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Xiaohu Tang (xiaohut@mtu.edu)  
Michigan Technological University  https://orcid.org/0000-0001-6367-4991

Tahiyat Alothaim  
Michigan Technological University

Morgan Charbonneau  
Michigan Technological University

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Recurrent triple-negative breast cancer from cysteine deprivation loses tumorigenicity via downregulation of the CST4 signaling

Tahiyat Alothaim¹, Morgan Charbonneau¹, Xiaohu Tang¹.²

¹Department of Biological Sciences, Michigan Technological University, Houghton, Michigan, 49931

²Corresponding author: Xiaohu Tang;
E-mail: xiaohut@mtu.edu
Telephone: 906-487-3068

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Running title: Sustained cysteine deprivation suppresses tumorigenesis of mesenchymal-type triple-negative breast cancer
Abstract

Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer with a high risk of recurrence following therapeutic treatments. Targeted cysteine therapy via inhibition of cysteine uptake by erastin effectively induces mesenchymal TNBC cells to ferroptosis. However, a small residual population of cancer cells exhibited the erastin-resistance and survived after the erastin treatment. This phenomenon is likely analogous to the event of tumor recurrence in patients after therapy. To characterize this resistance, we established the erastin-resistant/recurrent TNBC cell models in vitro by multi-cycles of erastin challenge. By an epigenetic compound library screen, the erastin-resistance be abolished by adjuvant epigenetic compounds. Intriguingly, the erastin-recurrent TNBC cells failed to grow in anchorage-independent conditions, implying a loss of tumorigenicity. By transcriptomic profiling analysis, the recurrent cells displayed many gene expression alterations and attenuated signaling processes, including K-Ras signaling. Three members of the cystatin gene family, including CST4, were significantly downregulated in recurrent cells. Knocking out of CST4 by CRISPR/Cas9 significantly suppressed the tumorigenic potential and K-Ras signaling. Our findings suggested that targeted cysteine therapy could be a valid treatment for mesenchymal TNBC with a low probability of tumor recurrence.
Introduction

Breast cancer is the first leading cause of cancer mortality in women globally, whereas triple-negative breast cancer (TNBC) is the most aggressive subtype with a high mortality rate. Surgery, chemotherapy, and emerging targeted therapies are therapeutic options for TNBC treatment [1-2]. Regardless of initial tumor responses to treatments, many tumors gain drug resistance and recurrence/relapse in situ from residual resistant tumor cells [2-3]. Recurrence is a major clinical manifestation of breast cancer and represents the major cause of cancer mortality. Particularly, TNBC frequently gains drug-resistance and distant recurrence within 3-5 years of surgery and therapeutic treatments [3-6].

Drug resistance is one of the major obstacles of cancer treatment. Generally, tumors acquire drug resistance by either intrinsic or acquired resistance mechanisms [7]. Intrinsic resistance involves innate molecular qualities of the tumor, including inter-tumor or intratumor heterogeneity [8]. On the other hand, the acquired resistance typically associates with gained molecular alterations in tumors during or after therapy, which is mediated by many different mechanisms, such as increasing drug efflux, alteration of drug metabolism, deregulation of cell signaling, changes in epigenetics and tumor microenvironment, et al. [9-14]. In TNBC, multidrug-resistant proteins are often upregulated to increase drug efflux. Mutations on cell death mediators and deregulated signaling have been reported to cause chemoresistance [15]. In addition, emerging evidences suggest that TNBC displays signatures of cancer stem cells at functional and molecular levels, which possesses tumor-initiating potential and self-renewal capacity, and increases the risks of drug resistance, metastasis, and recurrence [16-17].
Malignant tumor cells often exhibit altered cellular metabolisms, such as increased uptake of glucose or amino acids, which contribute to fast proliferation, tumorigenesis, and malignancy [18-20]. Targeting metabolic vulnerabilities in cancer is considered as a promising targeted therapeutic strategy. Since cysteine is required for the synthesis of glutathione (GSH), a major antioxidant against reactive oxidative species (ROS), cysteine deprivation leads to accumulation of lipid peroxidation and induces an iron-dependent ferroptosis [21-24]. Recently, targeted cysteine therapy by either inhibition of cysteine transporter (xCT) and GSH synthesis or inhibition of glutathione peroxidase 4 (GPX4) stands out as an effective option in treatment of a variety of cancers, including TNBC [25-32].

Breast cancer shows differentiated sensitivity to targeted cysteine therapy partially due to intertumoral heterogeneity. For example, the mesenchymal-type TNBC is highly sensitive to erastin, an inhibitor of xCT, while the epithelial-type TNBC is irresponsive [25, 33]. Epigenetic sensitizers have been identified to overcome the erastin-resistance in the epithelial TNBC [33]. Other mechanisms contributing to the erastin-resistance have been suggested in some cancers, such as activation of NRF2/CBS in ovarian cancer [34], degradation of VDAC2/3 melanoma [35], hypoxia, and extracellular cysteinyl glycine in glioblastoma [36], and deregulated cellular signaling in breast cancer [37-38]. It is necessary recapitulate molecular and cellular adaptations during or after targeted cysteine therapy and understand the underlying resistance mechanism.

In this study, we observed a small subpopulation of mesenchymal TNBC cells frequently escaping from cell death induced by cysteine deprivation. To characterize phenotypic and molecular changes, we established the erasin-resistant/recurrent TNBC
cell models in-vitro. We found that the recurrent TNBC cells lose the tumorigenic potential.

By analyzing transcriptomic alterations in recurrent TNBC cells, we identified the downregulation of the CST4 signaling as one of the mechanisms to suppress tumorigenesis in TNBC.

Results

Establish in vitro erastin-resistant TNBC cell models by multiple-cycle of challenges

The mesenchymal TNBC cells are extremely sensitive to the xCT inhibitor erastin with prompt ferroptosis [25, 33]. However, about 1 ~ 5% of cell population were frequently observed resistant to erastin. Subsequently, these cells resumed growth and proliferation once the stress was removed. Such phenomena might reflect the facts of drug resistance and tumor recurrence in patients after therapy. To that end, we established the erastin-resistant/recurrent cell models in vitro to examine cellular characteristics and understand the underlying resistance mechanism. Two mesenchymal-type TNBC cells, MDA-MB-231 and BT549, were challenged under a series of 6 to 8 cycles of treatment and recovery from erastin to establish the erastin-resistant/recurrent (E\textsuperscript{R}) cells (Fig. 1A). Notably, both lines of erastin-resistant cells exhibit distinct morphologies with long stretched and neuron-like cell bodies with slower proliferation rates than their parental erastin-sensitive (E\textsuperscript{S}) cells (Fig. 1B-C). As expected, both MDA-MB-231 E\textsuperscript{R} and BT549 E\textsuperscript{R} cells were strongly resistant to erastin-induced ferroptosis when compared with their parental E\textsuperscript{S} cells (Fig. 1D and S1A-B). Importantly, MDA231 E\textsuperscript{R} and BT549 E\textsuperscript{R} cells were also significantly resistant to RSL3, a direct inhibitor of GPX4 (Fig. 1E and S1C). In consistent
with resistant phenotypes, immunoblotting analysis showed strong reductions of the
death signaling (pho-p38) and DNA damage marker (pho-H2AX) in the E^R cells under
erastin (Fig. 1F). In addition, the erastin-induced lipid peroxidation in the E^R cells was
dramatically decreased when compared with the E^S cells (Fig. S1D).

**Epigenetic compounds abolish the erastin-resistance in recurrent cells**

It was reported that epigenetic compounds can render the non-mesenchymal TNBC
cells sensitive to ferroptosis inducers [33]. Using a similar strategy of epigenetic
compound library screening, three epigenetic compounds, targeting different epigenetic
enzymes, were identified to potentially overcome the erastin-resistance in MDA-MB-231
E^R cells (Fig. 2A). Tubacin, an inhibitor of histone deacetylase HDAC6, synergized with
erastin to induce an extensive ferroptosis in both MDA-MB-231 and BT549 E^R cells, but
tubacin or erastin alone did not (Fig. 2B-D and S2A). In line with phenotypical responses,
the death signaling and cell death markers were significantly activated in the E^R cells
upon erastin plus tubacin (Fig. 2E-F). Similarly, two inhibitors for histone
methyltransferases EZH2 (EPZ005687) or DOTL1 (SGC0946) abolished the erastin-
resistance and induced significant cell death in MDA-MB-231 and BT549 E^R cells when
combined with erastin (Fig. S2B-C). These results suggested that epigenetic inhibitors
can serve as adjuvants to overcome the drug-resistance in the erastin-resistant/recurrent
cells.

**Erastin-resistant TNBC loses the potential of anchorage-independent growth**
Intriguingly, we found that both MDA-MB-231 ER\textsuperscript{R} and BT549 ER\textsuperscript{R} hardly grew in an anchorage-independent condition, which indicated by a soft-agar colony formation assay (Fig. 3A-B). The colonies of ER\textsuperscript{R} cells on soft-agar were significantly decreased in comparison with the ES\textsuperscript{S} cells. Large colonies (≥ 25 µm) were dramatically reduced in the ER\textsuperscript{R} cells (Fig. 3B). Next, cell migration was more defective in the ER\textsuperscript{R} cells than the ES\textsuperscript{S} cells (Fig. 3C). In addition, the ER\textsuperscript{R} cells exhibited a distinct morphology with a relatively slow proliferation when growing in a three-dimensional (3D) low-attachment culture condition (Fig. 3D), in which the ES\textsuperscript{S} cells displayed invadopodia-like structures, while the ER\textsuperscript{R} cells mostly grew as spheroids. These data suggested that the ER\textsuperscript{R} cells lose tumorigenic potentials in spite of their erastin-resistance.

**Gene expressions and signaling are dramatically altered in recurrent ER\textsuperscript{R} cells**

To understand the underlying mechanism that contributes to loss of tumorigenicity in recurrent ER\textsuperscript{R} cell models, the transcriptomic profiling was analyzed in MDA-MB-231 ES\textsuperscript{S} and ER\textsuperscript{R} cells. The gene cluster analysis indicated that numerous genes were altered in ER\textsuperscript{R} cells in comparison with those in ES\textsuperscript{S} cells, in which 156 genes were up-regulated, and 334 genes were down-regulated in ER\textsuperscript{R} cells with at least 2-fold changes (Fig. 4A). Gene set enrichment analysis (GSEA) revealed that the K-Ras, interferon, and hypoxia signaling pathways were significantly downregulated in ER\textsuperscript{R} cells when compared with their parental counterparts (Fig. 4B). RT-qPCR analysis confirmed that gene expressions in the K-Ras and interferon pathways were significantly suppressed in ER\textsuperscript{R} cells (Fig. 4C and S3A). The genes related to glutathione biosynthesis (GCLM and GCLC) and lipid peroxidation removal (GPX4) were increased in ER\textsuperscript{R} cells (Fig. S3B). Interestingly, GSEA analysis
indicated that genesets associated with the neuronal cell type were enriched and highly expressed in E\textsuperscript{R} cells (Fig. 4D), which correlated with a neuron-like, multipolar, and stretched morphology in E\textsuperscript{R} cells (Fig. 4E and S3C). Taken together, these results indicated that the erastin-resistant/recurrent TNBC cells undergo many intrinsic changes in cellular signaling, metabolism, and structure to acquire the inability of tumorigenicity.

The cystatin genes are repressed in the E\textsuperscript{R} cells

Notably, we observed that the expressions of three cystatin family genes (CST1, CST2, and CST4) were significantly downregulated in both MDA-MB-231 and BT549 E\textsuperscript{R} cells (Fig. 5A-B), although the basal expression levels of cystatin genes in BT549 E\textsuperscript{S} cells were much lower than those in MDA-MB-231 cells. In agreement with the mRNA expression, the protein expression of CST4 was dramatically decreased in MDA-MB-231 E\textsuperscript{R} cells when compared with MDA-MB-231 E\textsuperscript{S} cells (Fig. 5C). It was reported that the expression of cystatin family genes contributes to poor prognosis, metastasis, and tumor relapse in a variety of cancers. Particularly, overexpression of CST1 or CST2 promotes tumor progression in gastric, breast, or colorectal cancer [39-43]. In TCGA breast invasive tumor samples, we found that the expression of CST4 is highly correlated with gene expressions of CST1 and CST2 (Fig. 5D).

CST4 regulates cellular signaling and tumorigenicity

The role of CST4 in either drug resistance or tumor progression, particularly in TNBC progression, is largely unknown. To that end, CST4 was knocked out in parental MDA-
MB-231 cells using two CST4-targeting CRISPR guide RNAs, and independent CST4-knockout (gCST4) cell clones were established (Fig. 6A). The gCST4 cells showed similar sensitivity to erastin as the wild-type cells (Fig. S4A), indicating that CST4 is not involved in the erastin-resistance. Intriguingly, the potential of gCST4 cells growing in the anchorage-independent condition was significantly reduced, in which the colonies were much smaller than their counterparts (Fig. 6B and S4B). Similar to the E\textsuperscript{R} cells, the genes involved in the K-Ras and interferon signaling were also significantly suppressed in the gCST4 cells (Fig. 6C and S4C). Interestingly, knockout of CST4 strongly repressed the transcription of CST1 and CST2, even its own gene transcription (Fig. 6D), indicating that CST4 is an upstream signaling mediator of CST1 and CST2. Reciprocally, we overexpressed CST4 in BT549 cells, with a low basal level, to examined whether CST4 promotes tumorigenesis (Fig. 6E). Indeed, increased CST4 expression significantly enhanced the ability of cell growth in soft-agar (Fig. 6F-G). Cell migration was slightly increased when CST4 was overexpressed (Fig. S4D). Taken together, our data suggested that CST4 regulates cellular signaling and tumorigenicity, but not the erastin-resistance.

Discussion

Targeting metabolic vulnerability in tumors has been suggested as a promising targeted therapeutic strategy. We and others have shown that targeted cysteine therapy by blocking the cysteine-glutathione-GPX4 pathway induces ferroptosis in many types of cancers [22, 26, 44-46]. Particularly, mesenchymal-type triple negative breast cancer (TNBC) cells are extremely sensitive to ferroptosis inducers. However, a very small
subpopulation of cells remains survival or sustains proliferation after cysteine deprivation. Since tumor recurrence or relapse often occurs in triple-negative breast cancer (TNBC) within three years after chemotherapy or surgery [1, 3, 5], and the recurrent cells escaping from targeted cysteine therapy may pose potential risks for its clinic application, we established and characterized these erasin-resistant/recurrent cells. The drug-resistant mechanisms, including Intra-tumoral heterogeneity and acquired resistance, are complex and vary with tumor types, genetics, and functional contexts [2, 5]. As we reported previously, HDAC6 inhibitors such as tubacin can be used as adjuvants to overcome the erasin-resistance in epithelial-type TNBC cells [33]. Similarly, the erasin-resistance in recurrent mesenchymal cells can be abolished by identified epigenetic inhibitors, such as histone deacetylase or methyltransferase inhibitors. Despite of morphological changes, the recurrent cells lack epithelial gene markers. The gene profiling and GSEA analysis showed a neuronal-like gene signature enriched in recurrent cells. This suggests that the underlying resistance mechanism in recurrent cells from mesenchymal-type TNBC may differ from that in epithelial-type TNBC.

Strikingly, the erasin-resistant/recurrent cells lose the ability of anchorage-independent growth, which may correlate with tumorigenic and metastatic potentials in vivo. This observation is further supported by the transcriptomic profiling analysis. GSEA analysis showed that the K-Ras, hypoxia, and interferon signaling gene signatures are impaired in recurrent cells, which are all known hallmarks of cancer. As well known, sustained K-Ras growth signaling and intra-tumoral hypoxia are important drive forces for breast cancer progression [47-49]. Recently, the interferon signaling in tumors has been
suggested as an inhibitory mechanism on immune destruction, which permits cancer cells to escape from immune clearance [50-53].

Notably, we observed a set of cystatin genes including CST1, CST2, and CST4 are dramatically downregulated in the recurrent cells. Cystatins belong to a superfamily of cysteine protease inhibitors. The expression of CST1, and CST2 has been reported to be positively correlated with cancer progression [39-40]. High expression of CST1 in lung, gastric, pancreatic, colorectal, and breast cancer leads to poor prognosis, metastasis, and tumor relapse, while knockdown of CST1 suppresses tumor growth [54-56]. In addition, the epithelial and mesenchymal transition (EMT) and TGF-β signaling can be induced by CST2 in gastric cancer, which can promote metastasis and tumor progression [41]. CSTs have also been suggested as a prognostic biomarker for various cancers [57-59]. CST4, known as cystatin SA, was also founded highly expressed in gastric and colorectal cancer to promote cell proliferation, invasion, and metastasis [42, 60]. The role of CST4 in breast cancer, particularly in TNB[61]C, remains unclear. We observed that downregulation of CST4 associates with loss of tumorigenicity in recurrent cells. Knockout of CST4 mediated by CRISPR/Cas9 in parental erastin-sensitive cells recapitulates this phenomenon and suppresses tumorigenicity. Intriguingly, CST4 knockout causes dramatic downregulation of gene expression in the K-RAS and interferon signaling pathways. Furthermore, CST4 expression is positively correlated with the expression of CST1 and CST2 in TCGA breast tumor samples, and knockout of CST4 represses the transcription of CST1 and CST2. All these observations suggest that CST4 acts as an upstream mediator of other cystatin genes and regulates multiple signaling pathways to promote tumorigenesis. Although similar downregulations were observed in another
recurrent cell model, these cystatins have relatively low basal expressions, suggesting that there are additional mechanisms to suppress tumorigenesis.

Taken together, our study suggests that targeted cysteine therapy via inhibition of the cysteine-GSH-GPX4 axis could be an effective strategy to treat mesenchymal TNBC with a low probability of tumor recurrence or relapse in patients. As an upstream mediator of multiple tumor-promoting signaling and its extracellular localization, CST4 could be a novel potential therapeutic target.

Materials and Methods

Cell culture and reagents

Breast cancer cells and 293T cells were all obtained from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotics (Penicillin/Streptomycin) in a 95% humidified incubator at 37 °C and 5% CO2. To establish in vitro erastin-resistant/recurrent cell models, MDA-MB-321 and BT549 cells were treated with serial concentrations of erastin for 6~8 challenging cycles: Cells were treated with either 2 µM or 5 µM of erastin for seven days then recovered in fresh DMEM media without erastin; When reaching enough cell population, cells were subjected to next erastin-challenge until the cells were able to grow or showed no significant cell death under erastin. All compounds including erastin, RSL3, tubacin, EPZ005687, and SGC0946 were obtained from Cayman Chemicals (Ann Arbor, Michigan, US).

RNA extraction and quantitative RT-PCR
Total RNA was extracted from the cells by PureLink™ RNA kit (Invitrogen), 2 µg RNA were reverse transcribed by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The cDNA was subjected to quantitative PCR using SYBR Green PCR master mix (Applied Biosystems). The relative difference in mRNA expression was normalized with actin using the ΔΔCT method. All primers in this study were listed in Supplementary Table S1.

**Gene expression profiling analysis**

The gene expression profiles of MB-MDA-231 E<sup>s</sup> and E<sup>r</sup> cells in triplicate were analyzed by Clariom™ S Assay, human (ThermoFisher Scientific). The data were deposited in the GEO database (GSE202514). Probe intensities were normalized by RMAExpress and then subjected to hierarchical clustering analysis by Cluster 3.0. The pathway enrichment was analyzed by Gene Set Enrichment Analysis (GSEA) module using the G2 annotated-genesets with default criteria of 1000 permutations.

**Immunoblot analysis**

Cell pellets were lysed in RIPA buffer (Sigma) supplemented with the protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). Protein concentrations were determined by the BCA protein assay. Equal amounts of protein were loaded in SDS-PAGE and transferred on the PVDF membrane for Western blot analysis. The signal was detected by the ECL plus Western blotting detection system (Bio-Rad) and visualized using LAS-4000 lumino image analyzer. The following antibodies were used in this study: PARP1 (9542S), phospho-p38 (4511S), phospho-H2AX (9718S), BNIP3 (44060S), β-actin (3700S), and β-Tubulin (86298S) were all obtained from Cell Signaling Technology. The CST4 antibody was obtained from GeneTex (GTX100690).
Lentiviral infection

Lentiviral particles were generated in 293T cells with lentiviral packaging plasmids using TransFectin™ Lipid Reagent (Bio-rad); the virus was collected from cell media after 48 hours of transfection. Cells were infected with indicated virus and followed by antibiotic selection. The plasmid pLX304-CST4 (Cat. #: HsCD00942127) was purchased from DNASU and the CRISPR/gRNA plasmids targeting CST4 were designed and purchased from VectorBuilder. pLX304-blasticidin and pLKO.1-puromycin vectors were used as controls.

Cell viability and epigenetic compound library screening

Cell viability and proliferation were measured by either trypan blue cell counting, CellTiter-Glo ATP assay kit (Promega), CytoTox-Fluor cytotoxicity assay kit (Promega), or Crystal Violet staining. The luminescent or fluorescent signals were measured by Synergy LX Multi-mode plate reader (BioTek). The epigenetic compound library screening was performed as described in the previous report [33].

Cell lipid peroxidation detection

Cells were stained with the Image-iT™ Lipid Peroxidation Kit (Invitrogen) for 30 min and then incubated with a live cell imaging solution (ThermoFisher Scientific). Cell images under bright-light and fluorescence conditions were recorded by ZOE™ Cell Imager (Bio-Rad).

Anchorage-independent cell growth and three-dimensional spheroid growth

Cells (3,000 cells/well) was resuspended in 2 mL DMEM plus 20% FBS with final 0.3% agar and layered on 0.6% basal agar. The feeding layer was added every 5 days.
After 20 days of incubation at 37°C, colonies were stained with crystal violet, colonies larger than 25 μm diameter were counted. Images were taken by camera and ZOE™ Cell Imager (Bio-Rad). To evaluate cell spheroid growth, 2000 cells per well were seeded into the ultra-low attachment 96-well plates (Corning). Cell morphology was monitored every two days and recorded by phase-contrast microscopy. Cell proliferation under three-dimension culture was examined by the CellTiter-Glo ATP assay kit at day 15.

**Cell migration assay**

Cells (5 x 10^5/well) were seeded in 6-well plates. When cells reached confluency, the wound was introduced by Pasteur pipette scratching; Floating cells were washed away by PBS, and then cells continued to grow in DMEM. Wound healing images were taken at indicated times using ZOE™ Cell Imager (Bio-Rad).

**Statistical analyses**

The significance of differences between data groups was determined using a student t test. Statistical analysis was performed using GraphPad Prism 8.0 Software. A p-value < 0.05 was considered statistically significant. Data was presented in charts as mean ± standard deviation (SD).

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Figure legends

**Figure 1. Establishment of in vitro erastin-resistant cells from mesenchymal TNBC**

(A) Scheme of establishing recurrent MDA-MB-231 and BT549 cells from multiple cycles of the erastin treatment.

(B) Cell morphology of MDA-MB-231 and BT549 parental cells (ES) and erastin-resistant cells (ER). Scale bar = 100 μm.

(C) Cell growth curves of MDA-MB-231 and BT549 ES vs. ER cells (n=3; *, p <0.001).

(D, E) Relative cell survival of MDA-MB-231 and BT549 ES vs. ER cells was measured by CellTiter-Glo ATP assay under either control, 5 μM erastin (D), or 1 μM RSL3 (E) treatments for 24 hrs (n=3; **, p <0.001).

(F) Immunoblotting analysis of pho-p38, pho-H2AX in ES and ER cells under either control (Con) or 5 μM erastin (E) treatments for 18 hrs, β-actin was used for the loading normalization.

**Figure 2. Epigenetic compound library screening identifies potent sensitizers for recurrent TNBC**

(A) Cell viability was measured in MDA-MB-231 ER cells treated with the epigenetic compound library under either the control (Con), or 5 μM erastin (E) conditions for 72 hrs.

(B, C, D) Relative cell survival of MDA-MB-231 ER or BT549 ER cells was assessed by either CellTiter-Glo assay or crystal violet staining under the control, 5 μM erastin (E), 5 μM tubacin (T), or erastin plus tubacin (E+T) treatments for 72 hrs (n=3; *, p<0.0001).
(E, F) Immunoblotting of indicated markers in MB-MDA-231 \( E^R \) or BT549 \( E^R \) cells treated as (B, C).

Figure 3. Recurrent TNBC \( E^R \) cells lose tumorigenic potential

(A) Anchorage-independent growth of \( E^S \) vs. \( E^R \) cells in soft agar. Upper panel represents full-well images; lower panel shows cell colonies with magnification. (n=3, Scale bar= 25 \( \mu m \)).

(B) Quantification of colonies according to the size (n=3; *, p<0.001; **, p<0.01).

(C) Wound healing of \( E^S \) vs. \( E^R \) cells at 48 hrs.

(D) 3D-spheroid living images of MDA-MB-231 \( E^S \) vs. \( E^R \) at the day 7 (Scale bar = 100 \( \mu m \)); Cell viability was measured under the low attachment condition at the day 15 (n=3, #, p<0.01).

Figure 4. Gene transcriptomic profile is altered in recurrent TNBC

(A) Volcanic and heatmap view of gene expression changes in MDA-MB-231 \( E^S \) vs. \( E^R \) cells.

(B) Gene set enrichments by GSEA analysis in MDA-MB-231 \( E^S \) cells.

(C) RT-qPCR expression analysis of genes in the K-Ras signaling pathway in MDA-MB-231 \( E^S \) vs. \( E^R \) cells (n=4; *, p<0.01).

(D) Gene set enrichments by GSEA analysis in MDA-MB-231 \( E^R \) cells.

(E) Cell morphology of MDA-MB-231 \( E^S \) and \( E^R \) cells at confluent stage.
Figure 5. Cystatin genes are downregulated in recurrent TNBC

(A, B) Relative expression of three cystatin-family genes in MDA-MB-231 and BT549 ES vs. ER cells (n=3; *, p<0.0001).

(C) CST4 protein expression in ES vs. ER cells.

(D) Correlation of gene expression between CST4 and either CST1 or CST2 in TCGA breast invasive carcinomas (p<0.001).

Figure 6. CST4 gene knockout suppresses the anchorage-independent growth

(A) Immunoblot analysis of CST4 protein expression in MDA-MB-231 vector (Vec) and CST4-knockout cell clones (gCST4).

(B) Anchorage-independent growth of MDA-MB-231 Vec and gCST4 cell clones (n=3, scale bar= 25 μm).

(C) RT-qPCR expression analysis of genes in the K-Ras signaling pathway in cells as (B) (n=4; #, p<0.0005).

(D) Relative expression of indicated cystatin genes in indicated cells as (B) (n=3; **, p<0.0001).

(E) Immunoblot analysis of CST4 protein expression in BT549 vector (Vec) and CST4-overexpressed cells.

(F, G) Anchorage-independent growth of BT549 Vec and CST4-overexpressed cells (n=3; *, p<0.01). Scale bar = 25 μm.
Establishment of in vitro erastin-resistant cells from mesenchymal TNBC (A) Scheme of establishing recurrent MDA-MB-231 and BT549 cells from multiple cycles of the erastin treatment. (B) Cell morphology of MDA-MB-231 and BT549 parental cells (ES) and erastin-resistant cells (ER). Scale bar = 100 μm. (C) Cell growth curves of MDA-MB-231 and BT549 ES vs. ER cells (n=3; *, p <0.001). (D, E) Relative cell survival of MDA-MB-231 and BT549 ES vs. ER cells was measured by CellTiter-Glo ATP assay under either control, 5 μM erastin (D), or 1 μM RSL3 (E) treatments for 24 hrs (n=3; **, p <0.001). (F) Immunoblotting analysis of pho-p38, pho-H2AX in ES and ER cells under either control (Con) or 5 μM erastin (E) treatments for 18 hrs, β-actin was used for the loading normalization.
Epigenetic compound library screening identifies potent sensitizers for recurrent TNBC (A) Cell viability was measured in MDA-MB-231 ER cells treated with the epigenetic compound library under either the control (Con), or 5 μM erastin (E) conditions for 72 hrs. (B, C, D) Relative cell survival of MDA-MB-231 ER or BT549 ER cells was assessed by either CellTiter-Glo assay or crystal violet staining under the control, 5 μM erastin (E), 5 μM tubacin (T), or erastin plus tubacin (E+T) treatments for 72 hrs (n=3; *, p<0.0001). (E, F) Immunoblotting of indicated markers in MB-MDA-231 ER or BT549 ER cells treated as (B, C).
Recurrent TNBC ER cells lose tumorigenic potential (A) Anchorage-independent growth of ES vs. ER cells in soft agar. Upper panel represents full-well images; lower panel shows cell colonies with magnification. (n=3, Scale bar= 25 μm). (B) Quantification of colonies according to the size (n=3; *, p<0.001; **, p<0.01). (C) Wound healing of ES vs. ER cells at 48 hrs. (D) 3D-spheroid living images of MDA-MB-231 ES vs. ER at the day 7 (Scale bar = 100 μm); Cell viability was measured under the low attachment condition at the day 15 (n=3, #, p<0.01).
Figure 4

Gene transcriptomic profile is altered in recurrent TNBC (A) Volcanic and heatmap view of gene expression changes in MDA-MB-231 ES vs. ER cells. (B) Gene set enrichments by GSEA analysis in MDA-MB-231 ES cells. (C) RT-qPCR expression analysis of genes in the K-Ras signaling pathway in MDA-MB-231 ES vs. ER cells (n=4; *, p<0.01). (D) Gene set enrichments by GSEA analysis in MDA-MB-231 ER cells. (E) Cell morphology of MDA-MB-231 ES and ER cells at confluent stage.
Cystatin genes are downregulated in recurrent TNBC (A, B) Relative expression of three cystatin-family genes in MDA-MB-231 and BT549 ES vs. ER cells (n=3; *, p<0.0001). (C) CST4 protein expression in ES vs. ER cells. (D) Correlation of gene expression between CST4 and either CST1 or CST2 in TCGA breast invasive carcinomas (p<0.001).
Figure 6

CST4 gene knockout suppresses the anchorage-independent growth (A) Immunoblot analysis of CST4 protein expression in MDA-MB-231 vector (Vec) and CST4-knockout cell clones (gCST4). (B) Anchorage-independent growth of MDA-MB-231 Vec and gCST4 cell clones (n=3, scale bar= 25 μm). (C) RT-qPCR expression analysis of genes in the K-Ras signaling pathway in cells as (B) (n=4; #, p<0.0005). (D) Relative expression of indicated cystatin genes in indicated cells as (B) (n=3; **, p<0.0001). (E) Immunoblot analysis of CST4 protein expression in BT549 vector (Vec) and CST4-overexpressed cells. (F, G) Anchorage-independent growth of BT549 Vec and CST4-overexpressed cells (n=3; *, p<0.01). Scale bar = 25 μm.

Supplementary Files

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- Supplementalfullblots.pdf
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