of cellular DNA damage with Comet assay—

Comet assay was carried out according to vendor's manual (Trevigen, Inc.). Briefly, cells were plated 1 day before the assay, and resuspended in PBS at 1×10^5 /mL. Cells were then embedded in low melting point agarose gel and lysed. The cells were immersed in alkaline solution to denature DNA, and gel electrophoresis was performed at 300 mA for 30 min. After washing in H₂O, gels containing the cells were treated in 70% ethanol, dried for 15 min, and were stained in SYBR Gold (Invitrogen). Gels were then washed in H₂O and dried. Three independent experiments were carried out, and the comet tails of 30 individual cells were analyzed for each assay.

2.6. Long amplicon (LA)-PCR assay

LA-PCR assay was carried out as described previously [31,32] with the following modifications. After the treatment with the alkylating agent MMS for 1 h, cells were harvested immediately or after incubation at 37 °C for 6 h. Genomic DNA extraction was carried out using the QIAGEN Genomic-tip 20/G kit (Qiagen) with the manufacturer's directions. This kit has the advantage of minimizing DNA oxidation during the isolation step and thus can be reliably used to detect endogenous DNA damage using LA-QPCR. After quantitation by Pico Green (Molecular Probes) in a 96-well plate, gene-specific LA-QPCR analysis for measuring DNA damage were performed using Long Amp Taq DNA Polymerase (New England Biolabs). LA-PCR was carried out to amplify a 6.5 kb region around the mouse PolB or 8.7 kb region around the mouse Beta globin gene in mouse genomic DNA using the following primers: 5'-TATCTCTCTTCCTCTTCACTTCTCCCC-3' and 5'-CGTGATGCCGCCGTTGAGGGTCTCC-3' for PolB and 5'-TTGAGACTGTGATTGGCAATGCCT-3' and 5'-CCTTTAATGCCCATC-CCGGACT-3' for β -globin. The number of cycles and DNA concentration was standardized in each case before the actual reaction so that the PCR reaction remains in the linear stage of

amplification [32–34]. The final PCR reaction condition was standardized at 94 °C-30 s; (94 °C-30 s, 55 °C-30 s, 65 °C-10 min) for 25 cycles; 65 °C-10 min 15 ng of DNA template was used in each case. Since amplification of a small region would be independent of DNA damage, a small DNA fragment for each gene (PolB and β -globin) was also amplified to normalize amplification of large fragments using the following primers: 5'-TATGGACCCCATGAGGAACA-3' and 5'-AACCGTCGGCTAAAGACGTG-3' for PolB and 5'-ACACTACTCAGAGTGAGACCCA-3' and 5'-ATACCCAATGCTGGCT-CCTG-3' for β -globin. The PCR reaction condition was 94 °C-30 s; (94 °C-30 s, 54 °C-20 s, 68 °C-30 s) for 25 cycles; 68 °C-5 min. The amplified products were then visualized on gels and quantitated with ImageJ automated digitizing system (NIH) with three replicate gels.

2.7. Fluorescence-activated cell sorting (FACS) analysis

After removal of debris and aggregates by centrifugation in cell strainers, 1×10^5 MEFs in PBS were labeled with MitoTracker green (200 nM; Invitrogen) for 25 min and with 4 μ M MitoSoxRed for 10 min and analyzed by FACS Core Facility at University of Kentucky, using FACSCalibur (Becton Dickinson, San Jose, CA). The total numbers of mitochondria were also determined by the MitoTracker green fluorescence in FACS.

2.8. Mitochondrial bioenergetics

Oxygen consumption was determined using a Seahorse Extra-cellular Flux (XF-96) analyzer (Seahorse Bioscience, Chicopee, MA) [35,36]. Data are presented as oxygen consumption rate (OCR) in pmols/min/ 10^4 and extracellular acidification rate (ECAR) in mpH/min/ 10^4 cells. For OCR, cells were cultured in the presence of 4.5 g/L D-glucose with 45,000 cells/well in Seahorse Bioscience XF microplates, cultured in the presence of 2 g/L D-glucose. When necessary, cells were incubated in the presence of N-acetylcysteine (NAC, Sigma) at concentration of 1 mM. Basal OCR was measured four times and plotted as a function of time starting from a normal condition followed by additions of inhibitors, i.e., oligomycin (an inhibitor of the ATP synthase, 1 μ g/mL), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (mitochondrial inner membrane pore opener, 1 μ M) and rotenone (OXPHOS complex I inhibitor, 1 μ M). The progress curve is annotated to show the relative contribution of basal, ATP-linked and maximal oxygen consumption after the addition of FCCP, and the reserve capacity of the cells. For the ECAR measurements, following overnight incubation, cells were washed and changed to assay media lacking glucose. Similar for OCR, ECAR were measured and plotted as a function of time.

2.9. Gene expression microarray analysis

The Mouse Gene 2.0 ST array (Affymetrix) was used to profile global gene expression of three isogenic cell lines expressing empty vector control (MEF^{la}/vec), hApe1 (MEF^{la}/hApe1), and mApe1 (MEF^{la}/mApe1). Three batches of total RNA were prepared (Qiagen RNeasy) from each cell line. The qualities of RNA were verified by Agilent 2100 Bioanalyzer (Agilent Technology) and then the set of nine RNA samples (three preparations from three cell lines) were analyzed using GeneChip FLUIDICS station 450 and Affymetrix GeneChip Scanner 7G (Affymetrix) at the Microarray Core of the University of Kentucky. Transcriptional intensities of 41,345 genes were processed using PARTEK, to obtain 7115 genes with false discovery rates less than 0.05. Genes of which expressions were over-

represented in MEF^{la}/hAPE1 and MEF^{la}/mApe1 compared to MEF^{la}/vec were then calculated based on *P* values less than 0.001 (Fisher's exact test) to obtain 557 and 766 genes for hAPE1 and mApe1, respectively. Functional annotations for these genes were then obtained using NCBI DAVID (Database for Annotation, Visualization and Integrated Discovery; <http://david.abcc.ncifcrf.gov>). The data will be available at NCBI Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE55765.

2.10. Statistical evaluation

All experiments were carried out at least three times, and Student's *t*-test was used for pair-wise comparison with asterisks (**P* < 0.05) or double asterisks (***P* < 0.005) unless noted otherwise.

2.11. Other chemicals and reagents

Other chemicals were purchased from Sigma–Aldrich (St. Louis, MS) and Fisher Scientific (Pittsburg, PA). Antibodies purchased in this study include for APE1 (SCBT, sc-55498), cleaved caspase 3 and PARP (Cell Signaling Technology, 9664S and 9532S respectively), PAR [poly(ADP-ribose)] (Millipore, MAB3192), PGC1α (SCBT, sc13067), (β-tubulin (SCBT, sc58884), and β-actin (Sigma, A5441). A polyclonal APE1 antibody raised in rabbit was generated by Proteintech (Chicago, IL) using purified full-length human APE1 protein [37]. XPA antibody was kindly provided by Dr. Mellon (University of Kentucky).

3. Results

3.1. Isolation of MEFs with extremely low APE1 expression

We previously reported a MEF line in which the mApe1 gene was homozygously knocked out, but was viable because of integration of the hAPE1 gene in the genome (mApe1^{−/−} hAPE1^{Tg}) [15]. Here, a MEF line of the same APE1 genotype in the C57BL/6 isogenic background was established (Fig. 1A and B, S1A), after back-crossing with C57BL/6 to the isogenic background (more than 10 times). The level of APE1 in the mApe1^{−/−} hAPE1^{Tg} MEF was found to be extremely low compared to the wild-type MEF (MEF^{wt}; mApe1^{+/+} hAPE1^{Tg}) (Fig. 1C, lanes 1 and 2). Another APE1 antibody with a different epitope specificity confirmed the large difference in APE1 levels (Fig. 1C, lanes 3 and 4; also in Fig. S1B and C). The level of APE1 expression in MEF^{wt} was similar to those in NIH3T3 mouse fibroblast (Fig. S1B, lane 4 v.s. lane 1), a commonly used mouse fibroblast cell line [38], validating the normal APE1 expression in the wild-type MEF. We thus concluded that the low APE1 abundance observed in the immunoblots was indeed due to the low intracellular level of APE1 in the mApe1^{−/−} hAPE1^{Tg} MEF. By normalizing the intensities with β-actin and β-tubulin, the levels of APE1 in the cells were estimated to be 0.195% (±0.029) compared to that in MEF^{wt}. Studies using shRNA have been successful in reducing APE1 levels down to approximately 5% of those in normal cells [16,17,19,20] (also this study below). The mApe1^{−/−} hAPE1^{Tg} MEF thus exhibited a much lower level of APE1 than any other cells previously described. The MEF was named MEF^{la} (low APE1) to distinguish it from cells established by RNA interference. MEF^{la} grew normally with a morphology that was indistinguishable from MEF^{wt} (Fig. 1D), and the level of APE1 expression in MEF^{la} has

been stable for longer than 2 years. The low expression of MEF^{la} was most likely due to transgene silencing, a gene-silencing phenomenon that frequently occurs during back-cross of transgenic mice [39,40].

The levels of endogenous DNA damage was examined with the comet assay in MEF^{la}/vec, a stable transfectant of empty vector (Fig. S1B, lanes 5 and 8), and in MEF^{la}/hAPE1, an isogenic hAPE1 expressing derivative (Fig. S1B lanes 3 and 7). A small but statistically significant increase in the comet tail lengths was observed with MEF^{la}/vec compared to MEF^{la}/hAPE1 (Fig. 2A), indicating that the amount of endogenously generated AP sites and SSBs reach near the maximum that can be repaired in the MEF^{la}. However, it was unexpected to us that the APE1 deficiency in MEF^{la} caused only a minor accumulation of DNA damage, considering the pivotal role of APE1 for the repair of AP sites that are continuously generated even in the normal growth condition. Indeed, MEF^{la}/vec grew slightly faster than MEF^{la}/hAPE1 (Fig. 2B), although the difference between the two cell lines was not highly significant ($P = 0.07$ by paired Student's t-test).

3.2. Low stress of SSB generation monitored by poly(ADP-ribosyl)ation activity

During BER, AP sites and damaged bases are converted to SSBs before completely repaired [41]. SSBs then activate poly(ADP-ribose) polymerases (PARPs) as a DNA damage response [9,42]. The comet assay could not reveal the level of endogenously generated SSBs which may have been converted from AP sites after cell lysis. To probe the stress level in the APE1-deficient cells due to spontaneously generated SSBs, we measured basal levels of poly(ADP-ribose) (PAR) in MEF^{la}/vec and MEF^{la}/hAPE1 (Fig. 2C). There was a statistically significant decrease of the intracellular PAR level in MEF^{la}/vec (lane 1) compared to that in the MEF^{la}/hAPE1 cells (lane 2). By measuring immunoblot signals of three independent experiments, we found that the level of PAR, normalized by that of PARP, was 3.4 (± 1.2) fold higher in MEF^{la}/hAPE1 than that in MEF^{la}/vec. Thus, while MEF^{la} showed a slightly increased endogenous DNA damage possibly due to accumulation of AP sites, the damage did not provoke the DNA damage response triggered by SSBs, suggesting that the basal level of SSBs was decreased in the MEF^{la} cells compared to MEF^{wt}. Levels of cleaved caspase-3 in both cell line were low, indicating that no noticeable apoptosis was activated in either cells, although a slight increase of the cleaved caspase-3 in MEF^{la}/hAPE1 (1.68 fold higher than in MEF^{la}/vec) was measured. In any case there was no enhancement of apoptosis due to the APE1 deficiency (Fig. 2D).

3.3. Deficiency of repair of AP sites in MEF^{la}

The results raised the possibility that the MEF^{la} cells increased an APE1-independent repair pathway for AP sites. We analyzed the repair activity of MEF^{la} to test whether the cells enhanced APE1-dependent repair pathways to compensate the APE1 deficiency. Cellular AP-endonuclease activity was measured using oligonucleotides containing an AP site analog THF [43]. The AP-endonuclease activity of MEF^{la} cells was undetectable in the conditions where MEF^{wt} clearly showed the activity (Fig. 3A; Fig. 3B lane 2 v.s. lane 3). The reaction condition, including the amount of nuclear extract, incubation time and the extract preparation, was previously optimized to minimize non-specific nuclease reaction [29]. However, the assay was also performed with 1 μ g of nuclear extracts ($\times 100$ folds more than

in Fig. 3A and B). Under this condition, some non-specific degradation was visible as expected (double arrow, Fig. 3C). However, there was still no detectable APE-specific endonucleolytic cleavage with the extract from the MEF^{la} cells (Fig. 3C). The low APE1 activity in MEF^{la} cells was consistent with a previous observation that most, if not all, APE activity could be attributed to the APE1 polypeptide in mammalian cells [44], and excluded the possibility that an uncharacterized AP-endonuclease activity was enhanced in the MEF^{la}. Next, to evaluate the ability of MEF^{la} to repair induced DNA damage, survival curve of the MEF^{la} cells treated with an alkylating reagent methyl methane-sulfonate (MMS) was examined. MMS alkylates purine bases in DNA and generates AP sites [9,45]. Both the colony formation and colorimetric cytotoxicity assays showed that MEF^{la} was significantly more sensitive to MMS than MEF^{wt} (Fig. 3D and E). The results were in agreement with previous studies in that APE1 has a critical role in the repair of MMS induced-DNA damage [46,47]. To determine the cellular activity of repair of MMS-induced DNA damage, Long Amplicon-PCR (LA-PCR) was carried out with the genomic DNA extracted from MEF^{la}/vec and MEF^{la}/hAPE1 cells [32,34]. After 1 h of MMS treatment, the LA-PCR amplification was significantly decreased with both genomic DNA compared to those untreated. This indicated that MMS treatment caused similar levels of DNA damage in MEF^{la}/hAPE1 and MEF^{la}/vec cells. (Fig. 4A and B). Then, cells were incubated for 6 h after the MMS treatment to allow the repair. A complete recovery of LA-PCR amplification was observed with the genomic DNA from MEF^{la}/hAPE1. However, there was no repair activity detected in MEF^{la}/vec at the same condition. It should be pointed out that the level of endogenous DNA damage in MEF^{la}/vec was noticeably higher than that in MEF^{la}/hAPE1 (amplifications of DNA from the untreated cells). The observation was consistent with the results in Fig. 2A, where comet tail length was also noticeably longer in MEF^{la}/vec than MEF^{la}/hAPE1. The clearer damage accumulation in MEF^{la}/vec indicated by LA-PCR than by the comet assay may be due to the difference of the sensitivities of the two assays, and currently we are not able to assess which result reflected the *in vivo* damage accumulation more accurately.

We examined expressions of DNA repair proteins that may function for repairing AP sites. Namely, expressions of PNKP, critical for APE1-independent repair [21,24], and XPA, that may function in repairing AP sites [11], were examined. The levels of PNKP and XPA in MEF^{la} were indistinguishable from those in MEF^{wt} (Fig. S1D), suggesting that activities of two possible APE-independent repair pathways were unchanged in the MEF^{la}.

3.4. Decreased OXPHOS activity in MEF^{la}

We examined the possibility that the MEF^{la} cells adapted to the low APE1-expressing condition by reducing endogenous DNA damage formation, rather than by enhancing alternative DNA repair activities. Oxidative phosphorylation (OXPHOS) in mitochondria is the predominant mode for O₂ consumption in cells, and the mitochondria are the primary source of ROS in cells due to leaked electrons [3]. While APE1's presence in and on the surface of mitochondria was previously shown [48], its effect on mitochondrial functions has not been investigated. FACS analyses using Mito-TrackerGreen indicated that the mass of mitochondria per cell was slightly decreased in the MEF^{la} compared to the MEF^{wt} cells (Fig. S2), which may be in agreement with PPARGC1A activation shown below. We then

examined OXPHOS levels in MEF^{la} v.s. MEF^{wt} using a Seahorse XF analyzer [49]. The rate of oxygen consumption per cell was significantly lower in MEF^{la} than that in MEF^{wt} (Fig. 5A). By stably expressing hAPE1, the OXPHOS activity in MEF^{la} was restored to almost the same level as that of MEF^{wt} (Fig. 5A). The most noticeable difference in the O₂ consumption was found in the presence of FCCP. FCCP is an inner membrane pore opener which resets the proton gradient between mitochondrial matrix and interspace, resulting in continuous transport of protons and consuming O₂ at the maximum potential [35]. Remarkably, while the FCCP treatment increased O₂ consumption in both MEF^{wt} and MEF^{la}/hAPE1, the treatment showed no effect on the O₂ consumption in the MEF^{la}. The result indicated that the low basal OXPHOS activity in MEF^{la} was due to unusually low OXPHOS potential. We examined glycolysis in these cells by measuring extracellular acidification (ECAR) [35] and the pattern exactly opposite to that of OCR was observed for glycolysis rates between MEF^{la} and MEF^{wt} (Fig. 5B). The result was supported by measuring cellular lactate concentration, an indicator for glycolysis [50], which was also significantly higher in MEF^{la} than in MEF^{wt} and MEF^{la}/hAPE1 (Fig. 5C). The reverse relation of oxidative phosphorylation and glycolysis rates indicated changes in the metabolism for energy generation in MEF^{la}. Intracellular O₂⁻ concentration was measured using MitoSoxRed fluorescence indicator specific for O₂⁻. MEF^{la} exhibited lower O₂⁻ concentration than the corresponding wild-type MEF lines (Fig. 5D). Hence, these results suggested that the lower APE1 levels in MEFs cause reduced mitochondrial respiration, and consequently decreased oxidative stress in the cells.

We tested whether the decrease in OXPHOS activity was unique in MEF^{la} because of extremely low APE1 expression. OXPHOS activities were determined with HCT116 and JHU28, a human colon carcinoma and an oral squamous cell carcinoma cell lines, respectively. Both cells expressing APE1 shRNA stably showed approximately 5% of control cells (Fig. 6A). The O₂ consumption assays demonstrated that APE1 knockdown decreased OXPHOS activities in both cell lines compared to their corresponding control cells, i.e., HCT116/shCtl and JHU28/shCtl (Table 1), although the differences between APE1 knockdown and the wild-type cells were lower than that observed in the MEF lines.

We considered that the OXPHOS change in MEF^{la} was reversible, because the stable expression of hAPE1 in MEF^{la} rescued the OXPHOS activity (Fig. 5A). This observation thus excludes the possibility that mutations in genes critical for OXPHOS caused the drastic decrease in the mitochondrial respiration in MEF^{la}. However, such mutations in MEF^{la} could have occurred during the period between the isolation of MEF^{la}/hAPE1 (Fig. S1A, I) and the measurement of O₂ consumption. To exclude this possibility, a cell line stably expressing hAPE1 (Fig. S1A, II) was isolated along with a vector control (MEF^{la}/vec). The newly isolated MEF^{la}/hAPE1 cells again exhibited higher OXPHOS than MEF^{la}/vec (Table 1). In addition, the OXPHOS enhancement was also achieved by stable expression of mApe1 in MEF^{la} (Table 1). Therefore, we concluded that the decreased OXPHOS activity in MEF^{la} cells was not due to gene mutations.

3.5. Influence of APE1 expression on the global gene expression pattern

Given the gene regulatory function of APE1 [51–53], it is possible that APE1 regulates a set of genes important for oxidative phosphorylation. To probe the importance of transcriptional regulation of OXPHOS genes in hAPE1 or mAPE1 expressing MEFs more in greater detail, we focused on relative changes of gene expressions between MEF^{la}/hAPE1 v.s. MEF^{la}/vec and MEF^{la}/mAPE1 v.s. MEF^{la}/vec. The difference in the expression of each mRNA was given by following calculations.

$$E_{hAPE1} = \log_2(\text{Level in MEF}^{la}/hAPE1) - \log_2(\text{Level in MEF}^{la}/vec)$$

$$E_{mAPE1} = \log_2(\text{Level in MEF}^{la}/mAPE1) - \log_2(\text{Level in MEF}^{la}/vec)$$

We focused on correlation coefficient (r) between E_{hAPE1} and E_{mAPE1} of genes belonging to a functional group. A group of genes systematically and similarly regulated by both hAPE1 and mAPE1 should yield an r value positive and closer to 1.0. This is a reasonable assumption, considering that the human and mouse APE1 share a high amino acid sequence similarity (94% identical) and that both hAPE1 and mAPE1 activated OXPHOS in MEF^{la}. Gene clusters defined in the KEGG database (<http://www.genome.jp/kegg/pathway.html>) were analyzed for their r values (Table S1). The majority of gene groups (186 out of total 193 groups) showed positive r values with statistical significance, and only seven groups resulted in negative r values and none of these showed statistical significance (Table S1), validating the consistency of the influence of hAPE1 and mAPE1 on the whole gene expression patterns in the cells. Then, a cluster of 138 genes were found to be involved in the OXPHOS pathway based on the KEGG database (mmu00190, highlighted in Table S1). The r value for the OXPHOS gene group was 0.43 ($P < 0.01$), implying a systematic regulation of OXPHOS function by APE1. Detailed analysis of individual genes revealed that PGC-1 α (peroxisome proliferator activated receptor gamma co-activator 1 alpha, PPARGC1A), a master regulator of mitochondrial metabolism [54], was activated by both hAPE1 and mAPE1 (Table 2), which was then confirmed by immunoblotting (Fig. 6B) and by immunocytochemistry (Fig. 6C).

4. Discussion

In this study, we described generation and analyses of the MEF^{la} cell line, that expresses APE1 far lower than those achievable by shRNA downregulation. Based on immunoblotting, the level of APE1 in MEF^{la} is only 0.2% of the normal MEF. Although cell death was observed by down-regulating APE1 [16], the present study indicates that conditions exist where the threshold level of APE1 to maintain cell viability can be much lower than that achieved by shRNA knockdown. It should be noted that in the earlier study, the down-regulation of APE1 by shRNA was achieved using an inducible promoter, while the MEF lines as well as shRNA knockdown in the present study involved constitutive suppression of APE1 expression. This observation led us to hypothesize that cells can adapt to the APE1 deficiency by decreasing intracellular ROS generation. However, previous studies indicate that cells would die when APE1 level approaches to zero. It is likely that the level of APE1 in MEF^{la} is near the threshold required for cell viability.

MEF^{la} was hypersensitive to MMS, a commonly used reagent that generates AP-sites in DNA [45]. This result was expected, because APE1 accounts nearly all AP-endonuclease activity in mammalian cells [44]. The *E. coli xthA nfo* double mutant, lacking AP endonuclease activities is hypersensitive to alkylating reagents [55,56]. Similarly, *Saccharomyces cerevisiae* without APN1 and APN2 showed higher cytotoxicity to MMS [57]. The fundamental difference of the mammalian cells from *E. coli* and yeast cells, however, is that while these unicellular organisms grow normally in the absence of external genotoxicants, mammalian cells lacking APE1 are not viable. Worth noting in this context is that most DNA damage assays, including the comet and LA-PCR assays, cannot discriminate AP sites and SSBs, as AP sites are easily converted to SSBs *in vitro*, and PCR reactions will be blocked at both AP sites and SSBs. However, considering that the basal PAR level (and thus PARP activity) was significantly lower in MEF^{la} than in MEF^{wt}, interesting possibility is that majority of AP sites in MEF^{la} are not converted to SSBs which may be more harmful. Although it has not been possible to assess conversion of AP sites to SSBs *in vivo* particularly for those generated spontaneously, MEF^{la} may provide a useful methodology to assess basal levels of AP sites and SSBs *in vivo* [3,9].

In this report, we found that reduced APE1 expression suppressed mitochondrial oxygen consumption, i.e., OXPHOS activity. Lowering APE1 also resulted in increased glycolysis, presumably to compensate for ATP production in the cells. Moreover, the low OXPHOS activity in MEF^{la} could be rescued by APE1 expression. We were able to eliminate the possibility that irreversible genetic changes in the MEF^{la} cells, caused by the repair deficiency, resulted in OXPHOS abrogation, because OXPHOS could be reactivated by ectopic expression of APE1 in the MEF^{la} cells. Although the mechanism by which APE1 activates OXPHOS is not clear, our results postulate that the activation of PGC-1 α by APE1 contributed to the mitochondrial activation. Although it is beyond the scope of the present study, it may be an interesting development to test whether APE1 up-regulates PGC-1 α expression via its redox-regulatory function [58]. It should also be noted that recently Fang et al. reported that PARP1 activation due to DNA damage led to inhibition of PGC-1 α , which in turn resulted in mitochondrial degeneration [59]. It should be noted that Fouquerel et al. very recently reported a detailed mechanism of PARP's role in the metabolic reprogramming [60]. In the study, PARPs were activated to generate PAR by DNA damage induced by an alkylating reagent 1-methyl-2-nitro-1-nitrosoguanidine (MNNG). The study then found that PAR directly blocked hexokinase I, therefore inhibiting the cellular energy production [60]. Therefore, PARP1 and APE1 may function in the same signaling pathway with opposite effects in maintaining the mitochondrial integrity. However, a possible role of APE1 in DNA damage-associated alteration of the mitochondrial activity remains to be tested, as the present study did not examine the energy metabolism after MMS or MNNG treatments.

Based on the gene array analysis, Nrf1 was also moderately activated by both hAPE1 and mApe1 (1.20 fold increase in both MEF^{la}/hAPE1 and MEF^{la}/mApe1 compared to MEF^{la}/vec), which may additively contributed to the mitochondrial activation [61]. In any case, mitochondrial metabolic reprogramming involves multiple factors that together influence the mitochondrial activity, and thus the omics approaches including the gene expression analysis in this study will help unravel the function of APE1 in modulating cellular redox balance.

We identified other KEGG pathways that were similarly regulated by the human and mouse APE1 (Table S1). Among them, the renin-angiotensin system (KEGG mmu04614) scored highly, which was reported to be regulated by APE1 [51,62]. It is also interesting that the TGF-beta signaling pathway (mmu04350) is one of the highly regulated gene set as APE1's role during fibroblast activation during tumorigenesis may be a novel research direction.

In a Huntington's disease model, immortalized human striatal cells were used to investigate the effect of the huntingtin protein on the cellular oxidative stress and mitochondrial DNA damage [63]. Accordingly, the cells with mutant huntingtin proteins showed increased oxidative stress, and its respiratory activity was decreased when APE1 was knocked-down by siRNA. Our present study provides two insights relevant to these previous observations. First, although the previous study could not unequivocally conclude that APE1 alone influenced the mitochondrial respiratory activity, because of the statistically small difference between the normal and APE1 knockdown-cells, our results indicate that lowering the APE1 level alone is indeed sufficient to decrease the OXPHOS activity in the cells. While we did not specifically test if specific mutations in huntingtin occurred in the MEF^{la} cells, this is an unlikely scenario, given the fact that the change in OXPHOS was detected in at least three different cell lines (MEF, JHU28, and HCT116). Second, the present results indicate that OXPHOS activation by APE1 is reversible. Thus, in Huntington's disease, although mutant huntingtin proteins may cause mtDNA damage to lead to irreversible respiratory change, the effect of APE1 on oxygen consumption may be additive to the huntingtin mutation. The current study thus leads to a model whereby APE1 plays a role in mitochondrial biology not limited to BER. This model warrants detailed investigation to establish APE1 as a factor influencing the etiology of many types of neurodegenerative diseases, because mitochondrial damage is associated not only to Huntington's disease but also to amyotrophic lateral sclerosis (ALS), Alzheimer's and Parkinson's disease [64–66].

In the past year, a remarkable progress has been made in methodology of effective gene inactivation using CRISPR (clustered regularly interspaced short palindromic repeats) [67–70], which should enable to inactivate APE1 in cultured cells and in animals. However, cells or animals targeted by CRISPR for homozygous APE1 deletions will not be viable [14,15]. It will also be difficult to suppress APE1 expression down to the level achieved in this study. MEF^{la} described in this study should allow a unique experimental design for analyzing effects of mutated APE1 at the extremely low background activity of the wild-type APE1, in order to understand precise roles of APE1 in DNA repair, redox, and gene regulations. For example, using MEF^{la} may allow mapping to identify amino acid residues in APE1 that are required for activating OXPHOS. Identifying a particular APE1 function for this process may be the key to understand the role of APE1 for modulating the mitochondrial function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AP site	apurinic/aprimidinic site
APE	AP endonuclease
hAPE1 or mApe1	human or mouse AP endonuclease 1
ARP	aldehyde reactive probe
CRISPR	clustered regularly interspaced short palindromic repeats
EDD	endogenous DNA damage
EDDR	EDD repair
kd	knockdown
ko	knockout
MMS	methyl methanesulfonate
NER	DNA nucleotide excision repair
NEIL	endonuclease VIII-like
OXPHOS	oxidative phosphorylation
PARP	Poly (ADP-ribose) polymerase
PNKP	polynucleotide kinase phosphatase
ROS	reactive oxygen species
SSB	DNA single-strand breaks
UK	University of Kentucky
wt	wild-type
XRCC1	X-ray cross complementation group 1

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.dnarep.2015.01.003>.

Fig. 1.

Isolation of MEF^{la}. (A) APE1 genotypes of MEF lines. PCR fragments amplified from genomic DNA of the MEF^{wt} (lane 1) and MEF^{la} (lane 2) cells with primer sets for the APE1 (left panel) and neomycin resistance genes (right panel, double arrows). PCR products with (h) human APE1 and (m) mouse Apex 1-specific primers are indicated. M: λ-HindIII size marker. (B) Detection of mouse and human APE1 transcripts in the MEFs. Total RNA from (1) MEF^{la}/hAPE1, (2) MEF^{wt}, (3) MEF^{la}, (4) MEF^{wt}, (5) MEF^{la}, (6) MEF^{la}/hAPE1 were analyzed by RT-PCR using primers specific to human APE1 (h) or mouse Ape1 (m). Details of isolation of MEF^{la} (mApe1^{-/-} hAPE1^{Tg}) and its derivatives expressing hAPE1 or mAPE1 are described in Fig. S1. (C) Immunoblot of cell extracts using a monoclonal (1 and 2) and a polyclonal (3 and 4) APE1 antibodies. The signals in lanes 1 and 2 were intensified (dashed arrow) to detect APE1 in MEF^{la}. None of the immunoblot signals were saturated. (D) Cell morphologies of the MEF^{WT} and MEF^{la} taken with a phase contrast microscope at ×20 magnification.

Fig. 2.

(A) Comet assay with (1) MEF^{la}/vec and (2) MEF^{la}/hAPE1 under a normal growth condition. (Upper panel) Triplicated; counts of more than 30 cells per experiments. $*P < 0.05$. (Lower panel) Representative comet tails for the corresponding cell lines. (B) Growth curves of MEF^{la}/vec (open circle) and MEF^{la}/hAPE1 (dotted). Cells were plated on 6 cm dishes at 5×10^4 /plate. Cell counting was started after 24 h (Time 0). Cells were trypsinized, suspended and mixed in Trypan Blue, and counted using a hemacytometer. Average of six independent preparations and standard deviations are shown. (C) Levels of poly(ADP-ribose)ylation in the protein extracts from (1) MEF^{la}/vec and (2) MEF^{la}/hAPE1 shown with PARP1 and β-tubulin as internal controls. (D) Levels of cleaved caspase 3 in (1) MEF^{la}/vec and (2) MEF^{la}/hAPE1.

Fig. 3.

(A–C) AP-endonuclease activity from nuclear extracts of the MEF. (A) Extracts (10 ng) from MEF^{la}/hAPE1 (filled circle) and MEF^{la}/vec (open circle) were incubated with the 43-mer double-strand oligonucleotide (100 μ M) for 0–8 min, and percentage of cleaved products v.s. input substrates were calculated. (B) Nuclear extracts (10 ng) from 2: MEF^{wt} 3: MEF^{la}, 4: MEF^{la}/hAPE1, 5: MEF^{la}/vec, 6: MEF^{la}/hAPE1 (another stable transfectant different from 4) were incubated with the substrate for 4 min, and analyzed after gel electrophoresis. 1: no extract and 7: purified APE1 (1 ng). S: substrate DNA (open arrow), P: product DNA (filled arrow). (C) Nuclear extracts (1 μ g) from MEF^{wt} (left) or MEF^{la} (right) were incubated with the THF containing oligo for the indicated time. (–): no extracts. Double filled arrow points products due to non-specific degradation. (D and E) Sensitivity of MEFs to MMS. Cytotoxicity of MMS analyzed with (D) colony formation assay and (E) cell proliferation assay based on NADH dehydrogenase activities in the cells. Symbols: diamond: MEF^{wt}, square: MEF^{la}, triangle: MEF^{la}/hAPE1. Error bars denote standard deviation of three experiments.

Fig. 4. DNA damage quantitation measured by long-amplicon PCR analysis (LA-PCR). (A) MEF^{la}/vec and MEF^{la}/hAPE1 were treated with MMS (1.0 mM) for 1 h. At 0 or 6 h of post treatment incubation (PTI), genomic DNA from the cells were subjected for quantitative PCR using primers for long-amplicon (LA) and short-amplicon (SA) DNA. PCR conditions for the two genomic loci, the polβ and globin genes, were standardized in previous studies [32,34]. (B) Quantitative demonstration of the LA-PCR in (A). The SA-PCR intensities on which the effects of DNA damage are negligible were used to normalize the LA-PCR results.

Fig. 5. Mitochondrial activities in MEF^{wt} (1), MEF^{la} (2), and MEF^{la}/hAPE1 (3). (A) oxygen consumption rate (OCR, pmol/min/10⁴ cells) determined with Seahorse analyzer for OXPHOS activity. F: FCCP, O: oligomycin, R: rotenone. (B) Extracellular acidification rate (ECAR) by Seahorse for glycolysis activity. (C) Cellular lactate concentrations. Error bars denote standard deviation of triplicated experiments. (D) Cellular O₂⁻ concentration probed with MitoSoxRed and analyzed by FACS. Relative intensities of MitoSoxRed values are shown. **P* < 0.05, ***P* < 0.005.

Fig. 6.

(A) Intracellular levels of APE1 in HCT116 and JHU28 with APE1 knockdown compared to their controls. (B) PGC-1α immunoblot with protein extracts from (1) MEF^{la}/hAPE1 and (2) MEF^{la}/vec. (C) Immunocytochemistry for PGC-1α. Equal number of MEF^{la}/vec and MEF^{la}/hAPE1 were mixed and plated on coverslips, and probed with APE1 and PGC-1α antibodies simultaneously and detected with Alexa-Fluor488 (APE1) and 568 (PGC1α). D: DAPI, A: APE1, P: PGC1α. The signals of PGC1-α are indicated by the arrow (APE1-deficient cells) and by the double-arrow (APE1-proficient cells). Experiments were repeated for three times. MEF^{la}/vec and MEF^{la}/hAPE1 were distinguished based on the APE1 signals, and the PGC1-α intensities in two cell types were measured for more than 30 cells per staining using Image-J, and plotted in the right panel (1: MEF^{la}/hAPE1, 2: MEF^{la}/vec). * $P < 0.05$ determined by Student's t-test.

Table 1

The maximal potential of O₂ consumption in cell lines.

Cell lines	OXPHOS activity (fold difference)
HCT116 shAPE1 v.s. shCtl	1.29 ± 0.02 **
JHU28 shAPE1 v.s. shCtl	1.41 ± 0.05 **
MEF ^{la} mApe1 v.s. vec	2.52 ± 0.08 **
MEF ^{la} hAPE1 v.s. vec	2.54 ± 0.17 **

Relative values of maximal OXPHOS activities of cells (measured after FCCP treatment) are shown for (1) HCT116/shAPE1 vs. HCT116/shCtl, (2), JHU28/shAPE1 vs. JHU28/shCtl, (3) MEF^{la}/mApe1 vs. MEF^{la}/vec, (4) MEF^{la}/hAPE1 vs. MEF^{la}/vec.

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P < 0.005.

Table 2

Transcription levels of the PPARGC1A gene in MEF^{la}/vec.

Cell	PGC-1 α mRNA relative intensity
MEF ^{la} /vec	1.00 $\pm$ 0.016
MEF ^{la} /hAPE1	2.45 $\pm$ 0.015
MEF ^{la} /mApe1	3.80 $\pm$ 0.015

MEF^{la}/hAPE1, MEF^{la}/mApe1 in gene array analyses (data deposited to <http://www.ncbi.nlm.nih.gov/geo/> with the accession number GSE55765).