RESEARCH ARTICLE

Respiratory chain complex I is related to oxidative phosphorylation in gastric cancer stem cells

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Abstract

Background: Cancer stem cells (CSCs) are the main cause of resistance to anti-cancer drug therapy and distant metastasis of tumors, including gastric cancer. The metabolism of CSCs is an important factor in the maintenance of its stemness. This study is intended to explore the role of oxidative phosphorylation in gastric CSCs.

Methods: EpCAM$^+$CD44$^+$ gastric CSCs were sorted from the SGC-7901 cell line. The oxidative phosphorylation and glycolysis were determined by Seahorse experiment, and the oxygen consumption of cells was determined by Clark's oxygen electrodes. Gene expression and protein levels of mitochondrial proteins belonging to five respiratory chain complexes were checked. Phenformin and siRNA-NDUFB8 were used to inhibit respiratory chain complex I to explore the biological effect of enhanced oxidation phosphorylation in gastric CSCs. Cell migration capacity, proliferation ability, and vascular endothelial growth factor (VEGF) levels were also evaluated.

Results: Compared with control cells, the oxidation phosphorylation in mitochondria increased in Ep-CAM$^+$CD44$^+$ gastric CSCs, although the respiration level remained the same, and no significant changes were observed in glycolysis. Moreover, mRNA and protein expression levels of NDUFB8 in complex I were significantly increased. However, oxidative phosphorylation decreased in EpCAM$^+$CD44$^+$ cells after the treatment of phenformin and siRNA-NDUFB8 compared to the untreated cells. siRNA for NDFUB8 and phenformin inhibition also decreased the ability of cell migration, cell proliferation, as well as the VEGF secretion of gastric CSCs.

Conclusion: These results suggest that the increased oxidative phosphorylation was related to respiratory chain complex I and NDUFB8 in gastric CSCs.

Keywords: *gastric cancer*; *cancer stem cell*; *OXPHOS*; *glycolysis*; *Warburg effect*

Gastric cancer is one of the leading causes of cancer-related deaths worldwide (1). At present, surgery combined with traditional chemotherapy is the main recommended treatment, and tumor resistance is a major problem remains to be solved. Many tumors grow dramatically arising from an embryonic cell-like population called the cancer stem cells (CSCs), which are tumor-causing, metastatic, and resistant to chemotherapy and radiotherapy (2). CSCs are a small subset of tumor cells that can form and maintain tumor growth and heterogeneity. These cells have differentiation and self-renewal capabilities, and the tumor microenvironment surrounding the CSCs has secretory factors that can promote tumorigenesis (3). These cells are the main cause of resistance to anti-cancer drug therapy and distant metastasis of tumors. Thus, they become the potential therapeutic targets for many cancers, including gastric cancer (3).

Chemotherapeutics was reported to induce the epithelial–mesenchymal transition (EMT) of gastric cancer cells SGC7901 into CSCs (4). Epithelial cell adhesion molecule (EpCAM) is a newly established early tumor-associated antigen that can induce the EMT (5, 6). Similar to CD44, a well-known CSC marker (7, 8), it is also considered to be a CSC biomarker. In cancer cells, EpCAM protein plays an important role in promoting differentiation, cell

proliferation, migration and other biological functions. It has been reported that CD44 and EpCAM are used as markers of gastric CSCs (9, 10). Our research study also uses these two markers to isolate gastric CSCs.

Active metabolism is the key to provide the energy needed for tumor growth; glycolysis and mitochondrial oxidative phosphorylation are two ways to produce energy (11). A study shows that mitochondria is one of the key organs for the growth of pancreatic tumor stem cells (12), and the process of oxidative phosphorylation plays a pivotal role in tumorigenesis. The metabolism of CSCs is an important factor in maintaining its stemness. Previous studies have found that the energy metabolism of many tumor stem cells is mainly oxidative phosphorylation rather than glycolysis (13). However, there are few research studies on energy metabolism in CSCs of gastric cancer. This study is intended to explore oxidative phosphorylation in gastric CSCs.

Methods and materials

Cell culture and treatment
The SGC-7901 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Gibco, Grand Island, NY). For silencing NDUFB8, siRNA-NDUFB8 and control siRNA were obtained from GENEWIZ (Suzhou, China). Lipofectamine 2000 and Lipofectamine RNAiMAX reagent (Invitrogen, Waltham, MA) were used for the experiment, and a standard transfection procedure was performed.

CSCs sorting
Gastric CSCs were identified and isolated following a previous protocol (10). Cell suspension was prepared shortly before flow cytometry. Anti-human CD44-FITC antibody (555478, BD Biosciences, Franklin Lakes, NJ) and anti-human EpCAM-eFluor®450 antibody (48-5791-82, Thermo Fisher, Waltham, MA) were used for fluorescence-activated cell sorting (FACS). Antibody staining was performed in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin, subsequently washed and stained with antibodies. Flow cytometry was conducted using a BD FACSAria™ III (BD Bioscience, Franklin Lakes, NJ).

Bioenergetic parameters detection
Bioenergetic parameters were analyzed by the Seahorse XF24 analyzer (Agilent, Santa Clara, CA) and Clark's oxygen electrode (Oxygraph System, Hansatech Instruments, Shanghai, China), following a published protocol (14). Seahorse XF Cell Mito Stress test kit (Seahorse Bioscience, North Billerica, MA) was employed to determine the oxygen consumption rates (OCRs). The cell culture medium to be measured is replaced with oxidative phosphorylation (OXPHOS) detection medium, and the plate is pre-cultured in a non-CO_2 incubator at 37°C for 1 h. OCR is measured under basic conditions to obtain a basic value, and then after continuous injection of different reagents: 1 mM oligomycin (respiratory complex V inhibitor, allowing the calculation of mitochondria produced by adenosine triphosphate [ATP]), carbonyl cyanide-p-trifluoromethoxy phenylhydrazone (an uncoupling agent that allows the determination of maximum respiration and reserve capacity), and finally, 3 mM metformin (CAS 1115-70-4, Sigma) was used to inhibit complex I to stop mitochondrial respiration to calculate the blank value. The OCR is calibrated for 10,000 seeded cells.

RT-qPCR
RNA was isolated using PicoPure™ RNA isolation kit (KIT0204, Thermo Fisher Scientific) following the manufacturer's protocol. qPCR was carried out using TB Green® Advantage® qPCR Premix (639676, TaKaRa, Dalian, China). Primers used were as follows:

ATP5A:
Forward sequence: GCTCCTTACTCTGGCTGTTCCA
Reverse sequence: GCGGAGCAACAGAGACATCTGA

UQCR2:
Forward sequence: CCGTGGAATTGAAGCAGTTGGTG
Reverse sequence: CTGTGGTGACATTGAGCAGGAAC

SDHB:
Forward sequence: GCAGTCCATAGAAGAGCGTGAG
Reverse sequence: TGTCTCCGTTCCACCAGTAGCT

COXH:
Forward sequence: CGGTGAAACTCTGGCTAGACAG
Reverse sequence: GCAAACCGTAGATGCTCAGGGA

NDUFB8:
Forward sequence: CCACACCTGTTTCTTGGCATGTC
Reverse sequence: ATCACCGCCTCGTTCCAGGTAC

GAPDH:
Forward sequence: GTCTCCTCTGACTTCAACAGCG
Reverse sequence: ACCACCCTGTTGCTGTAGCCAA

Western blot
Proteins were first extracted from the cells to be tested, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred to the polyvinylidene fluoride membranes. The membrane and the primary antibody (ATP5A, NBP2-99322, Novus Biologicals; UQCR2, ab203832, Abcam; SDHB, ER1803-63, HUABIO; COXH,
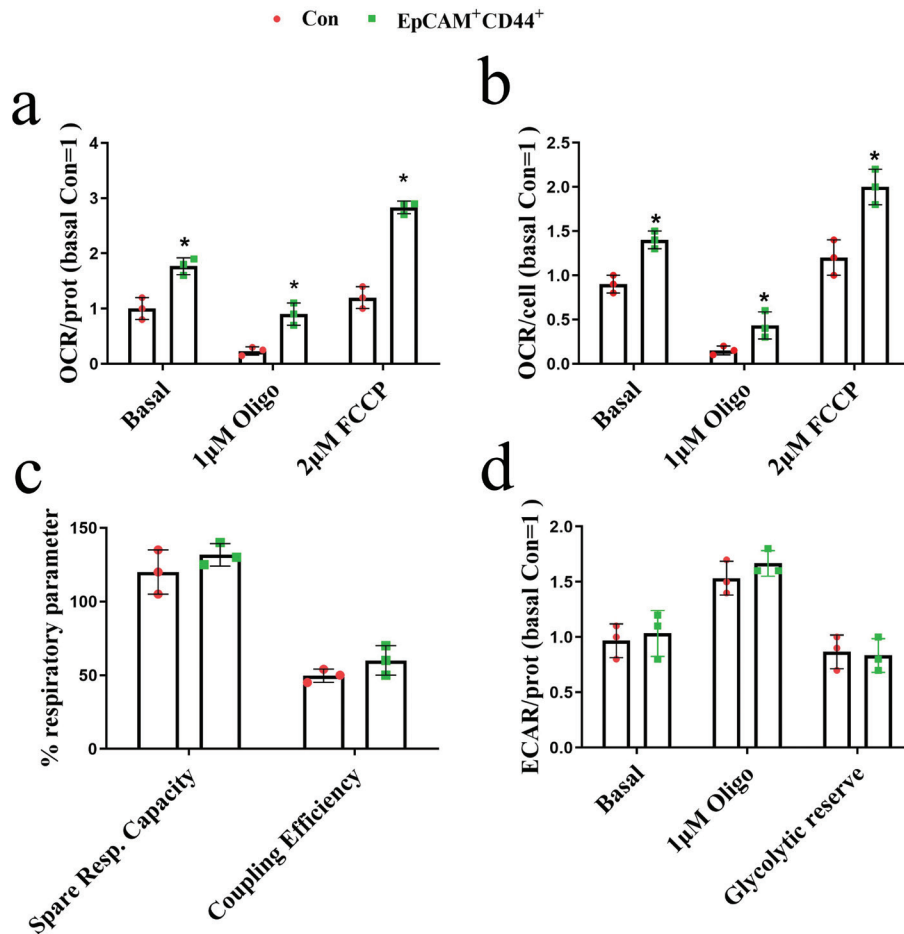
Fig. 1. Metabolic characteristics of EpCAM⁺CD44⁺ cells and control cells. OCR of EpCAM⁺CD44⁺ cells, determined by Seahorse Mito stress test (a) and oxygraph (b) analysis. (c) Spare respiratory capacity and coupling efficiency of EpCAM⁺CD44⁺ cells. (d) Extracellular acidification rate (ECAR) parameters derived from the analysis of Seahorse glycostress test, normalized on basal glycolytic parameters of control. Results are expressed as mean ± SD ($n = 3$). *$P < 0.05$ versus Con.

MABC757, Sigma-Aldrich; NDUFB8, 14794-1-AP, Proteintech; VEGF, BS-0279R, Thermo Fisher; GAPDH, R1108-1, HUABIO) were incubated overnight at 4°C and then washed three times. After that, the membrane was incubated with the secondary antibody (31460, Thermo Fisher). For protein expression level, the band was visualized using an ECL substrate kit (ab133408, Abcam).

Scratch test
The cells were seeded on a six-well plate. After the cells have reached 90% or higher confluence, a pipette tip was used to create a straight wound. Then remove debris if any by washing the cell layer with PBS and add 3 mL of appropriate medium. After 24 h of incubation in a tissue culture incubator, images were taken under a microscope.

Transwell assay
The gastric cancer cells in serum-free medium were placed in the upper chambers, while the lower chambers were filled with the medium containing fetal bovine serum

(10%, Gibco). After 24 h, the transwell insert was removed from the six-well plate, and the transwell membrane was allowed to dry for 15 min. The membrane was stained by adding 500 μL of 0.2% crystal violet, and the stained sections were observed under a microscope.

Statistical analysis
SPSS (version 21.0) was used for analyzing the data. Student's *t*-test, one- or two-way ANOVA with a post hoc test was performed to analyze the significance among the different groups. $P < 0.05$ was considered as statistically significant.

Results

Metabolic characteristics of EpCAM⁺CD44⁺ cells and control cells
We separated gastric CSCs from the gastric cancer cell line SGC-7901 by sorting out of EpCAM⁺CD44⁺ cells, while the remaining cells were used as the control group. The oxidative phosphorylation and glycolysis were determined
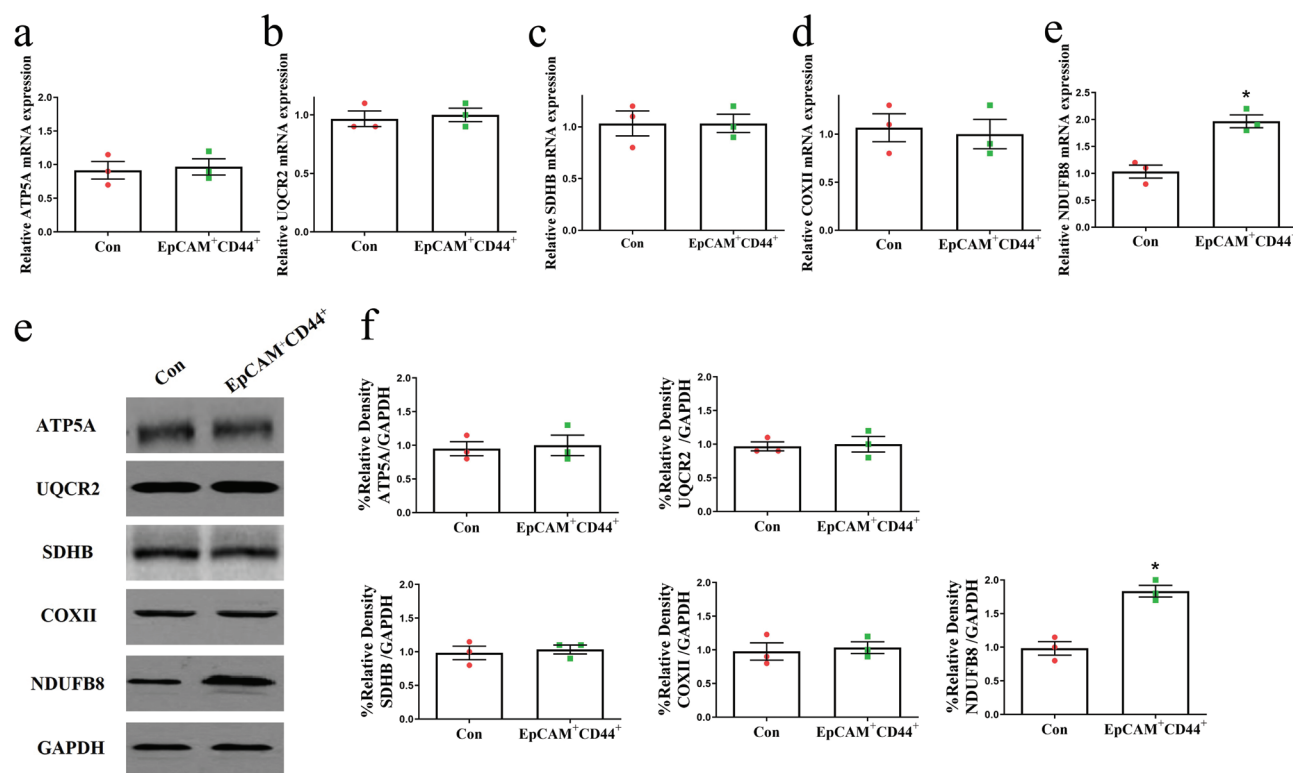
Fig. 2. mRNA and protein expression of five mitochondrial proteins of EpCAM+CD44+ cells and control cells. mRNA level of *ATP5A* (a), *UQCR2* (b), *SDHB* (c), *COXII* (d) and *NDUFB8* (e). (f) Representative Western blot analysis showing the abundance of five mitochondrial proteins (ATP5A, UQCR2, SDHB, COXII and NDUFB8) belonging to respiratory chain complexes (V, III, II, IV, and I, respectively). Results are expressed as mean ± SD (*n* = 3). *$P < 0.05$ versus Con.

by Seahorse experiment, and the oxygen consumption of cells was determined by Clark's oxygen electrodes. Compared with control group cells, the oxidative phosphorylation in mitochondria increased in EpCAM+CD44+ gastric CSCs (Fig. 1a, b), while the respiration levels in cells remained the same (Fig. 1c) and no significant changes were observed in glycolysis (Fig. 1d).

Comparison of mRNA and protein expression of mitochondrial proteins

As there was a change in oxidative phosphorylation, we checked several protein expressions in the respiratory chain. We tested the gene expression (Fig. 2a–e) and protein levels (Fig. 2f) of five mitochondrial proteins (ATP5A, UQCR2, SDHB, COXH, and NDUFB8) in gastric CSCs through qRT-PCR and Western blot analysis., which belong to five respiratory chain complexes (V, III, II, IV, and I, respectively). We found that the mRNA and protein levels of NDUFB8 in complex I were significantly increased.

Effect of phenformin or siRNA-NDUFB8 treatment in EpCAM+CD44+ cells

To test whether increased oxidative phosphorylation is associated with increased expression of NDUFB8,

we used phenformin, an anti-diabetic drug, to reduce OXPHOS in gastric CSCs (Fig. 3a, b). We also used the siRNA-NDUFB8 to silent NDUFB8, thus inhibiting respiratory chain complex I (Fig. 3c, d), and found that compared to the untreated gastric CSC, the oxidative phosphorylation decreased in EpCAM+CD44+ cells after treatment. These results suggested that the increase in oxidative phosphorylation was related to respiratory chain complex I and NDUFB8.

The biological effect of enhanced OCR in EpCAM+CD44+ cells

Next, to explore the biological effect of enhanced oxidation phosphorylation in gastric CSCs, we looked at cell migration capacity after using siRNA and phenformin inhibition complex I, where scratching experiments (Fig. 4a) and Transwell experiments (Fig. 4b) both reflect cell migration capacity, and the results revealed that the ability of gastric stem cell migration is enhanced compared to the control group, and the ability of stem cells to migrate is reduced after inhibiting phosphorylation and interfering with NDFUB8. As assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which looks at cell proliferation in each group (Fig. 4c), the ability of gastric CSCs to multiply significantly increased, while after intervention, the cell
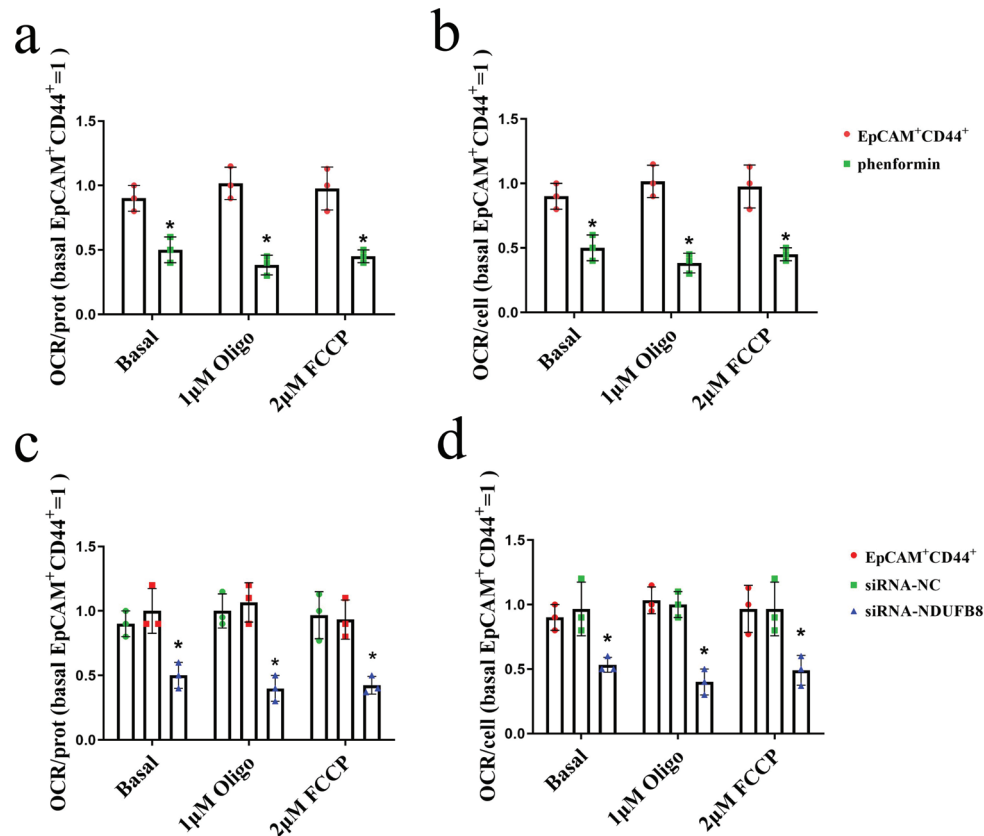
Fig. 3. OCR of EpCAM$^+$CD44$^+$ cells treated with phenformin or siRNA-NDUFB8. OCR of EpCAM$^+$CD44$^+$ cells treated with phenformin (0.5 mM) or not, as assessed by Seahorse Mito stress test (a) or oxygraph (b) analysis. OCR of EpCAM$^+$CD44$^+$ cells treated with siRNA-NDFUB8 or not, as assessed by Seahorse Mito stress test (c) or oxygraph (d) analysis. Results are expressed as mean ± SD (n = 3). *P < 0.05 versus Con.

proliferation decreased. Then looks at the expression of VEGF in the cells (Fig. 4d, e), reflecting the tumor stem cell's ability to promote angiogenesis increased, but after inhibiting phosphate oxidation, the ability of gastric CSCs to migrate and the expression of VEGF were significantly reduced. These results suggest that gastric CSCs promote the activity of complex I by promoting the expression of NDUFB8, enhance OXPHOS, thus promoting cell phenotypes, such as cell migration and proliferation and angiogenesis.

Discussion

At present, the origin of CSCs is not clear. One hypothesis is that CSCs originate from non-stem cells with a higher degree of differentiation. After transformation, they are mainly EMT (refers to epithelial or the process of transforming to mesenchymal cell phenotype under pathological conditions) after obtaining stem cell-like characteristics. Another hypothesis is that CSCs originate from non-malignant stem cells, and tumorigenic somatic mutations induce these non-malignant stem cells to transform into CSCs. At present, the isolation and identification of CSCs are still a challenge because tumors contain a variety of cell populations, and CSCs account for only a small portion (usually less than 1% in solid tumors). In theory, the use of new drugs to target CSCs with drug resistance has the potential to reduce cancer recurrence rates and improve the efficacy of tumor treatment. If drug development can be targeted at the metabolic characteristics of CSCs, it may be a favorable way to overcome drug resistance.

A key step in the process of tumorigenesis is the mechanism that drives tumorigenesis, which triggers their destiny to become tumor cells. So far, research studies have been mainly focused on the study of tumor suppressor genes (MYC, p53 or KRAS), mostly at the level of gene regulation. The role of metabolism has increasingly confirmed its importance. Compared with normal cells, it is well known that tumor cells undergo metabolic changes; however, it is not clear whether these changes are the result of tumor cell immortalization or the cause. A research study has shown that mitochondrial oxidative metabolism contributes to a CSC phenotype in cholangiocarcinoma (15). We observed that the OXPHOS also plays a role in maintaining the stemness of gastric CSCs in gastric cancer tumor cells, and the results are consistent with those in pancreatic cancer cells (14).
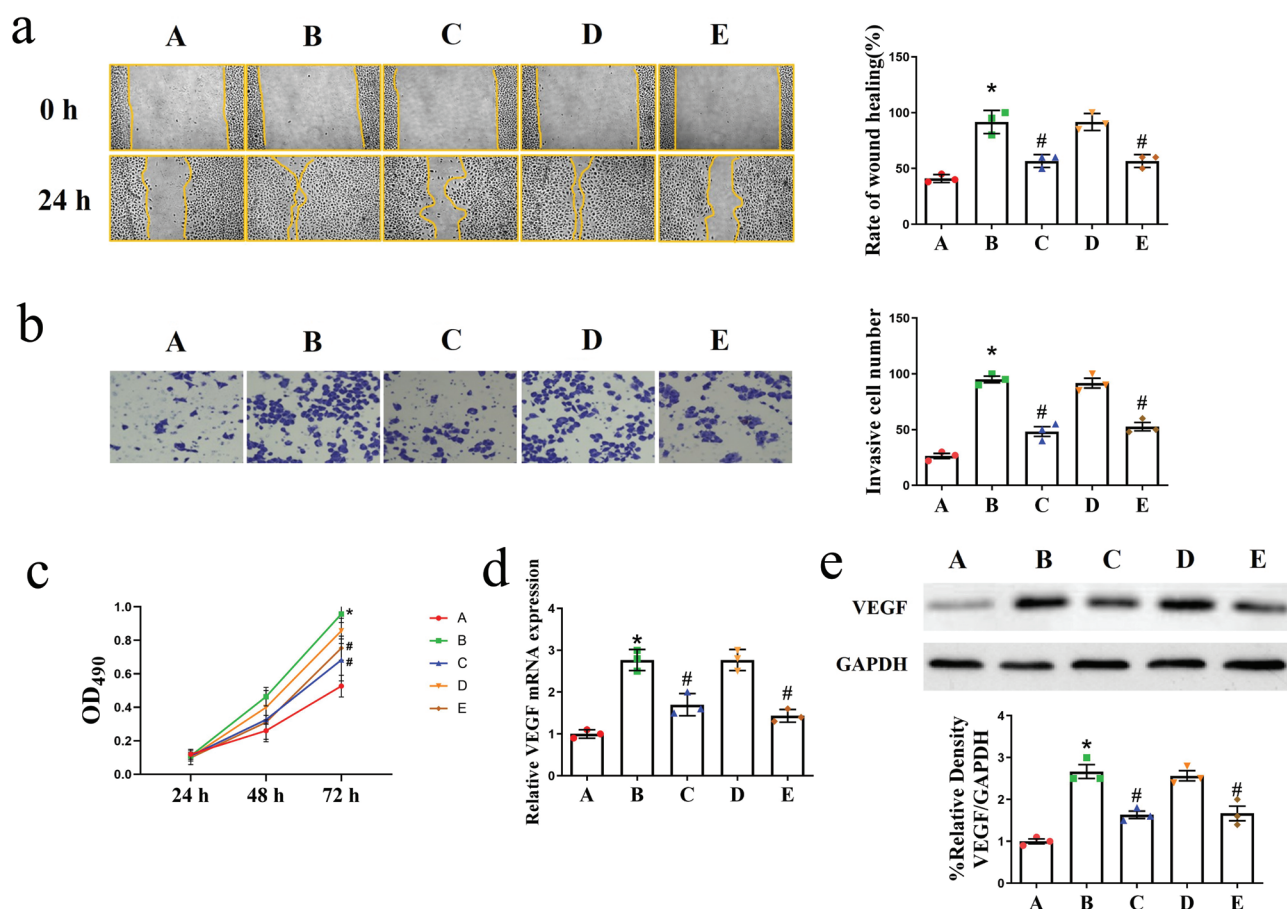
Fig. 4. Effect of enhanced OCR in EpCAM⁺CD44⁺ cells on cell migration, cell proliferation and the expression of VEGF. (a) Cell migration in each group, as assessed by wound-healing assay. (b) Cell migration in each group, as assessed by Transwell migration assay. (c) Cell proliferation in each group, as assessed by MTT assay through the OD at 490 nm. (d, e) mRNA and protein expression of VEGF in each group. A: Con; B: EpCAM⁺CD44⁺; C: EpCAM⁺CD44⁺ + phenformin; D: EpCAM⁺CD44⁺ + siRNA-NC; E: EpCAM⁺CD44⁺ + siRNA-NDFUB8. Results are expressed as mean ± SD ($n = 3$). $*P < 0.05$ versus A group. $^{\#}P < 0.05$ versus B group.

OXPHOS is a significant biochemical process occurring in cells and the final metabolic pathway of cellular respiration (16). This process occurs after glycolysis and the tricarboxylic acid cycle, and is the main step in the production of 'energy currency' ATP. Among the four respiratory chain complexes (complexes I, II, III and IV) that carry out electron transfer in mitochondria, complex I is the largest and most complex protein. In addition, complex I is the main site of reactive oxygen species (ROS) generation in mitochondria, except complex III (17). Therefore, many diseases, including cancer, have been shown to be related to complex I. Our study results suggest that the high expression of complex I in CSCs is related to the migration and proliferation of tumor cells.

Previous studies have suggested that tumor cells mainly undergo glycolysis and do not need oxidative metabolism. Recently, a new study found that Drosophila neural stem cell tumors are actually highly oxidative and have a higher OCR than normal cells (18). OXPHOS and increased NADH/NAD+ metabolism mediated by mitochondrial membrane fusion will promote tumor immortality. Oxidative metabolism is a mitochondrial oxygen-dependent bioenergy pathway, which plays a key role in the process of tumor cell immortalization. Studies have indicated that targeting mitochondrial complex I overcomes chemoresistance in high OXPHOS pancreatic cancer (14). Studies have found that the use of metformin under fasting conditions can significantly inhibit tumor growth, and proposed that the PP2A-GSK3β-MCL-1 pathway may be a new target for tumor treatment (19). Tumor cells will undergo metabolic changes that are different from normal cells. At the same time, tumor cells themselves can adapt to changes in the metabolic environment through the conversion between glycolysis and OXPHOS. This research study once again confirmed the possibility of this conclusion, indicating that metabolic pathways can be a new strategy for the treatment of tumors.

In the Drosophila model, the mitochondrial membrane is fused during tumorigenesis. This sharp change in mitochondrial morphology leads to an increase in the efficiency of OXPHOS. In the physiological condition, it is still unknown whether they share the same mechanism, which needs further investigation to reveal the underlying mechanism.

Conclusion

These results suggested that the increase in oxidative phosphorylation in gastric CSCs was related to respiratory chain complex I and NDUFB8, and hence, targeting metabolic pathways could be a new strategy in the treatment of gastric cancer.

Conflict of interest and funding

The authors report no conflict of interest.

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