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Method for depletion of mitochondria DNA in human bronchial epithelial cells

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ABSTRACT

Mitochondria are increasingly recognized to play a role in the airway inflammation of asthma. Model systems to study the role of mitochondrial gene expression in bronchial epithelium are lacking. Here, we create custom bronchial epithelial cell lines that are depleted of mitochondrial DNA. One week of ethidium bromide (EtBr) treatment led to ~95 % reduction of mtDNA copy number (mtDNA-CN) in cells, which was further reduced by addition of 25 μM 2′,3′-dideoxycytidin (ddC). Treatment for up to three weeks with EtBr and ddC led to near complete loss of mtDNA. The basal oxygen consumption rate (OCR) of mtDNA-depleted BET-1A and BEAS-2B cells dropped to near zero. Glycolysis measured by extracellular acidification rate (ECAR) increased ~two-fold in cells when mtDNA was eliminated. BET-1A ρ0 and BEAS-2B ρ0 cells were cultured for two months, frozen and thawed, cultured for two more months, and maintained near zero mtDNA-CN. Mitochondrial DNA–depleted BET-1A ρ0 and BEAS-2B ρ0 cell lines are viable, lack the capacity for aerobic respiration, and increase glycolysis.

- BET-1A and BEAS-2B cells were treated with ethidium bromide (EtBr) with or without 2′,3′-dideoxycytidine (ddC) to create cells lacking mitochondrial DNA (mtDNA).
- Cells' mtDNA copy number relative to nuclear DNA (nDNA) were verified by quantitative polymerase chain reaction (qPCR).
- Cells were also assessed for oxidative phosphorylation by measures of oxygen consumption using the Seahorse analyzer.

Specifications table

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More specific subject area:	Molecular Biology
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Name and reference of original method:	Khozhukhar N, Spadafora D, Rodriguez Y, Alexeyev M. Elimination of Mitochondrial DNA from Mammalian Cells. Curr Protoc Cell Biol. 2018;78(1):20 11 1–20 11 4.
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Method details

Introduction

In this article, we present a method to generate cell lines lacking mitochondrial DNA from human bronchial epithelial cells. These newly created cells will be useful in understanding how mitochondrial DNA contributes to airway diseases. Mitochondria have their own circular DNA (mtDNA) that encodes 37 genes (13 proteins, 22 tRNAs, and 2 rRNAs) [1]. Each human cell contains hundreds to thousands of mitochondria depending on tissue and cell type, and each mitochondrion has multiple copies of mtDNA [2]. Variation of mtDNA copy number (mtDNA-CN), which is associated with mitochondrial enzyme activity and ATP production, can be used as an indirect biomarker of mitochondrial function [3,4]. mtDNA encodes 13 proteins of the electron transport chain; however, the majority of mitochondrial proteins are encoded by nuclear DNA (nDNA). Mitochondria independently replicate, transcribe, and translate their mtDNA using their own ribosomal and transfer RNA, which are encoded by mtDNA. A decrease in mtDNA can result in low levels of mitochondria encoded proteins for oxidative phosphorylation and a mismatch of the levels of mtDNA-encoded proteins and nDNA-encoded proteins [3,4].

Decrease in mtDNA-CN is associated with cardiovascular disease, diabetes, and cancers [3,4]. Mitochondrial changes have been mechanistically linked to asthma [5,6]. Previously, we reported that asthmatic airway epithelium has greater expression of nDNA-encoded mitochondrial respiratory chain complexes and hyper-dense supercoiled mitochondria [5]. While the mtDNA-CN in platelets in a small group of asthmatics was not different than controls [7], others reported that mtDNA-CN of whole blood DNA was significantly higher in asthma as compared to controls in African Americans [8]. While variation of mtDNA-CN in blood has been used in population studies, there are limited systems for studying the effect of loss of mtDNA in a cell-specific manner, such as in the airway epithelium.

Others have created cell lines without functional mtDNA (ρ0 cells). Currently, there are several available ρ0 cells, e.g., HeLa ρ0 [9], but these ρ0 cell lines were generated from immortal cancer cells. Creating ρ0 cells lines from non-tumorigenic sources will enable understanding of mitochondria in nonmalignant diseases. Development of primary airway cell line models will enable investigation of mitochondria, metabolism, and bioenergetics in airway diseases.

Here, we describe detailed methods for deletion of mtDNA from BET-1A and BEAS-2B bronchial epithelial cells, human cell lines derived from bronchial epithelial sources.

Materials and methods

Cell culture conditions for BET-1A and BEAS-2B

BET-1A and BEAS-2B cells, a kind gift from Curtis Harris (NCI) [10], were grown in LHC-9 + 1 % penicillin G (Pen) and streptomycin (Strep) (serum-free) medium (Gibco) following established protocols under sterile conditions [11]. Cells were plated on tissue culture plates coated with a coating medium (31 ug/ml collagen (Vitrogen 100, Cohesion Technologies), 10 ug/mL of BSA, 10 ug/mL fibronectin in LHC Basal medium). Cells were passaged with trypsin/EDTA at ratio 1:10. Cells are sensitive to traces of trypsin/EDTA [12]. Several precautions were taken by neutralizing trypsin with TNS (Trypsin Neutralizer Solution) and refreshing the culture medium 2 h post plating.

Cell culture conditions for HeLa and A549

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) + 10 % fetal bovine serum (FBS) + 1 % pen/strep medium and sub-cultured every 3–4 days. A549 cell were grown in Minimum Essential Medium (MEM) + 10 % FBS + 1 % pen/strep + 2 mM L-Glutamine medium and sub-cultured every 3–4 days.

Phase contrast microscopy

Cells in culture were examined under Nikon TMS Inverted Phase Contrast Microscope, and pictures were captured with the Olympus CKX53 Inverted Phase Contrast Microscope with Lumenera Infinity 3S camera and software (Ontario, Canada).

Generation of ρ0 cells

To create mtDNA-free cells, cultured cells were treated with the mutagenic compound ethidium bromide (EtBr) alone [13,14] or with addition of a nucleoside reverse transcriptase inhibitor ± 2′,3′-dideoxycytidine (ddC) [9]. The mutagenic compound is non-specific in its function, but nuclear DNA has a greater ability to repair itself compared to mitochondrial DNA [15]. Reverse transcriptase inhibitors (like ddC) have been shown to specifically inhibit mitochondrial DNA [16].

BET-1A and BEAS-2B cells were initially plated at 100,000 cells on 100 mm tissue culture dishes in LHC-9 supplemented with 50 µg/ml uridine, 55 mg/ml pyruvate, and 17 mM glucose. Cells were exposed to 25 or 50 ng/ml EtBr and 0, 25, or 50 µM ddC. Media with EtBr and ddC was changed every other day. Cells were split when the cells became 80 % confluent, and 500,000 cells were then plated on a new 100 mm tissue culture dish. The remaining cells were used to assess mtDNA-CN. Cells were grown under the given treatment conditions and tested until mtDNA-CN reached near-zero. At that point, cells were switched to media supplemented

with uridine, pyruvate, and glucose without EtBr or ddC. The potential $\rho 0$ cells were then split into 150 mm dishes at 100, 500, or 1000 cells per plate for single colony selection/isolation to derive a homogenous cell population. Clones were picked from plates and isolated in two 35 mm dishes for expansion. mtDNA-CN of expanded clones were verified by quantitative real-time Polymerase Chain Reaction (qPCR) and by gel electrophoresis, cellular respiration by Seahorse, and morphology via microscopy. Cells that persistently had near zero mtDNA-CN for two or more months of culture were designated BET-1A $\rho 0$ or BEAS-2B $\rho 0$

DNA extraction from cells and tissues

Genomic DNA was extracted with the Qiagen DNeasy Blood & Tissue kit according to the manufacturer's protocol [Qiagen, Hiden, Germany]. DNA amounts and concentrations were determined using Invitrogen Qubit 3 system with Qubit dsDNA Broad Range kit (Thermo Fisher Scientific, Waltham, MA USA). DNA from human lung and heart samples were obtained from explanted tissues during transplantations under Cleveland Clinic IRB1546 with informed consent.

Determination of Optimal Primer Targets for mtDNA and nDNA We used five sets of previously reported primers that target either nuclear or mtDNA genes (Table 2); multiple annealing primer pair sets were purchased from Integrated DNA Technologies (IDT) [Coralville, Iowa USA] for mitochondrial / nuclear DNA targets. A mitochondrial DNA monitoring primer set kit from Takara Bio [San Jose, CA USA] was purchased for comparison. Dried oligos were hydrated with water to 100 nM concentration. In a new 1.5 ml tube, forward and reverse primer sets were mixed at a 1:1 ratio to make a 10x stock for qPCR reactions.

Monitoring mitochondrial DNA elimination by qPCR

$\rho 0$ cells have mtDNA-CN close to zero (0.1 is typically reported in HeLa $\rho 0$) [9]. We aimed to have a similarly low number in our BET-1A and BEAS-2B $\rho 0$ cells.

The qPCR reaction was performed in triplicate for each sample at a volume of 20 µl per well. Each qPCR reaction contained 2 µl diluted sample DNA (varying concentrations of DNA tested as described below), 10 µl Power-SYBR Green Polymerase Chain Reaction (PCR) Master Mix (Applied Biosystems), 6 µl PCR water, and 2 µl primer mix containing 10 nM forward and reverse primers. qPCR reactions were performed on Applied Biosystems QuantStudio5 using MicroAmp Optical 384-Well Reaction Plates. The real-time PCR conditions were the following: initial denaturation 2 min at 50 °C ramp to 95 °C for 10 min followed by 40 amplification cycles of (95 °C for 15 s denaturation ramp down to 60 °C for 60 secs for annealing and extension) followed by a final 95 °C for 15 second hold. The cycle threshold values (Ct values) were determined automatically via QuantStudio Design & Analysis software (Thermo Fisher Scientific).

To optimize the qPCR methods, we tested several primer sets detecting both mtDNA and nDNA. Primer sets tested are reported in Table 1. For this purpose, master mix was prepared by mixing DNA, SYBR Green and PCR water, and then added to the PCR plates which contain the 2 µL of primer sets.

Quantification of mtDNA copy number using qPCR

mtDNA copy number was quantified in relation to nDNA. The average Ct values of qPCR targeting mtDNA were subtracted from the average Ct values of qPCR targeting the nDNA; this was defined as ΔCt. mtDNA-CN is calculated with the following formula: 2 (each PCR cycle represents doubling the amount) to the power of the ΔCt value and multiply by the number of homologous chromosomes (2 in normal primary cells).

$$(Avg.\,Ct\,Value\,Nuclear) - (Avg.\,Ct\,Value\,mitochondria) = \Delta\,Ct\,Value$$

$$2X\left(2^{\Delta\,Ct\,Value}\right) = mtDNA\,Copy\,Number$$

Absence of mtDNA by gel electrophoresis

To confirm the loss of mtDNA, DNA from BET-1A $\rho 0$ and BEAS-2B $\rho 0$ cells were compared to the original non-treated source cell line (BET-1A and BEAS-2B) by PCR and gel electrophoresis. PCR reactions were performed in a volume of 25 µl per well on a NYX Technik A6 Thermal Cycler PCR machine with mtDNA tRNA-leu (MT-TL1) and nuclear DNA GAPDH primer sets. Each PCR reaction contained 2 µl annealing primer set, 12.5 µl MyTaq HS mix (Meridian Bioscience, Memphis, TN), 2 µl DNA template (5 ng total), and 8.5 µl PCR water. The PCR conditions were as follows: initial denaturation to 94 °C for 1 min followed by 25 amplification cycles of (94 °C for 30 s denaturation, ramp down to 55 °C 30 secs for annealing, and 72 °C for 30 s extension) followed by a final 72 °C for 5 min ramp down to 4 °C hold. A 1.5 % ultra-pure agarose gel in 1X TAE buffer was cast. 10 µl PCR sample and 5 µl 1 kb DNA ladder were loaded in each well. Gels were run for 45 mins at 80 Vs, and the image was documented with the Bio-RAD ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA USA).

Table 1
List of reagents, vendors, and catalog numbers.

Item	Company (Vendor)	Catalog Number	Protocol
LHC-9 Medium	Gibco	12680	Tissue Culture
Pen/Strep	Gibco	15070	
Vitrogen 100	Cohesion Technologies	FXP-019	
Fibronectin	Calbiochem	341631	
LHC Basal Medium	Gibco	12677	
Trypsin/EDTA	Lonza	CC-5012	
Trypsin Neutralizer Solution	Lonza	CC-5002	
Fetal Bovin Serum	Atlas Biologicals	EF-0500A	
Minimum Essential Medium	Gibco	11090	Generation of ρ0 Cells
GlutaMax	Gibco	35050	
Ethidium Bromide	Sigma	E7637	
2′,3′-dideoxycytidine	Sigma	D5782	
Uridine	Sigma	U3750	
Pyruvate	Sigma	P5280	
D-Glucose	Sigma	G7021	
DNeasy Blood & Tissue Kit	Qiagen	69504	DNA Extraction
Qubit dsDNA Broad Range Kit	Thermo Fisher	Q32853	
QuantStudio 5 qPCR System	Thermo Fisher		qPCR
Human Mitochondrial Monitoring Kit	Takara	7246	
Custom DNA oligos	Integrated DNA Technologies		
Power-SYBR Green	Thermo Fisher	4367659	
MicroAmp Optical 384-Well Plate	Thermo Fisher	4309849	
NYX Technik A6 Thermal Cycler	NYX Technik		PCR
MyTaq HS Mix	Bioline	BIO-25045	
Agarose	Thermo Fisher	16500	
XFe24	Agilent		Seahorse
Seahorse XFe24 FluxPaks	Agilent	102340	
XF Assay Medium	Agilent	103680	
HEPES	Lonza	CC-5024	
Sodium Pyruvate	Sigma	P2256	
Oligomycin	Sigma	O4876	
Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone	Sigma	C2920	
Rotenone	Sigma	R8875	
Antimycin A	Sigma	A8674	
2-deoxyglucose	Sigma	D8375	

Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in cells

When qPCR copy numbers indicated ρ0 sample, loss of electron transport chain activity was evaluated with a functional assay to measure cellular respiration and energy metabolism (bioenergetics).

Assessment of cellular bioenergetics was performed using the extracellular flux analyzer XFe24 (Seahorse Biosciences, Agilent, Billerica, MA) with a modified MitoStress Test protocol. BET-1A, BEAS-2B, BET-1A-ρ0, and BEAS-2B-ρ0 cells were plated on a precoated Seahorse XF24 V7 PS cell culture microplate with 30×10 [3] cells per well. Cells were cultured in the medium with supplements (uridine, pyruvate, and glucose) overnight. On the day of the assay, growth medium was removed, cells were washed with XF assay medium, and the media was replaced with XF assay medium. XF assay medium was serum-free DMEM supplemented with 5 mM HEPES, 5.6 mM D-glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate in the absence of sodium bicarbonate, and pH of the media was adjusted to 7.4. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at baseline, in response to 1 μM oligomycin (port A), 1 μM carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (port B), 1 μM rotenone and antimycin A (port C), and 100 mM 2-deoxyglucose (port D) for three measurements each. After extracellular flux analysis, cells were stained with 1.6 μM of Hoechst 33342 stain (Thermo Fisher Scientific, Waltham, MA USA), and cell numbers were determined with the Cytation 5 (Biotek Instruments, Agilent, Winooski, VT USA). OCR and ECAR data were expressed as pmol/minute/10^3 cells and mpH/minute/10^3 cells, respectively. Data are mean ± SEM of at least 5 replicates.

Method validation

Validity of mtDNA copy number quantification

Sets of primer pairs for mtDNA and nDNA targets for routine testing were established. There is expected variability in Ct value amounts for housekeeping nDNA genes when comparing to mtDNA genes. As the number of sets of primer pairs used increases, accuracy increases, but the quantity and cost of each PCR reaction also increases. qPCR was run with five different nuclear and mitochondrial targets (Fig. 1). Sets of primer pair combinations were compared with the average of all five annealing sets for both

Table 2
Primer sequences for target genes.

Genome	Target Gene Abbreviation	Gene Name	Primer	Primer Sequence	Reference
Nuclear (A)	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Forward Reverse	5′-GTCAGCCGCATCTTCTTTTG- 3′ 5′-GCGCCCAATACGACCAAATC- 3′	[17]
Nuclear (B)	B2MR	ß2-microglobulin Regulator	Forward Reverse	5′-TGCTGTCTCCATGTTTGATGTATCT- 3′ 5′-TCTCTGCTCCCCACCTCTAAGT - 3′	[9]
Nuclear (C)	RB1	Retinoblastoma RB Transcriptional Corepressor 1	Forward Reverse	5′-CCAGAAAATAAATCAGATGGTATGTAACA-3′ 5′-TGGTTTAGGAGGGTTGCTTCC-’3	[18]
Nuclear (D)	SLCO2B1	Solute Carrier Organic Anion Transporter Family Member 2B1	Forward Reverse	Takara mtDNA Kit* Takara mtDNA Kit	Takara Cat# 7246
Nuclear (E)	SERPINA1	Serpin Family A Member 1	Forward Reverse	Takara mtDNA Kit Takara mtDNA Kit	Takara Cat# 7246
mtDNA (a)	D-Loop	Mitochondrial Displacement Loop	Forward Reverse	5′-CCCCACATTAGGCTTAAAAACAGAT-3′ 5′-TATACCCCCGGTCGTGTAGCGGT-3′	[17]
mtDNA (b)	MT-TL1	tRNA-leuUUR	Forward Reverse	5′-CACCCAAGAACAGGGTTTGT-3′ 5′-TGGCCATGGGTATGTTGTTA-3′	[9]
mtDNA (c)	MT-CO2	Cytochrome C Oxidase subunit II	Forward Reverse	5′-CCACTGTAAAGCTAACTTAGCATTAACC-3′ 5′-GTGATGAGGAATAGTGTAAGGAGTATGG-3′	[18]
mtDNA (d)	MT-ND1	Mitochondrially Encoded NADH: Ubiquinone Oxidoreductase Core subunit 1	Forward Reverse	Takara mtDNA Kit Takara mtDNA Kit	Takara Cat# 7246
mtDNA (e)	MT-ND5	Mitochondrially Encoded NADH: Ubiquinone Oxidoreductase Core subunit 5	Forward Reverse	Takara mtDNA Kit Takara mtDNA Kit	Takara Cat# 7246

* Takara primers sets are from a commercial kit used in determining mitochondrial copy numbers Catalog # 7246 [*The Human Mitochondrial DNA (mtDNA) Monitoring Primer Set*].

nuclear and mitochondrial targets to determine which pair would be ideal for mtDNA-CN rigor and reproducibility. Based on Ct values in Fig. 2, the following set of primer pairs was chosen for mtDNA [tRNA-LeuUUR (MT-TL1) and Cytochrome C Oxidase subunit II (MT-COII)]. For nDNA, GAPDH and ß2-microglobulin (Glob) were chosen.

We also assessed whether variability of the DNA template amount would affect the calculated mtDNA-CN. DNA from six different source samples (BET-1A, BEAS-2B, HeLa, human lung tissue, human heart tissue, and the A549 cell line) were extracted and logarithmically diluted [0.1, 0.3, 1, 3, 10, 30, 100 ng] for mtDNA-CN determination (Fig. 2A). The calculated mtDNA-CN were consistent within each sample type from 0.1 to 30 ng DNA input. This indicates that any variation in DNA template that might occur within 0.1 and 30 ng will not significantly alter our mtDNA-CN calculations. Template dilution curves of the targets used to generate our mtDNA-CN show the DNA template amounts regarding Ct values are linear between 0.3 - 30 ng for our nDNA and mtDNA targets (Fig. 2B-E).

Generating ρ0 cells

The process for creating ρ0 BET-1A and BEAS-2B cells required treating the cells with both EtBr (a mutagen) and ddC (a nucleoside reverse transcriptase inhibitor). Two to three weeks of EtBr treatment led to ~95 % reduction of mtDNA copy numbers in both cell lines, which was further reduced by the addition of at least 25 μM ddC (Fig. 3A/B). While the loss of 95% mtDNA copy numbers is significant, creating long-term ρ0 cells requires near complete loss of mtDNA. This only occurred in cells that were treated with both EtBr and ddC, i.e., mtDNA-CN reached <1 copy per nDNA. EtBr treated cells did not maintain low mtDNA-CN when treatment was discontinued. Once a near loss of mtDNA-CN was achieved, cells were plated for single colonies and allowed to expand with continued testing for mtDNA-CN. To confirm cells lacked mtDNA by a method other than qPCR, DNA was amplified via PCR for mtDNA and nDNA targets and evaluated for PCR products by agarose electrophoresis (Fig. 3C/D). The band targeted for mitochondrial (MT-TL1) was absent in the newly created BET-1A ρ0 and BEAS-2B ρ0, while band targeted for nDNA (GAPDH) was still intact in BET-1A ρ0 and BEAS-2B ρ0. Established HeLa and HeLa ρ0 cells were used as controls.

Validation of ρ0 cell lines

To confirm loss of functional electron transport chain, we performed Seahorse extracellular flux analysis. BET-1A and BEAS-2B have OCR and ECAR profiles typical of airway cells [19]. However, the basal OCR values of mtDNA-depleted BET-1A and BEAS-2B cells were basically undetectable [OCR (pmol/min/10^3 cells), baseline vs. treatment: BEAS-2B, 4.68 ± 0.12 vs. 0.06 ± 0.07 $P<0.001$ and $N=4$ (baseline) 7 (treatments), technical replicates; BET-1A, 6.55 ± 0.30 vs. 0.02 ± 0.10 $P<0.001$ and $N=4$ (baseline), 7 (treatments), technical replicates]. ECAR values increased significantly in ρ0 cells [ECAR (mpH/min/1000 cells), baseline vs. treatment: BEAS-2B,

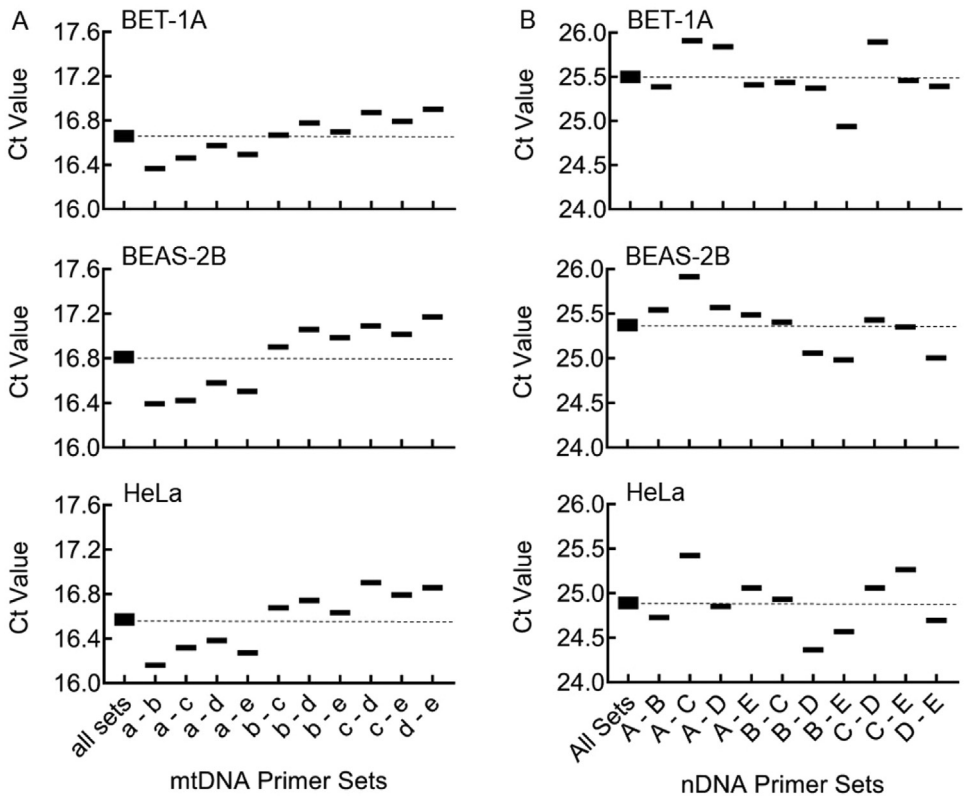

Fig. 1. Determining primers pairs for mtDNA-CN calculation. Cycle thresholds for various combination of primers were used to determine the ideal pair to represent the sample. (A) Average Ct values of five sets of mitochondrial primers (all sets) were compared to various combinations of two sets of Ct values on BET-1A, BEAS-2B, or HeLa mtDNA. (B) Average Ct values of five sets of nuclear primers (All Sets) were compared to various combinations of two sets of Ct values on BET-1A, BEAS-2B, or HeLa nDNA. A pair of primers were chosen as a representative value of the five sets, allowing future PCR to be performed with two pair sets for each nuclear or mitochondrial Ct value determination. Dotted line represents average Ct values of the five sets. See Table 1 for primer reference.

Table 3
Monitoring mtDNA-CNs of ρ0 cells over 4 months. Data are mean ±SD.

	ρ0 Generated	1 Month	2 Months	4 Months
BET-1A ρ0	1.0	0.16 ± 0.2	0.03 ± 0.02	0.08 ± 0.1
BEAS-2B ρ0	0.1	0.04 ± 0.02	0.04 ± 0.01	0.02 ± 0.01

0.50 ± 0.03 vs. 0.94 ± 0.10 P=0.005 and N=4 (baseline), 7 (treatments), technical replicates; BET-1A, 0.80 ± 0.04 vs. 1.14 ± 0.06 P=0.001 and N=4 (baseline), 7 (treatments), technical replicates]. Interestingly, injection of oligomycin (the ATP synthase inhibitor) did not increase the ECAR in BET-1A or BEAS-2B ρ0 cells (Fig 4A/B). These suggest that the cells were shifting energy metabolism from oxidative phosphorylation to glycolytic metabolism. These new ρ0 cells were multiplying and maintaining their shape and size typical of regular BET-1A cells (Fig 4C/D). Visibly, the cells in culture were proliferating and had no significant difference in viability. Newly created ρ0 cells were grown for 2 months and frozen for future expansion. Frozen cells were then thawed and cultured for 2 months to generate aliquots of cells for future experiments. Cells were tested monthly for mtDNA-CN by qPCR and were verified to have mtDNA-CN under 0.2, giving us confidence these BET-1A and BEAS-2B maintained their ρ0 status (Table 3).

Overview

Creating non-tumorigenic derived cell lines without mitochondrial DNA can be accomplished with common research tools and techniques, opening up the potential of generating novel ρ0 cell lines. qPCR measurement of mtDNA copy numbers is a quick and reliable method to determine loss of mitochondria, while quantitative measures of oxygen consumption can validate the mtDNA deletion. Furthermore, the BET-1A and BEAS-2B cells depleted of mtDNA are viable and utilize greater glycolysis for energy production.

Before creating ρ0 cells, establishing a protocol for determining mitochondrial copy numbers requires choosing annealing primer sets targeting mitochondrial DNA and nuclear housekeeping genes. While there will be variable Ct value results depending on the

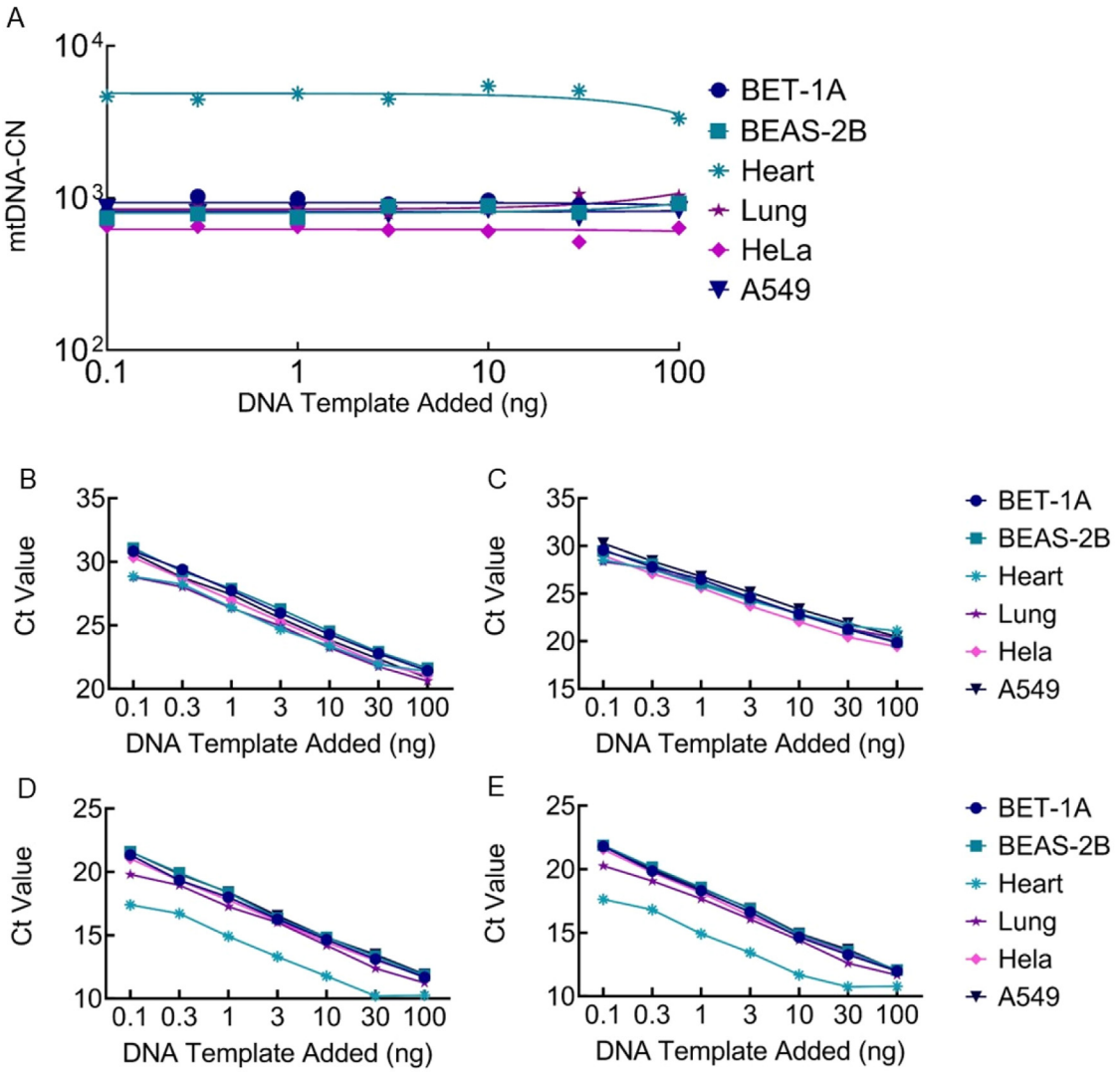

Fig. 2. mtDNA-CN with various amount of DNA template. BET-1A, BEAS-2B, human heart/lung tissue, HeLa and A549 DNA were logarithmically diluted [0.1, 0.3, 1, 3, 10, 30, 100 ng] (A) and used as a template for qPCR to determine mtDNA copy numbers. Template dilution curves of the Ct values of individual primer pairs for the following nDNA targets (B) GAPDH (C) B2MR and mtDNA targets (D) MT-TL1 (E) MT-CO2.

target choice, as long as those gene targets are constant, comparative sample analyses can be accomplished. There are several factors to consider when selecting which genes to target. For nuclear primer targets, standard single chromosome housekeeping genes that remain constant under multiple conditions are ideal [20–22]. Having a housekeeping gene not affected by treatments will allow different sample sets to be compared. For mitochondrial targets, we determined that looking at two targets would be prudent as opposed to a single mtDNA target. By testing multiple mtDNA targets, we confirm that the entire mtDNA is eliminated. When creating primer sets for both nuclear and mtDNA, melting temperatures of all primer sets should be similar (~60 °C). For our mtDNA targets, the average Ct values of MT-TL1 and MT-COII Ct values were consistently close to the average of five Ct values tested in three cell lines (BET-1A, BEAS-2B, and HeLa) and was the prime criteria for selecting mtDNA primers. For our nuclear targets, the average Ct values of GAPDH and ß2-microglobulin were used based on these being well-established stable housekeeping genes.

We optimized the minimum amount of DNA needed for mtDNA determination in order to retain the greatest number of cells for ongoing culture. When we performed a dilution curve on our DNA as a template, there was little difference in our calculated mitochondrial copy number in the six different sample types within the range of 0.1 to 30 ng DNA template. This provides confidence that despite any range of starting DNA, findings would still be accurate.

A limitation of SYBR-Green qPCR is less reliability and sensitivity as compared to TaqMan [23,24]. To address this, we used diluted DNA standards (HeLa and HeLa ρ0) to assess reproducibility across multiple qPCR runs (data not shown). The TaqMan chemistry system has a greater sensitivity and more consistent results, but the downside of TaqMan is greater cost of annealing primer

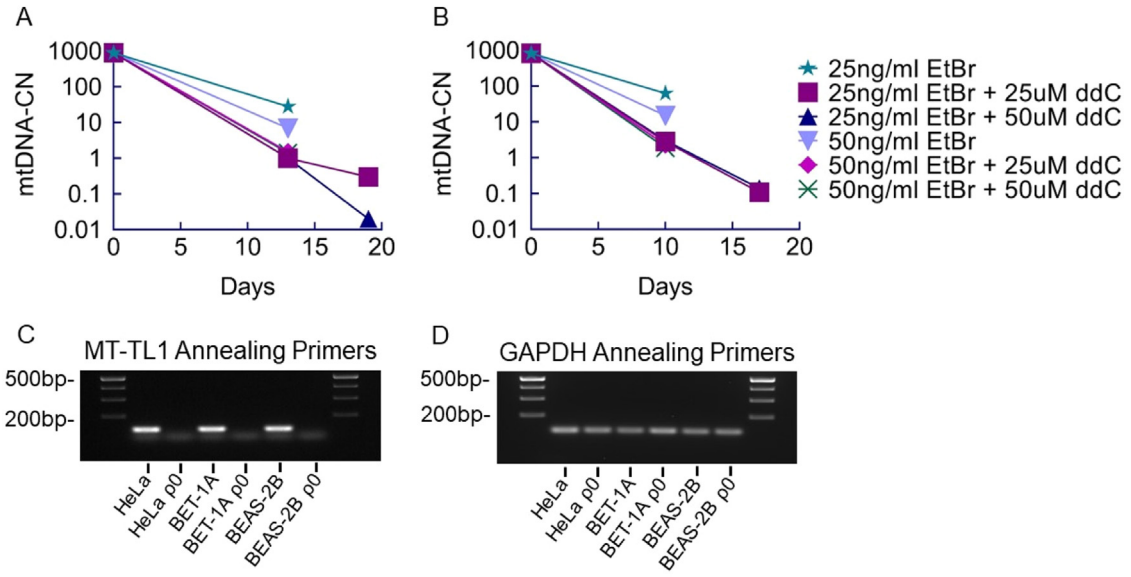

Fig. 3. Depletion of mtDNA-CN in BET-1A and BEAS-2B ρ0. Analysis of mtDNA-CN over a 2–3 week period with different treatment conditions. BET-1A (A) and BEAS-2B (B) cells were treated with either 25 or 50 ng/ml EtBr +/- 25 or 50 μM ddC on day 0. This graph tracks the progress of mtDNA copy numbers over a 2-week period under different treatment conditions. Some isolated cells were pelleted, and DNA was extracted, which in turn was used as the template for qPCR to determine mtDNA copy numbers. Amplification of MT-TL1 and GAPDH in cells detected by PCR. 1.5 % agarose gel from PCR using tRNA-leu^{UUR} (C) and GAPDH (D) annealing primers.

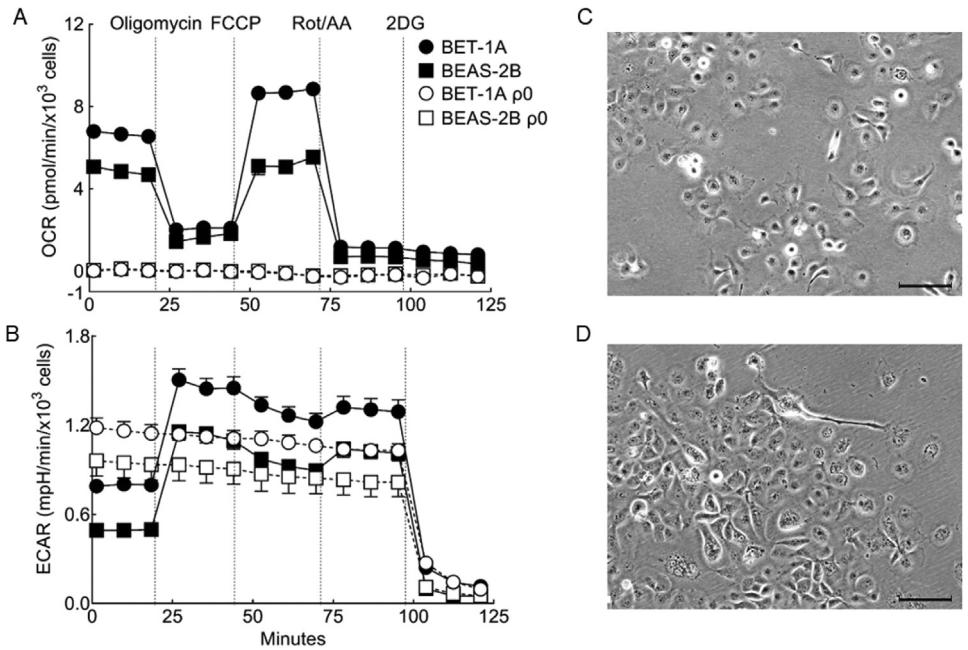

Fig. 4. Glycolysis replaces oxidative phosphorylation as the source of energy in BET-1A p0 and BEAS-2B p0 cells. Assessment of cellular energy metabolism was performed using the XFe24 extracellular flux analyzer (Seahorse). OCR (A) and ECAR (B) profiles are shown for BET-1A (dark circle), BEAS-2B (dark square), BET-1A p0 (open circle), and BEAS-2B p0 (open square) at baseline, in response to oligomycin, FCCP, rotenone/antimycin A, and 2-deoxyglucose (2DG). Data are mean ± SEM of at least three replicates. BET-1A (C) and BET-1A ρ0 (D) cells in culture (100x magnification: scale bar length = 100 μM, lower right).

sets. Thus, for qPCR to determine copy numbers in this methodology requiring multiple testing, SYBR-Green was a cost-effective method for determining loss of mtDNA. Unfortunately, methodology plays a critical role in quantitating mtDNA copy numbers, and different annealing targets and variable chemistry make it difficult to compare mtDNA copy numbers in cells across various reports. Nevertheless, the average numbers of mtDNA in BET-1A and BEAS-2B is ~800 mtDNA to nDNA gene, which is comparable to prior reports in cancer cell lines of ~1000 mtDNA to 1 nDNA [25]. All data shown in this manuscript were performed with SYBR-Green chemistry.

This report shows that it is possible to effectively delete mitochondrial DNA from transformed bronchial epithelial cells without compromising cell viability. In our first attempt to create BET-1A and BEAS-2B $\rho 0$, the treatment condition was only using EtBr to mutate the mtDNA. This did decrease mtDNA-CN by ~95 % in two to three weeks. However once EtBr treatment was discontinued, cells recovered mtDNA to original levels (data not shown). In the BET-1A and BEAS-2B cells, we determined that 25 ng/ml EtBr with 25 μM ddC was ideal to generate $\rho 0$ cells. This resulted in cells with near-zero mtDNA and oxygen consumption. Considering the nature of EtBr to cause lasting damage to nuclear DNA and since the results of both tested concentrations were similar, using a lower EtBr dose combined with ddC is prudent to avoid nuclear damage [26]. Once the treatment phase of creating $\rho 0$ cell was successful, plating for single colonies on larger dishes at various amounts led to stable clones of cells. Overall, this method is highly successful for creation of $\rho 0$ bronchial epithelial cells. These novel cell lines will be important in future studies to determine the role of mitochondria in bronchial epithelial functions.

Related research article

Khozhukhar N, Spadafora D, Rodriguez Y, Alexeyev M. Elimination of Mitochondrial DNA from Mammalian Cells. Curr Protoc Cell Biol. 2018;78(1):20 11 1–20 11 4.

Ethics statements

DNA from human lung and heart samples were obtained from explanted tissues during transplantations under Cleveland Clinic IRB1546 with informed consent.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Michael V. Novotny: Writing – review & editing. **Weiling Xu:** Writing – review & editing. **Anny Mulya:** Writing – review & editing, Formal analysis. **Allison J. Janocha:** Writing – review & editing, Investigation. **Serpil C. Erzurum:** Writing – review & editing.

Data availability

Data will be made available on request.

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