Frizzled-7 Identifies Platinum-Tolerant Ovarian Cancer Cells Susceptible to Ferroptosis

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Abstract

Defining traits of platinum-tolerant cancer cells could expose new treatment vulnerabilities. Here, new markers associated with platinum-tolerant cells and tumors were identified using in vitro and in vivo ovarian cancer models treated repetitively with carboplatin and validated in human specimens. Platinum-tolerant cells and tumors were enriched in ALDH+ cells, formed more spheroids, and expressed increased levels of stemness-related transcription factors compared with parental cells. Additionally, platinum-tolerant cells and tumors exhibited expression of the Wnt receptor Frizzled-7 (FZD7). Knockdown of FZD7 improved sensitivity to platinum, decreased spheroid formation, and delayed tumor initiation. The molecular signature distinguishing FZD7+ from FZD7− cells included epithelial-to-mesenchymal (EMT), stemness, and oxidative phosphorylation–enriched gene sets. Overexpression of FZD7 activated the oncogenic factor Tp63, driving upregulation of glutathione metabolism pathways, including glutathione peroxidase 4 (GPX4), which protected cells from chemotherapy-induced oxidative stress. FZD7+ platinum-tolerant ovarian cancer cells were more sensitive and underwent ferroptosis after treatment with GPX4 inhibitors. FZD7, Tp63, and glutathione metabolism gene sets were strongly correlated in the ovarian cancer Tumor Cancer Genome Atlas (TCGA) database and in residual human ovarian cancer specimens after chemotherapy. These results support the existence of a platinum-tolerant cell population with partial cancer stem cell features, characterized by FZD7 expression and dependent on the FZD7−/β-catenin−/Tp63−/GPX4 pathway for survival. The findings reveal a novel therapeutic vulnerability of platinum-tolerant cancer cells and provide new insight into a potential “persister cancer cell” phenotype.

Significance: Frizzled-7 marks platinum-tolerant cancer cells harboring stemness features and altered glutathione metabolism that depend on GPX4 for survival and are highly susceptible to ferroptosis.

Introduction

Ovarian cancer is the leading cause of death from female gynecologic cancers. Although initially a highly chemoresponsive tumor (1), most patients with ovarian cancer experience tumor relapse, and recurrent, resistant ovarian cancer is fatal (2). “Persister” or drug-tolerant cells have been described as cells surviving cytotoxic drug exposure (3) and represent a reservoir for the outgrowth of drug-resistant clones. Recent studies in various cancers have reported the molecular signature of “persister” cells, including upregulation of stemness factors, mesenchymal–like gene expression, enrichment in glutathione peroxidase 4 (GPX4) and other genes related to lipid peroxidation, which antagonize ferroptosis, allowing cells to survive after cytotoxic drug exposure (3–5). Small-molecule inhibitors targeting GPX4 were shown to induce lipid peroxidation and eliminate tyrosine kinase receptor inhibitor-tolerant cells through ferroptosis (3). It has been suggested that “persister” cells share characteristics with cancer stem cells (CSC), but also have distinct traits. Specific markers to allow their identification and early targeting remain elusive. Here we sought to characterize the ovarian cancer “persister” phenotype.

Our and other previous studies showed that although chemotherapy is effective at cytoreducing the mass of heterogeneous cancer cells, residual tumors persist and are enriched in CSCs (6, 7). Ovarian CSCs share some of the normal stem cells’ characteristics, including the ability to self-renew, differentiate, express specific stem cell–surface markers (6, 8, 9), and exhibit enhanced tumor initiation capacity (TIC; ref. 10). Importantly, ovarian CSCs possess a phenotype associated with drug resistance, including diminished apoptotic responses, increased efflux mechanisms, and antioxidation defense (4, 8, 11, 12). The boundaries between stemness and platinum-resistant (Pt-R) phenotypes remain blurry and, while an overlap exists, it is assumed that distinct pathways drive the two entities.

As platinum-tolerant (Pt-T) cancer cells drive tumor relapse, we aimed to identify specific markers, by using in vitro and in vivo models of repeated exposure to the cytotoxic agent. We observed that Pt-T cells and tumors contained an increased ALDH+ cell population, expressing stemness-related transcription factors (TF), and able to form more spheroids compared with chemotherapy-naive cells. We identified the Frizzled 7 receptor (FZD7) as a novel cell–surface marker significantly upregulated in the platinum-tolerant cell population. FZD7 knockdown increased sensitivity to Pt, decreased spheroid formation, and inhibited TIC. FZD7+ cells harbored a “persister cell”–like signature, including downregulated genes associated with...
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DNA damage response, upregulated epithelial to mesenchymal (EMT) and stemness, and decreased expression of genes associated with oxidative phosphorylation. Expression of the antioxidant enzyme GPX4 was increased in FZD7- Pt-T cells, rendering them sensitive to treatment with GPX4 inhibitors. Mechanistically, FZD7 caused activation of the transcriptional regulator, Tp63, which drove upregulation of glutathione metabolism genes, protecting cells from oxidative stress. In all, our results support the existence of a “persistent” Pt-T cell population, sharing traits with CSCs, marked by upregulation of the receptor FZD7- and harboring a dependency on FZD7-β catenin–Tp63–mediated GPX4 expression and antioxidant activity.

Materials and Methods

Human specimens

Deidentified high-grade serous ovarian tumors (HGSOC) and associated malignant ascites were collected and processed fresh from patients who provided written informed consent. Tumor tissues were enzymatically disassociated into single-cell suspensions and cultured as previously described (6, 8). A tissue microarray (TMA) was built from deidentified HGSOC specimens (n = 23) from patients who had undergone three to six cycles of platinum-taxane neoadjuvant chemotherapy [Institutional Review Board (IRB)-approved CSR protocol #1247]. Each specimen was entered in duplicate, and fallopian tube epithelium (n = 6) served as control. Patient characteristics are shown in Supplementary Table S1. Human subject studies were conducted in accordance with the Declaration of Helsinki and approved by the IRB (Northwestern University IRB#: STU00202468).

Cell lines and culture conditions

SKOV3 and OVCAR3 cells were purchased from the ATCC. OVCAR5 cells were a generous gift from Dr. Marcus Peter, Northwestern University, Chicago IL; COV362 cells were from Dr. Kenneth Nephew, Indiana University, Indianapolis, IN; immortalized human fallopian tube luminal epithelial cells (FT190) were from Dr. R. Drapkin of University of Pennsylvania, Philadelphia, PA; ref. (13). PEO1 and PEO4 cells were from Sigma-Aldrich. Cell culture conditions are in Supplementary Material. Low-passage cells were used, and all cell lines were tested of Pennsylvania, Philadelphia, PA; ref. (13). PEO1 and PEO4 cells were acquired from Indiana University, Indianapolis, IN; immortalized human fallopian tube epithelium (n = 6) served as control. Patient characteristics are shown in Supplementary Table S1. Human subject studies were conducted in accordance with the Declaration of Helsinki and approved by the IRB (Northwestern University IRB#: STU00202468).

Chemicals and reagents

RSL3 was purchased from Fisher Scientific (cat. #611810). ML210 (cat. #SML0521), cisplatin (cat. # 1134357), and carboplatin (cat. #C2538) were from Sigma-Aldrich. WNT3a was from Fisher Scientific (cat. # 5036WN010CF, R&D Systems), and IWR-1-endo was from Santa Cruz (sc-295215A).

In vitro development of Pt-R cells

To generate Pt-T ovarian cancer cells, SKOV3, OVCAR5, COV362, and OVCAR3 cells were treated with three or four repeated or increasing doses of cisplatin or carboplatin for 24 hours. Surviving cells were allowed to recover for 3 to 4 weeks before receiving the next treatment. Changes in resistance to platinum were estimated by calculating half maximal inhibitory concentration (IC₅₀) values as described below.

In vivo experiments

Animal studies were conducted according to a protocol (#IS00003060) approved by the Institutional Animal Care and Use Committee of Northwestern University and are described in Supplementary Material. Experiments using patient-derived xenograft (PDX) tumors were performed in the Developmental Therapeutics Core of the Lurie Cancer Center, as previously described (14) and following a similar protocol (see Supplementary Material).

Isolation of tumor cells

Tumors from patients or xenografts were minced and enzymatically dissociated in DMEM/F12 (Thermo Fisher Scientific, Ref #11320) containing collagenase (300 IU/mL, Sigma-Aldrich, cat. #C7657) and hyaluronidase (300 IU/mL, Sigma-Aldrich, cat. #H3506) for 2 to 4 hours at 37°C. The tissue digest was passed several times through a 16- to 18G needle using Cell Stripper (Corning, cat. #25–056-CL) to dissociate remaining cell aggregates. Red blood cell lysis used RBC lysis buffer (BioLegend, cat. #420301), followed by DNaseI (Sigma-Aldrich, cat. # DN25) treatment and filtering through a 40-µm cell strainer (Fisher Scientific, cat. #NC0147038) to yield single-cell suspension.

Aldefluor assay and flow cytometry

Aldehyde dehydrogenase (ALDH) activity was measured using an Aldefluor assay kit (STEMCELL Technologies, cat. #01700) following the manufacturer’s instructions and as described previously (6).

Cell survival assay

Cell survival was measured with a Cell Counting Kit 8 (CCK8, Dojindo Molecular Technologies, cat. #CK04), following the manufacturer’s protocol. Absorbances (450 nm) were measured with a microplate reader (BioTek ELX800, BioTek).

Detailed protocols for spheroid formation assay, clonogenic assay, RNA extraction, quantitative RT-PCR, Western blotting and IHC are included in Supplementary Material. Primers are included in Supplementary Table S2.

Extreme limited dilution assay

A serial dilution of OVCAR5_shControl or OVCAR5_shFZD7 cells (5, 10, 50, 100, 500, 1,000, and 5,000 cells) was sorted by FACS directly into 96-well low-attached plates and cultured in MammoCult medium for 14 days as described above. Each dilution included 10 replicates. The total number of wells containing spheroids for each dilution were counted. The CSC frequency and statistical significance were determined using ELDA software at http://bioinf.wehi.edu.au/software/elda/ (15).

Half maximal inhibitory concentration (IC₅₀)

The IC₅₀ values of the various treatment compounds were determined by the CCK8 assay as described in Supplementary Material. IC₅₀ values were determined by logarithm-normalized sigmoidal dose curve fitting using Prism 6 software (GraphPad Software Inc.).

Lipid peroxidation assay

Intracellular lipid peroxidation was determined by a lipid peroxidation assay (Sigma-Aldrich, cat. #MAK085) following the manufacturer’s protocol (see Supplementary Material).

Oxygen consumption rate

Cells were seeded on 96-well plates at 100,000 cells/well and incubated overnight. Ten microliters of extracellular O₂ consumption reagent (Oxygen Consumption Rate Assay kit, Abcam cat. #197243)
Figure 1.
Stemness and ferroptosis signatures are enriched in Pt-T ovarian cancer cells. A, Representative FACS side scatter analysis of the ALDH$^+$ population (left) and percentage (mean ± SD, n = 5) of ALDH$^+$ cells (right) in parental (WT), cisplatin-tolerant (CDDP), and carboplatin-tolerant (Carbo) SKOV3 cells. B, Representative images (top) and number (mean ± SD, n = 4) of spheroids (bottom) formed by 1,000 parental (WT), CDDP, and carboplatin-tolerant (Carbo) SKOV3 cells after 7 days of culture under non-attachment conditions. (Continued on the following page.)
was added to each well, and fluorescence was measured with a plate reader (Spectramax i3X, Molecular Devices) at 3-minute intervals for 180 minutes at excitation/emission = 380/650 nm. Alternatively, oxygen consumption was measured using a Seahorse assay. Briefly, OVCAR5 shControl and shFZD7 cell lines were seeded in Seahorse 96-well microplate (Agilent, cat. #102416-100) at a density of 10–80K per well. After incubation overnight, oxygen consumption was measured and calculated by Seahorse XFe96 Analyzer (Agilent).

**BODIPY staining for lipid peroxidation**

Cells were treated as described in Supplementary Material. After treatment, cells were stained with BODIPY 581/591 C11 (5 μmol/L) for an hour at 37°C, washed with PBS, and fixed with 4% PFA on ice for 30 minutes. The mean fluorescence intensity (minimum of 10,000 events per condition) was measured by FACS (LSRFortessa, BD). BODIPY emission was recorded on channels for FITC at 520 nm and PE at 580 nm. The data were displayed as histograms, and mean fluorescence intensity of FITC was calculated.

**RNA sequencing and data analysis**

The RNA sequencing (RNA-seq) libraries were prepared using the NEBNext Ultra II RNA library prep kit from Illumina (New England Biolabs Inc; see Supplementary Material). Trimmed reads were aligned to the ENSEMBL human genome version GRCh38 using STAR (2.5.2; ref. 16) and SAMtools (17). Mapped reads were then counted using HTSeq (18). Differentially expressed genes were determined by exact test analysis followed by multiple hypothesis correction using false discovery rate (FDR) on the edgeR package (19). Genes with FDR ≤ 0.05 were considered differentially expressed. Normalized counts for all genes were ranked and subjected to Gene Set Enrichment Analysis (20). Data are deposited in GEO (GSE148003).

Analysis of data from The Cancer Genome Atlas (TCGA) included correlation analysis between gene pairs and survival analysis, described in Supplementary Material.

**Statistical analyses of experimental data**

All data are presented as mean values ± SD of triplicate measurements. Two-tailed Student’s t test or ANOVA (one-way or two-way) was used to determine effects of treatments. P < 0.05 were considered significant. All analyses were performed using Prism 6.0 software (GraphPad Software).

**Results**

**Stemness and ferroptosis signatures are enriched in platinum-tolerant cancer cells**

Pt-T ovarian cancer cells were generated through repeated *in vitro* exposure of ovarian cancer cell lines (OVCAR3, OVCAR5, COV362, and SKOV3) to platinum at IC50 concentrations (Supplementary Fig. S1A), while Pt-R xenografts were obtained by treating tumor harboring mice with carboplatin for four to six weekly cycles (Supplementary Fig. S1B). Repeated platinum exposure of ovarian cancer cells induced a stable phenotype, with at least 2-fold increase in platinum IC50 (Supplementary Table S3) compared with parental chemotheraphy-naïve cells. Pt-T ovarian cancer cells were enriched in ALDH1a1 (Fig. 1A; Supplementary Fig. S2A), formed increased numbers of spheroids (Fig. 1B; Supplementary Fig. S2B), and contained cells expressing CSC-related TFs (Fig. 1C; Supplementary Fig. S2C). Similar observations were made in vivo, including in carboplatin-treated OVCAR3 (Fig. 1D and E) and SKOV3 (Supplementary Fig. S2F) xenografts. ALDH1a1 was enriched (Fig. 1E) and stemness-associated gene sets (ALDH1A1, Oct4, Nanog, and Sox2) were upregulated (Fig. 1F; Supplementary Fig. S2F) in carboplatin-treated compared with PBS-treated xenografts, as we noted previously (6, 7).

Given the possibility that CSCs would be more resistant to chemotherapy due to upregulated antiradex mechanisms (11) and considering a recently proposed association between oxidative stress and ferroptosis, a new form of cell death triggered by oxidized lipids, we examined a gene set related to “ferroptosis” (21) in Pt-T compared with parental ovarian cancer cells. Clear differences including upregulated genes involved in glutathione metabolism and antioxidant defense mechanisms were observed in Pt-T ovarian cancer cells versus chemotherapy-naïve cells (SKOV3, Fig. 1G; OVCAR5, Supplementary Fig. S2G). Interestingly, this molecular signature was observed in HGSC cells with higher baseline resistance to platinum (IC50 >5 μmol/L; COV362, OVCAR8, SNU119, and OVCAR4) compared with cells more sensitive to platinum (IC50 <5 μmol/L; TFKNU, IGROV1, OVCAR3; Supplementary Fig. S2H; refs. 7, 22), suggesting the pathway is a common signature of Pt-resistant cells. The selenoprotein glutathione peroxidase 4 (GPX4), a key protein regulating antioxidant response, was among the upregulated genes in Pt-T cells. GPX4 inhibitors impede antioxidant defense mechanisms and promote death of cells dependent on this pathway (3). Indeed, Pt-R

(Continued)
Figure 2.
Frizzled 7 (FZD7) is upregulated in Pt-T ovarian cancers. A, Fold change (mean ± SD, n = 3) of FZD7 mRNA expression levels measured by real-time RT-PCR in cisplatin-tolerant (CDDP) compared with parental OVCAR3, OVCAR5, and SKOV3 cells. B, Western blotting for FZD7 in parental and Pt-T SKOV3, OVCAR3, and OVCAR5 cells and Pt-T (-CDDP). Quantification shows fold change of FZD7 expression (n = 3 experiments). C and D, FZD7 mRNA expression levels (mean fold change ± SD, n = 3) and FZD7 protein levels measured by Western blotting (D, including quantification in three experiments) in Pt-R PEO4 vs. PEO1 cells. E, Fold change (mean ± SD) of FZD7 mRNA levels in carboplatin-treated (Carbo) and control (Ctrl) SKOV3 and OVCAR3 xenografts (n = 3 per group). G, FACS side scatter analysis (left) and average (±SD, n = 3) of FZD7+ cells dissociated from HGSOC tumors. H, FACS side scatter analysis of FZD7+ cells (left) and percentage (mean ± SD, n = 3) of FZD7+ cells (right) in WT and Pt-T SKOV3, OVCAR3, and COV362 cells. I, NSG mice carrying PDX received carboplatin (15 mg/kg weekly) to induce platinum tolerance. Arrows, carboplatin treatment. Mean volumes (±SD) are shown (n = 5). J, Mean fold change (±SD, n = 3) for Sox2, Nanog, Oct4, ALDH1A, and ALDH1A2 mRNA expression levels in Pt-T vs. control PDXs. K and L, Mean fold change of FZD7 mRNA levels (K) and representative images of FZD7 IHC staining (L) in Pt-T vs. control PDXs (Ctrl). M, FZD7 IHC staining and H-scores (mean ± SD; right) in sections of fallopian tube (n = 6), chemo-naïve ovarian cancer tumors (n = 117), and Pt-T tumors (n = 23) included in two tissue microarrays. For all comparisons: *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 3.
Functional role of FZD7 in ovarian cancer cells. **A**, FZD7 mRNA levels (mean fold change ± SD, n = 3–4) in FZD7⁺ and FZD7⁻ cells FACS selected from SKOV3, OVCAR5, and COV362 cells and HGSOC tumors. **B–D**, Cell viability (mean fold change ± SD, n = 4) of FZD7⁺ and FZD7⁻ cells from OVCAR5 (n = 3; B), SKOV3 (n = 3; C), or COV362 (n = 4; D) cells, plated, treated with the indicated doses of CDDP for 24 hours, and cultured for additional 3 days. **E and F**, Representative pictures and numbers (mean ± SD, n = 4–5) of spheroids formed after 7 days of culture by FZD7⁺ and FZD7⁻ cells FACS sorted from SKOV3 (**E**) and OVCAR5 (**F**) ovarian cancer cells. Spheroids were counted or cell numbers were estimated by CellTiter-Glo 3D cell viability assay. **G–I**, mRNA levels (fold change ± SD) of Nanog in FZD7⁺ compared with FZD7⁻ cells from SKOV3 (n = 3; G), OVCAR5 (n = 8; H), and COV362 (n = 3; I) cells. For all comparisons: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4.
FZD7 regulates stemness characteristics. A, Left, FZD7 mRNA levels (mean fold change ± SD, n = 3) in SKOV3 cells transduced with shRNAs targeting FZD7 (shFZD7) vs. control shRNAs (shctrl). Right, number of spheroids (mean ± SD, n = 4) formed by 2,000 shFZD7 or shctrl SKOV3 cells cultured for 14 days and counted under a microscope. (Continued on the following page.)
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Frizzled-7 (FZD7) mRNA expression levels were upregulated at mRNA (Fig. 2F) and protein level (Fig. 2L), as measured by IHC in Pt-T PDX versus control. Next, IHC assessed FZD7 expression in primary HGSOC specimens and in tumors collected after three to six cycles of neoadjuvant chemotherapy, containing surviving cells after standard platinum-taxane treatment, which are presumably Pt-T. Patient characteristics are included in Supplementary Table S1. Increased FZD7 staining intensity (measured as H-score) was observed in cancer cells residual after chemotherapy in these specimens (n = 23), when compared with chemotherapy-naïve tumors (n = 117, ovarian cancer cells were more sensitive to the GPX4 inhibitor, ML210, compared with control cells (SKOV3, Supplementary Fig. S3A; OVCAR5, Supplementary Fig. S3B; COV362, Supplementary Fig. S3C). ML210-induced inhibition of colony formation was inhibited by the iron chelator deferoxamine (DFOA), consistent with induction of a ferroptosis phenotype (Supplementary Fig. S3A–S3C). Furthermore, primary ovarian cancer cells derived from malignant ascites from patients with Pt-R ovarian cancer were found to be dependent on GPX4, as ML210 potently reduced colony formation in these cells (Fig. 1H), this inhibition being rescued by DFOA.

ML210 caused increased oxidized membrane lipid levels, as measured by flow cytometry using the C11-BODIPY dye in Pt-T cells compared with naïve cells, supporting increased susceptibility to ferroptosis of Pt-R ovarian cancer cells (SKOV3 and OVCAR5, Fig. 1I; COV362, Supplementary Fig. S3D). Additionally, Pt-T SKOV3 cells and parental normal cells (IC50 of 224 nmol/L and 342 nmol/L versus 889 nmol/L; Fig. 1J; Supplementary Fig. S3E and S3F). Primary ovarian cancer cells derived from malignant ascites associated with Pt-R ovarian cancer displayed resistance to platinum in vitro (Fig. 1K and L, left; IC50 of 82 and 16.52 μmol/L, and responded to low doses of the GPX4 inhibitors, ML210 (Fig. 1K and L, right; IC50 of 37.20 and 62.36 nmol/L) and RSL-3 (Supplementary Fig. S3G and S3H, IC50 of 14.20 and 17.72 nmol/L). Trypan blue staining of parental and Pt-T SKOV3 and OVCAR5 cells treated with ML210 showed that the inhibitor induced more cell death in resistant ovarian cancer cells (P < 0.05, Supplementary Fig. S3I and S3J). These results derived from multiple in vitro and in vivo ovarian cancer models, including primary human cancer cells, support the existence of a “persister” phenotype induced by Pt, sharing partial stenness characteristics, and highly susceptible to ferroptosis.

Frizzled-7 is upregulated in Pt-T ovarian cancer cells and tumors

To identify potential markers linked to the “persister” phenotype, an RT-PCR-based platform representing 90 cancer stemness-associated genes was used. A number of known CSC markers were found to be upregulated in the Pt-T cells (CD44, PROMI, and SOX2), along with membrane transporters known to be associated with Pt resistance (ABCG2 and ABCB5), and regulators of EMT (TGFBR1, SNAI1, BMP7, TWIST 1 and 2, and SNAI1 and 2; see Supplementary Table S4). Among transcripts representing membrane proteins, which could potentially be used as novel markers, Frizzled-7 (FZD7), a transmembrane receptor involved in canonical Wnt/β-catenin/TCF and non-canonical Wnt/planar cell polarity signaling (23, 24), was one of the top highly expressed transcripts (>8-fold) in Pt-R compared with control cells (Supplementary Table S4; Supplementary Fig. S4A). Increased FZD7 expression levels were confirmed in Pt-T models (SKOV3, OVCAR5, and OVCA23) generated as described, compared with control cells at mRNA (Fig. 2A) and protein level (Fig. 2B), but also in Pt-R PDOs compared with sensitive PEO1 ovarian cancer cells, an isogenic cell line pair, derived from the same patient at different times during the disease course (Fig. 2C and D; ref. 25). Likewise, FZD7 mRNA expression levels were upregulated in platinum-treated SKOV3 (Fig. 2E) and OVCAR5 (Fig. 2F) xenografts compared with vehicle-treated tumors.

Flow cytometry was used to determine whether an FZD7 high (FZD7+) cell population is detectable. FZD7+ cells were detected among cells dissociated from primary ovarian cancer, previously untreated with chemotherapy, and represented ~3% of all cells (Fig. 2G). In ovarian cancer cell lines, FZD7+ cells were identified as a distinct subpopulation representing ~25% to 35% of cells (SKOV3, OVCAR5, and COV362; Fig. 2H). Additionally, the FZD7+ cell population was detectable and was enriched in Pt-T compared with parental cells (SKOV3, OVCAR5, and COV362; Fig. 2H), suggesting that this cell membrane receptor may be a marker of “persister” cells, preexisting in unselected cell populations prior to Pt exposure, and enriched after exposure to the drug.

Further, we used FDX generated from newly diagnosed HGSOC (14), which were treated weekly with carboplatin. After initial response, recurrent tumors emerged (Fig. 2I), which were enriched in ALDH1+ cells (Supplementary Fig. S4B), CSC-related TFs (Sox2, Nanog, Oct4), as well as ALDH1A1 and ALDH1A2 (Fig. 2J). FZD7 expression levels were upregulated at mRNA (Fig. 2K) and protein level (Fig. 2L), as measured by IHC in Pt-T PDX versus control. Next, IHC assessed FZD7 expression in primary HGSOC specimens and in tumors collected after three to six cycles of neoadjuvant chemotherapy, containing surviving cells after standard platinum-taxane treatment, which are presumably Pt-T. Patient characteristics are included in Supplementary Table S1. Increased FZD7 staining intensity (measured as H-score) was observed in cancer cells residual after chemotherapy in these specimens (n = 23), when compared with chemotherapy-naïve tumors (n = 117, ovarian cancer cells were more sensitive to the GPX4 inhibitor, ML210, compared with control cells (SKOV3, Supplementary Fig. S3A; OVCAR5, Supplementary Fig. S3B; COV362, Supplementary Fig. S3C). ML210-induced inhibition of colony formation was inhibited by the iron chelator deferoxamine (DFOA), consistent with induction of a ferroptosis phenotype (Supplementary Fig. S3A–S3C). Furthermore, primary ovarian cancer cells derived from malignant ascites from patients with Pt-R ovarian cancer were found to be dependent on GPX4, as ML210 potently reduced colony formation in these cells (Fig. 1H), this inhibition being rescued by DFOA.

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Figure 5.
FZD7 regulates GPX4 and intracellular redox states. A, Fold change (mean ± SD, n = 3–4) of GPX4 mRNA levels in FZD7⁺ vs. FZD7⁻ cells FACS sorted from SKOV3, OVCAR5, and COV362 cells and cell suspensions from HGSOC tumors. B, Fold change (mean ± SD, n = 4) of GPX4 mRNA expression levels in OVCAR5 cells transduced with shRNAs targeting FZD7 (shFZD7) vs. control shRNAs (shctrl). C, Western blotting for FZD7, GPX4, and GAPDH in SKOV3 and OVCAR5 cells stably transduced with shctrl and shFZD7. (Continued on the following page.)
**Functional role of FZD7 in ovarian cancer cells**

FZD7 and FZD7 cells were FACS sorted from cell lines and human tumors. Differences in mRNA expression levels between FZD7+ and FZD7− cell lines are shown in Fig. 3A. FZD7+ ovarian cancer cells were less sensitive to cisplatin (CDPP; Fig. 3B–D; P < 0.05), supporting that the receptor marks a Pt-T population. Additionally, FZD7+ cells formed spheroids more efficiently compared with FZD7− cells (Fig. 3E and F, P < 0.01), and expressed higher levels of stemness-associated TFs (SKOV3, Fig. 3G, OVCAR5, Fig. 3H; COV362, Fig. 3I; P < 0.05), supporting that they share stemness-related features.

To further examine its functions, the receptor was knocked down (KD) by stable transduction of shRNA or was transiently overexpressed. Decreased FZD7 mRNA expression was confirmed by Q-RT-PCR in SKOV3 and OVCAR5 cells transduced with two shRNA sequences targeting the receptor (Fig. 4A and B). FZD7 KD decreased spheroid formation (Fig. 4A and B) and expression of stemness-associated TFs (Fig. 4C; Supplementary Fig. S5A). In vitro serial limited dilution assay showed that receptor KD decreased stem cell frequency, as calculated by the ELDA software (27) in FZD7 KD versus control cells (P = 0.034, Supplementary Fig. S5B). Likewise, FZD7 KD in Pt-T SKOV3_CDDP cells (Supplementary Fig. S5C) decreased sphere formation (Supplementary Fig. S5D). Stable FZD7 KD in primary Pt-R tumor cells caused decreased expression levels of the stemness-associated gene ALDH1A1 (Fig. 4D and E). Conversely, transient overexpression of FZD7 (Fig. 4E and F) promoted proliferation of SKOV3 and OVCAR5 cells as spheres (Fig. 4G) and increased expression of stemness-associated TFs (Supplementary Fig. S5E and S5F). FZD7 KD decreased IC50 to cisplatin by ~2-fold (SKOV3, Fig. 4H; SKOV3_CDDP, Fig. 4I; Supplementary Fig. S5G and S5H), while the receptor’s overexpression increased Pt resistance (Fig. 4J; Supplementary Fig. S5I).

To test the effects of FZD7 to tumor growth, a subcutaneous xenograft model was used. FZD7 KD in OVCAR5 cells delayed tumor initiation (sh-control 9.8+4.6 days versus sh-FZD7 25.3±4.9 days, Fig. 4K; P < 0.0001) and decreased tumor size (Supplementary Fig. S5J) and tumor weight (0.80±0.41 g versus 0.14±0.15 g, P = 0.04, Supplementary Fig. S5K, n = 4/group). FZD7 KD in xenografts was confirmed by IHC (Supplementary Fig. S5L). ALDH+ cells (16.5% ± 6.5% versus 5.9% ± 1.8%, P = 0.05; Fig. 4M, n = 3) and spheroid forming ability (Fig. 4M, P = 0.02) were decreased in cells dissociated from FZD7 KD versus control xenografts. Further, to test the effects of FZD7 KD to tumor initiation, an in vivo serial limited dilution assay was carried out by using 2,500, 5,000, and 10,000 FZD7 KD and control cells. FZD7 KD significantly inhibited Pt-T (Fig. 4N) and reduced stem cell frequency, as calculated by the ELDA software (Fig. 4O; Supplementary Fig. S5J).

**Molecular signatures of FZD7+ cancer cells**

Gene signatures distinguishing FZD7+ versus FZD7− cells, ovarian CSCs (ALDH4+CD133−) versus non-CSCs (ALDH−CD133−), and Pt-T versus platinum-naïve OVCAR5 cells were examined and integrated (Fig. 4P). FZD7− and + cells were sorted by FACS (Supplementary Fig. S5J). Ovarian CSCs and non-CSCs were FACS sorted by using dual stem cell markers, CD133 and Aldefluor activity (Supplementary Fig. SS and SSN). There were 666 differentially expressed genes (DEG) shared between FZD7+/FZD7− and CSCs/non-CSCs data sets, 5,404 DEGs being unique to CSCs and 536 DEGs unique to FZD7+ cells (Fig. 4P). Additionally, there were 927 DEGs overlapping between FZD7+/FZD7− and resistant/parental cells, 10,019 DEGs unique to the Pt-T cells, and 275 genes uniquely associated with FZD7− cells (Fig. 4P). Overlapping DEGs between FZD7+/FZD7− and OCSC/non-OCSCs were enriched in cancer stem cell, but enriched DNA-repair signatures between FZD7+/− and resistant/parental cells (Supplementary Fig. S6A and S6B).

Additionally, FZD7− versus FZD7+ cells displayed signatures enriched in stemness (Supplementary Fig. S6C), EMT (Supplementary Fig. S6D) and downregulated DNA damage response genes (Supplementary Fig. S6E). Together, these data suggest that FZD7+ cells possess both shared, but also distinct features, relative to stemness and chemoresistance, consistent with the phenotypes described above. Importantly, mitochondrial and oxidative phosphorylation gene sets were enriched among DEGs distinguishing FZD7− versus FZD7+ cells (Supplementary Fig. S6F–S6I) and Ingenuity Pathway Analysis identified oxidative phosphorylation and mitochondria dysfunction as the top enriched pathways in FZD7− cells, suggesting that the receptor marks a cell population harboring altered oxidative stress responses.

**FZD7 marks a cell population enriched in GPX4**

Given that GPX4, an antioxidant enzyme that reduces ROS, preventing formation of toxic lipid peroxides (28, 29), has been implicated in maintenance of normal mitochondrial function and oxidative phosphorylation (3, 28), we examined whether FZD7 expression affected GPX4 expression and function in Pt-T ovarian cancer cells. GPX4 levels were significantly increased in FACS-sorted FZD7− versus FZD7+ cells derived from SKOV3, OVCAR3, and COV362 cells or cancer cells dissociated from human tumors (Fig. 5A, P < 0.05). Furthermore, FZD7 KD by shRNA in OVCAR5 and SKOV3 cells resulted in repressed GPX4 mRNA (Fig. 5B, P < 0.001; Supplementary Fig. S7A, P < 0.05) and protein expression levels (Fig. 5C). GPX4 expression was decreased in Pt-T SKOV3 cells (Fig. 5D) and in primary Pt-R ovarian cancer cells (Fig. 5E) transduced with shRNA (Fig. 5O). Combined, these results support that FZD7 is linked to stemness and chemoresistance.
targeting FZD7. Conversely, FZD7 overexpression induced increased GPX4 mRNA and protein expression levels (Fig. 5F–H).

Furthermore, GPX4 and FZD7 mRNA expression levels were coordinately upregulated in Pt-T models, including Pt-T versus parental cells (SKOV3, OVCAR5, Fig. 5I, P < 0.05), Pt-T xenografts (Fig. 5J, P < 0.05) and PDxAs (Fig. 5K, P < 0.01). Increased GPX4 expression in Pt-T PDX versus controls was confirmed by IHC (Fig. 5L). IHC examined GPX4 in HGSOCS specimens collected after neoadjuvant chemotherapy, noting increased GPX4 staining in these residual tumors (n = 23) when compared with fallopian tube epithelium, Fig. 5M, P = 0.02). Together, the results confirm a positive correlation between FZD7 and GPX4 expression in ovarian cancer cells and in Pt-T models, supporting that FZD7+ cells have increased antioxidant capacity. To confirm the functional relevance, GPX4 enzymatic activity was measured by using the malondialdehyde (MDA) assay, which quantifies intracellular lipid peroxide levels. Lipid peroxides were found to be increased in FZD7 KD versus control cells (Fig. 5N, P < 0.05) and decreased in ovarian cancer cells overexpressing FZD7 (Fig. 5O, P < 0.05), supporting that FZD7+ cells clear these toxic products more effectively, due to higher levels of GPX4.

GPX4 participates in regulation of intracellular redox states by utilizing glutathione (GSH) as the critical antioxidant (21). GSH is synthesized from glutamate–cysteine under the action of glutamate–cysteine ligase (GCL; ref. 21). The cycling of reduced GSH to oxidized glutathione disulfide (GSSG) removes ROS derived from hydrogen peroxide and lipid hydroperoxides through various glutathione peroxidases (GPX), including GPX4 (21). GSH recycling from GSSG is catalyzed by glutathione reductase (GSR), using NADPH, whose synthesis is regulated by isocitrate dehydrogenase 2 (IDH2; ref. 21). FZD7 KD decreased the expression levels of multiple genes in this pathway, including GPX2, GSS, IDH2, GSR, GCL, and SLCA7A1 (OVCAR5, Fig. 6A; SKOV3, Supplementary Fig. S7B). Conversely, FZD7 overexpression caused increased expression levels of GSS, GSR, GCL and SLCA7A1 (OVCAR5, Fig. 6B; SKOV3, Supplementary Fig. S7C), suggesting a significant direct correlation between FZD7 and glutathione metabolism–related genes.

FZD7 marks a cell population susceptible to GPX4 inhibitors

Given the correlations between FZD7, upregulated in Pt-T cells, and GPX4–mediated cellular redox maintenance, we hypothesized that inhibition of this axis will eliminate resistant cells. Small-molecule inhibitors of GPX4 have been shown to increase 2-lipid oxidant stress and induce ferroptosis (3, 30–32). Thus, we examined the sensitivity of ovarian cancer cells with high versus low FZD7 expression levels to GPX4 inhibitors, ML210 and RSL3 (3, 32, 33). FZD7+ sorted cells were more sensitive to the GPX4 inhibitor ML210 compared with FZD7− cells (OVCAR5, Fig. 6C; COV362, Fig. 6D; SKOV3, Supplementary Fig. S7D). FZD7 KD in OVCAR5 and SKOV3 cells also slightly reduced sensitivity to GPX4 inhibitors (Fig. 6E; Supplementary Fig. S7E), while FZD7 overexpression slightly increased sensitivity to ML210 compared with vector-transduced cells (Fig. 6F; Supplementary Fig. S7F).

Through its antioxidant function, GPX4 protects mitochondria from damage, maintaining normal oxidative phosphorylation. To test whether these processes were altered in FZD7+ versus FZD7− cells, as a consequence of differential GPX4 expression, oxygen utilization was measured by using fluorescence–labeled oxygen uptake assay and the seahorse assays. FZD7 KD resulted in decreased oxygen uptake (Supplementary Fig. S7G) and consumption rate (Supplementary Fig. S7H), supporting the role of this pathway maintaining normal mitochondrial function. Additionally, intracellular ROS levels, quantified by DCFHDA staining, were decreased in FZD7 KD cells compared with controls (Fig. 6G). As increased ROS levels contribute to oxidation of polyunsaturated lipids, leading to ferroptosis, C11-BODIPY staining was used in cells expressing different levels of FZD7 and/or exposed to GPX4 inhibitors. Oxidation of the polyunsaturated butadienyl portion of the dye in the presence of ROS is reflected in a shift of the fluorescence emission peaks from red to green, a hallmark of ferroptosis. The mean green (FITC) fluorescence intensity caused by oxidized lipids was decreased in cells overexpressing FZD7 and increased in FZD7 KD cells (Fig. 6H), consistent with increased susceptibility to ferroptosis of FZD7+ cells. ML210-induced increase in fluorescence was higher in SKOV3 and OVCAR5 cells overexpressing FZD7 compared with controls and decreased in FZD7 KD cells (Fig. 6I and J). Likewise, baseline and ML210-induced intracellular ROS levels (rescued by DFOA) were higher in control versus FZD7 KD OVCA5 cells (Fig. 6K).

Collectively, the data suggest that cells marked by FZD7 are more susceptible to ferroptosis and could be eliminated by targeting GPX4.

FZD7 regulates GPX4 expression and glutathione metabolism by activating the canonical β-catenin/p63 pathway

As a classic Wnt receptor, FZD7 participates in both canonical β-catenin and noncanonical signaling. One of the known β-catenin targets is the transcription factor p63, directly transactivated by the TCF/LEF complex (34). Its most common isoform, ANT p63, lacking its N-terminus domain, has been implicated in maintaining intracellular redox homeostasis by regulating genes involved in glutathione metabolism, including GPX4 (21). We therefore hypothesized that in Pt-T ovarian cancer cells, FZD7 could alter glutathione metabolism and protect ovarian cancer cells from oxidative stress, through activation of β-catenin/TP63 signaling. Tp63 expression was upregulated in Pt-R PDX (Fig. 7A) and ovarian cancer cells compared with controls (Supplementary Fig. S8A). Tp63 expression was higher in FZD7+ versus FZD7− cells derived from SKOV3, OVCAR5, COV362, and human HGSOCS (Fig. 7B), and FZD7 KD caused decreased Tp63 expression in OVCAR5 (Fig. 7C and D) and SKOV3 cells (Supplementary Fig. S8B; Fig. 7D), while FZD7 overexpression led to increased Tp63 expression (Fig. 7E–G) at mRNA and protein levels. To demonstrate that the correlations between FZD7, TP63, and GPX4 were dependent upon the engagement of β-catenin, we used Wnt3A stimulation and the β-catenin inhibitor IWR-1-endo. Stimulation with Wnt3A induced p63 and GPX4 expression in control, but not in FZD7 KD, cells, while treatment with IWR-1-endo inhibited the expression of both p63 and GPX4 (Fig. 7H). OVCAR5 and SKOV3, similar observations were made when FZD7 was overexpressed (Supplementary Fig. S8F), supporting that GPX4 upregulation is directly regulated by β-catenin.

Lastly, to determine whether FZD7 regulates GPX4 expression by altering Tp63 function, the effects of Tp63 knockdown on GPX4 expression in cells expressing high versus low levels of FZD7 were tested. Overexpression of FZD7 and KD of p63 in OVCAR5 cells was confirmed at the mRNA level (Fig. 7I–K; SKOV3, Supplementary Fig. S8C–S8E). GPX4 upregulation induced by FZD7 overexpression was abrogated in cells in which p63 was KD (Fig. 7K; SKOV3, Supplementary Fig. S8E), supporting that this TF, engaged by β-catenin, downstream of FZD7, is an important regulator. Interestingly, Tp63 KD caused reduced expression of FZD7 in ovarian cancer cells transfected with either control vector or FZD7, indicating a feedback regulatory role of Tp63 on FZD7. These experimental results were validated by examining the TCGA HGSOCS database (35). FZD7 and Tp63 expression levels were positively correlated (Fig. 7L).
Figure 6. FZD7 marks a cell population susceptible to GPX4 inhibitors. **A**, Average fold change (± SD, n = 3) in mRNA expression levels of selected glutathione metabolism genes in OVCAR5 cells transduced with shRNAs targeting FZD7 (shFZD7) vs. control shRNA (shCtrl). **B**, Viability of FZD7⁺ and FZD7⁻ cells sorted from OVCAR5 (C) and COV362 (D) cells and treated with DMSO or ML210 (OVCAR5, 2 μM; COV362, 1 μM) for 72 hours. Data are presented as average fold change (± SD, n = 4) of absorbance values relative to control. **E**, Survival curves of OVCAR5 (left) and SKOV3 (right) cells transduced with control shRNA (shCtrl) or shRNAs targeting FZD7 (shFZD7) and treated with ML210 for 3 days (n = 3–4). ML210 IC₅₀ values are shown below. **F**, Survival curves of OVCAR5 (left) and SKOV3 (right) cells transduced with FZD7-pcDNA3.1 or control vector and treated with ML210 for 3 days. ML210 IC₅₀ values are shown below (n = 3–4). **G**, Images (left) and quantification (right) of intracellular ROS levels in OVCAR5 cells transfected with control shRNA (shCtrl) or shRNA targeting FZD7 (shFZD7). Data are presented as means (± SD) of DCF fluorescence intensity per cell area (n = 15). **H**, Histograms of fluorescence intensity (left) and mean (± SD, n = 3) fluorescence intensity of BODIPY 581/591-C11 in OVCAR5 (left) and SKOV3 (right) cells transfected with vector (ctrl), FZD7-pcDNA3.1 (FZD7), control shRNA (shCtrl), or shRNAs targeting FZD7 (shFZD7). **I and J**, Mean (± SD, n = 3) fluorescence intensity of BODIPY 581/591-C11 shows effects of ML210 (1 μM/L for 20 hours) on lipid peroxidation levels in SKOV3 (I) and OVCAR5 (J) cells transfected with empty vector (ctrl), FZD7-pcDNA3.1 (FZD7), control shRNA (shCtrl), or shRNAs against FZD7 (shFZD7). **K**, Intracellular ROS levels in OVCAR5 cells transfected with shCtrl and shFZD7 treated with DMSO, ML210 (2 μM/L, 24 hours), and ML210 + DFOA (800 nM/L, 24 hours), measured by assessing DCFHDA oxidation. Average intensity per cell area (± SD) is shown (n = 15).

For all comparisons: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 7.
FZD7 regulates GPX4 expression and glutathione metabolism by activating the canonical β-catenin/p63 pathway. A, P63 mRNA levels (fold change ± SD, n = 4) in P1-T PDXs (carbo) vs. controls (Ctrl). B, P63 mRNA levels (fold change ± SD, n = 3–6) in FZD7+ versus FZD7− cells sorted from SKOV3, OVCAR5, and COV362 cell lines, and cell suspensions from human tumors. C, P63 mRNA expression levels (fold change ± SD, n = 4) in OVCAR5 cells transduced with shRNAs targeting FZD7 (shFZD7) vs. control shRNA (shctrl). (Continued on the following page.)
Discussion

Our data support that a Pt-T ("persister") cancer cell population serving as a reservoir for resistant tumors shares common features with CSCs and is characterized by FZD7 expression. We demonstrate that the survival of these cells is dependent on an active FZD7–β-catenin–Tp63–GPX4 pathway, which renders these cells susceptible to inducers of ferroptosis. Our findings have several implications.

First, we identified FZD7 as a receptor enriched in Pt-T cancer cells and tumors. FZD7 is a transmembrane receptor that transduces signals involved in both the canonical and noncanonical Wnt pathways (36). Previous data indicated that FZD7 plays essential roles in stem cell biology and cancer (37). In breast and hepatocellular carcinoma, FZD7 has oncogenic functions, promoting cell proliferation, migration, and invasion (24, 38–41). Here we show that FZD7 marks a population representing ~2% to 25% cells in Pt-T cell lines or tumors. The receptor’s KD sensitized ovarian cancer cells to platinum, and its overexpression rendered cells resistant. Our group previously identified FZD7 as a receptor facilitating interaction of ovarian CSCs with the tumor niche (42) and the current findings corroborate the link between this receptor and cancer stemness. FZD7+ cells were shown to proliferate more robustly as spheroids, to express higher levels of stemness-associated TFs and display enhanced TIC. Interestingly, a related receptor, FZD10, was recently linked to PARP inhibitor resistance (43). Thus, it is likely that activation of the Wnt pathway through activation of one or more FZD receptors contributes to emergence of resistance to DNA-damaging agents.

Second, we report that FZD7 marks a population of cells highly susceptible to ferroptosis. Ferroptosis is a newly described type of cell death distinct from apoptosis and necrosis (44) characterized by iron-dependent accumulation of ROS resulting in increased lipid peroxidation and eventually leading to cell death (33). Ferroptosis is dependent on NADPH/H+–polysaturated fatty acid metabolism, and the mevalonate and glutaminolysis metabolic pathways (31). Class 1 (system Xc− inhibitors) and class 2 (GPX4) inhibitors are small molecules that induce ferroptosis (44). Here we observed that FZD7+ platinum-tolerant cells were highly sensitive to ferroptosis induced by small molecules targeting GPX4. Tyrosine kinase inhibitor–tolerant cells have been reported to be sensitive to ferroptosis (3); however, no markers to identify cells prone to ferroptosis have been described.

Third, we found GPX4 to be significantly upregulated in Pt-T cells, xenografts, PDXs, and ovarian tumors residual after neoadjuvant chemotherapy. GPX4 detoxifies lipid peroxides (L-OOHs) by converting them to corresponding alcohols (L-OH), preventing the buildup of toxic, membrane oriented, lipid ROS (L-ROS; ref. 45). Aside from GPX4, other glutathione metabolism-related genes, such as GSH and GSR, are also involved in antioxidant defense. Cancer cells with acquired drug resistance were reported to have increased cellular GSH levels (46). Lower levels of endogenous ROS and higher levels of antioxidants and GSH were found in temozolomide (TMZ)-resistant glioblastoma cells (47) and silencing the GSH biosynthesis pathway triggered ferroptosis in clear cell carcinoma (48). Thus, modulation of redox homeostasis by GSH/GSR appears to be an important key modulating sensitivity of cancer cells to chemotherapy (47). Interestingly, in our study, the expression of glutathione metabolism-related genes GSS, GSR, GPX2, and IDH were directly correlated with expression of FZD7.

Lastly, our results shed light on a potential mechanism explaining the connection between FZD7 and activation of the glutathione regulatory machinery. A recent study reported that expression of GPX4 and of other genes involved in glutathione metabolism, such as cysteine, is regulated transcriptionally by Tp63 (21). Tp63 (along with p53 and p73) belongs to the Tp53 family (49). Tp63 regulates the self-renewal of progenitor cells in epithelial tissues through its byproduct ΔNp53, which has dominant-negative effects on other p53 family isoforms and exerts tumorigenic functions (34). Unlike Tp53, inactivated in a majority of human cancers, including ovarian cancer, p63 is rarely mutated or inactivated. Overexpression of Tp63 was associated with poor survival in ovarian cancer (49, 50), similar to our findings exploring the TCGA database. Tp63 was shown to regulate the expression of FZD7 and enhance Wnt signaling in mammary tissue (49). A putative cross-talk between the Wnt/β-catenin pathway and ΔNp63, the Tp63 isoform lacking the N-terminus domain, was reported in skin, hair follicles, mammary glands, and limb buds during development (41). We observed a similar direct and strong correlation between Tp63 and FZD7 expression in ovarian cancer cells, supporting that expression of this receptor is regulated by this TF. Furthermore, GPX4 expression, increased in FZD7-overexpressing cells, was downregulated by Tp63 knockdown, supporting this mechanism.
In all, our results propose FZD7 as a new marker for cancer cell populations likely to survive exposure to platinum, enriched in antioxidant response mechanisms. These rare cells responsible for disease relapse after chemotherapy, share stemness features, and are susceptible to eradication through ferroptosis. Our data provide compelling evidence that targeting Pt-T FZD7⁺ cells by inducing ferroptosis is effective and could represent a new strategy in a disease of high unmet need.

Authors’ Disclosures

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Authors’ Contributions

Y. Wang: Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. G. Zhao: Data curation, software, formal analysis, validation, writing–original draft, writing–review and editing. S. Condello: Data curation. H. Huang: Data curation. H. Cardenas: Data curation, writing–review and editing. E.J. Tanner: Resources. J. Wei: Resources. J. Li: Software, formal analysis, methodology, writing–original draft. J. Li: Data curation, formal analysis, and validation. Y. Tan: Data curation, formal analysis, and validation. R.V. Davuluri: Software, formal analysis, supervision, and methodology. M.E. Peter: Resources, data curation, formal analysis, validation, methodology, writing–original draft, and writing–review and editing. J.-X. Cheng: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. D. Matei: Conceptualization, resources, data curation, supervision, funding acquisition, investigation, methodology, writing–original draft, project administration, writing–review and editing.

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