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ORIGINAL ARTICLE

Transfer of healthy fibroblast-derived mitochondria to HeLa ρ^0 and SAS ρ^0 cells recovers the proliferation capabilities of these cancer cells under conventional culture medium, but increase their sensitivity to cisplatin-induced apoptotic death

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Abstract

Mitochondrial dysfunction is known to contribute to cancer initiation, progression, and chemo-and radio-resistance. However, the precise role of mitochondria in cancer is controversial. Hence, here we tried to further clarify the role of mitochondria in cancer by transferring healthy mitochondria to cancer cells, and also to cells with depleted mitochondrial DNA (ρ^0). Healthy mitochondria were isolated from WI-38 cells and were transferred to HeLa, SAS, HeLa ρ^0, and SAS ρ^0 cells. Then, cell proliferation was verified. In addition, the cells were treated by different concentrations of cisplatin and assessed for apoptosis induction and quantifying the mRNA expression of apoptosis-related genes. Results revealed that incubation of the HeLa, SAS and HeLa ρ^0 cells with 5 µg/ml of the isolated mitochondria for 24 h significantly ($p < 0.001$) increased cell proliferation compared to non-treated controls. Interestingly, the mitochondria transfer rescued the ρ^0 cells and made them capable of growing under conventional culture medium. However, the number of apoptotic cells was significantly higher in the HeLa ρ^0 cells that received the mitochondria (HeLa-Fibro-Mit) compared to the HeLa ρ^0. Furthermore, the expression level of BCL-2 anti-apoptotic gene was down-regulated in both HeLa-Fibro-Mit and SAS-Fibro-Mit cell lines while the expression levels of the BAX, caspase8, caspase9, and AIF pro-apoptotic genes were upregulated. Our findings indicated that although the response of cancer cells to the mitochondria transfer is cancer-type dependent, but the introduction of normal exogenous mitochondria to some cancer cells might be considered as a potential novel therapeutic strategy.

Keywords Mitochondria transfer · Cancer · Cisplatin · Chemoresistance · Apoptosis

Introduction

Cancer is a leading cause of death worldwide, and in spite of current advances in cancer therapy, it is still incurable in many cases [1]. Therefore, a number of studies are focusing

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on finding out the exact pathophysiology of cancer initiation, and consequently, developing efficient therapeutic strategies [2, 3]. Cancer can be considered a genetic and/or a metabolic disorder [4–6]. However, currently it is a controversial issue since some scientists believe that deregulation of various genes initiates cancer, while others propose that cancer progression is a consequent of metabolic alternations in cells [4–6].

In 1920, Otto Warburg discovered metabolic differences between cancer and normal cells. He, for the first time, reported that cancer cells prefer glycolysis rather than oxidative phosphorylation even at a normal oxygen level and with normal mitochondria [7]. It is believed that metabolic alternations might be the initiation causes of cancer, especially by considering the hypoxic conditions occurring at the early stages of carcinogenesis [8]. Basically, hypoxia induces the Hif-1α, which subsequently activates pyruvate dehydrogenase kinase (PDK) and also inhibits pyruvate dehydrogenase (PDH) [9]. This process results in conversion of pyruvate to lactate by lactate dehydrogenase (LDH), and finally inactivates mitochondria, which consequently makes the cancer cells more aggressive by increasing their proliferation rate and inhibition of apoptosis [9]. On the other hand, further studies show that downregulation of LDH by siRNA, or inhibition of PDK by some molecules such as dichloroacetate (DCA), results in pyruvate uptake by the mitochondria which activate oxidative phosphorylation. This, consequently suppresses proliferation and induces apoptosis in the cells [10]. To further support the idea of mitochondrion role in cancer progression, some studies showed that cancer cells shut down their mitochondria to protect themselves against apoptosis initiation, and also to become refractory to radio- or chemo-therapeutic agents [11–13].

Furthermore, the implication of mitochondrial DNA (mtDNA) mutations, and mitochondrial dysfunction have been shown in many types of cancers [14]. Altogether, it is obvious that mitochondria are important in cancer biology and play critical roles in the initiation, progression, and invasion of cancers [15–18]. Therefore, targeting mitochondria might be a selective and effective therapeutic strategy against cancer [19].

Mitochondria are dynamic organelles of cells which not only produce energy, but also attribute to the other functions of cells such as apoptosis, proliferation, and migration [15]. These organelles not only move around the cytoplasm but also transfer between cells. It has been shown that mitochondria can transfer between cells through tunneling nanotube and extracellular vesicles [20]. It has been also revealed that the cells which are present in the microenvironment of cancer cells (such as mesenchymal stem cells, endothelial or non-cancerous cells) can transfer their mitochondria to cancer cells [18–21]. However, the behavior of the cancer cells following mitochondria transfer is controversial. In other words, it is not clear whether mitochondria transfer makes the tumor cells more aggressive, or suppress their cancerous potential. The precise role of mitochondria in terms of resistance/sensitization to chemotherapy or radiotherapy, and induction/suppression of apoptosis after mitochondria transfer is also a controversial issue [15].

Therefore, considering these controversies, in the present study we evaluated the effect of mitochondria transfer on cancer cell proliferation, chemotherapeutics sensitivity and induction of apoptosis. Our results show that mitochondria are important contributor to cancer cell proliferation and apoptotic death, and reviving mitochondrial function in cancer cells by transferring healthy exogenous mitochondria to them might be a novel therapeutic strategy for cancer treatment.

Materials and methods

Cell culture

Human HeLa cervical cancer (HeLa-Parental; HeLa-P) and oral SAS (SAS-Parental; SAS-P) cancer cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. They were grown in RPMI-1640 (189-02025; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum FBS (Biological Industries, USA). HeLa and SAS cells deficient for mt-DNA (HeLa ρ^0 and SAS ρ^0, respectively) were established using low concentration of ethidium bromide as previously described [22]. Then, the cells were cultured in RPMI-1640 with 10% FBS, 110 µg/ml pyruvate (P5280; Sigma-Aldrich, St Louis, MO, USA), and 50 µg/ml uridine (U0020; TOKYO Chemical Industry Co. Ltd, Tokyo, Japan). In addition, clinically relevant radioresistance (CRR) cell lines for HeLa (HeLa-CRR) and SAS (SAS-CRR) were established as previously described by Kuwahara et al. [23]. Human fibroblast cell line (WI-38) was obtained from Riken BRC (Tsukuba, Japan), cultivated in EMEM (051-07615; FUJIFILM Wako Pure Chemical Corporation) with 10% FBS, and used as mitochondrial donor cell.

Mitochondria isolation and transfer

Healthy mitochondria were isolated from WI-38 cells using mitochondrial isolation kit for cultured cells (ab110171, abcam Cambridge, UK) according to the manufacturer's instruction. Briefly, 3×10^7 cells were harvested using cell scratcher and pelleted. The pelleted cells were homogenized on ice using a Dounce Homogenizer in isolation reagent supplemented with proteinase inhibitor (25955-11; NACALAI TESQUE, INC. Kyoto, Japan). Then, the mitochondria were

harvested by centrifugation at 3000 g for 15 min to reduce their contamination with other cytosolic components. The isolated mitochondria were suspended in 0.5 ml respiration buffer, kept on ice, and immediately used to transfer to the cancer cells. Protein concentration of the isolated mitochondria was detected by Pierce™ BCA Protein Assay Kit (23227; Thermo Fisher Scientific Inc., MA, USA) according to a standard curve of bovine serum albumin. Membrane potential ($\Delta\Psi m$) of the donor cells mitochondria and also the isolated mitochondria were assessed by JC-1 staining (T3168; Invitrogen, CA, USA). Briefly, JC-1 was added to the fibroblast cells or the isolated mitochondria and incubated at 37˚C and 5% CO_2 for 30 min. Then, the mitochondria aggregate (red color) and monomer (green color) forms were observed under fluorescent microscope and photographed. Immediately after mitochondria isolation, the HeLa-P and SAS-P cells were incubated with 5, 10, and 20 µg/ml of the isolated mitochondria for 24 h. The HeLa ρ^0 and SAS ρ^0 cells were also incubated with 5 µg/ml of the isolated mitochondria for 24 h. Afterwards, the cells were washed 3 times with PBS and cultured at regular condition and medium. In order to verify the mitochondria internalization to the target cells, MitoTracker™ Green (M7514; Thermo Fisher Scientific) was used to label the donor cells mitochondria before the isolation procedure. In this regard, 24 h before isolation of the mitochondria the donor cells were incubated with 25 nM MitoTracker™ Green for 30 min.

Cell proliferation assay

The HeLa-P, SAS-P, HeLa ρ^0 and SAS ρ^0 cells were seeded in 96-well plates at a density of 7×10^3 cells/well. As described above, HeLa-P and SAS-P cells were incubated with 5, 10 and 20 µg/ml of the isolated mitochondria for 24 h. Also, 5 µg/ml of the isolated mitochondria were added to HeLa ρ^0 and SAS ρ^0 cells and incubated for 24 h. Then, the medium was replaced by fresh medium and the cells were grown for another 48 h. Then, proliferation of the cells was assessed by Cell Counting Kit-8 (CK04; Dojindo, Kumamoto, Japan) according to the kit guideline. Briefly, 5 µl of the kit reaction was added to each well and the plates were kept in incubator for 3–4 h.. Finally, the plates were subjected to absorbance read at 450 nm with a micro plate reader (Multiskan FC; Thermo Fisher Scientific).

Assessment of chemotherapy resistance/sensitivity of the cells following mitochondria transfer

First, to determine cisplatin IC50s on the HeLa-P, SAS-P, HeLa-CRR, SAS-CRR, HeLa ρ^0 and SAS ρ^0 cells, the cells were cultured in 96-well plates at a density of 7×10^3 cells/well and treated with different cisplatin concentrations (0.5–10 µg/ml) for 48hrs. Then, the cytotoxicity was evaluated by the Cell Counting Kit-8 as described above. The concentration at which 50% of the cells were killed by cisplatin was considered as IC_{50}. In addition, the HeLa ρ^0 and SAS ρ^0 cells were treated with 5 µg/ml of the isolated mitochondria for 24 h. Then, the cells were seeded in 96-well plates and treated with 1, 1.5 and 2 µg/ml cisplatin in case of HeLa ρ^0, and 0.5, 1, 1.5 and 2 µg/ml cisplatin in case of SAS ρ^0 cells for 48 h. Then, cell proliferation was determined by the Cell Counting Kit-8 as mentioned before.

In order to evaluate the effect of mitochondria activation on inhibition of the cancer cells proliferation, the HeLa-P, HeLa-CRR, SAS-P and SAS-CRR cells were treated with various concentrations of DCA. In this regard, the cells were seeded in 96-well plates and treated with 0, 10, 15, 20, 30 and 40 mM of DCA for 72hrs, then subjected to evaluation of its cytolethal effects by the Cell Counting Kit-8 as described above.

Apoptosis

Apoptosis was detected by DAPI staining and Real-time PCR for apoptotic genes. First, five µg/ml of the isolated mitochondria were added to HeLa ρ^0 and SAS ρ^0 cells and incubated for 24 h. Then, 5×10^4 of the mitochondria recipient cells were cultured in 35 mm glass bottom dishes and treated with 120 µg/ml cisplatin for 6hrs. Then, the cells were washed 3 times with PBS and fixed by 4% paraformaldehyde and incubated with 1 µg/ml DAPI (D9542; Sigma-Aldrich) for 15 min. Finally, the cells were observed under fluorescent microscope, and apoptotic cells were counted. In addition, mRNA expression of pro- and anti-apoptotic genes including Bcl-2, BAX, AIF, Caspase8 and Caspase9 were assessed by Real-time PCR. Briefly, total RNA of the treated cells was extracted using ISOGEN kit (Nippon gene, Toyama, Japan) as recommended by the manufacturer, and corresponding cDNAs were prepared by reverse transcription of 1 µg total RNA using oligo-dT adapter primer (0.4 µM at 50 µl final volume) and ReverTra Ace enzyme (TOYOBO CO Ltd., Osaka, Japan). Real-time PCR was performed with specific primers (Table 1) for the mentioned genes, and β-actin (as housekeeping gene) expression was assessed for normalization. PCR condition for these genes was 95ºC 10 min for initial denaturation followed by (95ºC 10 s, 60ºC 60 s) × 40 cycles.

mtDNA copy number

In order to assess the stability of the transferred mitochondria in recipient cells, the HeLa ρ^0 and SAS ρ^0 cells were incubated with 5 µg/ml of the isolated mitochondria for 24 h. Then, the cells were cultured in uridine and pyruvate free medium at least for 5 passages. Then, the presence of mtDNA was determined by Real-time PCR for ND1as

Table 1 Primer sequences used in this study

Primer name	Primer sequence	Tm
ND1 F	5′-ACCCCCGATTCCGCTACGACCAAC-3′	63
ND1 R	5′-GGTTTGAGGGGGAATGCTGGAGAT-3′	59
BAX-F	5′-TTGGGTGAGACTCCTCAAGCCTCC-3′	61
BAX-R	5′-TCTGAAGATGGGGAGAGGGCACCA-3′	61
BCL2-F	5′-GGAGGCTGGGATGCCTTTGTGGAA-3′	61
BCL2-R	5′-TAGGCACCCAGGGTGATGCAAGCT-3′	61
AIF-F	5′-CAAAGGTGTCATCTTCTACCTCAG GGAC-3′	61
AIF-R	5′-CTCACCGTCCTTAATGATCTTCCTTGCT-3′	60
CASP8-F	5′-AGAGCGATGTCCTCGAGGCGATGA TATT-3′	61
CASP8-R	5′-AAGTAGGCTGAGGCATCTGTTTCC CCAT-3′	61
CASP9-F	5′-CAAGAGTGGCTCCTGGTACGTTGAGA-3′	61
CASP9-R	5′-CTGTTTATAAATCCCTTTCACCGAAAC AGC-3′	59
Beta-actin F	5′-AGAGCTACGAGCTGCCTGAC-3′	56
Beta-actin R	5′-AGCACTGTGTTGGCGTACAG-3′	54

described previously [22]. PCR condition for ND1 was 95 °C 10 min for initial denaturation followed by (95℃ 10 s, 55 °C 30 s, and 72 °C 40 s) × 40 cycles.

Statistical analysis

All data are presented as Mean ± SD. A minimum of 3 individual replications of each experiment were performed. The data were analyzed using *t*-test or one-way ANOVA. $P < 0.05$ was considered as significant.

Results

Isolation, characterization and confirmation of the healthy mitochondria transfer to the cancer cells

Before mitochondria isolation and transfer, the quality of the mitochondria of the WI-38 human fibroblast cells was verified by JC-1 staining. As it is shown by Fig. 1a; (A–D), the higher intensity of red color (corresponding to the aggregate form of mitochondria) comparing to the green color (corresponding to the monomer form of mitochondria) confirmed the cells as a proper source for the mitochondria isolation. Furthermore, JC-1 staining of the isolated mitochondria revealed a higher intensity of red color than the green color which confirmed high membrane potential and quality of them (Fig. 1a; E–H). Finally, the protein concentration of the isolated mitochondria was 340 ± 20 µg/ml.

In order to verify the internalization of the isolated mitochondria, they were labeled with MitoTracker™ Green in the WI-38 cells, and the labeling was confirmed under a fluorescence microscope as is represented by Fig. 1a; I–J. Evaluation of the recipient cells following 24hrs of incubation with 5 µg/ml of the isolated mitochondria confirmed successful transfer by revealing fluorescent signals corresponding to the MitoTracker™ Green (Fig. 1b).

Mitochondrial transfer increased cell proliferation in dose- and cancer-type dependent manner

Next, the effect of mitochondria transfer on cell proliferation was evaluated on HeLa-P, SAS–P, HeLa ρ^0 and SAS ρ^0 Cells. As it is represented by Fig. 2a, incubation of the HeLa-P and SAS–P cells with 5 µg/ml of the isolated mitochondria for 24 h significantly ($p < 0.001$) increased cell proliferation compared to non-treated controls. However, treatment of the two cell lines with 10 µg/ml of the isolated mitochondria did not affect their proliferation rate (Fig. 2a; A, B). On the other hand, treatment of the mtDNA deficient cells (HeLa ρ^0 and SAS ρ^0) with 5 µg/ml of the isolated mitochondria for 24hrs significantly ($p < 0.001$) increased proliferation rate of the HeLa ρ^0, but it did not affect the proliferation rate of SAS ρ^0 cells (Fig. 2a; C).

Next, we evaluated cisplatin cytotoxicity on HeLa-P, HeLa-CRR, SAS-P and SAS-CRR cells by cell proliferation assay. HeLa-CRR and SAS-CRR cells showed to be significantly ($p < 0.05$ and $p < 0.01$, respectively) resistant to 48 h of treatment with different concentrations of cisplatin compared to their parental cell lines (HeLa-P and SAS-P) (Fig. 2b; A, B). The cisplatin sensitivity of the mtDNA deficient cells was also evaluated. As it is shown by Fig. 2b; C, D, the cytotoxicity of cisplatin on the HeLa ρ^0 cells was almost similar to the HeLa-P cells after 48 h of treatment. However, the SAS ρ^0 cells showed higher cisplatin resistance comparing to the SAS-P cells (Fig. 2b; C, D).

In order to assess the effects of DCA, as an activator of mitochondria, on cancer cells proliferation, the cells were treated with various concentrations of DCA followed by cell proliferation assay. Interestingly, 20 and 30 mM DCA significantly decreased cell growth in HeLa-CRR compared to HeLa-P (Fig. 2b; E). In addition, 10 mM DCA significantly decreased the SAS-CRR proliferation after 72hrs comparing to the SAS-P cells (Fig. 2b; F). In other words, activation of mitochondria in the CRR cells decreased their proliferation potential, especially in HeLa-CRR cells.

Altogether, these findings indicate the implication and contribution of mitochondrial characteristics to the proliferation of cancer cells, though this contribution depends on the cancer type.

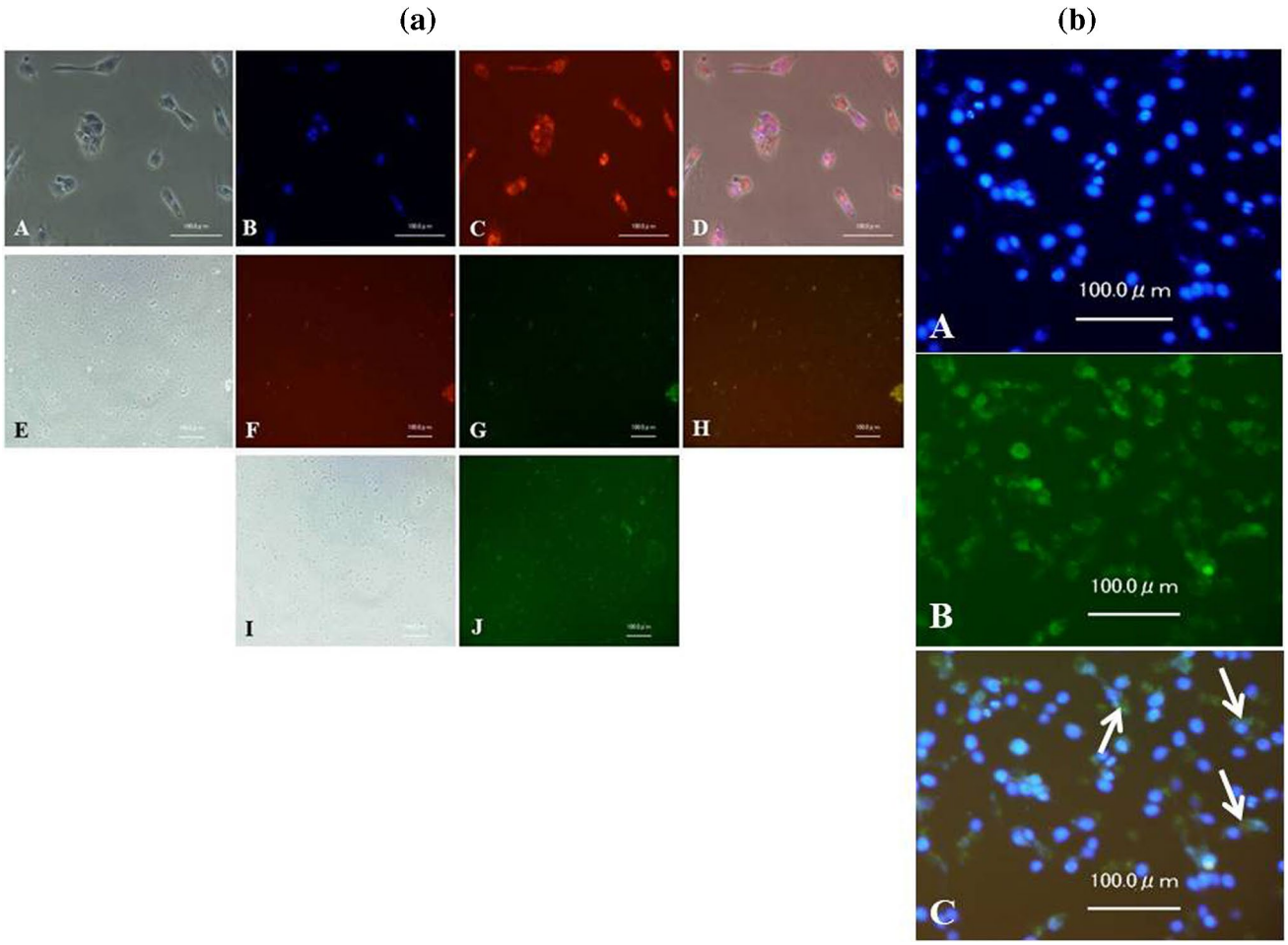

Fig. 1 **a** Staining of mitochondria donor cells (WI-38) and isolated mitochondria by JC-1 and MitoTracker™Green. (*A*) The **WI-38** human fibroblast cells under phase contrast microscope. (*B*) The **WI-38** cells were stained by DAPI. (*C*) The **WI-38** cells were stained by JC-1 and (*D*) the merged view of (*B*) and (*C*). This figure indicates high intensity of red color which confirms high membrane potential of the mitochondria in the **WI-38** cells. In addition, the isolated mitochondria were stained by JC-1. (*E*) Isolated mitochondria under phase contrast microscope. (*F*) The aggregate and (*G*) monomer forms of isolated mitochondria. (*H*) The merged view of F and G indicating high membrane potential of the isolated mitochondria by an intense red color. (*I*)The isolated mitochondria under phase contrast microscope and (J) the isolated mitochondria stained by MitoTracker™Green. **b** Internalization of the mitochondria to the cancer cells was verified. The fibroblast cells were treated by MitoTracker™Green 24 h before mitochondrial isolation. The labeled mitochondria were incubated with cancer cells and 24 h after mitochondria transfer, the cells were observed under fluorescent microscope. (A) The cells were stained by DAPI. (B) The mitochondria labeled with MitoTracker™Green. (*C*) Merged (*A*) and (*B*) confirming mitochondria internalization in the cells (white arrows). (Color figure online)

Mitochondrial transfer rescued the ρ^0 cells

In order to verify whether the transfer of healthy and normal mitochondria into HeLa ρ^0 and SAS ρ^0 cells would recover their normal expansion capacity, the cells were cultivated under conventional cell culture conditions (without uridine and sodium pyruvate) after the mitochondria transfer procedure. While all HeLa ρ^0 and SAS ρ^0 cells were died under the conventional cell culture condition, interestingly, the cells that received exogenous mitochondria (HeLa-Fibro-Mit, and SAS-Fibro-Mit) grew well, indicating that mitochondria transfer rescues the ρ^0 cells. The HeLa-Fibro-Mit and SAS-Fibro-Mit cells were expanded for seven passages, and the copy number of their mtDNA was detected after passages 3 and 5, although it was much lower than the parental cells, HeLa-P and SAS-P (Fig. 3a, b).

Exogenous mitochondria transfer increased cisplatin-induced apoptosis in cancer cells

The effect of mitochondria transfer on apoptosis induction in SAS-Fibro-Mit and HeLa-Fibro- Mit cells comparing to their parental counterpart (HeLa ρ^0 and SAS ρ^0) was also evaluated. In this regard, the cells were cultured on 35 mm

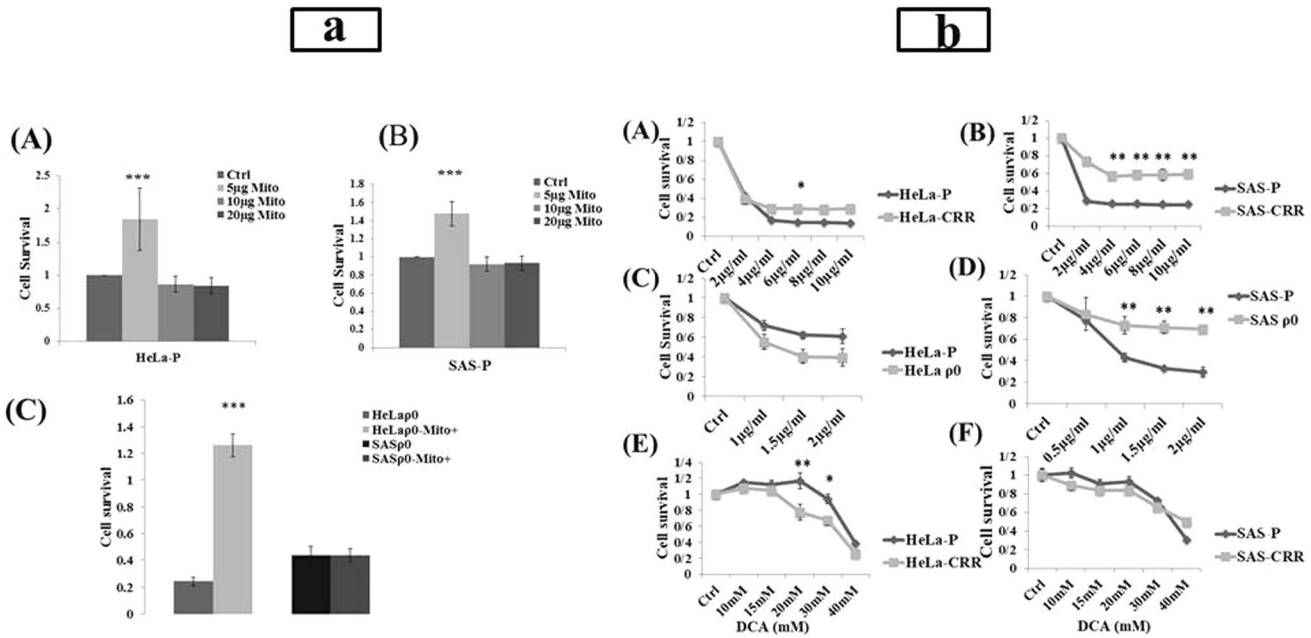

Fig. 2 **a** Cell proliferations after mitochondria transfer in HeLa-P, SAS-P, HeLa ρ^0, and SAS ρ^0 cell lines. **a**-**A**; Cell proliferation following transfer of 5, 10 μg/ml and 20 μg/ml of the isolated mitochondria to HeLa-P. **a-B**; Cell proliferation following transfer of 5, 10, and 20 μg/ml of the isolated mitochondria to SAS-P cells. **a-C;** Cell proliferation following transfer of 5 μg/ml of the isolated mitochondria to HeLa ρ^0 and SAS ρ^0 cell lines. *** $P < 0.001$, Ctrl; control, Mito; mitochondria, P; parental. (**b**) Cell survival of HeLa (P or CRR), SAS (P or CRR), HeLa ρ^0 and SAS ρ^0 after treatment with different concentration of cisplatin for 48 h **(A-D).** (*A*) HeLa-P and HeLa-CRR; (*B*) SAS-P and SAS-CRR (*C*) HeLa ρ^0; and (*D*) SAS ρ^0. **(b)** Proliferation of HeLa P, HeLa-CRR, SAS P and SAS-CRR cells after treatment with different concentrations of DCA for 72 h (*E–F*). (*E*) HeLa P, HeLa-CRR cells; (*F*) SAS P and SAS-CRR cells. *Ctrl* control, *P* parental, *CRR* clinically relevant resistance, *DCA* dichloroacetate. * $P < 0.05$, ** $P < 0.01$. (Number of replicates = 3)

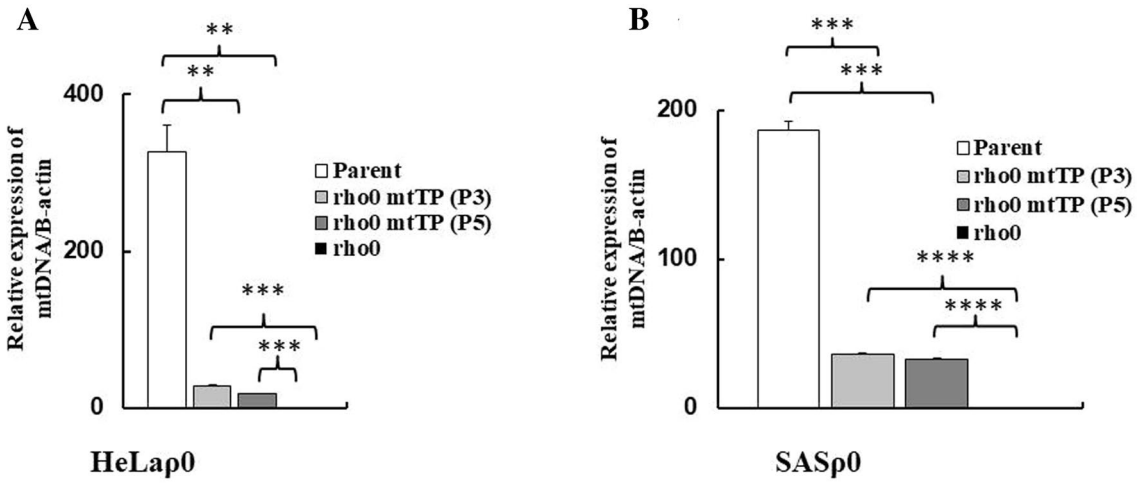

Fig. 3 Real time-PCR evaluation of mtDNA copy number of HeLa ρ^0 and SAS ρ^0 after mitochondrial transfer. **a** mtDNA copy number of HeLa ρ^0 cells following transfer of 5 μg/ml of the isolated mitochondria after passages 3 and 5 comparing to HeLa-P and non-recipient HeLa ρ^0. **b** mtDNA copy number of SAS ρ^0 cells following transfer of 5 μg/ml of the isolated mitochondria after passages 3 and 5 comparing to SAS-P and non-recipient SAS ρ^0. mtTP; Mitochondrial transfer, P3; Passage 3, P5; Passage 5. ** $P < 0.01$; *** P, 0.001; **** $P < 0.0001$

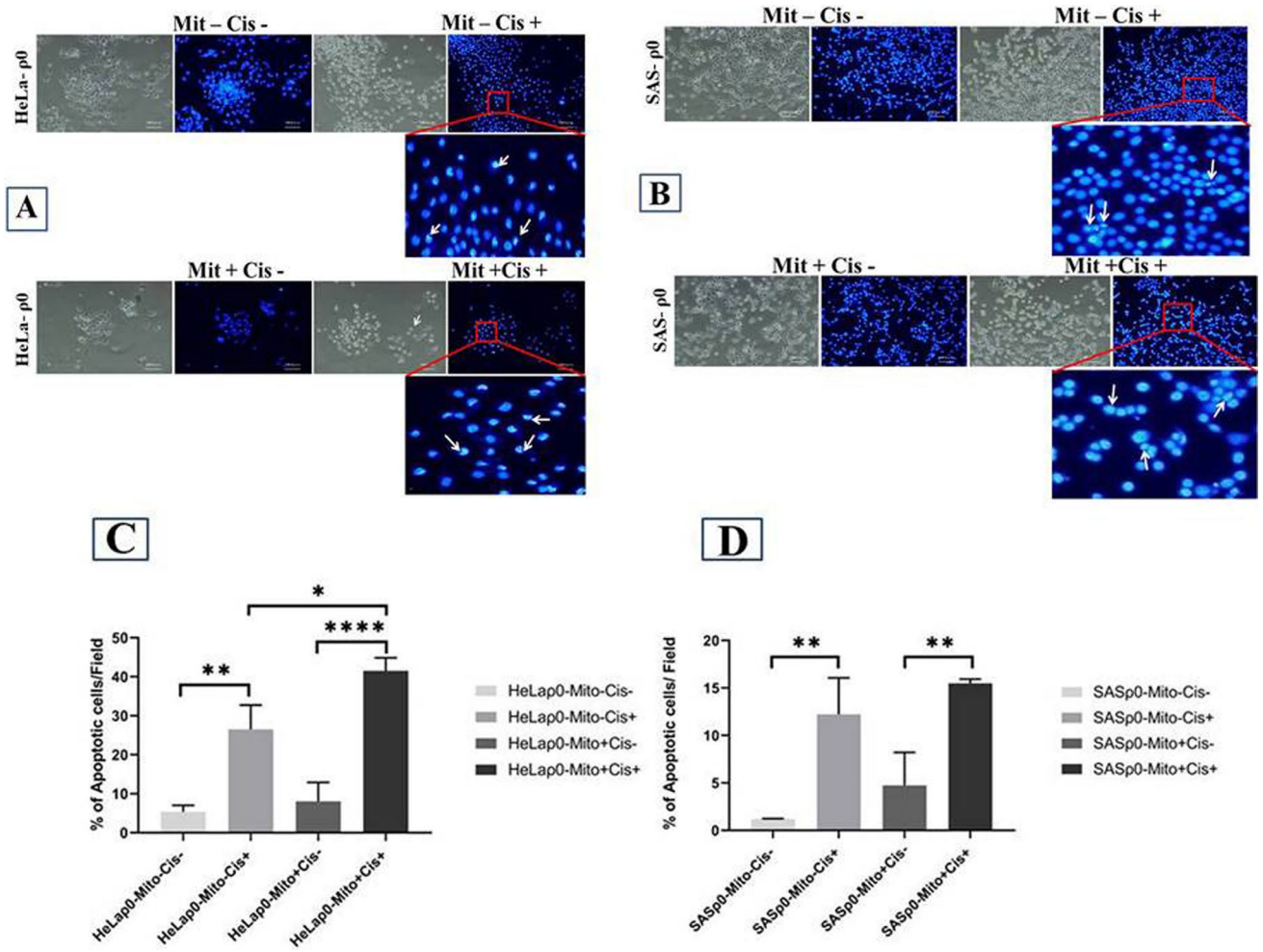

Fig. 4 **a**, **b** Evaluation of apoptotic cell percentage using DAPI staining. As it is shown the number of apoptotic cells in HeLa ρ^0 and SAS ρ^0 cells received 5 µg/ml of the isolated mitochondria (HeLa ρ^0 Mit + Cis + and SAS ρ^0 Mit + Cis +, respectively) are higher than the controls. **c**, **d** The number of apoptotic cells after mitochondrial transfer in HeLa ρ^0 and SAS ρ^0 verified by DAPI staining. * $P < 0.5$; ** P, 0.01; **** $P < 0.0001$. **Mit-**; the cells without mitochondrial transfer, **Cis-**; the cells without cisplatin traetment, **Mit +**; the cells treated by 5 µg/ml mitochondria for 24 h, **Cis +**; the cells were treated by 120 µg/ml cisplatin for 6 h, **Cis**; Cisplatin, **Mit**; mitochondria. (Number of replicates = 3)

cell culture plates and treated by 120 µg/ml cisplatin for 6 h followed by the detection of apoptosis by DAPI staining (Fig. 4a, b). As it is shown, the numbers of apoptotic cells were significantly higher in the HeLa–Fibro-Mit cells compared to the HeLa ρ^0 (Fig. 4c). The number of apoptotic cells in the SAS-Fibro-Mit cells was also higher, but not significant, compared to the SAS ρ^0 cells (Fig. 4d).

Next, the effects of transferring healthy mitochondria to the HeLa ρ^0 and SAS ρ^0 (HeLa–Fibro-Mit and SAS-Fibro-Mit) cells were evaluated on expression of the apoptotic genes including Bcl-2, BAX, caspase8, caspase9, and AIF by real-time RT-PCR and compared to their parental counterparts. As it is represented by Figs. 5a:A and 5b;A, while the expression level of BCL-2 anti-apoptotic gene was shown to be down-regulated in both HeLa-Fibro-Mit and SAS-Fibro-Mit cell lines, expression levels of the BAX, caspase8, caspase9, and AIF pro-apoptotic genes showed to be increased. Overall, these findings suggest that cancer cells shut down their mitochondria to adapt themselves to the tumor microenvironment. Hence transferring healthy mitochondria to cancer cells might be considered as a novel therapeutic modality.

Discussion

For many years, it has been believed that cancer is a genetic disease occurred by mutations in tumor suppressor genes and oncogenes [24]. However, some novel findings suggest cancer as a mitochondrion-related metabolic disease [7, 13]. In the present investigation, we tried to view cancer as a metabolic-based disease and to take some steps toward clarifying

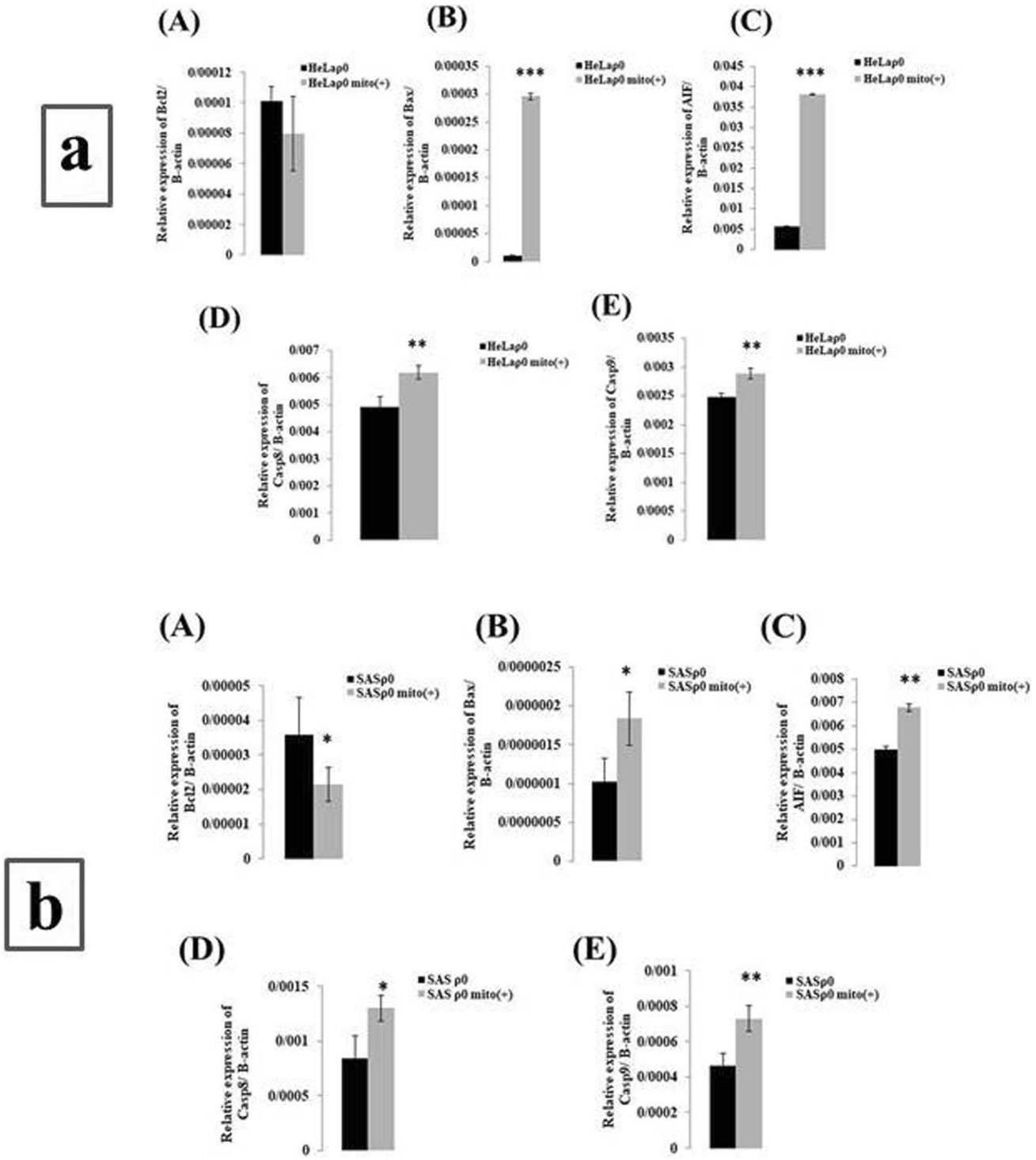

Fig. 5 **a** Evaluation of mRNA expression of pro- and anti-apoptotic genes in HeLa ρ^0 cells after mitochondrial transfer using real time PCR. (*A*) Bcl2, (*B*) BAX, (*C*) AIF, (*D*) caspase8, and (*E*) caspase9. B-actin considered as housekeeping gene. HeLa ρ^0 mito(+): the cells which received 5 µg/ml of the isolated mitochondria for 24 h. *AIF* Apoptosis-inducing factor. **b** Evaluation of mRNA expression of pro- and anti-apoptotic genes in SAS ρ^0 cells after mitochondrial transfer using real time PCR. (*A*) Bcl2, (*B*) BAX, (*C*) AIF, (*D*) caspase8 and (*E*) caspase9. B-actin considered as housekeeping gene. SAS ρ^0 mito(+); the cells which received 5 µg/ml of the isolated mitochondria for 24 h. AIF; Apoptosis-inducing factor. * **P < 0.5;** ** **P, 0.01;** ***$\mathbf{P < 0.001}$(Number of replicates = 3)

the role of mitochondria in cancer cells. Our results revealed that the transfer of mitochondria equal to 5 µg/ml protein to the cancer cells increased cell proliferation; however, the amount equal to the 10 µg/ml protein did not affect cell proliferation. In addition, our results showed that CRR cells were refractory to cisplatin-induced cytotoxicity. One of the potential mechanisms in this regard could be decreased mitochondrial function in the CRR cells. Mitochondrial dysfunction in the CRR cells has already been reported by our group. We have found that mitochondrial dysfunction in CRR cells contributed to docetaxel (DTX) and X-ray cross-resistance [25]. Supporting this notion, the implication of mitochondria in cisplatin resistance has been reported as well. Therefore, it seems that being radioresistant due to mitochondrial dysfunction might be also accompanied by chemoresistance. However, establishment of chemo resistance cell lines (especially resistant to cisplatin or similar drugs) instead of using radio resistant cells could be more helpful in this regard.

Our results also revealed that SAS ρ^0 cells were resistant to cisplatin-induced cytotoxicity when compared to the SAS-P cells. However, there was no difference between HeLa-P and HeLa ρ^0 cells in this regard.

In another experiment, when the mitochondria were further activated by DCA, the proliferation potential of CRR cells decreased comparing to the controls. This suggests that the mitochondrial shut down might be more advantageous to the CRR cells by making them resistant to the chemotherapeutic agents.

Mitochondrial DNA depleted cells (ρ^0 cells) are experimental models of mitochondria dysfunction for in vitro studies. Due to the impaired mitochondria, such cells are only capable to expand at the presence of uridine and pyruvate [22]. Lin et al. showed that co-culture of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) with human 143B osteosarcoma ρ^0 (mtDNA-depleted ρ0 cells) cells resulted in sieving out a surviving cell population due to the transfer of healthy mitochondria from WJ-MSCs. Remarkably, this population exhibited functional oxygen consumption and respiratory control. Moreover, their results revealed that cellular behaviors including anchorage-independent proliferation, OXPHOS-reliant cellular motility and aerobic viability were also reobtained. Interestingly, as they reported, the therapeutic effects of the mitochondrial transfer was sustained for 45 passages [26].

In the current study, we isolated healthy mitochondria from human fibroblast cells. A number of cells including endothelial and stem cells have been used as a source for isolation of healthy mitochondria [27]. More recently, we isolated mitochondria from human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) and transferred them into MDA-MB-231 breast cancer cells. There, we reported that the transfer of normal mitochondria to the MDA-MB 231 cells increased cell proliferation and enhanced their invasiveness, but in the group of cells with mitochondrial dysfunction decreased migration potency [15]. However, the precise roles of mitochondria in cancer cells still remain to be elucidated and even controversial. For example, in a study, Elliott et al. indicated a decreased proliferation rate in MCF-7 and NCI/ADR-Res cancer cells upon receiving normal mitochondria isolated from non-cancerous epithelial MCF-12A cells [28]. However, consistent with our findings, they reported increased cellular susceptibility to doxorubicin and paclitaxel chemotherapeutics following the mitochondrial transfer [29].

Recently, mitochondria transfer from homoplasmic 143B osteosarcoma cybrids to the MCF-7 cells has been reported, and showed to decrease oxidative stress and growth of the recipient cells [29]. Isolation of mitochondria from bone marrow-derived mesenchymal stem cells and their transfer to the MDA-MB-231 cells has also been reported [16]. Based on their findings, the transfer of healthy mitochondria to the cancer cells increased oxidative phosphorylation (OXPHOS) activity that favored cancer cell invasion and proliferation [16].

In this study, we transferred fibroblast-derived healthy mitochondria to the cancer cells (HeLa and SAS), and also to the HeLa ρ^0 and SAS ρ^0 cells in which mtDNAs were depleted. However, other controls such as cell lines with inactivated mitochondria could be considered. In this study, HeLa and SAS cell lines showed different responses to the cisplatin treatment following mitochondria transfer. The mechanism underlying mitochondria transfer effects on cell proliferation and/or on cisplatin sensitivity warrants further investigations. Moreover, further studies on other cell lines are required to better understand the role of transferring healthy mitochondria in cancer cells in terms of their proliferation and/or sensitivity to the chemotherapeutic agents.

Since the mitochondrial pathway of apoptosis is the target of a majority of chemotherapeutic agents including cisplatin [30] in the present study, we also investigated the effect of exogenous normal-cell derived mitochondria on apoptosis.. Our results showed that addition of normal mitochondria to the HeLa ρ^0 cells (HeLa-Fibro-Mit), but not SAS ρ^0 cells (SAS-Fibro-Mit) resulted in increased cisplatin-induced apoptosis. In addition, we evaluated mRNA expression of the apoptotic genes in the SAS-Fibro-Mit and HeLa-Fibro-Mit cells, and compared to their parental counterpart (HeLa ρ^0 and SAS ρ^0). Interestingly, expression of BAX, caspase 8, caspase 9, and AIF proapoptotic genes was up-regulated in both the SAS- Fibro-Mit (SAS ρ^0 + Mit) and HeLa-Fibro-Mit cells (HeLa ρ^0 + Mit). While expression of Bcl-2 anti-apoptotic gene was down-regulated. These results suggest that cancer cells prefer to shut down their mitochondria to be able to expand and also refractory to the apoptosis induction by chemotherapeutic agents.

More recently, we reported that the transfer of normal mitochondria to MDA-MB-231 cells did not affect the

cisplatin-induced apoptosis, and even decreased the sensitivity of the mitochondria recipient MDA-MB-231 cells to the cisplatin-induced apoptosis. However, when the endogenous mitochondria of the MDA-MB-231 cells were disrupted before the normal mitochondria transplantation, cisplatin-induced apoptosis was increased [15].

Kaipparettu et al. established cybrids by fusing mtDNA depleted 143B ρ^0 cells (an aggressive osteosarcoma cell line) with mitochondria from MCF10A cells (a benign breast epithelial cell line). The results of their study showed reversed oncogenic characteristics following the normal mitochondria transfer. In fact, the mitochondria transplantation resulted in regressed viability of the cells under hypoxic condition, decreased proliferation, reduced invasion, and limited colony formation in soft agar. It also decreased resistance of the cells to doxorubicin induced apoptosis and cell death [18].Similar to our findings, Chang et al. showed that mitochondria transfer to the MCF-7 cells enhanced the apoptotic cells by increasing the nuclear translocation of apoptosis-inducing factor (AIF) [29].

Conclusion

In the current study, we showed that mitochondria contribute an important role to cell proliferation and response to chemotherapeutic agents. It seems that the response of cancer cells to the mitochondria transfer is cancer-type dependent. In addition, our findings suggest that cancer cells might shut down their mitochondria to acquire advantages of the tumor microenvironment in favor of proliferation. Our results also suggest that the introduction of normal exogenous mitochondria to some cancer cells, at least in part, might be considered as a novel therapeutic strategy. However, further in vitro and in vivo investigations are required.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study only involves in vitro experiments and there was no involvement of Human Participants and/or Animals in it.

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