Research paper

NKX2-8 deletion-induced reprogramming of fatty acid metabolism confers chemoresistance in epithelial ovarian cancer

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A B S T R A C T
Background: Aberrant fatty acid (FA) metabolism is a unique vulnerability of cancer cells and may present a promising target for cancer therapy. Our study aims to elucidate the molecular mechanisms by which NKX2-8 deletion reprogrammed FA metabolism-induced chemoresistance in epithelial ovarian cancer (EOC).

Methods: The deletion frequency and expression of NKX2-8 in 144 EOC specimens were assayed using fluorescence in situ hybridization and immunochemical assays. The effects of NKX2-8 deletion and the fatty acid oxidation (FAO) antagonist Perhexiline on chemoresistance were examined by Annexin V and colony formation assays.

Findings: NKX2-8 deletion was correlated with the overall and relapse-free survival of EOC patients. NKX2-8 deletion inhibited the FAO pathway by epigenetically suppressing multiple key components of the FAO cascade, including CPT1A and CPT2. Loss of NKX2-8 resulted in reprogramming of FA metabolism of EOC cells in an adipose microenvironment and leading to platinum resistance. Importantly, pharmacological inhibition of FAO pathway using Perhexiline significantly counteracted NKX2-8 deletion-induced chemoresistance and enhanced platinum's therapeutic efficacy in EOC.

Interpretation: Our results demonstrate that NKX2-8 deletion-reprogrammed FA metabolism contributes to chemoresistance and Perhexiline might serve as a potential tailored treatment for patients with NKX2-8-deleted EOC.

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1. Introduction

Aberrant metabolism, an evolving hallmark of human cancer, plays vital roles in cancer progression and chemotherapy failure [1–3]. Various experimental and clinical studies demonstrated that metabolic reprogramming of cancer cells into dysregulated Warburg-like glucose metabolism, glutaminolysis and fatty acid metabolism may favor tumor cell growth and chemotherapy failure [4–6], suggesting that aberrant metabolism is a unique vulnerability of tumor cells and a promising target for cancer therapy.

Ovarian cancer metabolism is unique from that of other cancers because of its adipocyte-rich microenvironment, which relies markedly on fatty acid breakdown for the production of ATP and the NADPH biosynthetic intermediates that contribute to the growth and metastasis of ovarian cancer [7–9]. For instance, adipocytes promoted proliferation of metastatic ovarian cancer cells through transferring fatty acids into fatty acid oxidation (FAO)-hyperactivated tumor cells [9]. SIK2 coupled fatty acid metabolism with survival of ovarian cancer cells at the adipocyte-rich niche via activating both FAO and phosphatidylinositol-
4,5-bisphosphate 3-kinase (PI3K)/AKT kinase (AKT) pathways [8]. Meanwhile, the polyunsaturated fatty acids secreted by platinum analogs-activated mesenchymal stem cells (MSCs) conferred the resistance of ovarian cancer to multiple types of chemotherapy via augmenting FAO activity [10]. Moreover, pharmacologic inhibition of FAO using carnitine palmitoyltransferase CPT1 and CPT2 inhibitors such as Perhexiline, an FDA-approved drug approved for the treatment of angina and heart failure, significantly reduced NKX2–8 deletion-induced chemoresistance and enhanced the therapeutic efficacy of platinum in EOC.

2. Materials and methods

2.1. Cells

The OVCAR3, CAOV3 and OV90 EOC cell lines (ATCC) were cultured in the DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY). Human Ovarian Surface Epithelial Cell (HOSEpic) was obtained from ScienCell Research Laboratories and cultured in the specific medium according to the manufacturer’s instructions. The above-mentioned cell lines were identified by performing short tandem repeat fingerprinting in Sun Yat-sen University (Guangzhou, China).

2.2. Tumor specimens

EOC specimens (n = 144) used in the current study were collected from Sun Yat-sen University Cancer Center (Guangdong, China) between 2005 and 2016. The clinical pathological characteristics of EOC patients has been shown in Supplementary Table S1–3. All the EOC patients received standard platinum-based chemotherapy in Sun Yat-sen University Cancer Center. Chemoresistance or chemosensitivity defined as relapse or progression within six months or after six months from the last chemotherapy, respectively. Prior donor consent was obtained from all EOC patients and the approval of using the specimens was obtained from the Institutional Research Ethics Committee.

2.3. DNA/RNA extraction and real-time quantitative PCR and genomic PCR

In the current study, total RNA and genomic DNA were extracted from the EOC cell lines and EOC tissue samples by using TRIzol (Life Technologies) and QIAamp DNA Mini Kit, respectively. cDNAs/DNAs were amplified via using FastStart Universal SYBR Green Master kit (ROX; Roche, Toronto, ON, Canada) and quantified by using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The expression data were normalized to housekeeping gene GAPDH or Line1 to control the variability in expression levels. Deletion of NKX2–8 was analyzed by genomic PCR amplification. The primer sequences were obtained from the Genome database was shown in Primers and Oligonucleotides table in Supplementary Table S4.

2.4. Vectors, retroviral infection and transfection

The retroviral vector pMSCV-neo was used to generate pMSCV-neo/NKX2–8 recombinant plasmid. Small interfering RNA of CPT1A, CPT2, Sin3A, SAP18 and HDAC1 were used to transiently inhibit the gene expression. All the detailed sequences of siRNA oligonucleotides are shown in Supplementary Table S5. The promoter region of human CPT1A, including fragments covering nucleotides −853 to +475, and CPT2 promoter, including nucleotides covering −1450 to +483, were subcloned into the pGL3-Control vector luciferase reporter (Promega, USA), respectively. Recombinant plasmids or siRNA used in the current study were transfected by the Lipofermectin 3000 reagent (Invitrogen, Carlsbad, CA). Stable cell lines OV90/NKX2–8 was selected for 10 days with neomycin (1 μg/ml). The NKX2–8 protein level was detected by SDS–PAGE western blotting after ten-day selection to confirm stable expression of NKX2–8 in EOC cell lines. A list of primers used in the present study was shown in Supplementary Table S5.

2.5. Western blotting analysis

Western blotting analysis were performed in accordance with standard procedures. The antibodies used in this study were NKX2–8 (1:500, Abcam, ab125040), CPT1A (1:1000, Abcam, ab128568), CPT2
DNA fragments from cultured cell lines were subjected to sequence by density gradient (1% NKX2 fragments was conducted PCR assay to detect the enrichment of for 10 min. Anti-NKX2 (ChIP) assay kit (Upstate/Millipore, Billerica, Maine, USA) according to assay 2.9. Chromatin immunoprecipitation (ChIP) and ChIP-Immunobloting Table S4.

2.6. Immunohistochemistry (IHC) assay

IHC assay was performed to determine the protein expression of NKX2–8 (Abcam, ab125040), CPT1A (Abcam, ab128568) and CPT2 (Abcam, ab181114) in 144 EOC samples. The detailed processes of IHC assay were carried out according to previously published paper [22]. Axio Imager.Z2 system (Carl Zeiss Co. Ltd., Jena, Germany) was used to capture the immunohistochemistry images.

2.7. Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization assay was conducted to analyze the DNA deletion level of NKX2–8 in human EOC tissues and EOC cell lines, and carried out according to previously published standard protocol [23] using an NKX2–8-specific probe (Ribobio, Guangzhou, China). To determine the NKX2–8 deletion status, the number of red and green signals per nuclei was scored in 200 nuclei from each specimen, which NKX2–8 was considered deleted in EOC when the red/green ratio was <0.85.

2.8. CRISPR/Cas9 system

CAOV3/NKX2–8 and OVCAR3/NKX2–8 knockout cells were conducted with CRISPR/Cas9 system as described previously [24]. GeneChem (Guangzhou, China) designed and cloned the corresponding single guide RNAs into GV392 plasmid (NKX2–8 sgRNA sequences showed in Table S4). Briefly, 3 × 10^5 CAOV3 and OVCAR3 cells were infected with lenti-CRISPR virus. After 24 h, infected cells were selected for 7 days using 0.5μg/ml puromycin. Afterwards, CAOV3/Cas9 and OVCAR3/Cas9 cells were re-infected with GV392-GFP-NKX2–8 gRNA lentivirus to ensure >95% cells were positive. The infected cells were sorted using flow cytometry and single-cell cloned, the CAOV3/ NKX2–8−/− #1 and NKX2–8−/– #2; OVCAR3/NKX2–8−/− #1 and NKX2–8−/– #2 referred to single-cell clone 1 and 2, respectively. Genomic DNA was extracted from CAOV3/NKX2–8−/− #1 and NKX2–8−/– #2; OVCAR3/NKX2–8−/− #1 and NKX2–8−/– #2 cells using a QIAGEN genomic DNA Kit and the target sequence were amplified. Then, heterodimerization and digestion were performed using the Knockout and Mutation Detection Kit (GeneChem) according to the manufacturer’s instructions. Mutagenesis was finally confirmed by nucleic acid sequencing. Primers used in the current study is shown in Supplementary Table S4.

2.9. Chromatin immunoprecipitation (ChIP) and ChIP-Immunobloting assay

ChIP assay was performed by using chromatin immunoprecipitation (ChIP) assay kit (Upstate/Millipore, Billerica, Maine, USA) according to the manufacturer’s instructions. For ChIP-Immunobloting assay, the indicated EOC cells lysis were treated with or without formaldehyde (1%) for 10 min. Anti–NKX2–8 antibody (Abcam) and Sin3A (Abcam) was used for incubated the cell lysates overnight at 4 °C. The enriched DNA fragments was conducted PCR assay to detect the enrichment of NKX2–8 or Sin3A in the promoter of CPT1A and CPT2. Western blotting was performed to analyzed the protein fractions enriched by sucrose density gradient (1%-28% (W/V)). For ChIP-seq analysis, the enriched DNA fragments from cultured cell lines were subjected to sequence by using HiSeq 2000 (Illumina). The ChIP-seq data was available and downloaded from the NCBI’s Sequence Read Archive (PRJNA487880).

2.10. In vivo experiments

Female BALB/c nude mice and NOD/Shi-SCID/IL-2Rnull (NOG) were purchased from Vitalriver (Beijing, China) and housed under SPF level conditions. NOG mice were random assorted to different groups (n = 6/group) and embedded with fresh clinical EOC patient tissues (2–3 mm^3 pieces) coated with a 1:1 ratio of Matrigel (BD Biosciences, San Jose, CA, USA). Nude mice were injected subcutaneously with induced EOC cells (1 × 10^6) mixed with adipocytes (100 μl packed cell volume (PCV)) with growth factor-reduced Matrigel (50 μl). In the intraperitoneal tumor model, female nude mice were injected intraperitoneally with the indicated EOC cells (1 × 10^6). Tumor growth was monitored by living image software. The eq. (L*W^2)/2 was used to determine the tumor volume of mice in the current study. The mice were treated with CDDP (5 mg/kg every 3 days) or vehicle (control) when the tumor became palpable (subcutaneous model) or the luminescence signal reached 1 × 10^7 p/s/cm^2/sr (intraperitoneal model). Approved of the Institutional Animal Care and Use Committee of Sun Yat-sen University were obtained before all the in vivo experiment in this study.

2.11. Fatty acid oxidation (FAO) assay

To determine the FAO activity, the rate of carbon dioxide produced from the oxidation of [1^4C] palmitate (PerkinElmer) was measured in the Nuclear Medicine Department of Sun Yat-sen University. Briefly, tumor cells (2 × 10^6) were seeded in 24-well plates in the presence of 1 μCi [1^4C] palmitic acid-BSA complex and incubated for 3 h at 37 °C. The released [1^4C] carbon dioxide was trapped using filter paper soaked in 100 mM NaOH for 1 h at 37 °C. The rate of FAO activity was determined by calculating the amount of [1^4C] carbon dioxide in relative units produced per milligram of protein per hour.

2.12. Chemical reagents

Perhexiline was purchased from Sigma-Aldrich (Germany), and Trichostatin A (TSA,) purchased from Selleck Chemicals.

2.13. Metabolomic analysis

All the EOC samples (1 × 10^7 cells/sample, n = 6 per group) were harvested firstly by centrifugation (3500 rpm, 10 min). The residue was suspended by adding equal volume of PBS and low temperature methanol, then centrifuged at 4 °C (3500 rpm, 10 min) to absorb the supernatant and immediately stored at −80 °C for analysis on the LC/MS platform, according to the manufacturer’s instructions.

2.14. Adipocyte extraction and co-culture system

Adipocytes were extracted from omental tissues of ovarian cancer patient who undergo surgical procedures in the Sun Yat-sen University Cancer Center. Omental tissue was minced within DMEM/F12 medium which containing collagenase type I and bovine serum albumin. The above mixture was incubated and shake (80 g, 1 h) at 37 °C. Adipocytes cells was filtered by using a 250-μm mesh filter and removed the undigested tissue, and adipocytes were collected by centrifugation at 200 g and maintained in DMEM/F12 medium.

Adipocytes that extracted from omental tissues of ovarian cancer patient were co-cultured with ovarian cancer cell lines in vitro. Briefly, adipocytes (2 × 10^4) were cultured in DMEM/F12 medium containing collagenase type I, BSA and 50 μg/ml gentamicin reagent (Gibco) in the cell inserts (1.0 um-pores) coated with collagen type I. Meanwhile, EOC cell lines were seeded into the lower wells (2 × 10^4)/well coated
with collagenase type I and the above-mentioned adipocytes cells were transferred to the 24 well culture insert companion plates.

2.15. Mean optical density (MOD) analysis

Mean optical density (MOD) was used to determine the immunostaining of EOC specimen. Briefly, the stained slides were evaluated by using the ZEISS Axioscan.Z1 Digital Slide Scanner (Carl Zeiss, Germany) and the Image-Pro Plus 6.0 analysis software to measure the mean optical density (MOD). Ten random picked fields in each specimen were analyzed to determine the MOD of the specimen, based on which the mean MOD of a study group of samples was further generated for subsequent inter-group comparative analysis. The MOD data were statistically analyzed by using the Spearman's correlation analysis to compare the average MOD difference between different groups of tissues, and $P < 0.05$ was considered significant.

![Diagram](image-url)

Fig. 1. NKO2–8 deletion correlates with worse prognosis in patients with EOC. a. GISTIC2.0 analysis of 32 human cancer types (The Cancer Genome Atlas, TCGA) showing varying degrees of heterozygous deletions of NKO2–8. b. Loss of NKO2–8 was correlated with overall and relapse-free survival in the TCGA pan-cancer cohort ($P < 0.001; n = 9822; 32$ cancer types). c. EOC was analyzed by FISH for NKO2–8 deletion (left, upper), IHC for NKO2–8 protein (left, tower), and the correlation between NKO2–8 deletion and protein expression (right) ($P < 0.01$). d. Loss of NKO2–8 was associated with shorter relapse-free survival in EOC ($P < 0.001; n = 144$). e. Significant correlation between NKO2–8 protein expression and NKO2–8 deletion with the EOC clinical pathological parameters. NKO2–8 expression was stratified into three groups: (i) NKO2–8 del/low, (ii) NKO2–8 non-del/low and (iii) NKO2–8 non-del/high. g. Kaplan-Meier survival analysis of low protein expression/NKO2–8 deletion, low protein expression/ NKO2–8 non-deletion, and high expression/NKO2–8 non-deletion. h. GSEA analysis showing that NKO2–8 deletion and NKO2–8 mRNA levels was correlated with CDDP resistance-related gene signature in the TCGA ovarian cancer dataset ($P < 0.05; n = 419$).
Fig. 2. NKX2–8 deletion contributes to EOC chemoresistance in adipose microenvironment. 

- In vivo growth of subcutaneous tumors formed by the indicated EOC cells mixed with or without adipocytes injected in the dorsal flank of the mice. Representative images of tumor-bearing mice (left, upper) and quantification (right) of bioluminescence signal were measured on the indicated days (left, lower). (∗P < .05, ANOVA, n = 6 mice/group). 
- Representative images (left) and quantification (right) of intraperitoneal tumor-bearing nude mice in the indicated time (∗P < .05, ANOVA, n = 6 mice/group). 
- IHC staining for TUNEL and activated caspase-3 (left) and quantification of the apoptotic rate in the indicated xenograft tumors (right). ∗P < .05. 
- Kaplan-Meier survival of CDDP-treated intraperitoneal tumor-bearing nude mice. ∗∗P < .01.
2.16. XF24 extracellular flux analyzer for measurement of cellular oxygen-consumption rate

Oxygen-consumption rate (OCR) was measured by using a Seahorse XF96 Extracellular Flux Analyzer according to the published protocol [25]. In brief, EOC cells (2 × 10^5) were plated on XF96 cell culture microplates (Seahorse Bioscience, Cat. No. 103010–100) and OCR assay was measured in the indicated cells treated with oligomycin (1.0 μM), FCCP (0.5 μM), 2-DG (10 mM) and Antimycin (2 μM) on a Seahorse XF96 bioanalyzer.

2.17. Annexin-V assay

For evaluation of apoptosis, Annexin-V assay was performed by using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer’s instructions. Brieﬂy, OVCAR3 cells (5 × 10^7) which transfected with FB-dCas9 plasmid were collected and washed with PBS and the Annexin-V binding solution, and added 150 μl Annexin-V antibody,1.5 μl of PI at 1 mg/ml in binding buffer and incubated for 15 min at room temperature in the dark, subsequently analyzed with an EPICS XL flow cytometer (Beckman-Coulter).

2.18. ROS analysis

Intracellular ROS levels were determined by using the ROS detection kit (Beyotime, Jiangsu, China) according to the manufacturer’s instructions. ROS levels was detected by using flow cytometry system (FACScan, Becton Dickenson, USA) and analyzed with FlowJo 7.6 software. Fluorescence values were normalized to protein load.

2.19. Streptavidin affinity purification of dCas9-captured DNA and proteins

Streptavidin affinity purification of dCas9-Captured DNA and proteins was performed following the published protocol [26]. Brieﬂy, OVCAR3 cells (5 × 10^7)) which transfected with FB-dCas9 plasmid and CPT1A or CPT2 promoter sgRNAs were harvested when treated with 1% formaldehyde to cross-link the proteins to DNA. Anti-streptavidin antibody was used for incubated the cell lysates. The ChIP DNA was puriﬁed by Qiagen quick Spin columns (Qiagen) and proteins were separated by SDS-PAGE gel, subsequently analyzed by IP-MS analysis (Shanghai Applied Protein Technology Co.Ltd.).

2.20. Assessment of ATP, NADH and NADPH level

The indicated EOC cells were seeded in 96-well plates (2 × 10^3 cells) and treated with 10 μM Perhexiline for 24 h. The ATP, NADH and NADPH levels were determined after treatment with perhexiline (Sigma-Aldrich) by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), NAD/NADH Assay kit (Abcam, ab65348) and NADP/ NADPH Quantitation Kit (Sigma-Aldrich, MAK038-1KT) according to the manufacturer’s instructions, respectively.

2.21. Cell survival assay

The indicated cells were plated in 6-well plates (2 × 10^3 cells) and treated with CDDP (5 μM) and cultured for 10 days. The colonies were ﬁxation with 10% formaldehyde for 5 min and washed three times, then stained with 1% crystal violet for 30 min. The number of colonies (deﬁned as cell clusters consisting of at least 50 cells) was quantiﬁed by Analysis software (Olympus Biosystems).

2.22. Drug combination analysis

To evaluate the effects of CDDP and perhexiline combination treatment, NKKX2–8+/− and NKKX2–8+/− cells were incubated with different ratios of CDDP and perhexiline and cell viability was analyzed using MTT assays. Brieﬂy, 3000 cells/well were seeded into 96-well plates, grown for 24 h, and treated with CDDP and perhexiline alone, different weight ratios of CDDP and perhexiline combination (CDDP/perhexiline = 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10). After incubation for 24 h, 100 ml sterile MTT dye (0.5 mg/ml, Sigma) was added to each well for 4 h at 37 °C. Then, the media were removed and 150 μl dimethyl sulfoxide (DMSO, Sigma) was added. Finally, the absorbance of each well was measured at 570 nm using an EPICS XL ﬂow cytometer (Beckman-Coulter). To evaluate the synergistic effects of CDDP and perhexiline combinations, Synergistic drug interactions were analyzed using the Chou and Talalay method [27]. The combination index (CI) for drug combinations is derived according to the equation below where n = number of drugs

\[
CI = \sum_{j=1}^{n} \frac{(f_j)}{(f_0)}
\]

According to the equation, CI < 1 indicates synergy, CI = 1 indicates additivity, and CI > 1 indicates antagonism. The above median effect equation and CI allow a plot of CI values at different effect levels ( fees) to be determined using CalcuSyn software (Biosoft).

2.23. Statistical analysis

In the current study, all the data analysis was conducted by using SPSS 21.0 statistical software (IBM, Armonk, NY, USA). Student’s t-test was performed to analysis differences between two variables. The relationship between NKKX2–8 expression and the clinicopathological characteristics and prognosis of EOC patients were analyzed by using pearson’s Chi-square test, log-rank analysis, Kaplan–Meier and Cox regression model. Data represented the mean ± SD. P values <.05 were considered statistically signiﬁcant.

3. Results

3.1. Heterozygous deletion of NKKX2–8 correlates with a worse prognosis in patients with cancer

Previously, it has been shown that NKKX2–8+/− or NKKX2–8+/− mice spontaneously developed multiple tumors, suggesting a tumor suppressive role of NKKX2–8 [21]. To decipher the molecular pathogenesis of NKKX2–8 deletion in tumorigenesis, we examined the patterns of NKKX2–8 deletion in 9855 tumors spanning 32 human tumor types (TCGA dataset). As shown in Fig. 1a, all 32 tumor types displayed het- erozygous deletions to varying degrees of NKKX2–8 and 16 types of tumor exhibited above 20% heterozygous deletion of NKKX2–8. Importantly, patients with NKKX2–8-deleted cancer had signiﬁcant shorter overall and relapse-free survival compared with patients without Fig. 3. NKKX2–8 deletion activates FAO signalling and reprograms fatty acid metabolism. a. Distribution of NKKX2–8 binding sites in the promoter (−3 kb to +3 kb), gene body, and intergenic regions (top). NKKX2–8 binding regions were enriched in the gene promoters (bottom). Heatmap of the ChIP enrichment signal in NKKX2–8–binding regions (right). b. GO enrichment analysis of the NKKX2–8 regulated-transcripts identiﬁed in ChIP-seq analysis in OVCAR3/NKKX2–8 cells treatment with adipocyte-CM. c. The results of GSEA analysis associated with NKKX2–8–deletion and fatty acid metabolism in the TCGA EOC dataset. d. Fold change in metabolite levels in NKKX2–8 deleted EOC cells. The values are presented as minimum-to-maximum box plots for six experiments per group. e. Relative FAO activity and level of NADPH, ATP and ROS in adipocyte-CM-treated control cells. f. Relative FAO activity and level of NADPH, ATP and ROS in the indicated adipocyte-CM-treated EOC cells treatment with Perhexiline (10 μM) for 24 h. h-j. Effects of Perhexiline (H), 2-DG (I), or glutamine withdrawal (J) on the oxygen consumption rate (OCR) in the indicated adipocyte-CM-treated EOC cells. Each bar in Fig. 3e–j represents the mean ± SD of three independent experiments. *P < 0.05.
NKX2–8 deletion (P < .001; P < .001; Fig. 1b), suggesting that loss of NKX2–8 results in a worse prognosis for patients with cancer.

3.2. Loss of NKX2–8 is associated with chemoresistance in EOC

One of the major reasons for poor prognosis in cancer patients is chemotherapy resistance [28]. More than 75% of patients with EOC quickly become resistant to standard platinum-based treatment and suffer recurrence, therefore EOC was selected as our study model. Consistent with the data presented in the TCGA EOC file (37.9%), fluorescence in situ hybridization (FISH) analysis of our EOC cohort (38.1%) revealed that the frequency of NKX2–8-deletion increased with increasing FIGO stage and shorter relapse-free survival (Fig. 1c and d; Supplementary Fig. S1a and b). We found that 81.8% of NKX2–8-deletion EOC...
cases exhibited chemoresistance whereas 70.8% of cases without NKX2–8 deletion were chemosensitive (Fig. 1e), suggesting that loss of NKX2–8 might be involved in chemotherapeutic failure.

Complementing NKX2–8 expression with NKX2–8 gene copy alterations revealed that 90.9% of NKX2–8-deleted EOC cases displayed low NKX2–8 expression whereas 62.9% of EOC cases without NKX2–8 deletion exhibited high NKX2–8 expression (Fig. 1c), suggesting that heterozygous deletions represent a major mechanism of NKX2–8 protein reduction. Next, 144 EOC specimens from patients who received CDPD-based chemotherapy were divided into three groups according to NKX2–8 deletion/protein expression patterns: (1) non-del/high, (2) non-del/low and (3) del/low. The NKX2–8–del/low group was characterized by a higher FIGO grade and failure of CDPD-based chemotherapy and had the shortest relapse-free survival time (Fig. 1f and g; Supplementary Table S1–3), which suggest that loss of NKX2–8 is involved in EOC chemoresistance and recurrence. This hypothesis was further confirmed by Gene Set Enrichment Analysis (GSEA) that NKX2–8-deletion and NKX2–8 mRNA levels were significantly associated with gene signature-related CDPD-resistance in EOC (Fig. 1h).

3.3. The adipose microenvironment enhances chemoresistance of NKX2–8-deleted EOC cells

To determine the effect of NKX2–8 deletion on EOC chemoresistance, two EOC cell lines CAOV3 and OVCAR3, which exhibited no NKX2–8 gene copy alterations, were selected to establish NKX2–8+/− and NKX2–8−/− cells via CRISPR/Cas9 genome editing technology (Supplementary Fig. S2a and b). Interestingly, genetic ablation of NKX2–8 only slightly enhanced the resistance capability of EOC cells to CDPD treatment, while co-cultured with adipocytes or treatment with adipocyte-derived conditioned medium (adipocyte-CM) resulted in increased tolerance of NKX2–8–deleted EOC cells to CDPD treatment, which was indicated by decreased numbers of apoptotic cells and an increased CDPD IC50 value (Supplementary Fig. S2c–e). On the other hand, co-cultured with adipocytes or treatment with adipocyte-CM significantly decreased the cytotoxic effect of CDPD in OV90 cells, which showed a heterozygous deletion of NKX2–8 (Supplementary Fig. S2a, g and h). However, overexpression of NKX2–8 significantly abrogated the effect of adipose microenvironment-induced CDPD resistance of OV90 cells (Supplementary Fig. S2f–h). These results suggest that adipose microenvironment is involved in the NKX2–8–deletion-induced chemoresistance.

3.4. NKX2–8 deletion contributes to EOC chemoresistance in the adipose microenvironment

In agreement with the in vitro results, in vivo subcutaneous tumor model experiments showed that mixing CDPD with adipocytes significantly abolished its anti-tumor effect on subcutaneous tumor growth of NKX2–8−/−EOC cells, as evidenced by rapid tumor progression, and lower proportions of TUNEL+ and activated caspase-3+-apoptotic cells. However, these effects were not observed using NKX2–8–nondeleted EOC cells (Fig. 2a and Supplementary Fig. S3a–c). Furthermore, adipose microenvironment-induced CDPD resistance of OV90 cells was dramatically reduced by restoring NKX2–8 expression (Fig. 2b and Supplementary Fig. S3d). These results indicate that the adipose microenvironment enhanced the chemoresistance of NKX2–8–deleted EOC cells.

Given that the omentum and mesentery are major repositories of visceral white adipose tissues and are frequently involved in the progression of ovarian cancer, an intraperitoneal xenograft model was then further employed (Fig. 2c). When the bioluminescence signal of the intraperitoneal tumors reached 1 × 107 p/s/cm2/sr, nude mice were treated with vehicle or CDPD for 6 weeks. Consistently, in the intraperitoneal adipose-enriched microenvironment, genetic deletion of NKX2–8 dramatically abolished the anti-tumor effect of CDPD on the intraperitoneal growth of EOC cells, as indicated by rapid tumor growth, lower proportion of TUNEL+ and caspase-3+-apoptotic cells and shorter survival time of tumor-bearing mice (Fig. 2c–e). These results demonstrate that NKX2–8+/−/EOC possesses a stronger capability of chemoresistance in the adipose microenvironment.

3.5. NKX2–8 deletion activates FAO signalling and reprograms fatty acid metabolism

To further investigate the mechanism by which the adipose microenvironment enhances chemoresistance of NKX2–8–deleted cells, NKX2–8–associated gene promoters were profiled using ChIP-Seq (Fig. 3a). Gene ontology (GO) enrichment analysis revealed that genes with GO biological process terms “Intrinsic apoptotic signaling pathway”, “Apoptotic signaling pathway” and “Response to CDDP” were enriched (Fig. 3b). Strikingly, genes with GO terms such as “Long-chain fatty acid import”, “fatty acid beta-oxidation” and “fatty acid β-oxidation using acyl-CoA dehydrogenase” were also enriched. Similar enrichments were also observed in public dataset via GSEA and GO enrichment analysis (Fig. 3c and Supplementary Fig. S4a). The above results suggest an important role of NKX2–8 in regulation of FAO signalling. This hypothesis was further confirmed by multiple assays, in which the presence of adipocyte-CM, metabolite analysis revealed a higher amount of long-chain acylcarnitine intermediates in NKX2–8–deleted EOC cells compared with that in control cells, and the FAO activity and production levels of NADPH and ATP were also significantly increased in NKX2–8–deleted EOC cells but decreased in NKX2–8–transduced cells compared with those in control cells (Fig. 3d–f). Conversely, the ROS level was significantly decreased in adipocyte-CM-treated NKX2–8+/−/cells but increased in adipocyte-CM-treated NKX2–8–transduced cells (Fig. 3e and f).

Moreover, we observed that inhibition of FAO signalling with Peroxhexilin, a FAO inhibitor, in the adipocyte-CM-treated NKX2–8+/−/cells resulted in significantly decreasing of oxygen consumption rate (OCR), FAO activity, ATP and NADPH production and but led to more increased ROS level compared to control cells (Fig. 3g and h). However, treatment with glycolysis inhibitor 2-DG or withdrawing glutamine had no obvious effect on OCR in adipocyte-CM-treated NKX2–8+/−/cells (Fig. 3i and j). These results indicate that loss of NKX2–8 induces the reprogramming of fatty acid metabolism of EOC cells in the adipose microenvironment.

3.6. NKX2–8 transcriptionally represses multiple key components of the FAO cascade

Analysis of the NKX2–8 ChIP-Seq results suggested that the two key components (CPT1A and CPT2) in the FAO pathway might be downstream targets of NKX2–8 (Fig. 4a), which was further confirmed by a ChIP assay in which NKX2–8 was most significantly associated with
the CPT1A and CPT2 promoters in adipocyte-CM-treated EOC cells (Fig. 4b). Moreover, we found that expression of CPT1A and CPT2, at both the mRNA and protein levels, and the luciferase activity driven by the promoters with a NKX2–8–specific binding site (NBS) of these genes was increased in NKX2–8–deleted cells but decreased in NKX2–8–transduced cells in the presence of adipocyte-CM (Supplementary Fig. 54b and c; Fig. 4c and d). However, no effect was shown on the luciferase activities of the promoter with a deleted NKX2–8–binding site (Fig. 4d). The adverse association of NKX2–8 with FAO activity was further confirmed via IHC analysis in EOC specimens, in which NKX2–8 levels correlated with the levels of CPT1A and CPT2 (Fig. 4e). Therefore, our results suggest that NKX2–8 regulates FAO-related genes via direct association with their promoters in the adipose microenvironment.

Additionally, individually silencing CPT1A and CPT2 not only significantly abolished the effects of NKX2–8–deletion on FAO activity and ATP production, but also drastically abrogated NKX2–8–deletion-induced chemoresistance in the adipose microenvironment (Supplementary Fig. S5d and Fig. 4f and g). Importantly, statistical analysis revealed that the expression levels of CPT1A and CPT2 correlated significantly with shorter relapse-free survival in patients with EOC (Supplementary Fig. S5a). ChIP-immunoblotting assay showed that genetic deletion of Sin3A, HDAC1, or SAP18 dramatically decreased the interaction between NKX2–8 and Sin3A/HDAC1/SAP18 complex in the adipose microenvironment (Supplementary Fig. S4d and Fig. 4f and g). Importantly, statistical analysis revealed that the expression levels of CPT1A and CPT2 genes was increased in NKX2–8–deleted cells but decreased in NKX2–8–transduced cells in the presence of adipocyte-CM (Fig. 5h and i and Supplementary Fig. S5c). Furthermore, treatment with Trichostatin A (TSA), a general histone deacetylase (HDAC) inhibitor, in NKX2–8–transduced cells not only increased CPT1A and CPT2 transcriptional levels but also elevated the FAO activity and ATP levels in the adipose microenvironment (Supplementary Fig. S5d and e). More importantly, the effect of NKX2–8–induced chemosensitivity in the adipose microenvironment was drastically abolished by individually silencing of Sin3A, HDAC1, SAP18, or TSA treatment (Supplementary Fig. S5f), which provide further evidence that the Sin3A/HDAC1/SAP18 repressor complex is essential for NKX2–8–mediated inhibition of FAO signalling and chemoresistance in the adipose microenvironment.

3.8. Blocking FAO signalling abrogated the adipose microenvironment-induced chemoresistance of NKX2–8–deleted EOC cells in vitro

Furthermore, drug combination index analysis indicated a synergistic anti-tumor effect of FAO inhibition and CDDP in NKX2–8–deleted EOC cells but not in NKX2–8–nondeleted cells in an adipose microenvironment (Fig. 6a). We found that blocking FAO signalling using Perhexiline led to inhibition of Akt and mTOR/S6K pathways but increased levels of p-Bad expression in NKX2–8–deleted cells, which resulted in the release of more cytochrome c and increased activated caspase 3 (Fig. 6b). Interestingly, we found that the intracellular glutathione (GSH) level was much lower in NKX2–8–/− cells treated with Perhexiline, but the content of genomic DNA-bound CDDP was significantly increased in NKX2–8–/− cells when treated with Perhexiline (Fig. 6c and d), suggesting that Perhexiline treatment led to inhibited CDDP efflux in NKX2–8–/− cells. Consistently, Perhexiline treatment significantly decreased the IC50 values for CDDP in -treated NKX2–8–/− EOC cells under the adipose microenvironment (Fig. 6e), which further confirmed by colony formation and Annexin V assays that Perhexiline treatment significantly augmented the cellular toxic effects of CDDP in NKX2–8–/− cells but only had a minor effect in NKX2–8+/− cells in the adipose microenvironment (Fig. 6f and g). Collectively, our results demonstrated that inhibition of the FAO pathway reversed the resistance capability of NKX2–8–deleted EOC cells to platinum treatment in the adipose microenvironment.

3.9. Inhibiting FAO signalling sensitizes NKX-2-8-deleted EOC to CDDP therapy in vivo

The therapeutic efficacy of inhibiting FAO with Perhexiline was further tested using intraperitoneal xenograft model. As shown in Fig. 7a–c, combined therapy with Perhexiline significantly enhanced the curative efficacy of CDDP on the growth of NKX2–8+/− tumors and prolonged the survival of the tumor-bearing mice of NKX2–8+/− tumors after CDDP therapy, providing further evidence that activation of FAO signalling plays a vital role in EOC recurrence. Furthermore, two NKX2–8+/− and two NKX2–8+/− patient-derived EOC cells, isolated from fresh clinical EOC tissues, were tested (Supplementary Fig. S6a and b). As shown in Supplementary Fig. S6c, treatment with adipocyte-CM significantly decreased the cytotoxic effect of CDDP in NKX2–8+/− patient-derived ovarian cancer (PDOC) cells but not in NKX2–8+/−/PDOC cells. Consistently, the intraperitoneal xenograft model also showed that tumors formed by NKX2–8+/−/PDOC cells
were more sensitive to co-treatment with Perhexiline and CDDP (Supplementary Fig. S6d and e). Taken together, our results demonstrate that NKX2–8-deletion contributes to chemoresistance via activation of FAO signalling in EOC cells in an adipose microenvironment (Fig. 7d).

**4. Discussion**

Metabolic energy changes have recently emerged as an important determinant of chemotherapy resistance [29,30]. Numerous studies linking aberrant energy metabolism to chemoresistance have revealed that elevated ATP and NADPH levels, which are produced from aerobic glycolysis in the cytoplasm and oxidative phosphorylation (OXPHOS) in the mitochondria, confer resistance of cancer cells to multiple types of chemotherapeutic drugs [11,31–33]. A series of recent reports suggested that mitochondrial FAO plays a major role in sustaining ATP levels and NADPH production [34–39], suggesting that targeting FAO might be an alternative therapeutic approach for cancer. Herein, we reported that pharmacologically inhibiting FAO using Perhexiline, an anti-angina drug that inhibits carnitine palmitoyltransferases 1 and 2 (CPT-1 and CPT-2), considerably decreased energy metabolism to enhance the therapeutic efficacy of CDDP-chemotherapy in NKX2–8-deleted EOC. Therefore, these findings not only provide mechanistic and clinical insights into EOC chemoresistance, but also provide evidence that the FAO pathway is a druggable target to prevent chemoresistance.
The relationship between EOC progression and fatty acid metabolism has long been known. Previously, it was reported that an increased intake of foods with high levels of saturated fats or cholesterol leads to altered systemic lipid metabolism and elevated serum lipid levels, which are positively associated with the risk of ovarian cancer [6,40]. Recently, analysis of the ovarian metabolome showed dramatically increased FAO in primary and metastatic EOC [41]. In addition, a large-scale plasma metabolomics study indicated that the metabolic pathways in patients with EOC are mainly characterized by aggressive FAO and abnormal phospholipid metabolism [42]. Taken together, these studies provided fundamental insights into the activation of FAO in EOC. However, the precise mechanism underlying FAO hyperactivation in EOC is unknown. The current study provided several lines of evidence that loss of NKX2–8 induces FAO activity in EOC. First, NKX2–8-deletion was significantly associated with FAO activity and NADPH and ATP levels. Second, NKX2–8 transcriptionally repressed key components of the FAO cascade, such as CPT1A and CPT2, by recruiting the Sin3A/HDAC1/SAP18 transcriptional repression complex. Third, the PDX model indicated that inhibition of FAO was sufficient to sensitize NKX2–8-deleted EOC, but not NKX2–8-nondeleted EOC, to chemotherapy, resulting in longer overall and relapse-free survival. Therefore, our results uncover a novel molecular mechanism for FAO hyperactivation in chemoresistant EOC, and suggest that combined Perhexiline and chemotherapy could be a tailored treatment for patients with NKX2–8-deleted EOC.

Hyperactivation of the FAO pathway was recently reported to contribute to cancer metastasis and the maintenance of stem cells and cancer stem cells. For instance, activation of FAO via promyelocytic leukemia (PML) or antioxidants dramatically precluded the loss of matrix attachment-induced anoikis in breast cancer cells [43,44]. Metastatic EOC cancer cells exhibit elevated FAO activity [9,39]. Meanwhile, inhibiting FAO affected leukemia-initiating cells (UCs) and hematopoietic stem cells (HSCs), which resulted in the exhaustion of the stem cell pool [38,45–48]. These studies provided fundamental insights into activated FAO in cancer progression and tumorigenesis. Interestingly, a previous study reported that heterozygous or homozygous NKX2–8-knockout mice had bronchial hyperplasia at birth, which developed to lung carcinoma and progressed to invasive lung cancer latterly. Meanwhile, the NKX2–8-knockout mice also exhibited increased numbers of putative tracheobronchial stem/progenitor cells throughout life [21]. However, the mechanism by which loss of NKX2–8 induced tumorigenesis, stem cell proliferation and cancer invasion/metastasis is remains unclear. In the current study, we demonstrated that loss of NKX2–8 induced FAO activity by directly regulating FAO components and inhibiting FAO remarkably enhanced the efficacy of chemotherapeutic drugs in NKX2–8-deleted EOC. Therefore, we speculate that FAO activation might also contribute to NKX2–8 loss-induced tumorigenesis and invasion/metastasis, which is under investigation in our laboratory.

Another key finding in the current study is the mechanism by which NKX2–8 mediates transcriptional repression. It has been reported that NKX2–8 inhibits the epithelial-mesenchymal transition phenotype of bladder urothelial carcinoma cells via transcriptionally repressed Twi1 expression via directly targeting to the Twi1 promoter region [49]. Our previous study also showed that NKX2–8 directly associated with the AKIP1 promoter and transcriptionally repressed the expression of AKIP1, which resulted in inhibition of NF-κB signalling pathway [19]. However, the molecular mechanism underlying NKX2–8-mediated transcriptional repression remains incompletely understood. Herein, we demonstrated that NKX2–8 transcriptionally repressed CPT1A and CPT2 via directly recruiting Sin3A/HDAC1/SAP18 transcriptional repressor complex to the promoters of CPT1A and CPT2. SIN3 complex-mediated repression of target genes was through recruiting the histone deacetylase 1/2 (HDAC1/2), which, resulted in deacetylation of histone H3 and H4 and blocking transcription [50–52]. For instance, Ji and colleagues also reported that the SIN3A-HDAC complex transcriptionally repressed CDKN1A by increasing levels of deacetylated H3 and H4.

Consistently, we found that enrichment of H3K27ac on the promoters of CPT1A and CPT2 was decreased in NKX2–8/−/− cells but increased in NKX2–8/−/+ EOC cells, and silencing SIN3A-HDAC complex significantly reduced the level of H3K27ac in the promoters of CPT1A and CPT2 genes, suggesting the important role of Sin3A/HDAC1/SAP18 in NKX2–8-mediated transcriptional repression of CPT1A and CPT2. Therefore, the current study reveals a novel molecular mechanism of NKX2–8-mediated transcriptional repression of FAO signalling in EOC.

In conclusion, our findings reveal a precise mechanism by which loss of NKX2–8 activates the FAO signalling pathway and confers chemoresistance in EOC. A combination treatment using an FAO inhibitor and CDPP might serve as a targeted therapy option for NKX2–8-deleted EOC. Since NKX2–8 deletion was tightly associated with chemoresistance and shorter relapse-free survival of patients in multiple distinct cancer types via TCGA dataset analysis, which promoted us to investigate currently whether NKX2–8 deletion can also confer chemoresistance in other cancer types via activation of the FAO pathway. In this scenario, inhibition of the FAO pathway using a pharmacological inhibitor might serve as a novel and efficient approach to counterbalance NKX2–8 deletion-mediated drug resistance. Therefore, our results demonstrated that NKX2–8 might have the potential role to assist with prognosis evaluation and personalized treatment selection in multiple types of cancer.

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Declaration of interests

The authors declare no conflicts of interest.

Author contributions

Concept and design of study: Jinrong Zhu, Geyan Wu, Libing Song and Jun Li; Performed the xenograft tumor experiments: Jinrong Zhu, Geyan Wu, Lixue Cao; Performed the in vitro cell line studies: Jinrong Zhu, Geyan Wu, Lixue Cao, Zhanhao Tan; Designed and established the mouse models: Zhang, Zhiwen Li, Dongni Shi, Shuxia Zhang; Wrote the manuscript with input from the other authors: Jun Li.

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