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Mitochondrial Transfer Drives Stem Cell–Like Phenotypes in Gastric Cancer Cells

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Abstract

Background and Objective: Mitochondrial dynamics have a crucial role in the development of cancer and the plasticity of cancer cells. The role of mitochondria isolated from parental cells in regulating mitochondrial content and cancer stem cell (CSC) properties of MKN45 gastric cancer cells was examined.

Methods: Mitochondria were isolated from parental MKN45 cells and transferred into recipient cells using a co-incubation approach. Mitochondrial DNA (mtDNA) copy number was analyzed by quantitative real-time PCR (qPCR). Fluorescence microscopy was used to confirm the presence of mitochondria stained with Rhodamine 123 in the recipient cells following the transfer of mitochondria. Self-renewal capacity was evaluated using a sphere formation assay. The expression levels of stemness (*OCT4, NANOG, BMI1*), mesenchymal and epithelial (*SNAIL, SLUG, VIMENTIN, E-CADHERIN*) markers were determined by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

Results: The transfer of mitochondria caused a significant increase in the number of mtDNA copies and a higher mitochondrial membrane potential in the recipient cells. Furthermore, these mitochondrial alterations were functionally associated with an increase in the ability to form spheres and the expression of genes related to stemness, mesenchymal marker expression, and epithelial marker expression.

Conclusion: These results suggest that the augmented mitochondrial content enhances the CSC-like traits that could be a factor in tumor aggressiveness.

Keywords: mitochondria, mitochondria isolation, mitochondria transfer, mitochondrial DNA, gastric cancer

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Mitokondriyal Transferin Gastrik Kanser Hücrelerinde Kök Hücre Benzeri Özelliklerin Ortaya Çıkışındaki Rolü

Öz

Amaç: Mitokondriyal dinamikler, kanserin gelişiminde ve kanser hücrelerinin plastisitesinde kritik bir rol oynamaktadır. Bu çalışmada, ebeveyn hücrelerden izole edilen mitokondrilerin MKN45 mide kanseri hücrelerinin mitokondriyal içeriği ve kanser kök hücresi (CSC) özellikleri üzerindeki rolü incelenmiştir.

Yöntemler: Mitokondriler, ebeveyn MKN45 hücrelerinden izole edilerek alıcı hücrelere birlikte inkübasyon yöntemiyle aktarıldı. Mitokondriyal DNA (mtDNA) kopya sayısı eş zamanlı qPCR ile analiz edildi. Mitokondri transferinden sonra alıcı hücrelerde Rhodamine 123 ile boyanmış mitokondri varlığını doğrulamak için floresan mikroskopi kullanıldı. Öz yenilenme kapasitesini değerlendirmek için küre (sphere) oluşum analizi yapıldı ve kök hücre belirteçleri (*OCT4, NANOG, BMI1*) ile mezenkimal ve epitelyal belirteçlerin (*SNAIL, SLUG, VIMENTIN, E-KADERİN*) gen ekspresyon düzeyleri RT-qPCR ile belirlendi.

Bulgular: Mitokondrilerin aktarımı, alıcı hücrelerde mtDNA kopya sayısında dikkat çekici bir artışa ve daha yüksek bir mitokondriyal membran potansiyeline neden olmuştur. Ayrıca, bu mitokondriyal değişikliklerin, küre (sphere) oluşturma yeteneğinde ve kök hücreliliğe (stemness) ilişkin genlerin (*OCT4, NANOG, BMI1*) yanı sıra mezenkimal ve epitelyal belirteçlerin (*SNAIL, SLUG, VIMENTIN, E-KADERİN*) ekspresyonunda artışla işlevsel olarak bağlantılı olduğu görülmüştür.

Sonuç: Bu sonuçlar, artan mitokondriyal içeriğin CSC özelliklerini güçlendirdiğini ve bunun tümör agresifliğinde etkili bir faktör olabileceğini düşündürmektedir.

Anahtar kelimeler: mitokondri, mitokondri izolasyonu, mitokondri transferi, mitokondriyal DNA, mide kanseri.

INTRODUCTION

Mitochondria, the powerhouse of the cell, are dynamic organelles that play essential roles in cellular homeostasis by regulating energy production, redox balance, and calcium signaling[1]. Impairments of mitochondrial function are increasingly associated with a wide spectrum of pathological conditions, including aging, cancer, metabolic syndromes, neurodegenerative disorders, and skeletal muscle abnormalities[2,3].

Impairment in the function of mitochondria leads to oxidative stress, perturbs intracellular signalling, and alterations in gene expression[4]. Under conditions of severe oxidative stress, ATP depletion can shift cell death from apoptosis to necrosis[5].

Cancer cells frequently exhibit metabolic reprogramming characterized by a shift toward aerobic glycolysis, known as the Warburg effect, which reduces their reliance on oxidative phosphorylation, although functional mitochondria remain essential for biosynthetic and signaling pathways[6]. Increased mitochondrial DNA (mtDNA) mutations along with disturbances in respiratory chain function, and dysregulated mitochondrial dynamics further exacerbate the malignant phenotype and promote therapy resistance[7].

However, in addition to mitochondrial dysfunction, there is growing evidence that mitochondrial hyperfunction also plays a critical role in cancer progression by meeting increased bioenergetic and biosynthetic demands. Mitochondrial hyperfunction has been implicated in cancer progression. Enhanced mitochondrial biogenesis and oxidative phosphorylation can sustain tumor cell proliferation and provide survival advantages under metabolic stress[8].

There are several strategies for enhancing mitochondrial function, such as mROS scavenging, mitochondrial repairment,

reprogramming, stimulating mitochondrial biogenesis and signaling, and healthy mitochondria transfer[9]. Repairing and reprogramming have limitations related to genetic lesions correction. The latest studies have indicated that the transplantation of functional or engineered mitochondria can rejuvenate bioenergetic activity and cellular phenotypes in the damaged cells[10,11]. Accordingly, mitochondrial transplantation has emerged as an effective option for therapies to mitochondrial dysfunction.

Mitochondria transfer between the healthy or engineered ones into the tumor has been linked to the restoration of oxidative metabolism, thus leading to less accumulation of reactive oxygen species (ROS) and impacting the cancer cell's response to treatment with chemotherapeutics[12,13].

In addition, the production of non-cancerous mitochondria can act as a tumor-suppressor and lead to encouraging metabolic homeostasis, which implies that the mitochondrial transfer can be used alongside the existing anticancer methods[14]. Furthermore, the investigation of the impact of mitochondrial transfer on cancer-associated mitochondrial function could not only bring new understanding to the control of malignant cells with excessive bioenergetic and biosynthetic capacity but also create new therapeutic intervention possibilities.

This study aimed to investigate whether mitochondrial transfer from parental MKN45 cells increases mitochondrial content in recipient MKN45 cells, thereby promoting cancer stem cell-like and mesenchymal properties. We hypothesised that increased mitochondrial content would enhance CSC traits and contribute to tumour aggressiveness.

Therefore, we isolated mitochondria from MKN45 parental cells and then transfer them into MKN45 cells and hereafter referred to as mitochondria-transferred cells. We used a straightforward technique for mitochondrial transfer to increase mitochondrial content of the cells, which involves co-incubation of isolated mitochondria with recipient cells. Mitochondrial transfer was confirmed through Rhodamine 123 staining and analysis of mtDNA copy number. The effect of the increase in the mitochondria on the gene expression and spheroid formation was analyzed.

METHODS

Cell Culture

The MKN45 cell line (Accession CVCL_0434), derived from liver metastasis of a poorly differentiated gastric adenocarcinoma, was employed for the experiments. Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cultures were maintained at 37 °C under 5% CO_2 in a humidified incubator and were routinely passaged at 85–90% confluence using 0.25% Trypsin-EDTA.

DNA Extraction and Quantification of mtDNA Copy Number

Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Germantown, USA) according to the manufacturer's instructions. Relative changes in mtDNA copy number were determined using SYBR Green-based quantitative real-time PCR. Reactions were performed on a Bio-Rad CFX96 real-time PCR system using SYBR green master mix (Abmgood-G891, Richmond, BC, Canada). The mtDNA-encoded tRNA leucine 1 gene (tRNA-Leu1 gene, *MT-TL1*) (forward primer: CACCCAAGAACAGGGTTTGT, reverse primer: TGGCCATGGGTATGTTGTTAA) served as the target, while the nuclear 18S rRNA gene (forward primer: TAGAGGGACAAGTGGCGTTC, reverse primer: CGCTGAGCCAGTCAGTGT) was used as a reference. Data were analyzed using the 2^−ΔΔCt method[15,16].

Mitochondrial Transfer

$1x10^6$ cells were trypsinized or harvested using a cell scraper from T25 culture dish. Cells are resuspended in 1ml of 0.25 M sucrose in cold (4ºC) TE buffer. Cells were homogenized using ice cold Dounce Homogenizer (70-100 strokes). Homogenate was centrifuged at 2000g and 4ºC for 5 minutes. The pellet contained nuclei and cellular debris. The supernatant was then centrifuged at 8000 × g for 5 min at 4 °C to obtain a pellet enriched with intact mitochondria. Intact mitochondria were found in this final pellet. The isolated mitochondria were resuspended in the same TE buffer and subsequently used for mitochondrial transfer experiments. 1 µg/ml Rhodamine 123 staining, a mitochondria-specific fluorescent dye, was performed on isolated mitochondria. Following 10 min of incubation at 37 °C, the mitochondria were washed three times with PBS. Mitochondrial transfer was performed by co-incubating the isolated mitochondria with recipient cells (1×10^6 cells per T25 flask) in complete culture medium (RPMI supplemented with 10% FBS, 1% antibiotic mixture, and 1% L-glutamine) at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h. The mitochondria were subsequently internalized by recipient cells. The control cells were cultured under identical conditions for the same duration but were not exposed to the isolated mitochondria.

Confirmation of mitochondrial transfer was performed with mtDNA copy number analysis through quantitative real-time PCR. Cells were examined using a fluorescence microscope under both bright-field and green fluorescence channels. Fluorescence intensity was subsequently quantified using ImageJ software. Mitochondrial transfer and control experiments were conducted in three independent biological replicates. Each replicate was conducted on a different day using a separate cell culture (n = 3 per group).

RNA Extraction and Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Parental and mitochondria-transferred MKN45 cells were collected by trypsinization. Following centrifugation at 400 × g for 5 min, the supernatant was discarded. RNA isolation was performed using an RNeasy Mini Kit (Qiagen, Germantown, USA). A cDNA synthesis was carried out using a Wonder RT-cDNA Kit (Euroclone, Pero, Italy). A total of 3 stemness-related genes, *OCT4* (forward: 5'-GATCACCCTGGGATATACAC, reverse: 5'-GCTTTGCATATCTCCTGAAG), *NANOG* (forward: 5'-AAAGAAGCCAACACTAAACC, reverse: 5'-TGGTCATTTCGTTAAAGGC), *BMI1* (forward: 5'-AAATGCTGGAGAACTGGAAAG, reverse: 5'-CTGTGGATGAGGAGACTGC), *SNAIL* (forward: 5'-GCTGCCAATGCTCATCTGGGACTCT, reverse: 5'-TTGAAGGGCTTTCGAGCCTGGAGAT), *SLUG* (forward: 5'-GTGATTATTTCCCCGTATCTCTAT, reverse: 5'-CAATGGCATGGGGGTCTGAAAG), *VIMENTIN* (forward: 5'AGGCAAAGCAGGAGTCCACTGA, reverse: 5'-ATCTGGCGTTCCAGGGACTCAT) *and E-CADHERIN* (forward: 5'-GCCTCCTGAAAAGAGAGTGGAAG, reverse: 5'-TGGCAGTGTCTCTCCAAATCCG) were selected and analyzed using a FluoCycleII SYBR Master Mix Kit (Euroclone, Pero, Italy). *ACTB (β-actin)* (forward: 5'-CACCATTGGCAATGAGCGGTTC, reverse: 5′-AGGTCTTTGCGGATGTCCACGT) was used as a reference gene, and gene expression changes were calculated using the Livak Method[16].

Spheroid Formation Assay

Spheroid bodies were derived by plating 4 × 104 cells in 24-well plates coated with 2% agarose. MKN45 cells were cultured in RPMI-1640 medium with 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). After the 10th day of the culture, spheroid bodies were counted under an

inverted microscope Paula (Leica, Wetzlar, HE, Germany) at 100× magnification.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 8.0.2. Comparisons between two groups were conducted using an unpaired, two-tailed Student's t-test with Welch's correction. A p-value of less than 0.05 was considered statistically significant (*p < 0.05).

RESULTS

mtDNA levels and mitochondrial membrane potential increased upon mitochondrial transfer

Mitochondria were obtained from parental MKN45 cells and transferred into sibling MKN45 cells. One day after mitochondrial transfer, mtDNA levels significantly (p=0.02) increased 1.9-fold over parental counterparts for MKN45 (Figure 1A).

Mitochondrial transfer resulted in an augmentation in mitochondrial membrane potential as revealed by measurement of ΔΨm using the Rhodamine 123 method. Mitochondria-transferred MKN45 cells exhibited increased rhodamine uptake as compared with parental cells (Figure 1B, C).

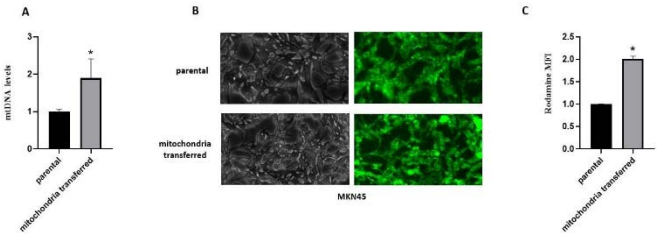

Figure 1. Relative mtDNA content and mitochondrial membrane potential. The relative level of mitochondrial DNA (mtDNA) was assessed by real-time PCR amplification of human t-RNA leucine 1 gene and nuclear-encoded 18S rRNA gene. Relative mtDNA levels of parental and mitochondria-transferred MKN45 cells. Mitochondrial membrane potential was analyzed by Rhodamine 123 staining. B) Upper panel shows unstained (left) and stained (right) images of parental cells. The lower panel shows unstained (left) and stained (right) images of mitochondria-transferred cells. C) Rhodamine 123 mitochondrial membrane potential MFI values of parental and mitochondria-transferred cells. Data are reported as mean ± SD based on three independent biological replicates (n = 3). The statistical significance of differences between the two groups was assessed using an unpaired, two-tailed Student's t-test with Welch's correction. * p < 0.05.

Mitochondrial transfer increased spheroid formation capacity

The ability of spheroid formation was demonstrated through culturing cells with serum-free medium on agarose-coated plates. Mitochondria-transferred MKN45 cells showed increased spheroid formation capacity by 1.5 fold (p=0.05) than parental counterparts (Fig.2).

Mitochondrial transfer augmented stemness and EMT-related gene expression

Mitochondrial transfer altered stem cell and epithelial-mesenchymal transition related genes in MKN45. Mitochondria-transferred cells had increased expression of stemness related genes; *OCT4* (1.12 fold, p=0.13), *NANOG* (2.78, p=0.0003), *BMI1* and EMT related genes *SNAIL* (1.57 fold, p=0.0091), *SLUG* (1.01 fold, p=0.66), *VIMENTIN* (2.86, p=0.0001). Mitochondria transfer led to a slight decrease in *E-CADHERIN* (0.98 fold, p=0.39) expression (Fig.2).

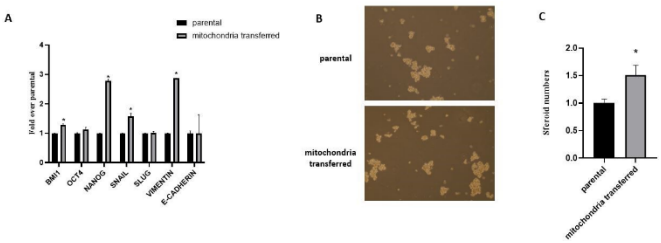

Figure 2. Alterations in the stemness-related gene expression and spheroid formation capacity in parental, and mitochondria-transferred cells of MKN45. A) mRNA levels were normalized with respect to *ACTB*. B) Representative images of parental and mitochondria-transferred cells grown in 3D cultures. Microscopic images were obtained 10 days following cell seeding and treatment with

a PAULA Cell Imager. C) Spheroid numbers for MKN45. Data are reported as mean ± SD based on three independent biological replicates (n = 3). The statistical significance of differences between the two groups was assessed using an unpaired, two-tailed Student's t-test with Welch's correction. * $p < 0.05$.

DISCUSSION

The isolation and transfer of intact mitochondria are a crucial and promising approach for mitochondrial-related studies, including investigations of mitochondrial function, mtDNA depletion, the mitochondrial genome, proteome, lipidome, mitochondria-associated cell-to-cell communication, mitochondria-organellar interactions, dynamics, and cellular homeostasis[17,18].

This study demonstrates the effect of parental mitochondrial transfer through an effective and straightforward approach on the recipient cells. We successfully isolated mitochondria from MKN45 gastric cancer cells using differential centrifugation and demonstrated successful transfer into recipient cells. This approach provides a straightforward and efficient method for manipulating mitochondrial content and examining related biological outcomes. Differential centrifugation is still the most popular and widely used technique for mitochondrial isolation because of its simplicity and accessibility. Though more advanced methods like magnetic-activated cell sorting (MACS) or fluorescence-activated mitochondrial sorting (FAMS) have been introduced[19,20], a two-step centrifugation protocol still gives good yield and purity for most downstream applications[21]. In the present study, the low-speed centrifugation step effectively removed all the intact cells, nuclei, and debris, while the high-speed step enriched the intact mitochondria with the membrane potential still intact, which was indicated by the results of the Rhodamine 123 staining.

Mitochondrial transfer has been an area of research in the context of several problems like mitochondrial dysfunction, metabolic disorders, neurodegeneration, and cancer. Research has demonstrated that exogenous mitochondria can become part of the mitochondrial network of the host cells and take part in ATP production, regulating the level of oxidative stress, and restoring some cellular functions[22,23]. Although it has been shown that the exogenous delivery of mitochondria through centrifugation can greatly enhance metabolic cell function 22, our findings indicate that a centrifugation-free delivery strategy can also provide highly functional mitochondria in the recipient cells. The successful staining of transferred mitochondria with Rhodamine 123 in our study supports the viability and functionality of the isolated mitochondria post-transfer. This suggests that the preservation of mitochondrial integrity and activity does not necessarily depend on a centrifugation step, and that alternative approaches may be equally effective in supporting downstream applications. Furthermore, increasing mitochondrial and mtDNA content in recipient cells can be particularly useful for studying mitochondrial biogenesis, mitochondrial-nuclear crosstalk, and mtDNA-dependent gene expression[24,25]. The method also has the potential to model diseases related to mitochondrial defects or investigate therapeutic interventions aimed at mitochondrial enhancement or rescue.

In this study, we examined how exogenous mitochondrial transfer derived from parental cells can modulate mitochondrial content and functionality in recipient gastric cancer cells. Our results show that recipient cells acquire increased mtDNA copy number, restored mitochondrial membrane potential, and enhanced functional capacity, which in turn correlate with elevated sphere formation and upregulation of stemness (*OCT4, NANOG, BMI1*),

mesenchymal (*SNAIL, SLUG, VIMENTIN*) and a decrease in epithelial (*E-CADHERIN*) marker expression. Collectively, these observations support that an increase in the mitochondrial content can drive CSC-like phenotypes and may thus contribute to tumor progression.

The upregulation of genes such as *OCT4, NANOG, BMI1*, and mesenchymal markers *SNAIL, SLUG, VIMENTIN* in recipient cells is consistent with enhanced stemness and EMT-like plasticity. The coupling of mitochondrial augmentation and EMT/CSC programs is conceptually coherent: EMT is a recognized route toward increased plasticity, invasiveness, and acquisition of stem-like behavior[26]. In many cancer types, metabolic reprogramming toward oxidative metabolism favors EMT-related transcriptional networks, and vice versa. In effect, enhanced mitochondrial capacity may facilitate the metabolic flexibility needed for cells to engage the EMT–CSC axis.

Mechanistically, several possibilities may underlie the linkage between mitochondrial augmentation and CSC phenotypes. Enhanced mitochondrial respiration may lead to increased ATP, alteration in $NADH/NAD^+$ balance, or biosynthetic precursors that support the energy-intensive processes of self-renewal and sphere formation[27]. Second, increasing or stabilizing mitochondrial membrane potential may reduce mitochondrial stress and may limit the generation of excessive ROS and help maintain an optimal ROS homeostasis that a moderate ROS homeostasis is often favorable for stemness, whereas excessive ROS is deleterious, as has been reported in CSC settings[28,29]. The increase in mtDNA copy number itself may enhance mitochondrial efficiency, reduce dysfunction, and support sustained metabolic output required by CSC[30].

In this study, the findings demonstrate the effects of mitochondrial content in the gastric cancer cells. The findings underscore the role of mitochondrial transfer as a functional driver of cancer stem cell–like properties in gastric cancer cells. From a clinical perspective, these results suggest that blocking CSC-associated tumor aggressiveness and therapy resistance by targeting mitochondrial augmentation or intercellular mitochondrial transfer may represent a novel therapeutic strategy. Future studies should investigate the molecular mechanisms governing mitochondrial uptake and integration, and evaluate whether inhibiting mitochondrial transfer could make gastric cancer cells more sensitive to conventional treatments. Extending these findings to in vivo models and patient-derived systems will be crucial in assessing the relevance of mitochondrial modulation in gastric cancer translationally.

In conclusion, our study shows that the transfer of cells' own parental mitochondria augments mitochondrial content and function in recipient gastric cancer cells, thereby potentiating CSC-like phenotypes through metabolic and signaling reprogramming. These findings emphasize the centrality of mitochondrial dynamics in cancer plasticity and highlight the increase in the mitochondrial content as a putative contributor to tumor aggressiveness. Future work dissecting mechanisms, in vivo relevance, and translational inhibition will be essential to further validate this paradigm and exploit it therapeutically.

Ethical Approval: Ethical approval was not required.

Conflict of Interest: The authors declare that there is no conflict of interest.

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