MOLECULAR CANCER RESEARCH | CELL FATE DECISIONS

A TAZ–ANGPTL4–NOX2 Axis Regulates Ferroptotic Cell Death and Chemoresistance in Epithelial Ovarian Cancer
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ABSTRACT

Ovarian cancer is the deadliest gynecologic cancer. Despite recent advances, clinical outcomes remain poor, necessitating novel therapeutic approaches. To investigate metabolic susceptibility, we performed nutrigenetic screens on a panel of clear cell and serous ovarian cancer cells and identified cystine addiction and vulnerability to ferroptosis, a novel form of regulated cell death. Our results may have therapeutic potential, but little is known about the determinants of ferroptosis susceptibility in ovarian cancer. We found that vulnerability to ferroptosis in ovarian cancer cells is enhanced by lower cell confluency. Because the Hippo pathway effectors Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) are recognized as sensors of cell density, and TAZ is the predominant effector in the tested ovarian cancer cell lines, we investigated the role of TAZ in ferroptosis of ovarian cancer. TAZ removal confers ferroptosis resistance, while TAZS89A overexpression sensitizes cells to ferroptosis. In addition, we found that lower TAZ level in chemo-resistant recurrent ovarian cancer is responsible for reduced ferroptosis susceptibility. The integrative genomic analysis identified ANGPTL4 as a direct TAZ-regulated target gene that sensitizes ferroptosis by activating NOX2. Collectively, cell density–regulated ferroptosis in ovarian cancer is mediated by TAZ through the regulation of the ANGPTL4–NOX2 axis, suggesting therapeutic potentials for ovarian cancers and other TAZ-activated tumors.

Implications: This study reveals that TAZ promotes ferroptosis in ovarian cancers by regulating ANGPTL4 and NOX, offering a novel therapeutic potential for ovarian tumors with TAZ activation.

Introduction
The impact of ovarian cancer and the critical need for novel therapeutics

Epithelial ovarian cancer is the deadliest gynecologic cancer and claims the lives of approximately 150,000 women every year worldwide (1). The symptoms of ovarian cancer are vague and often attributed to other more common ailments. As a result, the correct diagnosis usually only occurs after cancer has spread beyond the ovaries. Standard therapy involves surgical debulking followed by chemotherapy with a platinum-taxane doublet (2). Although many patients initially respond favorably to this combined treatment, most patients relapse with a recurrent disease that is often resistant to platinum and taxane drugs. Other chemotherapeutic options are used mainly in an effort to prolong survival. Recently, platinum-PARP inhibitor combinations have been proven to be beneficial for ovarian cancer regardless of the BRCA1/2 mutation status (3, 4). However, the outcomes for most women with ovarian cancer are still unsatisfactory, therefore novel therapeutic options are still urgently needed.

Ferroptosis as a novel cell death involving lipid peroxidation

One possible therapeutic approach is the induction of ferroptosis, a novel and distinct form of iron-dependent programmed cell death (5, 6). Ferroptosis sensitivity is found to be affected by various biological processes, such as loss of p53 (7), DNA damage pathway (8), metabolisms (9–11), or epithelial–mesenchymal transition (EMT; refs. 12, 13), which are often dysregulated in ovarian cancer. Ferroptosis can be induced by the small molecule, erastin (14), that reduces cellular glutathione levels. Glutathione is a cofactor for glutathione peroxidase (GPX4), an enzyme that resolves the accumulation of lipid-based reactive oxygen species (ROS). Therefore, ferroptosis and lipid peroxidation can also be induced by chemical or genetic inhibition of GPX4 (6). A previous study has indicated that the levels of GPX4, regulated by the EMT-activator ZEB1, may dictate ferroptosis sensitivity of drug-resistant cancer cells, implicating GPX4 as a major determinant of ferroptosis (12, 13). On the other hand, accumulation of lipid-based ROS and ferroptosis can also be induced by the generation of superoxide and hydrogen peroxide upon upregulation of NADPH oxidases (NOX; ref. 5).

In this study, we perform a nutrigenetic screen and show that most ovarian cancer cell lines are addicted to cystine and sensitive to ferroptosis. Furthermore, we found that ferroptosis susceptibility of ovarian cancer cells is affected by cell density. Low density, but not high-density ovarian cancer cells, were highly susceptible to erastin-induced ferroptosis. The density-dependent phenotypes of cancer cells are sensed and regulated by the evolutionarily conserved Hippo pathway (15) converging into two transcriptional coactivators, Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). YAP/TAZ activities are regulated by their phosphorylation and intracellular localization. When grown at high cell density, YAP/TAZ are phosphorylated, retained in the cytosol, and subjected to proteasomal degradation. Upon shifting to low cell density, YAP/TAZ become dephosphorylated and translocate into the nucleus to associate with transcriptional factors to drive

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Mol Cancer Res 2020;18:79-90
doi: 10.1158/1541-7786.MCR-19-0691
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AACRJournals.org | 79
Yang et al.

gene expression regulating cell proliferation, differentiation, and migration (16). Recent studies have also identified the novel role of YAP and TAZ in regulating ferroptosis (17, 18). However, the relevance of these findings for ovarian cancer remains unknown. Here, we have established the role of cell density and TAZ in regulating ferroptosis of ovarian cancer. In addition, we found that TAZ regulates erastin-induced ferroptosis through the induction of ANGPTL4, which in turn activates NOX2, resulting in ferroptosis. Thus, these data support the role of TAZ in regulating ferroptosis through ANGPTL4–NOX2 and that inducing ferroptosis may be a novel therapeutic strategy for ovarian cancer and other TAZ-activated tumors.

Materials and Methods

Materials and reagents

Erastin was obtained from the Duke University Small Molecule Synthesis Facility. The following antibodies, their catalog numbers, sources, and dilutions were indicated below: YAP/TAZ (#8418; Cell Signaling Technology, 1:1,000), β-tubulin (#86289; Cell Signaling Technology, 1:2,000), vinculin (sc-73614; Santa Cruz Biotechnology, 1:2,000), V5 tag (#13202; Cell Signaling Technology, 1:2,000), H3 (#4499; Cell Signaling Technology, 1:2,000), GAPDH (sc-25778; Santa Cruz Biotechnology, 1:2,000), ANGPTL4 (#40-9800; Thermo Fisher Scientific, 1:1,000), NOX2 (sc-130543; Santa Cruz Biotechnology, 1:1,000), anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (#7074; Cell Signaling Technology, 1:2,000–1:4,000), and anti-mouse IgG, HRP-linked Antibody (#7072; Cell Signaling Technology, 1:2,000–1:4,000). Plasmids were obtained from Addgene (TAZ5891A #52084; ANGPTL4-V5 #102446). The NOX2 inhibitor, gp91 des-tat, was purchased from Eurogentec (catalog no. A7S89A #52084; ANGPTL4-V5 #102446). The NOX2 inhibitor, VAS2870 (Calbiochem-492000), and recombinant human ANGPTL4 protein was purchased from Novus Biologicals (#4987–AN9). VAS2870 (Calbiochem-492000), GKT136901 (Calbiochem-534032), Z-VAD-FMK (SML0583), ferrostatin-1 (SML0583), and liproxstatin-1 (SML1414) were purchased from Calbiochem and Novus Biologicals.

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Cell culture and transfection

Cell lines were maintained as part of the cell line repository within the Division of Reproductive Sciences at Duke University. For the details, please refer to ref. 17. Short tandem repeat profiling was performed at the Duke University DNA Analysis Facility each time new stocks were prepared; they were last genotyped on June 5, 2018. Mycoplasma testing was conducted at the Cell Culture Facility, Duke University on June 5, 2018. For nutrigenetic screens, all ovarian cancer cell lines were cultured in a humidified incubator at 37°C and 5% CO2 using custom-made DMEM (11995-DMEM; Thermo Fisher Scientific) with 10% heat-inactivated and dialyzed FBS (HyCloneTM FBS; GE Healthcare Life Sciences, #SH30070.03HI) with the indicated amino acid removed as described previously (19, 20). Transfections were performed according to the manufacturer’s instructions with TransIT-LT1 transfection reagent (Mirus Bio) or RNAiMax transfection reagent (Thermo Fisher Scientific).

siRNA-mediated gene knockdown

All human siRNAs were purchased from Dharmacon or Qiagen: nontargeting control, siNT (Qiagen, SI03650318); siITAZ (Dharmacon, M-016083); siANGPTL4 (Dharmacon, M-007807); siNOX2 (Dharmacon, M-011022); siTAZ#1 (target sequence: AGA CAT GAG ATC CAT CAC TAA); siTAZ#2 (target sequence: ACA GTA GTA CCA AAT GCT TTA); siANGPTL4 #1 (target sequence: CTG CGA ATT CAG CAT CTT CAA); siANGPTL4 #2 (target sequence: CAT GTT GAT CCA GCC CAT); siNOX2 #1 (target sequence: GAA GAC AAC TGG ACA GGA A); and siNOX2 #2 (target sequence: GAA ACT ACC TAA GAT AGC G). Knockdown efficacy was validated by qRT-PCR and/or Western blot analysis.

RNA isolation and qRT-PCR

Total RNAs from cultured cells were extracted using the RNeasy Mini Kit (Qiagen, #74104) with DNase I treatment (Qiagen, #79254). cDNAs were synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, #18064) with random hexamers following protocols from the manufacturer. The levels of gene expression were measured by qPCR with Power SYBR Green PCR Mix (Applied Biosystems; Thermo Fisher Scientific, #4367659).

Primers used included (listed 5’–3’): β-actin-F: GGG GTG TTG AAG GTG TCA AA; β-actin-R: GCC ATC GTC ACC CTG AAG TA; TAZ-F: TGC TAC AGT GTC CCC AGA AC; TAZ-R: GAA ACG GGT CTG TTG GGA AT; ANGPTL4-F: GCC TCA GTG GAC TTC AAC CG; ANGPTL4-R: CCG TGA TGC TAT GCA CCT TGT; NOX2-F: TGG AGT TGT CAT CAC GCT GTG; NOX2-R: CTG CCC ACG TAC AAT TCG TTC; and 18S-F: CTG GAT ACC GCA GCT AGG AA; 18S-R: CCA TCT TAA TCA TCA TGG CCT CA.

Western blot analysis

For immunoblotting, please refer to previous publication for details (17). In short, cells were collected and quantified by BCA protein assay. After separating by SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes that were further blocked with 5% nonfat milk or BSA and probed with indicated antibodies following by HRP-conjugated secondary antibodies. The signals were developed and detected by Amersham ECL prime Western blotting detection reagent and the Bio-Rad ChemiDoc Imaging System.

Cell viability and cytotoxicity assays

After seeded and transfected with indicated siRNAs for 2 days, cells (~60–70% confluence) were treated with erastin or cysteine deprivation for another 24 to 48 hours. Cell viability was evaluated using crystal violet staining or the CellTiter Glo Luminescent Cell Viability Assay Kit (Promega G7571) according to the manufacturer’s instructions. The CellTiter Glo Luminescent Cell Viability Assay is based on quantitation of the cellular ATP levels as an indicator of metabolically active cells and cellular viability. The cytotoxicity and cell death of treated cells were determined by CytoTox-Fluor Cytotoxicity Assay (Promega G9260), which measured the released DNA as indicator of cell death according to the manufacturer’s instructions.

ELISA

Quantification of secreted ANGPTL4 in the culture media from cells following a 2-day incubation was performed by Human ANGPTL4 ELISA Kit (RAB0017; Sigma-Aldrich) according to the manufacturer’s instructions.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP)–qPCR experiment was carried out according to the Myers Lab ChIP-seq protocol (21). Please refer to the previous publication for the experimental details (17). Primers targeting chromosome 10 was used as a negative control for potential nonspecific binding (22). Primer sequences are as follows (listed 5’–3’): CTGF-F: GCC AAT GAG CTG AAT GGA GT; CTGF-R: CAA TCC GGT GTG AAT TGA TG; ANGPTL4-F: GTC TCC CAC GGT TCG TAG AG; ANGPTL4-R: TAT AAG TTG GGT GCG
GAG TGG; and Ch10-F': ACC AAC ACT CTT CCC TCA GC; Ch10-R': TTA TTT TGG TTC AGG TGG TTG A.

**Lipid ROS assay using flow cytometry**

Ten μmol/L of C11-BODIPY dye (D3861; Thermo Fisher Scientific) was used for lipid ROS staining according to the manufacturer’s instructions. Please refer to the previous publication for the experimental details (17). In short, cells were collected after siRNAs treatments for 2 days, 10 μmol/L erastin treatment for 18 hours, and 10 μmol/L C11-BODIPY-containing medium for 1 hour and the ROS levels were determined by flow cytometry analysis (FACSCanto II; BD Biosciences).

**Statistical analyses**

The results were evaluated with two-tailed Student t test or ANOVA (one- or two-way) using GraphPad Prism version 8.0.1 (GraphPad Software). Statistically significant differences were set to P < 0.05 between experimental groups (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The statistical data were represented as mean ± SEM. The number of biological replicates is listed in each figure.

**Data availability**

RNA-sequencing (RNA-seq) for transcriptional profiles of CAOV2 primary and CAOV2 recurrent cells (GSE133663) and CAOV2 ovarian cancer cells with TAZ silencing (GSE133664) have been deposited in the NCBI Genome Expression Omnibus under SuperSeries GSE 133673.

**Results**

**Ovarian cancer cells are sensitive to cystine deprivation**

Many tumor cells have dysregulated metabolisms that render them addicted to certain nutrients, such as amino acids (23). To reveal such nutrient addiction in ovarian cancers, we established a nutrigenetic screen by removing glucose or each of the 15 individual amino acids usually included in DMEM (19, 24). The nutrigenetic screen was...
applied to a panel of eight ovarian cancer cell lines including four cell lines of clear-cell subtype (TOV-21G, ES-2, RMG-2, and RMG-V) and four cell lines of serous subtype (OVCA432, OVCA429, OVCA420, and 41M). The screen revealed that the removal of cystine, the dimeric form of cysteine, dramatically reduced the viability of most clear-cell and serous subtypes of ovarian cancers (Fig. 1A). Glutamine or glucose deprivation also decreases cell viability, especially in the clear-cell subtype, reminiscent of the glutamine addiction of basal-type breast cancer cells (25). Knowing that cystine deprivation is reported to trigger ferroptosis (5), we determined whether ferroptosis inhibitors can rescue the cell death triggered by cystine deprivation. Both liproxstatin-1 and ferrostatin-1, two ferroptosis inhibitors, significantly rescued cystine deprivation-induced cell death of TOV-21G cells (Fig. 1B and C), indicating that cystine deprivation indeed induces ferroptosis. We further confirm that the erastin-induced cell death can be rescued by ferrostatin-1, but not apoptosis inhibitor, Z-VAD-FMK (Fig. 1D). Therefore, we used erastin, a canonical inducer of ferroptosis, to investigate the ferroptosis mechanisms of ovarian cancer in our subsequent studies.

**Cell density affects the sensitivity of ovarian cancers to erastin-induced ferroptosis**

While investigating ferroptosis in ovarian cancer cells, we observed that cell density affects the ferroptosis sensitivity of CAOV2, a serous ovarian cancer cell line. When CAOV2 cells were plated at low density, they were highly sensitive to erastin based on CellTiter Glo assay (Fig. 2A) and crystal violet staining (Fig. 2B; Supplementary Fig. S1A). On the other hand, when plated at higher densities, CAOV2 cells were much less sensitive to erastin-induced death using CellTiter Glo and crystal violet assays (Fig. 2A and B; Supplementary Fig. S1A). To exclude the possibility that these differences are caused by different levels of available erastin per cell, the same number of TOV-21G cells were seeded in larger or smaller areas to recreate lower or higher cell densities. We found that cells plated at lower cell density were consistently more sensitive to ferroptosis (Supplementary Fig. S1B). Therefore, cell density impacts ferroptosis sensitivity.

Because the two closely related Hippo pathway paralogues, YAP/TAZ, sense and mediate cell density-dependent responses (15), we investigated the potential role of YAP/TAZ in regulating ferroptosis in ovarian cancers. Among the two paralogues (YAP and TAZ), Western blot analysis reveals that TAZ is the predominantly expressed protein in both TOV-21G (clear cell subtype) and CAOV2 cells (serous subtype; Fig. 2C). In contrast, YAP is the predominant Hippo effector protein in the breast cancer cell, MDA-MB-231, which serves as a control for YAP expression and detection (Fig. 2C). We further performed cytosolic and nuclear fractionations of CAOV2 cells under low or high cell densities. Consistently, TAZ is the main protein whose nuclear levels were elevated when grown in low cell density (Fig. 2D). Therefore, we focused on the potential role of TAZ as the major effector of the Hippo pathway in regulating density-dependent ferroptosis in ovarian cancers.

**TAZ regulates sensitivity to erastin-induced ferroptosis**

To test the possibility that TAZ regulates susceptibility of ovarian cancer cells to erastin, we first reduced TAZ expression (>85% knockdown) by siRNAs in CAOV2 cells (Supplementary Fig. S1C and S1D). Knockdown of TAZ by pool siRNA as well as two more independent siRNAs all reduce erastin-induced cell death based on CellTiter Glo assays (Fig. 2E). To determine whether TAZ also regulates ferroptosis in TOV-21G cells, we found that TAZ knockdown by siRNAs reduces sensitivity to erastin (Fig. 2F; Supplementary Fig. S1E). Collectively, these data strongly indicate that TAZ regulates the sensitivity of ovarian cancer cells to erastin-induced ferroptosis.

**Resistance to ferroptosis following treatment with carboplatin in vivo**

We performed our experiments in CAOV2 because we have established paired CAOV2 xenograft cells from a mouse model of ovarian cancer that mimics the course of disease (26). In short, the CAOV2 cells were stably transduced with a construct containing GFP and luciferase (pGreenFire plasmid constructs, SBI) to generate CAOV2-GFP/LUC cells, allowing us to monitor tumor growth using *in vivo* bioluminescence imaging. CAOV2-GFP/LUC cells (3.5 × 10⁶ per mouse) were injected intraperitoneally into female nude mice with tumor formation monitored weekly using the IVIS in *in vivo* imaging system. Once tumor formation was evident, we initiated treatment with carboplatin (60 mg/kg i.p. twice a week for 2 weeks) and monitored tumor volume based on the luciferase flux. In this model, tumor signal was reduced following carboplatin treatment, but then recurred approximately 2 to 4 weeks after carboplatin treatment had stopped. Therefore, we refer to the residual tumor cells that eventually emerged after carboplatin treatment as CAOV2R (recurrent). We then compared cystine dependency (Fig. 3A and B) and erastin sensitivity (Fig. 3C and D) between the CAOV2 and CAOV2R cells. These experiments revealed that CAOV2R was more resistant to ferroptosis based on crystal violet staining (Fig. 3A and C), cytotoxicity assay (Fig. 3B), and CellTiter Glo viability assay (Fig. 3D). Because TAZ regulates ferroptosis sensitivity (Fig. 2), we compared TAZ protein abundance in CAOV2 and CAOV2R cells and found that CAOV2R contained a lower level of TAZ protein (Fig. 3E). We, therefore, overexpressed the constitutively active TAZS89A (27) in the CAOV2R cells (Fig. 3F) and found that increased TAZ significantly sensitized the CAOV2R cells to erastin (Fig. 3G). These findings suggest that comparisons of CAOV2 versus CAOV2R can be used to elucidate the molecular mechanisms of TAZ-regulated ferroptosis.

**ANGPTL4 is a direct target gene of TAZ that regulates sensitivity to ferroptosis**

TAZ is a transcriptional coactivator that affects cellular phenotypes through regulating the expression of target genes upon association with transcriptional factors such as transcriptional enhanced associate domain (TEAD). Assuming that knockdown of TAZ may repress the relevant target genes essential for ferroptosis, we determined the transcriptional response to knockdown of TAZ in CAOV2 by RNA-seq (Supplementary Fig. S2A; GSE133673). Next, we integrated these TAZ-affected genes with the genes that were downregulated in CAOV2R when compared with CAOV2 (Fig. 4A) to identify 1,179 candidate genes. To identify the TAZ-regulated genes that are essential for ferroptosis across different cells, we further narrowed the list of candidate genes by comparing with the gene lists identified from our renal cell studies through both RNAi screen and TAZ-affected genes (Supplementary Fig. S2B; ref. 17). Among the 3 candidate genes from these analyses, ATP6V1B2 was not pursued because it was only one subunit of a multicomponent protein complex. The knockdown of GPSM3 did not confer erastin resistance (Supplementary Fig. S2C and S2D). Therefore, Angio-poietin-Like 4 (*ANGPTL4*) emerged as the most promising candidate gene based on the following evidence. First, *ANGPTL4* mRNA was downregulated upon TAZ knockdown, which was confirmed by

82 Mol Cancer Res; 18(1) January 2020

MOLECULAR CANCER RESEARCH
qRT-PCR (Fig. 4B). Second, there is a significant correlation between the expression of ANGPTL4 and TAZ (encoded by WWTR1) in the TCGA ovarian tumor data set. The expression of WWTR1 is also consistently correlated with CTGF (28) and CYR61 (29), 2 canonical YAP/TAZ target genes (Supplementary Fig. S2E). Third, the knockdown of ANGPTL4 in both CAOV2 and TOV-21G protects cells from erastin-induced death (Fig. 4C and D; Supplementary Fig. S2H and S2I). This protective effect was further validated by using two more independent ANGPTL4-targeting siRNAs in CAOV2 cells (Fig. 4E; Supplementary Fig. S2J). Consistently, overexpression of V5-tag ANGPTL4 sensitized TOV-21G cells to ferroptosis (Fig. 4F; Supplementary Fig. S2K). Collectively, these data indicate that ANGPTL4 is a prominent TAZ-regulated determinant of ferroptosis in ovarian cancer.

Next, we analyzed previous ChIP-seq studies and found the regulatory regions of ANGPTL4 were physically associated with YAP/TAZ/TEAD complexes (22, 30). To further validate that ANGPTL4 is a direct target gene of TAZ, we performed ChIP-qPCR using an antibody specific for endogenous TAZ protein. As shown in Fig. 4G, we found that the promoter region of ANGPTL4, similar to the CTGF (positive control), was enriched in the TAZ pull-down, indicating that the ANGPTL4 promoter is associated with TAZ. Thus, ANGPTL4 is a direct downstream target gene of TAZ, which may contribute to TAZ-regulated ferroptosis.

Figure 2.
TAZ regulates the sensitivity of CAOV2 to erastin-induced ferroptosis. A, The relative cell viability of CAOV2 cells grown at low/medium/high (4,000/8,000/16,000 cells per 96-well) cell densities after treated with indicated concentrations of erastin. Data are represented as mean ± SEM with three biological replicates per group; two-way ANOVA (***, P < 0.001). B, Crystal violet staining of CAOV2 cultured at low/medium/high (1.6 × 10⁵/3.2 × 10⁵/6.4 × 10⁵ cells per 6-well) cell densities and treated with 16 μM/L erastin. C, Western blot analysis of YAP and TAZ proteins in the ovarian cancer cells (TOV-21G, CAOV2, and CAOV2R) and one breast cancer cell line (MDA-MB-231). Representative data of at least three independent experiments. D, Western blot analysis measuring YAP and TAZ protein levels in the cytosolic (Cyto)/nucleic (Nuc) fractions of CAOV2 extracts when grown at low (L) or high (H) cell density. Histone H3 and GAPDH serve as nuclear and cytosolic marker, respectively. E, The relative cell viability of CAOV2 cells after silencing of control (siNT), TAZ (siTAZ), or two individual TAZ-targeting siRNAs (siTAZ #1 and siTAZ #2) for 1 day before they were treated with indicated dosages of erastin for 2 days (n = 2; mean ± SEM; two-way ANOVA; ***, P < 0.001). F, The relative cell viability of TOV-21G cells after silencing of control (siNT) or TAZ (siTAZ) for 2 days before they were treated by indicated dosages of erastin treatment for 1 day (n = 3; mean ± SEM; two-way ANOVA; ***, P < 0.001). See also Supplementary Fig. S1.
We next determined whether the differential expression of ANGPTL4 could explain the different ferroptosis sensitivity between CAOV2 and CAOV2R cells. From the results of qRT-PCR and Western blot analysis, we found CAOV2R has a lower ANGPTL4 expression at both mRNA level and protein level (Fig. 5A and B). Because ANGPTL4 encodes a secreted protein, we also examined the level of extracellular ANGPTL4 in the culture media by ELISA. Consistently, extracellular ANGPTL4 proteins are less abundant in the CAOV2R cells as compared with the CAOV2 cells (Fig. 5C). To test the possibility that the lower expression of ANGPTL4 in CAOV2R may contribute to its relative ferroptosis resistance, we determined whether the addition of recombinant ANGPTL4 proteins may enhance the ferroptosis sensitivity of CAOV2R. As shown in Fig. 5D, soluble ANGPTL4 sensitized CAOV2R, but not CAOV2, cells to ferroptosis.

**ANGPTL4 regulates ferroptosis through NOX2**

With regard to the mechanistic link between ANGPTL4 and ferroptosis, it is interesting to note that ANGPTL4 has been reported to activate the NADPH oxidase 1, NOX1, in the keratinocyte...
ANGPTL4 is a direct target gene of TAZ that regulates ferroptosis sensitivity. A, Venn diagram showing genes that are both downregulated upon TAZ knockdown in CAOV2 cells (3,370 + 1,179 genes) and downregulated genes (2,540 + 1,179 genes) in CAOV2R cells compared with CAOV2 cells by RNA-seq. B, Validation of downregulated ANGPTL4 mRNA level by qRT-PCR upon TAZ knockdown (* n = 3; mean ± SEM; Student t test; ** P < 0.05). The relative cell viability of CAOV2 (C) and TOV-21G (D) cells after knockdown of ANGPTL4 following by treating with indicated dosages of erastin. Data are represented as mean ± SEM, n = 3 biological replicates (** P < 0.001). E, The relative cell viability of CAOV2 cells after silencing of ANGPTL4 by two individual siRNAs for 2 days before treated with indicated dosages of erastin for 1 day. Data are represented as mean ± SEM, n = 3 biological replicates (** P < 0.001). F, The relative cell viability of TOV-21G cells after overexpression of V5-tagged ANGPTL4, followed by treating indicated dosages of erastin. Data are represented as mean ± SEM, n = 3 biological replicates (** P < 0.001). G, The relative levels of TAZ-associated genomic DNA in the indicated promoters associated with endogenous TAZ protein using ChIP-qPCR with TAZ antibody in CAOV2 protein lysate. CTGF promoter serves as a positive control for a TAZ target gene, whereas Ch10 serves as a negative control. See also Supplementary Fig. S2.
carcinoma cells (31). The NADPH oxidases are recognized to generate ROS (32), promoting lipid peroxidation and ferroptosis (5). Therefore, we investigated the expression levels of all seven members of the NOX protein family (NOX1-5 and DUOX1-2) in ovarian cancer. Among different NOXs, we found that NOX2 is the most abundantly expressed member from the analysis of RNA-seq data from the TCGA data set (Supplementary Fig. S3A). We also noticed NOX2 protein was lower in CAOV2R when compared with CAOV2 cells (Supplementary Fig. S3B). Therefore, we focused on the role of NOX2 protein. First, we found a pan NOX inhibitor, VAS2870, protected CAOV2 cells from ferroptosis (Fig. 6A). Another NOX inhibitor, GKT136901, also protected cells from ferroptosis (Fig. 6B). We further used a NOX2-specific inhibitor, gp91dstat peptide, in the CAOV2 cells and found it protected ferroptosis as well (Fig. 6C). In addition, silencing of NOX2 by siRNA also conferred ferroptosis resistance in both CAOV2 cells (Fig. 6D; Supplementary Fig. S3C) and TOV-21G cells (Fig. 6E; Supplementary Fig. S3D). To examine the relevance of TAZ–ANGPTL4–NOX2 for the varying ferroptosis sensitivity between CAOV2 and CAOV2R, we found that gp91dstat protected CAOV2, but not CAOV2R, from ferroptosis (Fig. 6F). Furthermore, NOX inhibitor GKT136901 also abolishes the ferroptosis-sensitizing effect when TAZS89A was overexpressed in CAOV2R cells (Fig. 6G). Therefore, NOX2 activity is essential for ferroptosis regulation by TAZ. Finally, we measured the lipid-based reactive oxygen species (lipid ROS), the hallmark of ferroptosis, by C11-BODIPY staining and validated that knockdown of TAZ, ANGPTL4, or NOX2 decreases the erastin-induced lipid peroxidation (Fig. 7A). Taken together, we propose a signaling mechanism (Fig. 7B) by which TAZ is a cell density–dependent determinant of ferroptosis sensitivity in ovarian cancer through regulating levels of ANGPTL4, which in turn regulates NOX2 activity and ferroptotic death.

Discussion

Here, we employed nutrigenetic screens and identified cystine addiction and ferroptosis susceptibility of ovarian cancer cells,
Figure 6.
ANGPTL4 regulates ferroptosis through NOX2. A–E, The relative cell viability of erastin-treated CAOV2 cells (A–D) or TOV-21G cells (E) when NOXs was inhibited by 20 μmol/L pan-NOX inhibitor VAS2870 (A); 20 μmol/L NOX inhibitor, GKT136901 (B); specific NOX2 inhibitor, gp91dstat (33 μg/mL; C); or pooled siRNAs (siNOX2) and/or two independent NOX2-targeting siRNAs (siNOX2 #1 and #2; D–E, n = 3, mean ± SEM, two-way ANOVA; ***, P < 0.001). F, The relative cell viability of CAOV2 or CAOV2R cells was determined by CelltiterGlo after 24 hours of 8 μmol/L erastin with or without NOX2 inhibitor, gp91dstat (33 μg/mL). Data are represented as mean ± SEM, n = 3 after normalized to the DMSO controls (two-way ANOVA; **, P < 0.01; *** P < 0.001; ns, not significant). G, The relative cell viability of CAOV2R expressing control vector or TAZS89A was determined by CelltiterGlo after 24 hours of 10 μmol/L erastin with or without 20 μmol/L NOX inhibitor, GKT136901. Data are represented as mean ± SEM, n = 6 after normalized to the DMSO controls (two-way ANOVA; ***, P < 0.001). See also Supplementary Fig. S3.
implying that ferroptosis-inducing agents may hold therapeutic potential for ovarian cancer. However, little is known about ferroptosis in ovarian cancer other than being briefly mentioned in two recent articles (33, 34) without mechanistic investigation. By studying how cell density regulates ferroptosis sensitivity, as described for other cancer types, we have elucidated the role of TAZ in regulating ferroptosis through the ANGPTL4–NOX2 axis in ovarian cancer.

This study enhances our understanding of the role of TAZ and how it regulates ferroptosis in ovarian cancer.

YAP/TAZ and other components of the Hippo signaling pathway are important in oncogenesis and migration of ovarian cancer cells (35). In our experiments, TAZ, rather than YAP, is the dominant effector in the tested ovarian cancer cells. Although the role of YAP was the first recognized, many studies have also supported the role of TAZ in regulating ferroptosis.
in ovarian cancer. For example, increased expression of TAZ mRNA is correlated with poor prognosis and TAZ manipulation affects migration, proliferation, treatment response, and EMT of ovarian cancer (36, 37). Therefore, our findings suggest that inducing ferroptosis will be an effective strategy for eradicating TAZ-activated tumors, which are particularly aggressive and resistant to current standard treatments. In the future, it will be interesting to test whether YAP or other regulators of the Hippo pathway also play a role in regulating ferroptosis.

ANGPTL4 is a member of the angiopoietin family and the members of which act as regulators of lipid and glucose metabolism (38, 39). ANGPTL4 also plays a role in tumor biology. ANGPTL4 is upregulated in several human cancers and this is associated with metastasis and poor outcome (40, 41). In addition, ANGPTL4 is induced by various oncogenic pathways to promote angiogenesis, invasion, and metastasis (42). Especially relevant is a study that ANGPTL4 stimulates oncogenic ROS and anoikis resistance through the activation of NADPH oxidase (31). Our current study reveals that ANGPTL4 is a direct transcriptional target of TAZ, consistent with the repression of ANGPTL4 by the TAZ/YAP inhibitor, verteporfin (43). On the basis of the previous studies, ANGPTL4 is expected to increase ROS and predispose cells to signal that induces ferroptosis. Therefore, our findings implicate high ANGPTL4 levels and anoikis resistance as novel and highly relevant oncogenic properties that can be targeted by inducing ferroptosis. Because anoikis resistance is essential for tumor metastasis (44), our results imply that ferroptosis may target tumor cells at different stages of tumor progression. Anoikis resistance is one of the phenotypic changes that occur during EMT (45, 46). Our findings are also in agreement with other studies showing increased ferroptosis sensitivity during EMT that is in part due to the regulation of GPX4 by ZEB1 (12).

We have found that the carboplatin-treated CAOV2R cells are less sensitive to ferroptosis and have a lower level of TAZ. These results seemingly contradict previous reports on the ferroptosis sensitivity of persister cells (12) due to increased GPX4 expression. However, our results are consistent with the data in Hangauer et al. (13), which show cells regrowing at 2 months, similar to the timeframe of carboplatin-treated CAOV2R cells in our studies, are much less responsive to GPX4 inhibitor RSL3-induced cell death. Of course, the discrepancy between results may also be due to the different cell lines used, different cancer drugs, or different ferroptosis inducers in these studies.

Our results may have therapeutic implications. While inducing ferroptosis may have substantial antitumor potential, challenges remain. It is not clear which tumors would respond to ferroptosis-inducing agents. Our results indicate that TAZ-activated tumors may be particularly sensitive to various ferroptosis-inducing therapies. Thus, TAZ activation and induction of its canonical target genes may serve as predictive biomarkers. In addition, it is important to consider the compounds or therapeutic agents that would be most effective for inducing ferroptosis. Although erastin and various GPX4 inhibitors can induce ferroptosis, their toxicity and stability may limit in vivo application and translation potential. One promising agent is recombinant human cyst(e)ine that can trigger ferroptosis by depleting plasma cystine (47). Because its antitumor efficacy and in vivo safety have been demonstrated in murine models for multiple tumors, it may have the potential to trigger in vivo ferroptosis of ovarian cancer and improve clinical outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: W.-H. Yang, C.-K.C. Ding, J.-T. Chi
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.-H. Yang, J. Wu, C.-K.C. Ding, S.K. Murphy
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W.-H. Yang, J. Wu, J.-T. Chi
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Acknowledgments

We thank David Corcoran for RNA seq data analysis and Carole Grenier for technical assistance with experiments. This work was supported by the Duke Cancer Institute Pilot Project, Department of Defense grants (W81XWH-17-1-0143, W81XWH-15-1-0486, and W81XWH-19-1-0842) and NIH (1R01GM124062 and 1R01NS11588) to J.-T. Chi.

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Received July 4, 2019; revised September 17, 2019; accepted October 17, 2019; published first October 22, 2019.

References

Yang et al.


Molecular Cancer Research

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