Molecular Cancer

Research

Nuclear genes involved in mitochondria-to-nucleus communication in breast cancer cells

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Abstract

Background: The interaction of nuclear and mitochondrial genes is an essential feature in maintenance of normal cellular function. Of 82 structural subunits that make up the oxidative phosphorylation system in the mitochondria, mitochondrial DNA (mtDNA) encodes 13 subunits and rest of the subunits are encoded by nuclear DNA. Mutations in mitochondrial genes encoding the 13 subunits have been reported in a variety of cancers. However, little is known about the nuclear response to impairment of mitochondrial function in human cells.

Results: We isolated a Rho^0 (devoid of mtDNA) derivative of a breast cancer cell line. Our study suggests that depletion of mtDNA results in oxidative stress, causing increased lipid peroxidation in breast cancer cells. Using a cDNA microarray we compared differences in the nuclear gene expression profile between a breast cancer cell line (parental Rho^+) and its Rho^0 derivative impaired in mitochondrial function. Expression of several nuclear genes involved in cell signaling, cell architecture, energy metabolism, cell growth, apoptosis including general transcription factor TFIIH, v-maf, AML1, was induced in Rho^0 cells. Expression of several genes was also down regulated. These include phospholipase C, agouti related protein, PKC gamma, protein tyrosine phosphatase C, phosphodiesterase 1A (cell signaling), PIBF1, cytochrome p450, (metabolism) and cyclin dependent kinase inhibitor p19, and GAP43 (cell growth and differentiation).

Conclusions: Mitochondrial impairment in breast cancer cells results in altered expression of nuclear genes involved in signaling, cellular architecture, metabolism, cell growth and differentiation, and apoptosis. These genes may mediate the cross talk between mitochondria and the nucleus.

Background

Mitochondria participate in numerous functions in the cell. In addition to producing energy, mitochondria are involved in intermediary metabolism, ion homeostasis, synthesis of lipids, amino acids, and nucleotides, active transport processes, cell motility, and cell proliferation [1–4]. Recent developments also demonstrate that mitochondria are key regulators of programmed cell death [4]. Mitochondrial dysfunction is one of the most profound features of cancer cells. Several distinct differences between the mitochondria of normal cells and cancer cells have been observed at the microscopic, molecular, biochemical, metabolic and genetic levels [5]. Microscopic study of oncocytic tumors revealed mitochondrial hyperplasia [6], and differential expression of mitochondrial cytochrome oxidase II in benign and malignant breast tissues has also been reported [7]. Furthermore, mutations in mitochondrial DNA (mtDNA) are commonly found in a variety of cancers including the ovarian, thyroid, salivary, kidney, liver, lung, colon, gastric, brain bladder, head and neck, leukemia and breast cancers [8].

Mitochondrial DNA encodes two rRNA, 22 tRNA and 13 proteins [2]. Each of the polypeptides form subunits of four respiratory enzyme complexes localized to the inner mitochondrial membrane [2]. These subunits include seven subunits of respiratory enzyme complex I, one subunit of complex III, three subunits of complex IV, and two subunits of complex V [2]. All other mitochondrial proteins, including those involved in the replication, transcription and translation of mtDNA, are encoded by nuclear genes and are targeted to the mitochondrion by a specific transport system [2]. Although the mitochondrial and nuclear genomes are physically distinct, there is constant communication between the two genomes to carry out many of the mitochondrial functions. For example, in the yeast *Saccharomyces cerevisiae*, RTGproteins monitor the functional state of mitochondria and mediate the intergenomic communication between the mitochondria and the nucleus. In this organism, RTG regulates transcription of numerous genes involved in several pathways that help cells adapt to mitochondrial dysfunction [9]. In addition, yeast cells shift their metabolic profile by altering expression of a number of genes that respond to depletion of the mitochondrial genome [9]. As in the case of yeast, the depletion of mitochondrial DNA from human cell lines leading to the generation of cell lines devoid of mtDNA (denoted Rho[0]) provides an opportunity to study nuclear responses to impairment of mitochondrial function. In the present study we isolated a Rho[0] derivative of a breast cancer cell line. Our results demonstrate that Rho[0] derivative experience increased oxidative stress and has impaired mitochondrial function. Using cDNA microarray we compared differences in the nuclear gene expression profile between parental Rho[+] breast cancer cell line and

its Rho[0] derivative. Our study suggests a variety of structural genes as well as genes involved in cellular signaling and transcriptional regulation are differentially regulated in Rho[0] breast cancer cells.

Methods

Reagents

The plasmid containing the mitochondrial DNA-specific probe, pTZ19/K5, was a generous gift from Dr. Michael King (Thomas Jefferson University, USA). The probe has bases 2578–4122 of the human mitochondrial genome corresponding to a portion of the NADH dehydrogenase I gene, subcloned into the pTZ19 plasmid. Chemical reagents were obtained from Sigma-Aldrich, Corp. (St. Louis, MO) unless otherwise indicated.

Cell Culture

MDA-MB-435 cells were obtained from American Type Culture Collection, and maintained in Dulbecco's modified Eagle's medium/ Ham's F-12 (50:50 mix) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (GIBCO/Invitrogen Life Technologies, Carlsbad, CA), 2 mM L-glutamine, penicillin, streptomycin, and 50 μg/ml uridine. For measurement of cell growth in glucose and galactose, a 50:50 mix glucose-free Dulbecco's modified Eagle's medium (Sigma-Aldrich) and glucose-free Ham's F-12 (Biofluids, Rockville, MD) was used. Either glucose or galactose was added to 3.15 g/L, and the medium was supplemented as described above. Cells were cultured in a water-humidified incubator at 37°C in 10 % CO_2/90% air. Medium was replenished every 2 days.

Generation of ρ^0 Cell Line

Cells were cultured in the routine growth medium containing 50 ng/ml ethidium bromide (0.22 μm-filtered) with regular replenishment of medium. After a minimum of 30 days culture, single cell clones were isolated by limiting dilution in 96 well culture clusters, in the presence of ethidium bromide. The mtDNA status of clones was determined by Southern blotting, after which Rho[0] clones were maintained in medium without ethidium bromide. Cells were screened after a minimum of 30 population doublings in the absence of ethidium bromide to verify continued Rho[0] status.

MtDNA Southern blotting

Total cellular DNA was isolated from washed exponentially growing cells extracted in 10 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 320 mM sucrose, 1% Triton X-100, 0.1 mg/ml proteinase K, 0.58% sodium dodecyl sulfate at 37°C for 2 h. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (Fisher Scientific, Pittsburgh, PA) was added to the extracts, and DNA was isolated from the aqueous phases after phase separation by centrifugation. After a second round of phenol/chloroform/isoamyl alcohol ex-

traction, DNA was cleaned with chloroform/isoamyl alcohol (25:1), twice with diethyl ether, and precipitated. Three μg of total DNA were digested with PvuII, which cuts a single site in the mitochondrial genome. Linearized DNA was electrophoresed on a 0.8% agarose gel in TAE containing ethidium bromide, and photographed. The DNA was denatured and transferred to Nytran SuPer-Charge membrane (Schleicher & Schuell, Keene, NH) using TurboBlotter (Schleicher & Schuell, Keene, NH) rapid downward transfer with the alkaline transfer conditions recommended by the manufacturer. DNA was crosslinked by UV (1200 joules) and the membrane was prehybridized at 65°C for 4 h in 3X SSC, 0.25% milk powder, 0.25% SDS. Oligonucleotide probe was excised from pTZ19 (K5) by digestion with HindIII and EcoRI and isolated from low melting point agarose gel using β-agarase (New England Biolabs, Beverly, MA). Probe (25 ng) was labeled with ^{32}P-deoxycytidine (New England Nuclear/ Perkin Elmer Life Sciences, Boston, MA) by RadPrime random primer labeling (Invitrogen) and unincorporated nucleotides were removed by Concert ™ PCR purification kit (In Vitrogen). After hybridization with labeled probe for a minimum of 12 h, the membrane was washed twice with 2X SSC, 0.25% milk powder, 0.25% SDS for 30 min, and twice for 30 min with 0.5X SSC, 0.25% milk powder, 0.25% SDS. All washes were performed at 65°C. Hybridization was visualized by autoradiography with X-ray film (Kodak) at -80°C.

Cytochrome oxidase immunoblotting
Exponentially growing cells were washed with ice-cold PBS, and collected in AG Buffer(0.3 M PMSF,0.9% NaCl,0.1% Triton X,1 mM EDTA,0.5% NP40,50 mM Na_2HPO_4), PMSF (100 μg/ml), leupeptin(2 μg/ml), and aprotinin (2 μg/ml). Extracts were homogenized for 20 strokes on ice and protein concentration was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE on 12% polyacrylamide gels under denaturing conditions. Proteins were transferred to Immobilon-P nylon membrane by electrophoresis, and protein transfer visualized by staining with Ponceau S and destaining with deionized water. The membrane was blocked in 5% milk in TBST overnight at 4°C, washed with TBST and hybridized with mouse anti-cytochrome oxidase II (Molecular Probes Inc., Eugene, OR) 1:1000 in 3% milk/TBST for 1 h at room temperature. After washing, membranes were hybridized with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:5000 in 3% milk/TBST for 1 h at room temperature, washed, and incubated with ECL chemiluminescence solution. Antibody binding was visualized by exposure of membrane to X-ray film and subsequent processing. Membranes were stripped at 50°C in, washed extensively with TBST, blocked and hybridized as

described above using a mouse anti-actin primary antibody at 1:1000 and HRP-conjugated goat anti-mouse IgG secondary antiserum as described above.

Cell growth in glucose or galactose
The Rho$^+$ and Rho^0 cells were plated in 24 well tissue culture clusters (50,000 cells/well) in growth medium containing 3.15 g/L glucose. After overnight culture, cells were washed three times with PBS, and growth medium containing either glucose or galactose (3.15 g/L) was added back to the cells. MTT at a final concentration of 1 μg/ml was added to triplicate wells of both cell lines grown in both carbon sources immediately (t = 0) and at 24, 48, and 96 h following medium change. MTT was also added to one well containing each medium only, as a control. After 4 h incubation with MTT, medium was aspirated, the converted MTT-formazan extracted with DMSO, NaCl, glycine, pH 10.5, and the absorbance at 553 nm was measured by spectrophotometer.

Measurement of lipid peroxidation
Thiobarbituric acid reactivity was measured by a modification of Ohkawa et al., [10] as described by Bowie et al. [11]. Subconfluent cultures were collected by trypsinization, washed with ice cold PBS and lysed by three freeze/ thaw cycles in sterile deionized water. Protein concentration of samples was estimated using Bio-Rad Protein Assay Reagent compared with BSA standards. 1',1',3',3'-tetramethoxypropane (TMP) was diluted to 10 mM in 20 mM Tris-Cl, pH 7.5, and serially diluted for standards. Equal volumes (200 μl) of lysate or TMP standards were mixed with 800 μl of solution containing 4 mg/ml 2-thiobarbituric acid (TBA), 0.5% SDS, and 9.4% glacial acetic acid, and heated at 95°C for 1 h. Samples were cooled to room temperature, centrifuged for 10 min at 16,000 × g, and the absorbances of the supernatants were read at 532 nm. Concentrations of TBA-reactive substances were estimated by comparing values to TMP standard curve.

Microarray analysis
Total RNA was extracted from exponentially growing cells with Trizol. Precipitated RNA was resuspended in DEPC-treated deionized water, and concentration was estimated by absorbance at 260 nm. Labeled cDNA was prepared by RT-PCR according to the Array-Advantage UA protocol from Ambion (Ambion Inc., Austin, TX). Briefly, 5 μg experimental RNAs and 1 μl alignment control RNA were denatured at 70°C for 10 min and allowed to anneal to oligodT by cooling to room temperature for 5 min. M-MLV reverse transcriptase (400 units), [α-^{33}P] dATP, dCTP, dGTP, dTTP and 2 μl 10X RT buffer were added to a final reaction volume of 20 μl, and RT reaction proceeded for 2 h at 42°C. Unincorporated nucleotides were removed by passing reaction through NucAway™ Spin Column and radiolabel incorporation of the reaction determined by liq-

uid scintillation counting. Human LifeGrid Array membranes (Incyte Genomics, Palo Alto, CA), which contain 8000 cloned genes spotted in two positions on each filter, were used for analysis. Membranes were blocked with heat denatured herring sperm DNA and prehybridized for 1 h with 15 ml ULTRArray Hybridization Buffer at 68°C. Labeled cDNA from each RT reaction was heat denatured at 95°C for 5 min, added to the prehybridization buffer, and hybridized to an array for 16 h at 68°C. Membranes were washed twice 30 min each with 2X SSC, 0.5% SDS, followed by 0.5X SSC, 0.5% SDS, at 60°C. Membranes were exposed to phosphorimager screen for 3 hr, and imaged as .gel files using a phosphorimaging scanner with TyphoonScan (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ) software. Spot intensities were measured using ArrayVision software (Imaging Research, Inc., St. Catharines, Ontario, Canada), and the values. Genes corresponding to differentially expressed RNAs were identified by Incyte Genomics clone identification numbers. Membranes were stripped and reconstituted according to the manufacturer's instructions, and hybridizations repeated using freshly labeled cDNAs. In repeats, each cDNA was hybridized to the opposite membrane from that to which it had been previously hybridized. Only those genes that were consistently differentially expressed in 3 hybridizations were selected for analysis.

RT-PCR analysis

Trizol reagent (In Vitrogen) was used to isolate RNA from 10^6 cultured cells according to the manufacturer's instuction. One and half micrograms of total RNA was reverse transcribed using Superscript II Rnase H^- reverse transcriptase (In Vitrogen). Two microlitres of the reverse transcribed products was used in the PCR reactions. Primers were made for a twenty of the differentially expressed genes and for G3PDH a housekeeper gene. Twenty five microlitres of the PCR reactions contained 20 mM Tris-HCL, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP and 10 picomoles of each primer and one unit of Taq DNA polymerase (Invitrogen). Each gene was amplified by PCR using various cycle numbers ranging from 30 to 55 cycles to ascertain its linear amplification. In general the PCR profile consisted of an initial denaturation at 94°C for 5 minutes and a variable number of cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 1 min and extension for 2 min at 72°C with a final extension at 72°C for 10 min. To aid comparison five microliters of the PCR products of the differentially regulated genes were mixed with an equal amount of the housekeeper gene products and electrophoresed on a 1% agarose gel and visualized after staining with ethidium bromide (0.5 μg/ml) under ultra violet.

Results

Generation of Rho⁰ breast cancer cell line

The rarity of recombination in the replication of mtDNA is prohibitive to the possibility of selectively inactivating mtDNA encoded genes by homologous recombination [12]. However, by culturing cells in the presence of low concentrations of ethidium bromide, it is possible to preferentially inhibit the replication of mtDNA, generating a cell line that are devoid of the mitochondrial genome [13]. These cells, denoted Rho⁰, have altered metabolic requirements due to the loss of components of the electron transport chain, and require both uridine and pyruvate for growth [13]. Cells with this engineered mitochondrial dysfunction require exogenous uridine because a functional electron transport chain is needed for pyrimidine biosynthesis [13]. We generated a mtDNA-depleted variant of the breast cancer cell line MDA-MB-435 by culturing cells with EtBr (50 ng/ml) for approximately 90 cell generations, then isolating clones by limiting dilution. Total DNA from this Rho⁰ clone (clone 7) was digested with PvuII, which cuts at a single site in mtDNA. Southern blotting, and hybridization with a mtDNA-specific probe showed the presence of a 16.6 kb band in the MDA-MB-435 Rho⁺ cell line. The 16.6 kb fragment was absent in the Rho⁰ cell line (Figure 1A,1B). To further verify loss of mtDNA encoded protein, the expression of mtDNA-encoded cytochrome oxidase II (COX II) was assessed by western blotting. Expression is not observed in Rho⁰ cell lysates as compared to the parental cells (Figure 2A and 2B). To assess the growth characteristics of the Rho⁰ clone, cell growth was measured in glucose and galactose media. Cells with dysfunctional mitochondria are unable to efficiently utilize galactose as a primary carbon source for energy production (14). Following an initial growth lag, parental MDA-MB-435 cells grown in galactose medium proliferated at a rate comparable to the rate observed in glucose (Figure 3A). However, Rho⁰ cells were unable to grow in galactose media, indicating an impairment of mitochondrial function (Figure 3B). These results demonstrated that we had isolated Rho⁰ breast cells that do not express protein encoded by mtDNA, and which lack proper mitochondrial function.

Increased lipid peroxidation in Rho⁰ cells

Mitochondria are the major site of reactive oxygen (ROS) production [1,2] OS cause oxidative damage. It is conceivable that altered mitochondrial function in Rho⁰ leads to increased oxidative stress. We therefore measured lipid peroxidation in parental and Rho⁰ breast cancer cells. Malondialdehyde, a product of the catabolism of peroxidized lipids, forms a pigmented reaction product with thiobarbituric acid that can be measured spectrophotometrically [10,11]. Levels of thiobarbituric acid-reactive species were measured in extracts of parental Rho⁺ and Rho⁰ cells to determine if altered mitochondrial function
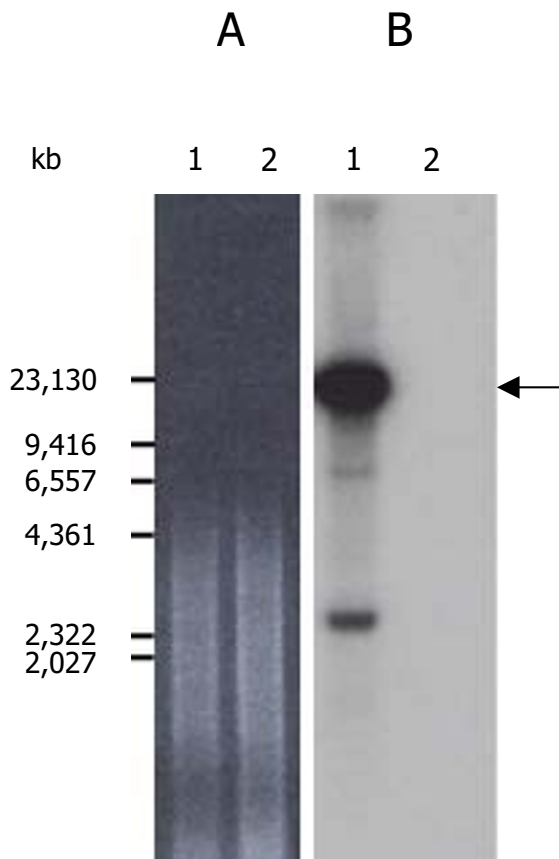
Figure 1
Southern blot analysis of mtDNA. Total DNA was extracted from parental MDA-MB-435 cells (Lanes 1A, 1B) and the clone 7 mtDNA-depleted variant of MDA-MB-435 cells (Lanes 2A, 2B), (A): Total DNA was then digested with PvuII (which cuts a single site in mtDNA generating a single 16.6 Kb fragment), separated by electrophoresis on agarose gel containing ethidium bromide. (B) DNA was transferred to nylon membrane, and the membrane was hybridized to a ^{32}P-labeled mtDNA-specific DNA probe. The 16.6 Kb mtDNA is present in parental MBA-MD-435 cells (Lane 1B), but absent in Clone 7 (Lane 2B). The 16.6 Kb genome is indicated by arrow.

Figure 2
Rho^0 cells do not express mtDNA-encoded COX II protein. Equal amounts of total cell lysates from parental MDA-MB-435 (Lane 1) and Clone 7 Rho^0 variant (Lane 2) cells were separated by SDS-PAGE and transferred to a membrane. (A) Blot was hybridized with a monoclonal antibody against cytochrome oxidase II (COXII), and (B) then stripped and rehybridized with a monoclonal antibody against ??-actin. COXII staining demonstrates the loss of expression of the mtDNA-encoded COXII (Lane 2A). Actin staining shows that protein loading is approximately equal (Lanes 1B, 2B).

results in lipid peroxidation. Figure 4 shows that thiobarbituric acid-reactive species were increased in Rho^0 cells (solid bar) compared to the Rho$^+$ parental cells (open bar). We conclude that an increase in lipid peroxidation is associated with the impairment of mitochondrial function.

Microarray analysis
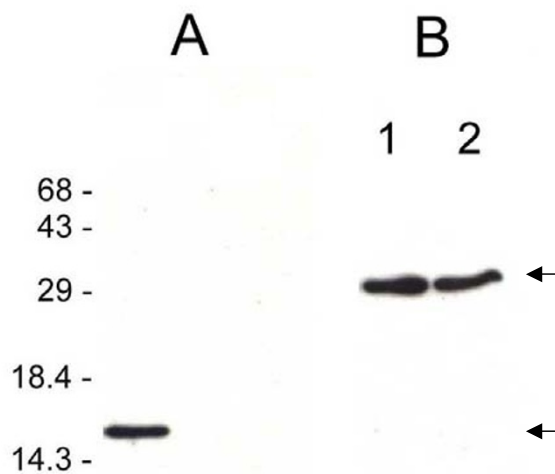Nuclear and mitochondrial genomes interact at least in two possible ways. First they contribute essential protein subunits to important mitochondrial function; second they collaborate in the synthesis of and assembly of mitochondrial proteins [14]. To identify genes that respond to lack of mtDNA encoded genes, we compared the mRNA level between the Rho^0 cell line and parental Rho$^+$ cell line by cDNA microarray analysis. We analyzed 8000 genes present in the array. The microarray analysis revealed that 35 genes were significantly up regulated and 22 genes were down regulated in Rho^0 cells (Table 1 and 2). The differentially expressed genes were classified into various classes including transcription factors and architectural proteins (Table 1 and 2). Eight genes were selected randomly to confirm the microarray results by RT-PCR. The messages for each particular gene needed different cycle numbers to assess the exponential amplification accurately indicating the relative abundance of the individual messages. The RT-PCR results confirmed differential expression of the genes indicated by the microarray results (Figure 5, Tables 1 and 2). RT-PCR results show (Figure 5) that p19 (cyclin dependent kinase inhibitor) and v-maf were maximally up regulated followed by phospholipase C epsilon, a gene involved in signalling by increasing the intracellular levels of calcium ions. Among the down reg-
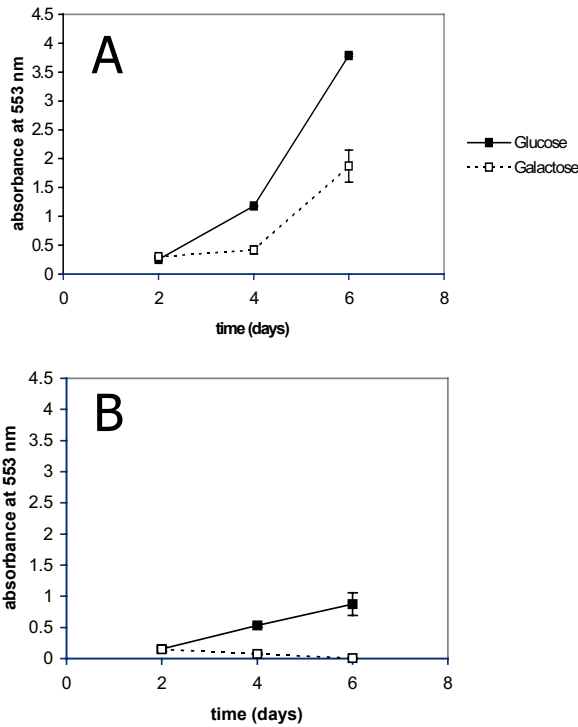
Figure 3
Rho⁰ cells do not grow in galactose medium. Rho⁺ (MDA-MB-435) cells and Rho⁰ cells were grown in custom-made medium containing galactose (instead of glucose, purchased from Sigma). While MDA-MB-435 cells continue to grow in this medium (A), Rho⁰ clone 7 cells began to die after 48 hours (B).
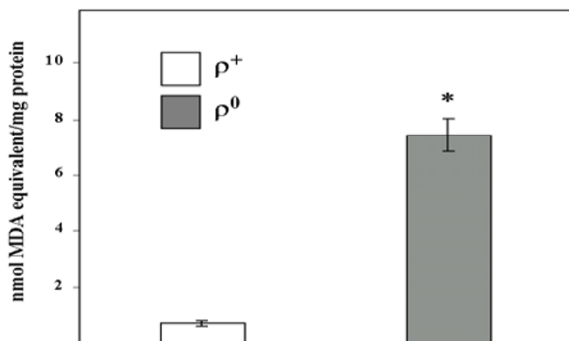

Figure 4
Lipid peroxidation is increased due to dysfunction in mitochondria: Subconfluent cultures were collected by trypsinization, washed with ice cold PBS and lysed by freeze/thaw cycles in sterile deionized water. Lipid peroxidation was measured as described in material methods.
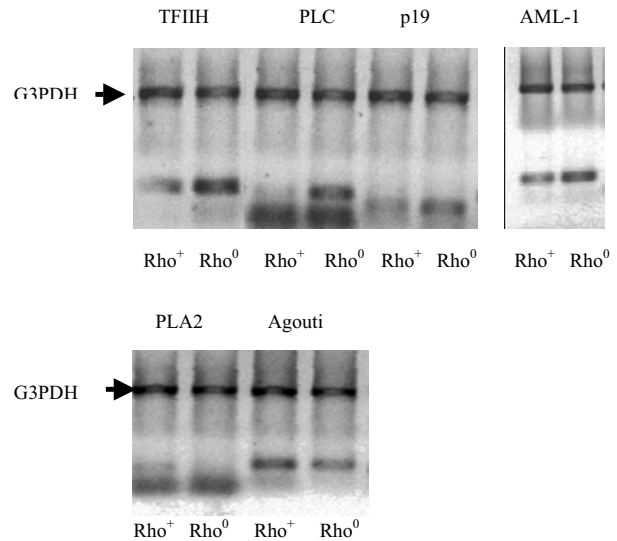

Figure 5
RT-PCR analysis: RT-PCR analysis of up-regulated or down regulated genes was carried out as described in material methods.

ulated genes phospholipase A2 was maximally down regulated, followed by progesterone-induced blocking factor 1 (PIBF1) a protein that acts on the phospholipase A2 enzyme and interferes with arachidonic acid metabolism. These results suggest that impairment of mitochondrial function leads to altered expression of nuclear genes involved in communication between mitochondria and nucleus.

Discussion

This paper reports on a comprehensive analysis of the nuclear gene expression in response to the absence of mtDNA in breast cancer cells. We demonstrate that expression of a number of nuclear genes is altered in response to the absence of mtDNA in breast cancer cells. These genes ranged from transcription factors to genes that are involved in metabolism, cell architecture and signaling. Our results are consistent with previous studies in yeast and mammalian cells that reported altered pattern of nuclear gene expression due to elimination of mtDNA [15–17].

We conducted comparative microarray analysis on Rho⁺ MDA-435 cell line and its Rho⁰ derivative. Rho⁰ cells have been used extensively to study the role of mitochondria in a variety of cellular processes that are affected by mitochondrial dysfunction (16, 17, 18). Our results showed that 35 genes are up regulated (> 3 fold) and 22 are down regulated (>3 fold) in Rho⁰ breast cancer cells. We chose 10 genes randomly to confirm the results of microarray

Table 1: Genes Up-regulated in Rho^0 cells ($\geq$ 3 fold)

Function	Gene
Transcription	v-maf protein G
	General transcription factor IIH polypeptide 3
	IFN consensus sequence binding protein 1
	Nuclear autoantigen
	Runt-related transcription factor 1(AML-1Oncogene)
Cell Signalling	Slit(Drosophila) homolog 3
	Phospholipase C, epsilon
	Syanaptojanin 2
	HLA DR beta 5
Cell Architecture	Tubulin specific chaperone D
	Hyaluronglucosaminidase 1
	Collagen Type IV
	Gap Junction Protein 43
Metabolism	Apolipoprotein D
	Arylacetamide deacytylase (esterase)
	Neuromedin U
	ATP5A1
	UDP-galactose:N-acetylglucosamine beta-1, 4-galactosyltransferase 1
	Cysteine dioxygenase type 1
Cell growth and differentiation	Cyclin-dependent kinase inhibitor P19
	Microtubule-associated protein 4
	CDC28 protein kinase
	CD34
Apoptosis	TNF superfamily member 6, (Fas Ligans)
	Ectodermal dysplasia 1, anhidrotic.
Unknown	FBR89
	FLJ23231
	ESTs, Weakly similar to AAB47496 NG5 [H. sapiens]
	EST
	FLJ13164
	Uncharacterized Bone marrow protein, BM033
	FJ 10407

analysis with RT-PCR (Figure 5). Among genes that are up regulated in Rho^0 cells p19 (INK4d), a cyclin dependent kinase inhibitor, is focally expressed during fetal development and plays a role in terminal differentiation [19]. Members of the group of INK proteins are involved in arresting cells in G1 phase of the cell cycle. Conceivably, p19 may be involved in the observed slow growth rate of the Rho^0. A number of genes that influence gene transcription are differentially expressed after mitochondrial impairment. The Maf oncoprotein, a basic leucine zipper-bearing transcriptional activator that recognizes the Maf recognition element (MARE) DNA sequence (20) is noticeably up regulated in the Rho^0 cell line. c-MAF is highly expressed in developing skeletal tissues, cells where mitochondria play a central role in their functioning [20,21]. The transcription factors TFIIH and AML1 were also up regulated in the Rho^0 cell lines. TFIIH is associated with the RNA polymerase II transcription complex, which is involved in transcription and transcription-mediated DNA repair [22]. The transcription factor AML1 is frequently found translocated in leukemic cells [23]. Conceivably, altered expression of these transcriptional regulators in response to mitochondrial impairment could directly or indirectly lead to the changes in expression of other genes seen in Rho^0 cells.

Phosphoinositide-specific phospholipase C (PLC) $\in$ was among the signaling molecules that were up regulated in the Rho^0 cell line. PLC is a critical signaling enzyme that hydrolyzes membrane phospholipids to generate inositol trisphosphate (IP$_3$), which binds to IP$_3$ receptors and increases intracellular Ca^{2+}[24]. The $\in$ isoform of PLC is a novel effector of *ras*[25,26]. Another signaling molecule of significance that is up regulated in the Rho^0 cells is the Slit (Drosophila) homolog 3. Three vertebrate orthologs of the fly slit gene, Slit1, 2, and 3, have been isolated. Each displays overlapping, but distinct, patterns of expression in the developing vertebrate central nervous system, im-

Table 2: Genes Down regulated in Rho^0 ($\geq$ 3 fold)

Function	Gene
Transcription	Dachshund (Drosophila) Homolog
Cell Signaling	LIM binding domain 2
	Phospholipase A2 Group IB
	Phospholipase C, epsilon
	Lipopolysaccharide response-68 protein
	Agouti related protein
	PKC, gamma
	Protein Tyrosine Phosphatase, Receptor Type c, Polypeptide-Associated Protein
	Neurotensin Receptor I
	Phosphodiesterase 1A, calmodulin dependent
Cell Architecture	Adaptor related protein complex 4 mu 1 subunit
	Synuclein gamma
	Thymopoietin
Metabolism	PIBF1
	Carboxypeptidase B2 (plasma)
	Cytochrome P 450 subfamily VIIB, polypeptide 1
	Protective protein for beta-galactosidase
Cell growth and differentiation	Cyclin-dependent kinase inhibitor P19
	GAP-43
Intracellular Protein degradation	Huntingtin interacting protein B
	Ubiquitin-like 4

plying conservation of function [27]. Slit3 gene product is the least evolutionarily conserved of the vertebrate Slit genes. The fact that Slit3, but not Slit2, is predominantly localized within the mitochondria makes its up regulation in the Rho^0 cells an intriguing phenomenon [28].

One feature of the Rho^0 cell line is the differential expression of certain genes that are involved directly or indirectly in metabolism of fatty acids or signaling (Figure 5 and Table 1 and 2). Among those that are up regulated are apolipoprotein D (ApoD) and arylacetamide deacetylase. While ApoD may have a similar function as the other apoproteins, it does not share a similar protein structure to the other family members, and does not bind cholesterol with high affinity [29]. Hence, it may have a unique effect via binding of different ligands. ApoD may be involved in the binding of steroids or fatty acids, Apo D interacts with Ob-Rb, but not Ob-Ra, in hypothalamic neurons *in vivo*[29]. In the central nervous system it was found that Apo D may be activated through its interaction with a leptin-stimulated Ob-Rb and may bind a specific ligand in hypothalamic neurons, where it exerts signaling functions [30]. Arylacetamide deacetylase may also function as a lipase during the process of lipoprotein secretion [31,32]. Other genes involved in fatty acid signaling were down regulated in Rho^0 cells. These include phospholipase A2, a protein closely involved with arachidonic acid metabolism and also involved with cell signaling [33–35], that

was found to be maximally down regulated in Rho^0 cells. Phospholipase A2 also plays a role in the translocation of PKC-mediated by fatty acids. Another gene with functions closely related to the arachidonic acid metabolism that is down regulated in the Rho^0 cells is the progesterone-induced blocking factor 1 (PIBF1). PIBF1 is known to act on the phospholipase A2 enzyme, and so interferes with arachidonic acid metabolism, inducing a Th2 biased immune response [36]. PIBF1 exerts an anti-abortive effect by controlling NK activity [37]. A gene related to lipid signaling that encodes agouti-related protein was also down regulated in the Rho^0 cells. Agouti is a paracrine-signaling factor that acts to block melanocortin action [38,39]. Disruption of melanocortin signaling with antagonist administration leads to an increase in feeding and eventually to obesity. Such blockage has been observed in both lipopolysaccharide-induced animals and also during illness [40]. The data exhibiting down regulation of the agouti gene is further strengthened by the down regulation of the LPS response-68 protein in the Rho^0 cells.

The ATP synthase 5A1 subunit gene that is directly involved in mitochondrial function was up regulated in the Rho^0 cell lines. ATP synthase is a multimeric complex composed of at least 16 different polypeptides, two of which are mitochondrially encoded, and the remainder encoded by nuclear genes [2]. Certain leukemic cells show an increased production of the ATP5A1 subunit [41]. In-

terestingly, a down regulated gene CYP7B1 encoding an oxysterol 7 alpha-hydroxylase (Cytochrome P450 subfamily VII BI) is involved in many metabolic processes including bile acid synthesis and neurosteroid metabolism [42].

Mitochondria control Ca^{++} homeostasis [2]. Protein kinase C γ, neurotensin receptor, and neuromodulin (GAP-43) are among the genes that are involved in calmodulin regulation or related to calmodulin function [43–45] which are down regulated in Rho^0 cells. Neuromodulin is also a substrate for phosphorylation by protein kinase C [46] which may reflect an influence of mitochondrial function on multiple members of a signal transduction pathway. Another interesting feature in the expression profile of cells lacking mtDNA is the low expression of many genes that play a role in the development and maintenance of the nervous system [47,48]. Of particular interest is the gene that encodes neurotensin receptor I, a short peptide receptor for neurotensin that exerts neuromodulatory functions in the central nervous system and endocrine/paracrine actions in the periphery [49]. Neuromodulin is also involved in the growth and regeneration of axons, and in the elongation of axons as an axonal transport membrane protein [45]. Synuclein is expressed in the nervous tissues and mutations in this gene are associated with rare familial cases of early-onset Parkinson's disease [50]. Gamma synuclein is also involved in tumorigenesis [42]. PKC-g mutation has been shown to lead to neurodegeneration [51]. Dachshund, a putative transcription factor, plays a role in retinal development [52]. Huntingtin-interacting protein-2 is involved in the neurodegenerative Huntington disease [53].

In summary, our data provides an overview of the genes that are involved in the nuclear and mitochondrial cross talk and respond to oxidative stress manifested due to impaired mitochondrial function. The identification of a large number of genes whose expression is influenced by mitochondrial function provides a foundation for future investigation of the pathways affected by the impairment of mitochondria in pathological conditions. An understanding about the role of the proteins in the maintenance of proper mitochondrial function and in oxidative stress response is critical for understanding the role of mitochondria in carcinogenesis.

Authors' contributions

RD isolated and analyzed the Rho^0 derivative of breast cancer cell line. RD, SK and AR performed microarray experiments in EG's laboratory. EG contributed to the analysis of microarray data and writing the manuscript. SK and RD, wrote the manuscript. KKS conceived, coordinated, critiqued and designed the study.

All authors read and approved the final manuscript.

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