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Transplantation of gastric epithelial mitochondria into human gastric cancer cells inhibits tumor growth and enhances chemosensitivity by reducing cancer stemness and modulating gastric cancer metabolism

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Abstract

Background Gastric cancer is the malignant disease. The problems associated with cancer stemness and chemotherapy resistance in gastric cancer therapy remain unresolved. Glucose-regulated protein 78 (GRP78) is a biomarker of gastric cancer and modulates cancer stemness and chemoresistance. Previous studies have shown that mitochondrial transplantation from healthy cells is a promising method for treating various diseases and that the regulation of mitochondrial metabolism is crucial for modulating the stemness and chemoresistance of cancer cells. The aim of this study was to investigate the therapeutic effect of mitochondrial transplantation from normal gastric epithelial cells into gastric cancer and the associated mechanisms.

Methods The expression of cancer stemness markers, intracellular oxidative stress, or apoptotic-related proteins were evaluated via flow cytometry. Western blotting was used to investigate the molecular mechanism involved in MKN45 or AGS human gastric cancer cells after transplantation with human gastric epithelial mitochondria. The mitochondrial metabolic function of gastric cancer cells was determined via a Seahorse bioanalyzer, and extracellular lactate was evaluated via bioluminescent assay. The viability of 5-fluorouracil (5-FU)-treated gastric cancer cells was detected via a CCK-8 assay. Furthermore, a xenograft tumor animal study was performed to validate the therapeutic effects of human gastric epithelial mitochondrial transplantation in gastric cancer. Immunohistochemistry and Western blotting were then used to assess the expressions related to cancer stemness and mitochondrial metabolism-related proteins in tumor tissues.

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Results Transplanting human gastric epithelial mitochondria downregulates gastric cancer mitochondrial biogenesis, glycolysis, GRP78-mediated cancer stemness, and increases oxidative stress, cell apoptosis under hypoxic conditions and chemosensitivity in response to 5-FU treatment. Moreover, the transplantation of epithelial mitochondria into gastric tumors inhibited the tumor growth in vivo tumor graft animal models. Therefore, mitochondrial transplantation can be considered for the treatment of gastric cancer.

Highlights

- Transplanted human gastric epithelial mitochondria suppressed gastric cancer cell stemness.
- Transplanted human gastric epithelial mitochondria modulate cancer metabolism.
- Transplantation of human epithelial mitochondria into gastric cancer cells inhibited gastric tumor growth.
- Transplanted human gastric epithelial mitochondria promoted gastric cancer apoptosis under hypoxic conditions and enhanced chemosensitivity.

Keywords Gastric cancer, Mitochondrial transplantation, Cancer stemness, GRP78, Chemoresistance, Metabolism, Apoptosis

Background

Gastric cancer is the malignant disease. In particular, the resistance of in solid gastric tumors to chemotherapeutic agents remains an unresolved problem in clinical treatment [1]. Recent studies have shown that cancer cells with self-renewability and stem capacity contribute to cancer recurrence and chemotherapy resistance [1–4].

Glucose regulated protein 78 (GRP78) was reported to be a biomarker of malignant human stomach tumors [5–6]. Previous clinical and experimental studies have confirmed that GRP78 is increased in the tissues of human gastric tumors, enhances the cancer stemness, regulates tumor microenvironments, and plays a crucial role in gastric cancer chemotherapy [7–8]. The cell surface markers CD24, CD44, and LGR5 can be determined by examining the transcription factors associated with cancer stemness, such as NANOG and SOX2. NANOG and SOX2 are widely expressed in cancer and are involved in chemoresistance. Another functional marker is aldehyde dehydrogenase 1 (ALDH1), an oxidative detoxification enzyme as poor prognosis predictor of cancer. The elimination of GRP78 expression eliminates the activity of CD24, CD44, LGR5, SOX2, NANOG and ALDH1 in human gastrointestinal cancer. Our previous animal study of tumor xenografts revealed that inhibiting GRP78 in gastric cancer cells also inhibits tumor growth [7]. These findings suggest that the suppression of cancer stemmess is a new target for the treatment of gastric cancer in precision medicine.

Mitochondria not only are important for the production of most of a cell's energy but also contribute to the synthesis, metabolism and maintenance of cell homeostasis [9]. It is necessary to control cell growth and cell death. In addition, there is increasing evidence that mitochondria play a key role in tumor development. Deregulation of mitochondria is involved in cancer progression, as demonstrated by the Warburg effect [10]. Targeting mitochondrial dynamics and redox control has been reported to overcome the toxicity of cancer chemotherapy [11]. The regulation of mitochondrial dynamics and metabolism is crucial for modulating the stemness and chemical resistance of cancer cells, as it promotes survival pathways in response to hypoxia or chemical therapies [12]. Several studies have shown that mitochondria can be exchanged between different types of adjacent cells by regulating the fate and function of these cells through tunnel nanotubes, connexins, or extracellular vesicles [13–15]. Mitochondrial transfer between cells has been shown to play a role in regulating the development of cancer. The transfer of mitochondria from astrocytes enhances glioblastoma tumorigenesis [16], and endothelial mitochondria induce tumor growth and increase chemotherapy resistance [17]. In contrast, osteocytes impede tumor development by transmitting mitochondria to cancer cells [18]. The efficiency of mitochondrial transmission in inhibiting tumor growth and reducing chemical resistance depends on the cell type. Thus, specific mitochondria that inhibit the progression of gastric tumors must be identified and investigated.

In 1982, the first mitochondrial transplant was carried out, involving the joint cultivation of isolated healthy mitochondria [19]. Recently, transplantation of mitochondria has been a promising approach for treating various diseases [20–22]. Especially, mitochondrial transplantation has become a method of evaluating the function of tumor cells after the intake of mitochondria from donor cells in tumor microenvironments [23]. A common approach to this assessment is to co-cultivate the recipient cells with mitochondria isolated from the donor cells [21, 24].

GRP78 is required for glycolytic proliferation and adenomagenesis in cancer [25] and is involved in the modulation of aerobic glycolysis in tumors [26]. It has also been proposed to be involved metabolic alterations and during tumor progression. Mitochondrial transfer techniques have been introduced into cell therapy and have

had strong therapeutic effects. However, how mitochondria affect GRP78 expression, metabolism, stemness, chemosensitivity, and tumor growth in gastric cancer after mitochondrial transplantation remains unclear. Previous studies have shown that mitochondrial transplantation from healthy cells is a promising method for treating various diseases [20–22] and that the regulation of mitochondrial metabolism is crucial for modulating the stemness and chemoresistance of cancer cells. However, whether normal gastric epithelial cells are suitable for mitochondrial transplantation in gastric cancer treatment is unclear. Therefore, the aim of this study was to investigate the therapeutic effect of mitochondrial transplantation from normal human gastric epithelial cells into human gastric cancer and the associated mechanisms.

In the present study, we investigated the antitumor mechanisms triggered when normal human gastric epithelial mitochondria are transplanted into human gastric cancer cells. Signaling pathways and tumor xenograft animal models were used to investigate the role of gastric epithelial mitochondrial transfer in gastric cancer metabolism and stemness, chemoresistance, and tumor progression. Our results indicate that the transplantation of human gastric epithelial mitochondria has potential for use in therapy for gastric cancer.

Materials and methods

Cell culture and reagents

The human gastric cancer cell line AGS (ATCC No.CRL-1739) was purchased from ATCC (America Type Culture Collection), under a Material Transfer Agreement (https://www.atcc.org/policies/product-use-policies/material-transfer-agreement). The human gastric cancer cell line MKN45 (DSMZ No. ACC409) was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), under a Material Transfer Agreement (https://www.dsmz.de/collection/catalogue/microorganisms/cbd-and-material-transfer-agreements). AGS or MKN45 cells were cultured in RPMI1640 (Gibco, Waltham, MA, USA) medium with 10% fetal bovine serum (FBS) (Gibco) under 5% CO_2 at 37 °C. The normal human gastric epithelium cell GES-1 (Cytion No. 305428) was obtained through Cytion (Cell Lines Service, Eppelheim, Germany), under a Material Transfer Agreement (https://www.cytion.com/Our-Approach/Terms-and-Conditions). GES-1 was cultured in Dulbecco's Modified Eagle Medium (Gibco) with 10% FBS (Gibco) under 5% CO_2 at 37 °C. Stock solutions of 0.1-M $CoCl_2$ solution (Sigma-Aldrich, St. Louis, MO, USA) and 5-fluorouracil (5-FU; Sigma-Aldrich) were diluted and prepared in culture medium before use.

Isolation of mitochondria

Mitochondria were isolated from GES-1 cells via a Thermo Scientific Mitochondria Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) for use with mammalian cells. The isolated mitochondria were then suspended in 100 μL of phosphate-buffered saline (PBS) and performed protein quantification by using the Bio-Rad protein assay kit before use (Bio-Rad, Hercules, CA, USA). The mitochondrial transplantations in vitro or in vivo were performed according to the previous studies [20, 27–28]. For in vitro test, mitochondria are freshly isolated before each use and mitochondria (25 μg/ml) are treated directly into the culture medium for 24 h. For in vivo testing, mitochondria are freshly isolated before each use and mitochondria (25 μg/100 μl) are directly intratumoral injection into mice every 2 days for 6 days.

Western blot analysis

Western blot analysis was performed according to previous study [29]. The human gastric cancer cells and tumor tissue samples were washed with PBS. Total protein samples were extracted using a lysis buffer (Thermo Fisher Scientific), and protein concentrations were measured via the Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Equal quantities of total proteins were separated by performing BOLT BISTRIS PLUS 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Thermo Fisher Scientific) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with a blocking buffer (Bio-Rad) for 30 min at room temperature and incubated at 4 °C with the following primary antibodies: SOX2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), NANOG (1:1000, Merck Millipore, Darmstadt, Germany), GRP78 (1:1000; Cell Signaling Technology), JNK (1:1000; #9252; Cell Signaling Technology), p-JNK (1:1000; #9251; Cell Signaling Technology), NOTCH1 (1:1000; #3608; Cell Signaling Technology), PKM2 (1:1000; #4053; Cell Signaling Technology), MCT1 (1:1000; A3013; ABclone, Woburn, MA, USA), MCT4 (1:1000; A10548; ABclone), PGC-1α (1:1000; NB-100-60955; Novus Biologicals, LLC, CO, USA), YY-1(1:1000; ab109228; Abcam, Cambridge, UK), MCP-1 (1:1000; NBP1-07035; Novus Biologicals, LLC, CO, USA), HIF-1α (1:1000; #14179; Cell Signaling Technology), Drp-1 (1:1000; ab184247; Abcam), Caspase3 (1:1000 #9662; Cell Signaling Technology), cleaved-Caspase3 (1:1000 #9661; Cell Signaling Technology), BAX (1:1000 #2772; Cell Signaling Technology), p-AKT (1:1000; Enzo, Farmingdale, NY, USA), AKT (1:1000 #9272; Cell Signaling Technology), and β-actin (1:1000, Merck Millipore). The membranes were washed, incubated with secondary antibodies at room temperature for 1 h, and evaluated using an electrochemiluminescence

detection system. All data were quantified using Image J software.

Flow cytometry analysis

Flow cytomertry analysis was performed according to previous study [29]. The human gastric cancer cells were stained with a surface marker antibody for 45 min. After staining, the cells were washed twice with cold PBS before analysis. The expressions of stemness markers (CD24, CD44, and LGR5; BD Biosciences, San Jose, CA, USA) were analyzed through flow cytometry. ALDH1 analysis was conducted by staining the cells with the AldeRed ALDH Detection Assay kit (Sigma-Aldrich). To detect superoxide in the mitochondria, the cells were stained with MitoSOX (Invitrogen, Waltham, MA, USA). To evaluate intracellular oxidative stress, the cells were stained with dihydroethidium (DHE) (Invitrogen). For caspase activity analysis, the cells were stained with a Cleaved Caspase-3 Staining Kit (Abcam) and then analyzed via flow cytometry.

Metabolic flux analysis

The extracellular oxygen consumption rate (OCR) was assessed via the Seahorse XFp Analyzer (Agilent Technologies, Santa Clara, CA, USA) [30]. After undergoing mitochondrial treatment for 24 h, the human gastric cancer cells were washed using XF DMEM-Base Medium (Agilent Technologies) supplemented with 10-mM glucose (Gibco) and 2-mM L-glutamine (Gibco). The cells were seeded in XFp FluxPak (Agilent Technologies) at a density of 20,000 cells/well. The OCR was evaluated using the XFp Cell Mito Stress Test Kit (Agilent Technologies) in real time following injections of oligomycin A (2 μM), FCCP (0.6 μM), and rotenone/antimycin A (5 μM).

Measurement of ATP levels

ATP level measurement was performed according previous study [29]. The human gastric cancer cells were seeded in 6-well dishes in quadruplicate and cultured for 24 h before treatment. The ATP level was analyzed by ATPlite Luminescence Assay System, Assay Kit (PerkinElmer, Baesweiler, Germany) and measured the luminescence using a microplate reader.

Measurement of lactate levels

Lactate level measurement was performed according to previous study [31]. Conditioned media were collected from both human GES-1 cells and gastric cancer cells. Extracellular lactate was measured via the Lactate-Glo Assay kit (Promega, Madison, Wisconsin, USA) in accordance with the manufacturer's instructions.

Xenograft tumor animal study

Male nude mice (Nude Mice-BALB/c) were purchased from LASCO Biotechnology at the age of 5 weeks and kept in the Laboratory Animal Center of the Faculty of Medicine of I-Shou University under special care. The 6-week-old BALB/c nude mice were anesthetized by inhalation of isoflurane (2% of isoflurane) before injection. The cancer cells were then harvested and subcutaneously inoculated (1×10^7 cells/0.1 mL PBS) into mice. For MKN45, the mice were randomly divided into three groups, namely a control group ($n = 5$), a pre-GES-1 group (where MKN45 cells pretreated with GES-1 mitochondria (25 μg) for 24 h). The mitochondrial transplantation in animal model was performed according previous studies [20, 27–28]. The treated MKN45 cells were then implanted in nude mice [$n = 5$]), and a GES-1 MITO group (The cancer cell subcutaneously inoculated into mice, and after the tumor grows, normal saline containing GES-1 mitochondria (25 μg/100 μl) is through intratumoral injections every 2 days for 6 days to observe the growth of the tumor [$n = 5$])). For AGS, the mice were randomly divided into two groups, namely a control group ($n = 5$) and a pre-GES-1 MITO group (where AGS cells pretreated with GES-1 mitochondria (25 μg) for 24 h. The treated AGS cells were implanted in nude mice [$n = 5$]). The tumor volume was measured every 2 days by applying the formula $V = (L \times W^2)/2$ (L, length; W, width). The mice were euthanized by inhalation of overdose isoflurane (approximately 5%) and the tumor was harvested. The procedures for animal care and handling adhered to the guidelines of the Laboratory Animal Center (IACUC-EDAH-112015) at the E-Da hospital. The work has been reported in line with the ARRIVE guidelines 2.0.

Immunohistochemistry and hematoxylin plus eosin staining

The obtained tissues were embedded in paraffin wax after they were cut and dehydrated via a series of alcohol solutions. The paraffin-embedded tissues were cut into 3-μm sections and placed on slides, after which staining was performed via the GRP78 antibody (1:1000; #3183; Cell Signaling) or ki-67 (1:500; SP6; Spring Bioscience, Pleasanton, CA, USA) to conduct immunohistochemical (IHC) analysis in accordance with the manufacturer's instructions. For hematoxylin and eosin (H&E) staining, the paraffin embed slides were dewaxed, gradually hydrated via graded alcohol, stained with hematoxylin solution, and differentiated in 1% hydrochloric alcohol. After the slides were rinsed with distilled water, they were dehydrated in 95% ethanol, counterstained in 1% eosin solution, washed with 70% ethanol followed by absolute ethanol, and then cleared through two rounds of treatment with xylene.

Cell viability analysis

AGS or MKN45 cells were seeded in 96-well plates in sextuplicate at a density of 6000 cells/well and cultured for 24 h before treatment. Cell viability was analyzed via the Sulforhodamine B (SRB) Assay Kit (Abcam), and measured at 565 nm via a microplate reader in accordance with the manufacturer's instructions.

Statistical analysis

The data were analyzed via GraphPad Prism version 8 (GraphPad Software, version 8, San Diego, CA, USA). The results are presented as the means ± standard errors of the means (SEMs). The significance of the results was assessed via Student's *t* tests or one-way analyses of variance, with a *p* value of < 0.05 indicating significance. The statistical results are labeled with $^{*;\#}p < 0.05$, $^{**;\#\#}p < 0.01$, or $^{***;\#\#\#}p < 0.005$.

Results

Transplanted GES-1 gastric epithelial mitochondria suppress gastric cancer cell stemness via GRP78, JNK, and NOTCH pathway inhibition

To investigate the intracellular consequences of gastric epithelial mitochondrial transplantation, isolated GES-1 mitochondria (from 5×10^6 cells) were incubated with 5×10^6 MKN45 human gastric cancer cells. After 24 h of co-incubation, gastric cancer cell stemness was evaluated. The results indicated that epithelial mitochondria treatment led to downregulation of the expression of the stemness-related transcriptional factors SOX2 and NANOG (Fig. 1A, B). Our previous study indicated that gastric cancer stemness, which is dependent on SOX2 and NANOG is mediated by GRP78 expression [7]. The JNK and NOTCH1 pathways contribute to cancer stemness [32–33]. In the present study, the transplantation of epithelial mitochondria downregulated the expression of GRP78 (Fig. 1C, D) and the stemness-related markers CD44, CD24, LGR5, and ALDH1 (Fig. 1E–L). Furthermore, the NOTCH1 and JNK signaling pathways were suppressed by the administration of epithelial mitochondria (Fig. 1M–P).

Transplanted GES-1 gastric epithelial mitochondria increased intracellular ROS and mitochondrial superoxide levels

To evaluate intracellular oxidative stress and mitochondrial metabolic function after gastric epithelial mitochondrial transplantation, isolated GES-1 mitochondria (from 5×10^6 cells) were incubated with 5×10^6 MKN45 or AGS human gastric cancer cells for 24 h. The intracellular ROS level was assessed via DHE staining, and mitochondrial metabolic function was evaluated via the mitochondria-targeting superoxide indicator MitoSOX and Seahorse bioanalyzer. The results indicated that the intracellular and mitochondrial ROS levels were elevated after GES-1 epithelial mitochondrial transplantation (Fig. 2A–D). The oxidative phosphorylation (OXPHOS) profiles of MKN45 gastric cancer cells after GES-1 mitochondria administration were evaluated by using the Seahorse bioanalyzer to measure their mitochondrial OCR, spare respiratory capacity, maximal respiration, and basal respiration. The results (Fig. 2E–H) revealed that the transplanted gastric epithelial mitochondria reprogrammed intracellular energy metabolism toward mitochondrial OXPHOS. Additionally, ATP production (Fig. 2I) was suppressed after the transplantation of GES-1 mitochondria into the MKN45 gastric cancer cells.

Glycolytic and mitochondrial biogenesis proteins were downregulated after GES-1 mitochondrial transplantation

To further investigate the molecular mechanisms underlying the suppression of glycolysis after GES-1 mitochondria administration, the expression of various proteins (pyruvate kinase muscle isozyme M2 [PKM2], monocarboxylate transporter 1 [MCT-1], monocarboxylate transporter 4 [MCT-4], peroxisome proliferator-activated receptor-gamma coactivator [PGC]-1α) and the secretion of lactate in MKN45 human gastric cancer cells were evaluated. PKM2 is a key regulator of glycolysis, and MCT-1 and MCT-4 play roles in maintaining glycolytic metabolism by regulating lactate transporters in gastric cancer cells [34–36]. In addition, PGC-1α regulates mitochondrial biogenesis. The results of the study indicated that the expression of PKM2, MCT1, MCT4, and PGC-1α and the lactate metabolism were decreased (Fig. 3A–F), suggesting that glycolysis-related proteins were inhibited after GES-1 mitochondrial transplantation.

Transplantation of GES-1 mitochondria into gastric cancer cells inhibited gastric tumor growth in a tumor xenograft animal model

MKN45 human gastric cancer cells were pre-treated for 24 h with mitochondria isolated from GES-1 cells (Fig. 4A). The mice were then incubated with MKN45 human gastric cancer cells with or without GES-1 mitochondria. After 9 days, the tumors in the mice injected with MKN45 cells with GES-1 mitochondria were larger than those in the mice injected with MKN45 cells without GES-1 mitochondria (Fig. 4C, E, F). For the treatment groups, after the MKN45 gastric cancer cells were incubated for 3 days, the mice were intratumorally injected with PBS or mitochondria isolated from GES-1 human gastric epithelial cells (1×10^7) every 2 days until they were sacrificed (Fig. 4B). The results revealed that GES-1 cell mitochondrial treatment inhibited tumor growth (Fig. 4C, E, F). To determine the antitumor effects of epithelial mitochondria on cancer stemness in human gastric cancer cells, the expression of cancer stemness–related

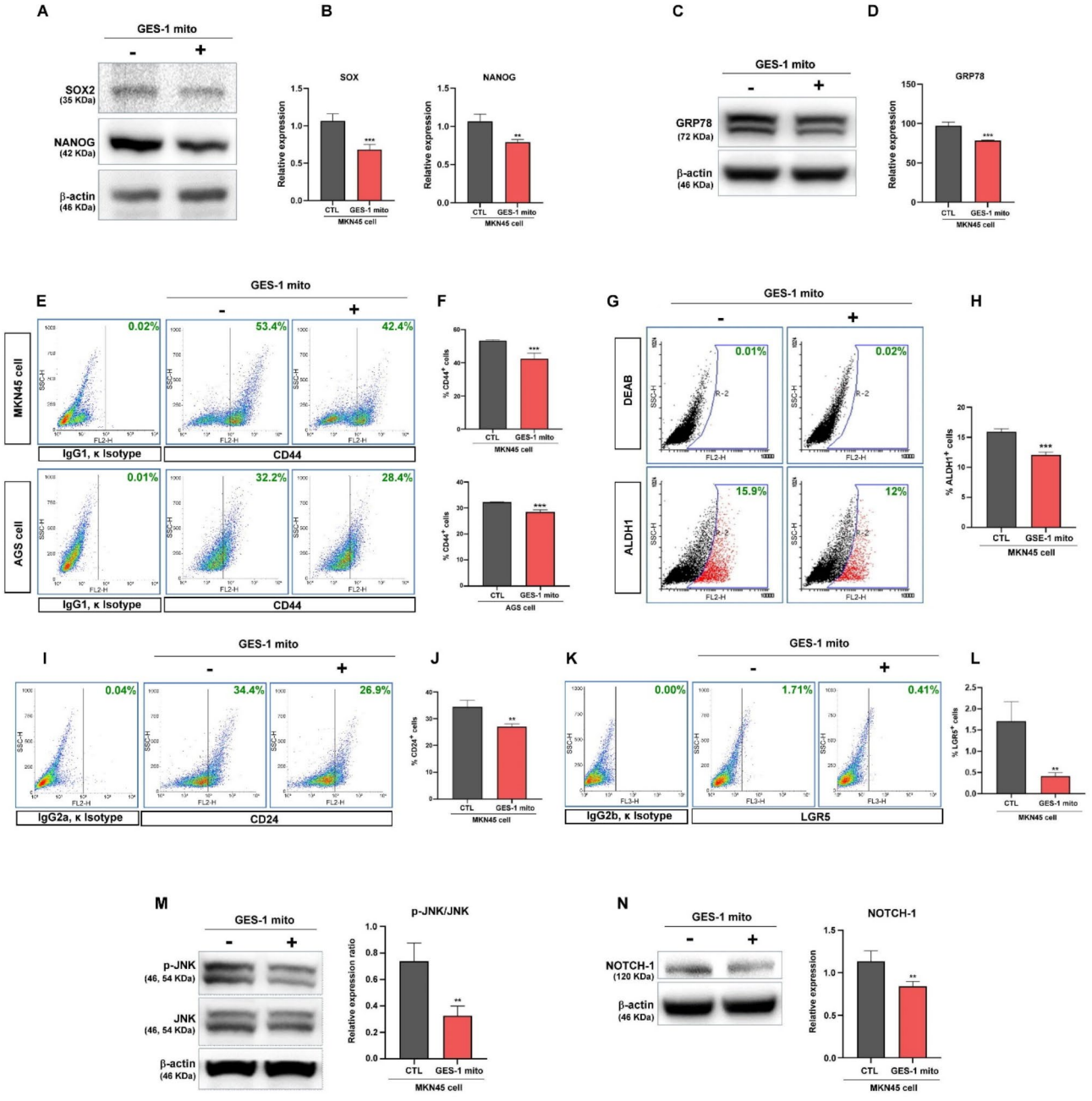

Fig. 1 Transplanted GES-1 gastric epithelial mitochondria suppress gastric cancer cell stemness. (**A**) Protein expression of stemness markers (SOX2 and NANOG) after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**B**) Quantification of SOX2 and NANOG expression. (**C**) GRP78 expression after GES-1 mitochondrial transplantation for 24 h. (**D**) Quantification of GRP78 expression. (**E**) CD44 positive cells on MKN45 or AGS cells after GES-1 mitochondrial transplantation for 24 h. (**F**) Quantification of CD44 positive cells. (**G**) ALDH1 activity after mitochondrial transplantation for 24 h in MKN45 cells. (**H**) Quantification of ALDH1 activity. (**I**) CD24 positive cells after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**J**) Quantification of CD24 positive cells. (**K**) LGR5 positive cells after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**L**) Quantification of LGR5 positive cells. (**M**) Expression of p-JNK and JNK after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**N**) NOTCH1 expression after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. The data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{*}p < 0.05$ and $^{**}p < 0.01$

proteins was evaluated through Western blotting. The results revealed that the cancer stemness–related transcription factors SOX2 and NANOG were downregulated in the GES-1 mitochondria-treatment groups. YY1 is a transcriptional factor that is required for GRP78 induction [37] and contributes to cancer stemness [38]. MCP-1, GRP78, NOTCH1, and PGC-1α promote cancer stemness [7, 32, 39, 40]. The expression of proteins in the tumors of these mice was similar to that observed in the MKN45 human gastric cancer cells after the uptake of epithelial

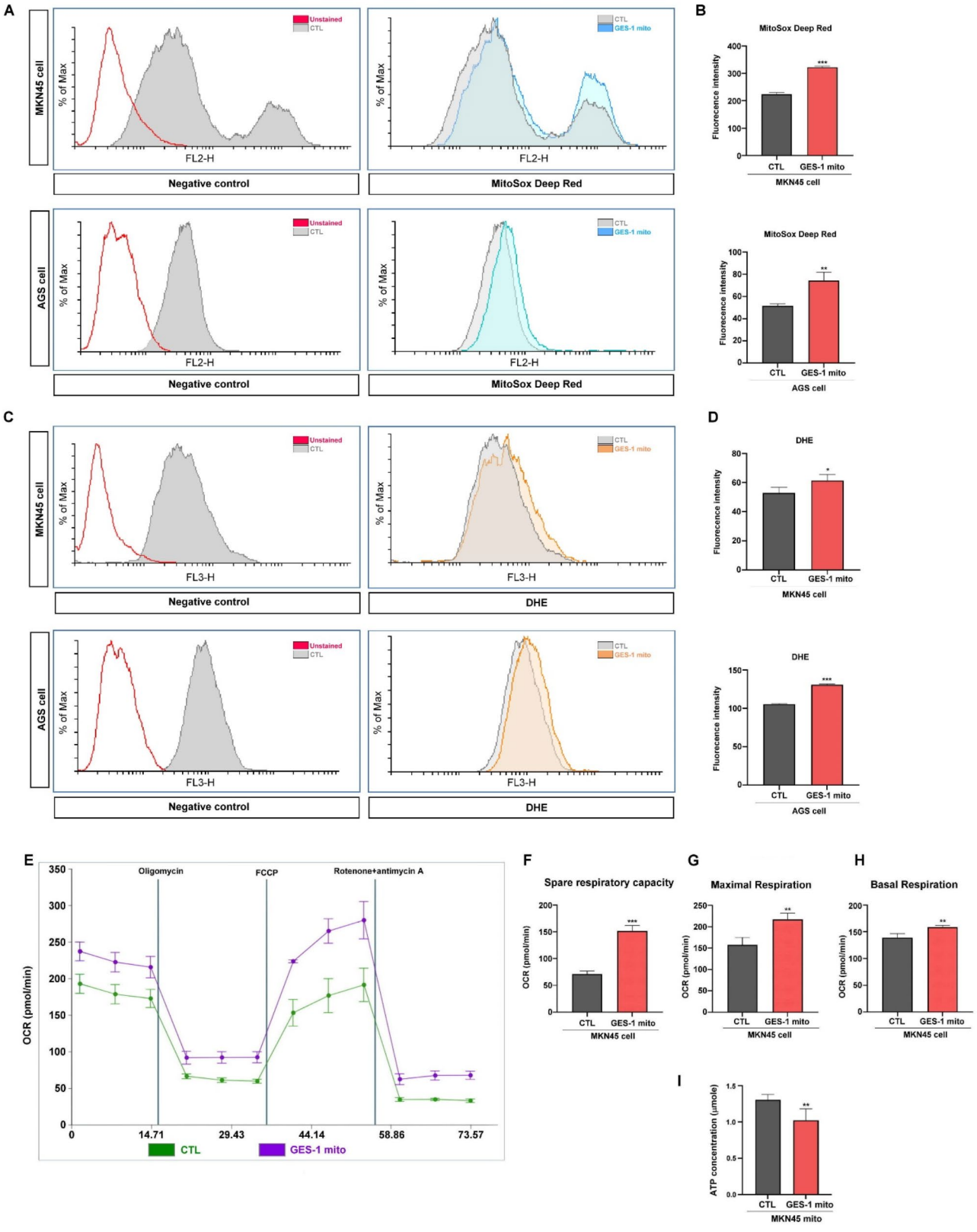

Fig. 2 (See legend on next page.)

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Fig. 2 Transplanted GES-1 gastric epithelial mitochondria into gastric cancer cells increased the level of intracellular ROS and modulated mitochondrial metabolism. (**A**) Superoxide generation in mitochondria was analyzed by performing mitoSOX Deep Red staining through flow cytometry after GES-1 mitochondrial transplantation for 24 h in MKN45 or AGS cells. (**B**) Quantification of mitochondrial superoxide generation. (**C**) Intracellular superoxide level was analyzed by performing DHE staining through flow cytometry after GES-1 mitochondrial transplantation for 24 h in MKN45 or AGS cells. (**D**) Quantification of intracellular superoxide level. (**E**) OXPHOS profiles, (**F**) spare respiratory capacity, (**G**) maximal respiration, and (**H**) basal respiration of MKN45 cells after GES-1 mitochondrial transplantation for 24 h were evaluated via a Seahorse bioanalyzer. (**I**) ATP concentration was analyzed via an ATPlite kit. Data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{*}p < 0.05$ and $^{**}p < 0.01$

mitochondria (Fig. 4G–H). The H&E staining results indicated that the cell proliferation marker Ki67 and the cancer stemness marker GRP78 were also eliminated in the tumors (Fig. 4K–M). In the GES-1 pre-treatment study, similar results were observed in mice incubated with AGS human gastric cancer cells (Fig. 5A). The tumors in these mice were larger than those in the mice injected with AGS cells without GES-1 mitochondria (Fig. 5B–E). The H&E staining results indicated that Ki67 and GRP78 were eliminated in the tumors (Fig. 5F–H). These results indicate that the uptake of epithelial mitochondria by gastric cancer cells inhibits tumor growth.

Gastric epithelial mitochondria promoted gastric cancer cell apoptosis under hypoxic conditions and enhanced chemosensitivity in response to 5-FU treatment

The hypoxic tumor microenvironment was revealed to contribute to tumor chemoresistance by upregulating hypoxia-inducible factor-1 α (HIF-1α). The present study used hypoxia-mimicking $CoCl_2$ to further investigate whether the administration of gastric epithelial mitochondria can overcome HIF-1α-mediated chemoresistance in gastric cancer cells. Exposure to $CoCl_2$ for 48 h reduced the viability of AGS human gastric cancer cells but not that of MKN45 cells. However, cell viability was significantly inhibited in both AGS cells and MKN45 human gastric cancer cells after the transplantation of GES-1 mitochondria (Fig. 6A, B). The results indicated that HIF-1α was upregulated under $CoCl_2$ treatment in both MKN45 and AGS cells but downregulated after epithelial mitochondrial transplantation (Fig. 6C, D). Hypoxia upregulates dynamin-related protein-1 (Drp1), and mitochondrial fission mediated by Drp1 contributes to gastric cancer chemoresistance [41]. The findings presented in Fig. 6E and F indicate that HIF-1α expression was correlated with Drp1 level. Furthermore, the transplantation of epithelial mitochondria enhanced cell apoptosis by activating caspase-3 under hypoxic conditions in both AGS and MKN45 gastric cancer cells (Fig. 6G–K). To determine whether the transplantation of GES-1 epithelial mitochondria increased chemosensitivity after the modulation of metabolic activity and the inhibition

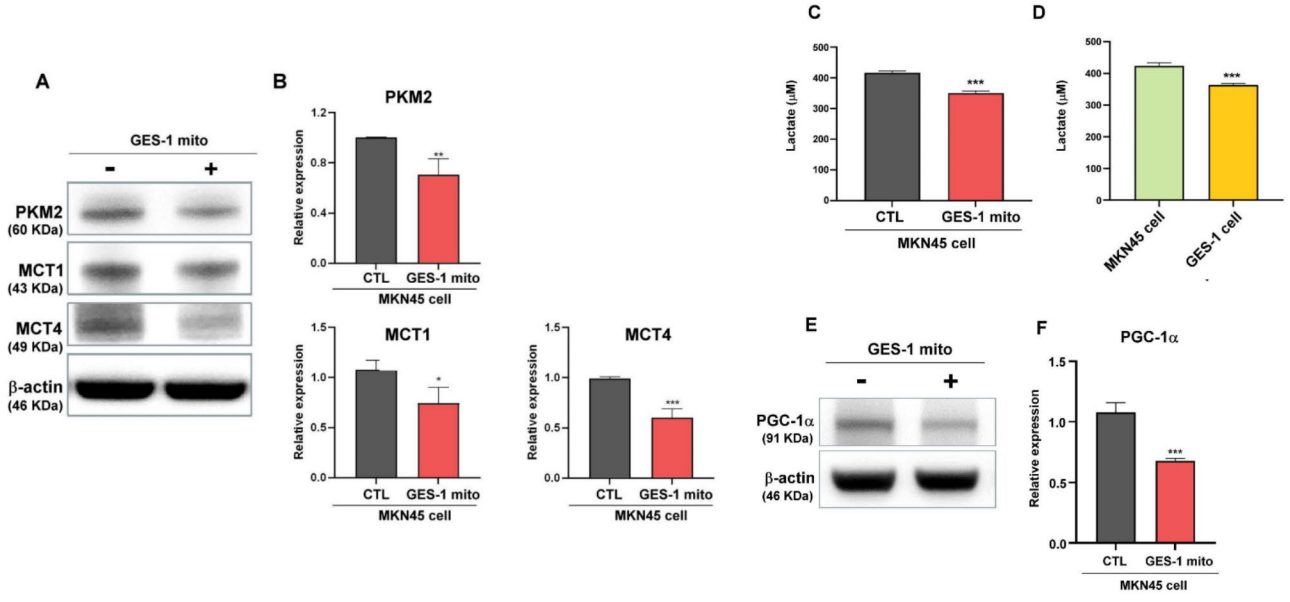

Fig. 3 Glycolytic and mitochondrial biogenesis proteins were downregulated after epithelial mitochondrial transplantation. (**A**) Protein expression of PKM2, MCT1, and MCT4 after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**B**) Quantification of PKM2, MCT1, and MCT4 expression after GES-1 mitochondrial transplantation for 24 h. (**C**) Lactate metabolism were analyzed using a Lactate-Glo Assay Kit after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**D**) Lactate secretion by MKN45 and GES-1 cells was analyzed using a Lactate-Glo Assay Kit. (**E**) Protein expression of PGC-1α after GES-1 mitochondrial transplantation for 24 h in MKN45 cells. (**F**) Quantification of PGC-1α. The data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{*}p < 0.05$ and $^{***}p < 0.005$

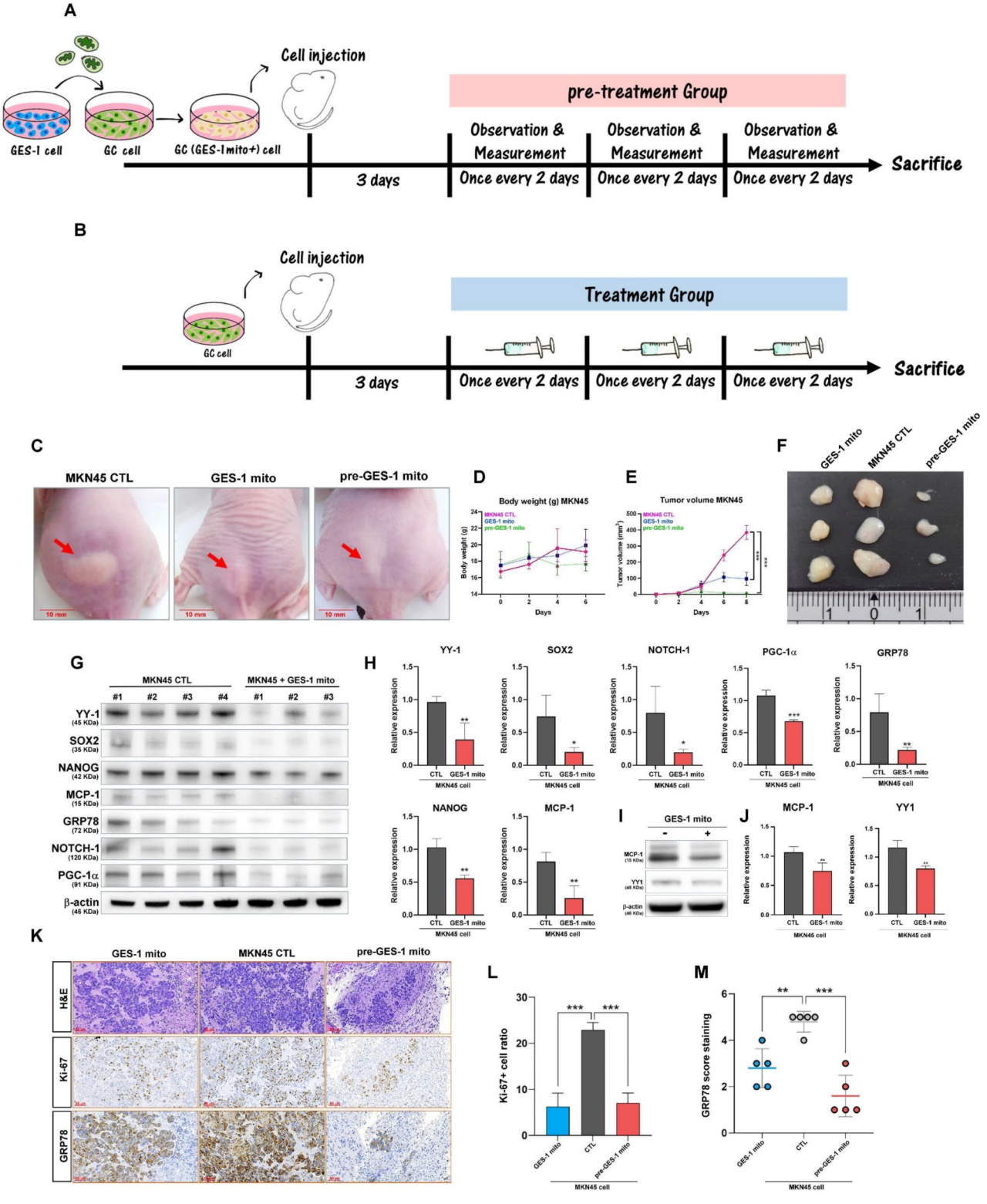

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Fig. 4 Transplantation of epithelial mitochondria into gastric cancer cells inhibited gastric tumor growth in a tumor xenograft animal model (**A**) Experimental design and flowchart of the mouse xenograft models and mitochondrial treatment. MKN45 cells were pretreated with epithelial mitochondria for 24 h before tumor xenografting. (**B**) Experimental design and flowchart of mouse xenograft models and mitochondrial treatment. Xenograft tumors were treated with epithelial mitochondria once every 2 days. (**C**) Schematic of gastric tumor xenografts. (**D**) Body weight of each group. (**E**) Tumor growth curves of each group. (**F**) Gastric tumors harvested from each group. (**G**) The protein expression of YY-1, SOX2, NANOG, MCP-1, GRP78, NOTCH1, and PGC-1α in each group was analyzed via Western blotting. (**H**) Quantification of YY-1, SOX2, NANOG, MCP-1, GRP78, NOTCH1, and PGC-1α protein expression. (**I**) Protein expression of MCP-1 and YY-1. (**J**) Quantification of MCP-1 and YY-1. (**K**) Representative images of hematoxylin and eosin and IHC staining of Ki-67 or GRP78 in each group. (**L**) Representative IHC analysis of ki-67 staining in each group. (**M**) Representative IHC analysis of GRP78 in each group. The data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{**}p < 0.01$ and $^{***}p < 0.005$

of cancer stemness and HIF-1α expression, combined treatment with GES-1 mitochondria and the clinical chemotherapy drug 5-fluorouracil (5-FU) was administered to MKN45 and AGS cells. The results indicated that GES-1 mitochondria reduced human gastric cancer cell chemoresistance by upregulating BAX-caspase 3–mediated apoptosis and inhibiting the AKT signaling pathway (Fig. 7).

Discussion

Mitochondrial reprogramming of energy metabolism is essential in the development of gastric cancer [42] and plays a pivotal role in cancer stemness [43]. Cancer cells that maintain stemness may be able to self-renew and thereby contribute to chemoresistance and cause cancer recurrence. The drug resistance of cancer cells reportedly results in the deregulation of mitochondrial function

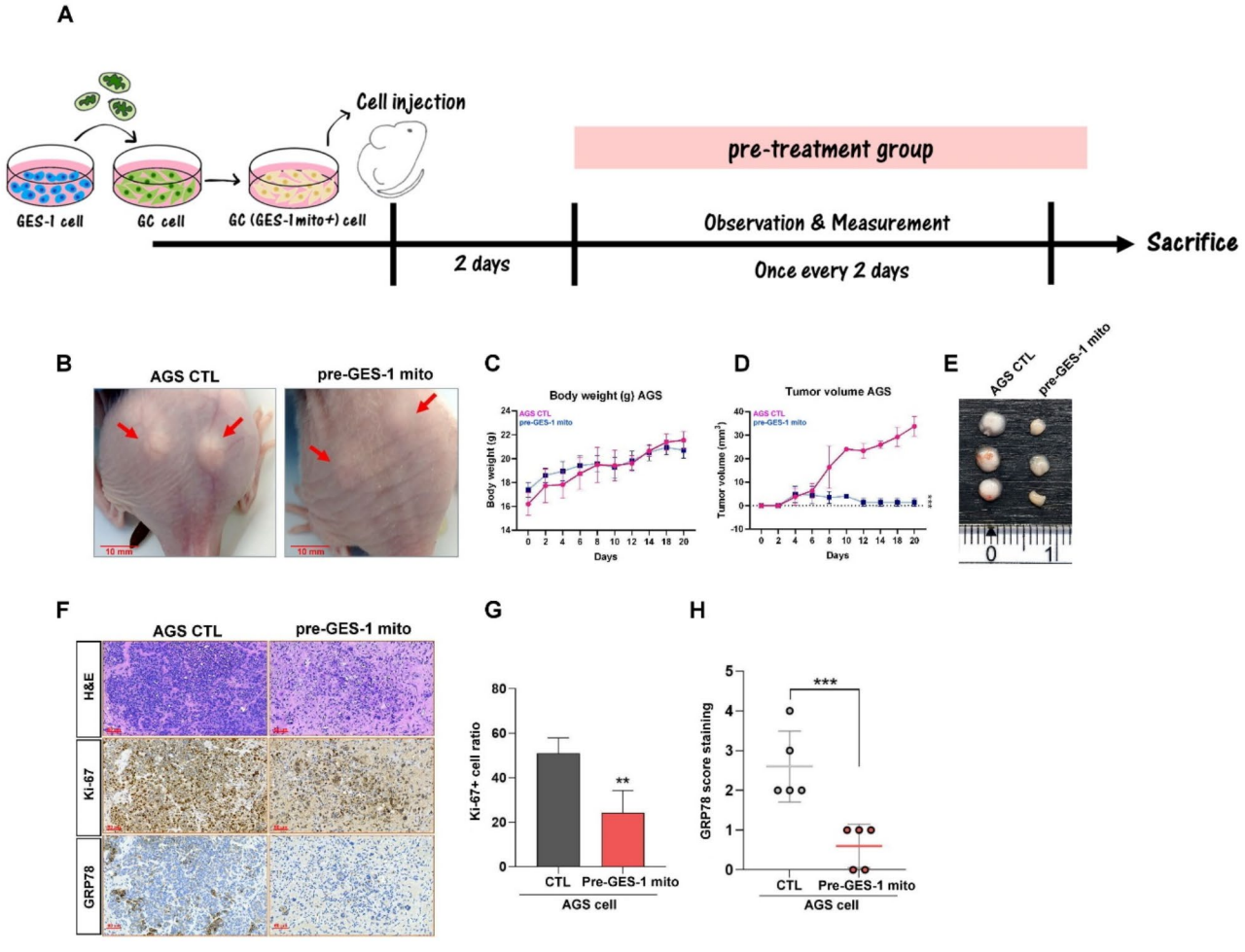

Fig. 5 Pretreatment of epithelial mitochondria into gastric cancer cells suppressed tumor growth in a mouse tumor xenograft model. (**A**) Experimental design and flowchart of the mouse xenograft model and mitochondrial treatment. AGS cells were pretreated with GES-1 mitochondria for 24 h before injection. (**B**) Schematic of the gastric cancer xenografts in each group. (**C**) Body weights of mice in each group. (**D**) Tumor growth curves of each group. (**E**) Gastric tumors harvested from each group. (**F**) Representative images of hematoxylin and eosin and IHC staining of Ki-67 and GRP78 in each group. (**G**). Representative IHC analysis of ki-67 staining in each group. (**H**) Representative IHC analysis of GRP78 in each group. The data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{**}p < 0.01$ and $^{***}p < 0.005$

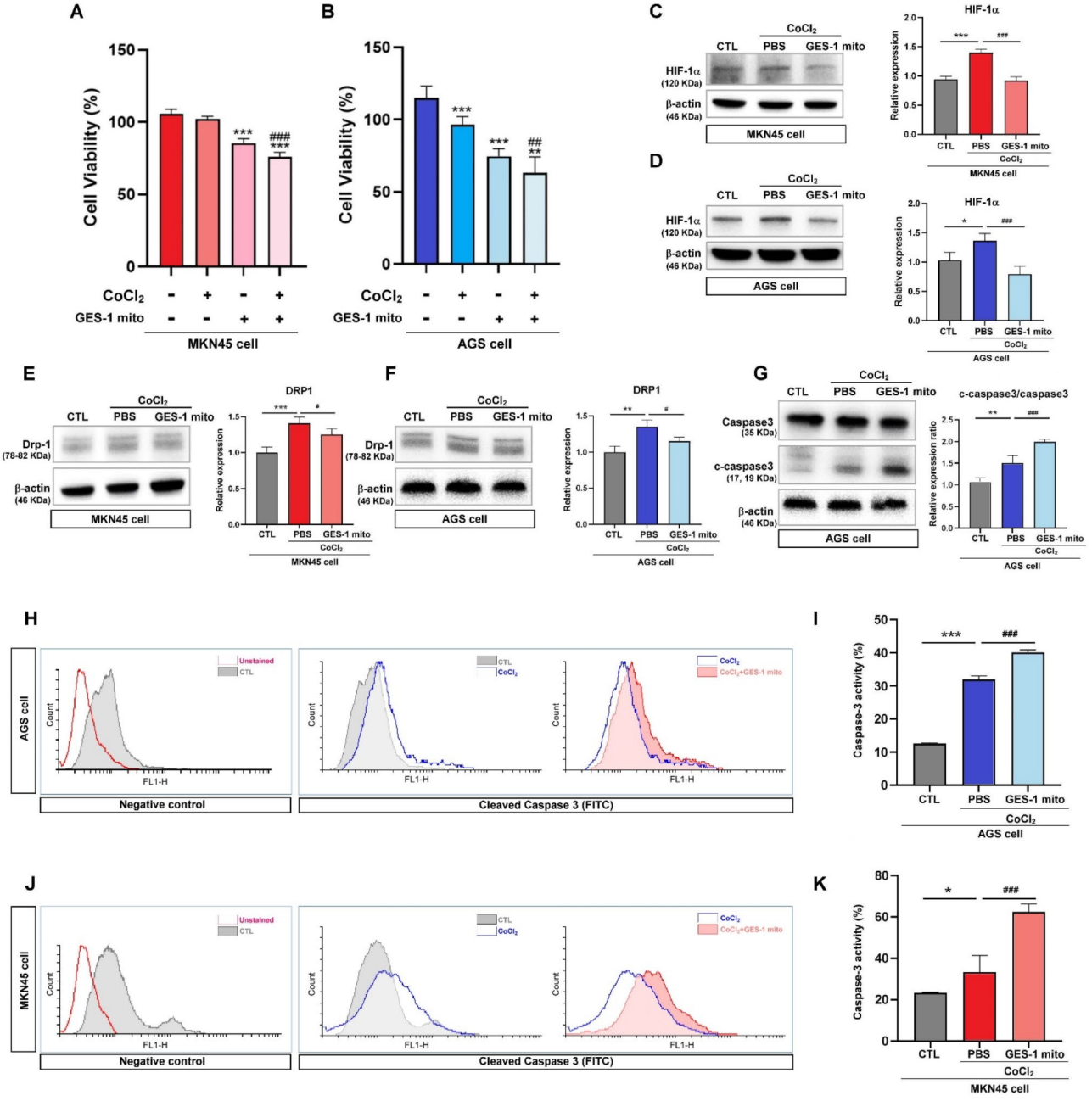

Fig. 6 Transplantation of gastric epithelial mitochondria promoted gastric cancer cell apoptosis under hypoxic conditions and enhanced chemosensitivity. (**A**) MKN45 or (**B**) AGS cells (**C**) were treated with $CoCl_2$ and GES-1 mitochondria for 48 h. Cell viability was analyzed via an SRB assay. Protein expression and quantification of HIF-1α in (**C**) MKN45 or (**D**) AGS cells were measured after GES-1 mitochondrial transplantation under hypoxic conditions for 24 h. (**E**) Protein expression and quantification of Drp1 in (**C**) MKN45 or (**D**) AGS cells were analyzed after GES-1 mitochondrial transplantation under hypoxic conditions. (**G**) Apoptotic protein expression was quantified on the basis of the c-csapase3/caspase3 ratio via Western blotting. (**H**,**I**) Caspase activity in AGS cells was analyzed through flow cytometry. (**J**,**K**) Caspase activity in MKN45 cells was analyzed through flow cytometry. The data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.005$

[44, 45]. Alterations in mitochondrial biogenesis contribute to the development of drug resistance in cancer [12]. Although surgery and chemotherapy are standard therapies for treating gastric cancer, tumor recurrence and chemotherapeutic resistance can occur. Therefore, advanced strategies targeting mitochondria are needed for gastric cancer therapy. In previous studies, mitochondria from the extracellular environment were effectively incorporated into cancer cell lines [46]. Our previous studies also revealed that the transplantation of mitochondria regulates cell fate programs and functions [17],

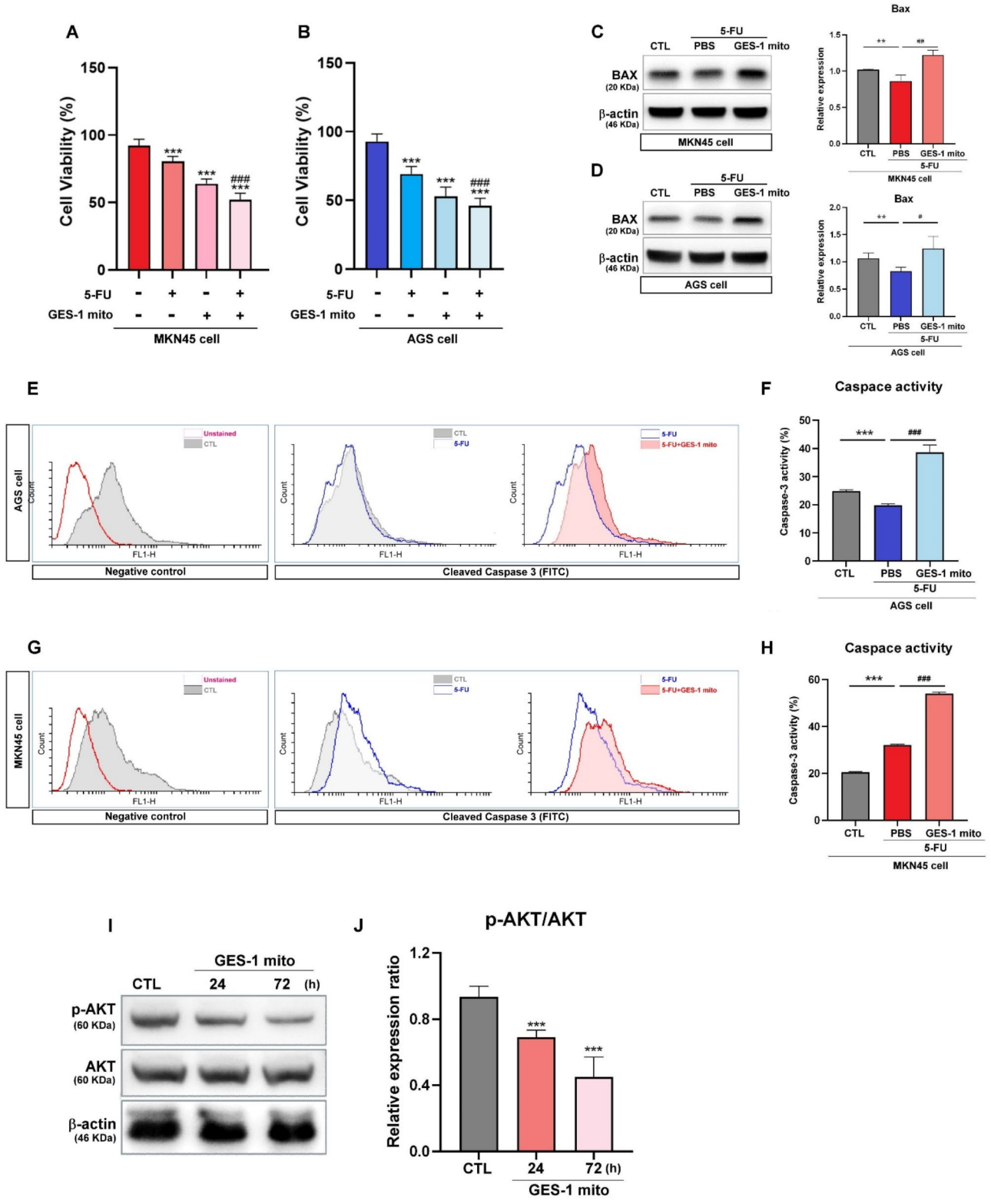

Fig. 7 Human gastric epithelial mitochondria reduce gastric cancer cell chemoresistance by promoting apoptosis. (**A**,**B**) MKN45 or AGS cells were treated with 5-FU or GES-1 mitochondria or both in combination for 24 h. Cell viability was analyzed via SRB Assay. (**C**,**D**) Protein expression and quantification of BAX in MKN45 or AGS cells were analyzed after treatment for 72 h. (**E**–**H**) Caspase activity in MKN45 or AGS cells was analyzed through flow cytometry. (**J**) Quantification of protein expression (p-AKT/AKT ratio) in MKN45 cells after GES-1 mitochondrial transplantation. The data are presented as the means ± SEMs; $n \geq 3$ for independent experiments; two-tailed Student's *t* test: $^{**}p < 0.01$ and $^{***}p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.005$

and mitochondrial transplantation has been proposed as a potential strategy for cancer therapy [23].

With the development of mitochondrial transplant therapy, many studies have shown that mitochondria can be transferred from various types of cells to pathological cells to prevent apoptotic cell death. Various mitochondrial transplant models have yielded effective results through mitochondrial transfer [13, 21, 22, 24]. Several ongoing clinical trials in humans are aimed at using mitochondrial transplantation to treat heart failure and acute respiratory disorders [21, 47]. Mitochondrial transplantation can be considered a promising therapeutic approach for treating diseases other than cancer. However, cancer is complex, and different cancers exhibit metabolic heterogeneity. Research has reported that melanoma cells that acquire endothelial mitochondria exhibit increased stemness and chemoresistance [17]. Although exogenous normal epithelial mitochondria were reported to inhibit the proliferation of human breast cancer cells [48], transfer of mitochondria from mesenchymal stem cells to glioblastoma cells was shown to confer chemoresistance [49]. Transferring healthy mitochondria isolated from healthy human embryonic kidney cell or umbilical cord mesenchymal stem cells or to PC-3 cells was demonstrated to increase cell proliferation and promote chemoresistance [50]. However, further investigations are needed to identify the specific types of mitochondria that would be effective for particular types of cancer.

In gastric cancer, mitochondrial abnormalities in energy metabolism reprogramming are crucial to the progression of tumors. Gastric cancer cells are based mainly on glycolysis rather than OXPHOS. The glycolytic pathway is preferred in tumors because glycolytic ATP can be obtained faster than the mitochondrial OXPHOS metabolism [10]. In the present study, we evaluated the role of gastric epithelial mitochondrial transplantation in regulating gastric tumor growth. These results indicate that human gastric epithelial mitochondrial transplantation modulate human gastric cancer cell metabolism by enhancing OXPHOS, inducing oxidative stress, promoting apoptosis, and inhibiting cancer stemness, thereby reducing chemoresistance. In our study, gastric tumor growth was dramatically reduced in the mitochondrial pretreatment and treatment groups in vivo.

GRP78 is a biomarker of gastric tumors, and it is upregulated in human gastric tumor tissues [6]. Our studies highlighted the relevance of GRP78-mediated cancer stemness. Inhibiting GRP78 expression in gastric cancer cells suppresses cancer stem cell characteristics, reduces malignancy, and enhances chemosensitivity [7]. Cancer stemness of gastric tumor can be assessed by validating the cell surface CD24, CD44, LGR5 and stemness-related transcription factors expressions. SOX2, NANOG, and ALDH1 are involved in self-renewal, and chemoresistance [4, 51]. In addition, ALDH1 serves as an indicator of poor prognosis in patients with gastric cancer [52]. Our previous study demonstrated that the downregulation of GRP78 reduced CD24, CD44, LGR5, SOX2, and NANOG expressions in gastric cancer cells [7]. Furthermore, the inhibition of GRP78 was reported to reduce CD44 or CD24 expression and the chemoresistant breast cancer cells, thereby increasing the sensitivity of these cells to the chemotherapy agent oxaliplatin in colorectal cancer [53, 54]. The JNK signaling pathway contributes to cancer stemness through NOTCH1, SOX2 or NANOG expressions [33]. In our study, transplantation of GES-1 mitochondria downregulated GRP78 expression and JNK activation, suggesting that the transplantation of human gastric epithelial cell mitochondria contributed to GRP78/JNK-mediated cancer stemness and chemoresistance inhibition in human gastric cancer cells.

Energy metabolism is essential for the development of gastric cancer. PGC-1 is one of the main regulators of mitochondrial energy metabolism [55]. Furthermore, PKM2 is an enzyme involved in glycolysis and is associated with poor prognosis in patients with gastric cancer [56]. The AKT signaling pathway promotes glycolysis and contributes to the energy metabolism of gastric cancer [57, 58]. MCT4 mediates the efflux of lactate, whereas MCT1 promotes the absorption of lactate in cancer cells. MCT1 and MCT4 play essential roles in maintaining the glycolytic metabolism of cancer, and inhibitors of MCT1 have been studied in clinical studies [59]. In the present study, our results revealed that mitochondrial transplantation led to an increased OCR, inhibition of AKT signaling, and a reduction in the expression of several glycolytic enzymes (i.e., PKM2, MCT1, and MCT4) and the mitochondrial biogenesis marker PGC-1α. These findings suggest a metabolic shift to OXPHOS from glycolysis in human gastric cancer cells. The increase in glycolysis promote cancer stemness via a positive feedback loop. PGC-1 reportedly maintains cancer stemness [40] and CD44 promotes breast cancer stemness through glycolysis [60]. SOX2 mediates glycolysis to promote the stem cell function of NSCLC cells [61]. In addition, it has been reported that increased regulation of GLUT1 contributes to glycolysis mediated by SOX2 [62]. YY1 regulates the proliferation and tumor formation of gastric cancer cells by activating signaling pathways associated with JNK, HIF-1α and GRP78 [7, 63]. Reports show that YY1 not only contributes to the stemness of cancer cells, but also induces glycolysis in cancer cells [64]. NOTCH1 signaling increases proliferation, prevents apoptosis, and contributes to cancer stemming and chemotherapy resistance in gastric cancer cells [65]. NOTCH1 is regulated by MCP-1 or AKT signaling and promotes cancer stemness and chemotherapy resistance [66, 67]. In the present study, transferring epithelial mitochondria to gastric cancer cells inhibited AKT activation in our cell model. The expression of YY1, SOX2, NANOG, MCP-1, GRP78, NOTCH1, and PGC-1α was inhibited in our tumor xenograft animal model after mitochondrial treatment, suggesting that the transplantation of human gastric epithelial cell mitochondria downregulated cancer stemness and the glycolysis pathway in the human gastric cancer cells.

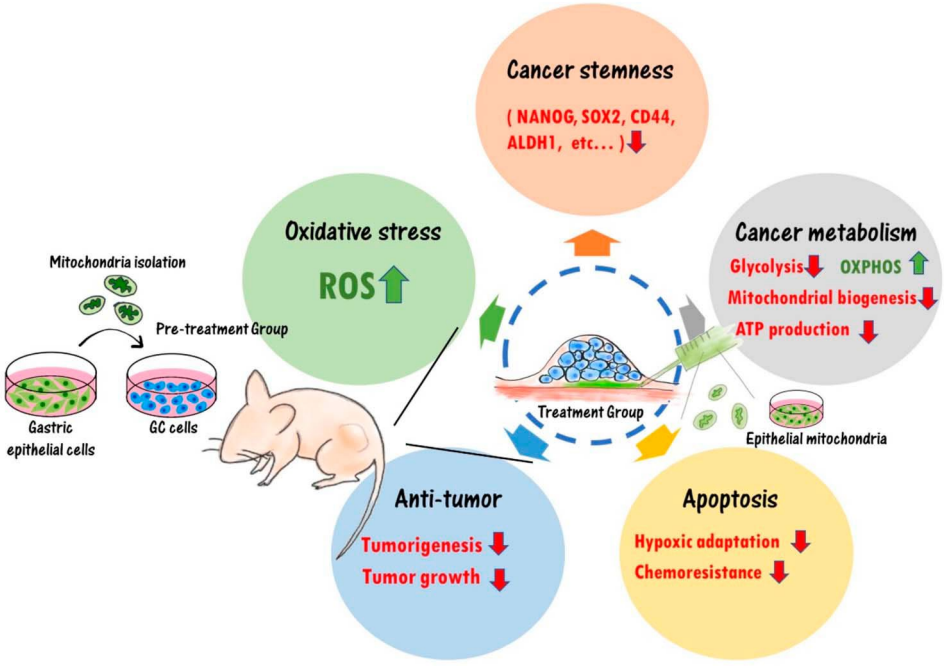

Fig. 8 The potential mechanisms of transplanting human epithelial mitochondria inhibit human gastric tumor growth through suppressing cancer stemness, modulating cancer metabolism, promoting oxidative stress, and inducing apoptosis-mediated pathways

Regarding to Notch1 signaling in mitochondrial transplantation, the Notch1 signaling pathway is found to play a key role in bone marrow stromal cell mitochondria-induced endothelial tube formation [68]. However, transplantation of normal gastric epithelial mitochondria into gastric cancer cells inhibits Notch1 signaling in this study. The different signal pathways regulation may dependent on receipt cells and the mitochondria of donor cells.

Gastric tumors are highly enriched in hypoxic niches, and HIF-1α is essential for cell survival under hypoxic conditions. The expression of HIF-1α is correlated with poor survival in patients with gastric cancer, and it is a crucial mediator of tumor progression, cancer stemness, and chemoresistance to therapy [69, 70]. With respect to mitochondrial dynamics during hypoxic stress, DRP1-dependent mitochondrial fission has been demonstrated to promote chemoresistance in ovarian cancer cells [71]. HIF-1α is a key molecule in glycolysis [72], and it was reported to regulate mitochondrial fission by increasing DRP1 expression [73]. Inhibition of DRP1-mediated mitochondrial fission increases mitochondria-dependent apoptosis and chemosensitivity in cancer cells during hypoxia [74]. Furthermore, YY1 can increase cancer stemness by stabilizing the hypoxia-inducible factor HIF-1α in a hypoxic microenvironment [38]. Transfer mitochondria from cancer-associated fibroblasts was reported to promote prostate cancer malignancy via HIF-1α pathways [75]. Hence, targeting the mitochondrial dynamics of cancer cells as they adapt to a hypoxic tumor microenvironment holds promise for anticancer therapy. Our results confirmed that the expression of HIF-1α and DRP1 increased in hypoxic gastric cancer cells. As expected, transplanted gastric epithelial mitochondria reversed the upregulation of these proteins.

The activation of AKT signaling promotes gastric cancer stemness and chemoresistance [67]. In the present study, mitochondrial transplantation inhibited AKT signaling and activated the mitochondria-dependent apoptotic pathway in gastric cancer cells, as confirmed by the BAX expression and caspase-3 activity detected in the study. Similar to our results, Liu et al.., demonstrated that mitochondrial transplantation inhibits cholangiocarcinoma cells growth through AKT signaling pathways [76]. Mitochondrial-targeting therapeutic strategies have been reported to help reduce cancer progression and enhance chemosensitivity [45, 77]. These results suggest that the transplantation of gastric epithelial mitochondria influences the fate of metabolic programming and the stemness status of gastric cancer cells both in vitro and in vivo, thereby increasing chemosensitivity and promoting apoptosis. Awareness regarding the importance of cell therapy for human health has been increasing. Our study indicates that the transplantation of human epithelial mitochondria is a promising therapeutic strategy for gastric tumor treatment in a clinical setting.

Conclusion

In the present study, human epithelial mitochondrial transplantation suppressed mitochondrial biogenesis and glycolysis in gastric cancer cells. Metabolic modulation resulted in inhibited gastric cancer stemness, induced mitochondrial apoptosis, and reduced tumor growth (Fig. 8). Our results help to clarify the molecular

mechanisms of epithelial mitochondrial transplantation in gastric tumor therapy and provide insights into its therapeutic potential.

Abbreviations

5-FU	5-fluorouracil
ALDH1	aldehyde dehydrogenase 1
DHE	dihydroethidium
Drp1	dynamin-related protein-1
FBS	fetal bovine serum
GRP78	glucose-regulated protein 78
H&E	hematoxylin and eosin
HIF-1α	hypoxia-inducible factor-1α
IHC	immunohistochemical
MCT-1	monocarboxylate transporter 1
MCT-4	monocarboxylate transporter 4
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator [PGC]-1α
PBS	phosphate-buffered saline
PKM2	pyruvate kinase muscle isozyme M2
SRB	Sulforhodamine B

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04223-7.

Supplementary Material 1

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Author contributions
Hsin-Yi Tsai: writing-original draft, project administration, investigation, data curation, formal analysis, visualization; Kuen-Jang Tsai: resources, methodology; data interpretation; Deng-Chyang Wu: resources, data interpretation, conceptualization. Yaw-Bin Huang: supervision, validation, data interpretation; Ming-Wei Lin: writing-original draft, writing-review and editing, conceptualization, validation, supervision, funding acquisition.

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Data availability
The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethics approval and consent to participate
The studies involving animal participants were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of E-Da Hospital. The approved project: The application of mitochondrial cell therapy in gastric cancer treatment. (Approval number: IACUC-EDAH-112015; date of approval: 2023/8/1-2025/7/31). The human gastric cancer cell line AGS was purchased from ATCC, under a Material Transfer Agreement (https://www.atcc.org/policies/product-use-policies/material-transfer-agreement). The human gastric cancer cell line MKN45 cell was purchased from DSMZ, under a Material Transfer Agreement (https://www.dsmz.de/collection/catalogue/microorganisms/cbd-and-material-transfer-agreements). The human normal gastric epithelial cell line GES-1 cell was obtained through Cytion (Cell Lines Service, Eppelheim, Germany), under a Material Transfer Agreement (https://www.cytion.com/Our-Approach/Terms-and-Conditions). The original source has confirmed that there was initial ethical approval for the collection of human cells, and that the donors had signed informed consent.

Artificial intelligence
The authors declare that they have not used Artificial Intelligence in this study.

Consent for publication
Not applicable.

Conflict of interest
The authors declare no conflict of interest.

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