A Unique Morphological Phenotype in Chemoresistant Triple-Negative Breast Cancer Reveals Metabolic Reprogramming and PLIN4 Expression as a Molecular Vulnerability

Isabelle Sirois1,2, Adriana Aguilar-Mahecha1, Josiane Lafleur1, Emma Fowler1,2, Viet Vu1, Michelle Scrivener1, Marguerite Buchanan1, Catherine Chabot1, Aparna Ramanathan1, Banujan Balachandran1,2, Stéphanie Légaré1,2, Ewa Przybytkowska1, Cathy Lan1, Urszula Krzemien1, Luca Cavallone1, Olga Aleynikova1,2, Cristiano Ferrario1,3, Marie-Christine Guilbert4, Naciba Benlimame1, Amine Saad1,2,3, Moulay Alaoui-Jamali1,2,3, Horace Uri Saragovi5,6,7, Sylvia Josephy5,6,7, Ciara O’Flanagan8, Stephen D. Hursting8,9, Vincent R. Richard10, René P. Zahedi10,11, Christoph H. Borchers10,11,12, Eric Bareke13, Sheida Nabavi14, Peter Tonellato14, José-Ann Roy15, André Robidoux16, Elizabeth A. Marcus17, Catalin Mihalciou18, Jacek Majewski1,3,19, and Mark Basik1,2,3

Abstract

The major obstacle in successfully treating triple-negative breast cancer (TNBC) is resistance to cytotoxic chemotherapy, the mainstay of treatment in this disease. Previous preclinical models of chemoresistance in TNBC have suffered from a lack of clinical relevance. Using a single high dose chemotherapy treatment, we developed a novel MDA-MB-436 cell-based model of chemoresistance characterized by a unique and complex morphologic phenotype, which consists of polyploid giant cancer cells giving rise to neuron-like mononuclear daughter cells filled with smaller but functional mitochondria and numerous lipid droplets. This resistant phenotype is associated with metabolic reprogramming with a shift to a greater dependence on fatty acids and oxidative phosphorylation. We validated both the molecular and histologic features of this model in a clinical cohort of primary chemoresistant TNBCs and identified several metabolic vulnerabilities including a dependence on PLIN4, a perilipin coating the observed lipid droplets, expressed both in the TNBC-resistant cells and clinical chemoresistant tumors treated with neoadjuvant doxorubicin-based chemotherapy. These findings thus reveal a novel mechanism of chemotherapy resistance that has therapeutic implications in the treatment of drug-resistant cancer.

Implications: These findings underline the importance of a novel morphologic–metabolic phenotype associated with chemotherapy resistance in TNBC, and bring to light novel therapeutic targets resulting from vulnerabilities in this phenotype, including the expression of PLIN4 essential for stabilizing lipid droplets in resistant cells.
Introduction

The most aggressive form of breast cancer remains triple-negative breast cancer (TNBC) for which chemotherapy remains the cornerstone therapeutic. Indeed, TNBCs can be sharply divided into two prognostic groups: chemo-resistant and chemo-sensitive TNBCs (1). Even in early stages of TNBC, chemoresistance augurs a poor prognosis. Almost all studies into the mechanisms of chemoresistance have focused on few cell line models (mostly MDA-MB-231) and have used traditional in vitro drug selection approaches (e.g., increasing drug dosage over months) to uncover molecular factors responsible for chemoresistance (2–5). Typically, these approaches revealed a role for drug efflux pumps such as ABCB1 and related ATP-binding cassette (ABC) drug transporters (5). Unfortunately, few actual clinical cancers show these changes, and targeting them in cancer revealed limited benefits in part due to their vital role in normal physiology (6, 7). Chemotherapy resistance may also be due to the selection of drug-resistant cancer stem cells or tumor-initiating cells. In breast cancer, these cells were characterized by Al-Hajj and colleagues (8), and shown to be CD44+ and CD24− by flow cytometry. However, there has been little characterization of these cells in actual clinical drug-resistant breast cancers, and no successful attempts to target these cell variants in the clinical setting (9).

To identify factors that contribute to chemoresistance in TNBC tumors, we developed a clinically relevant model of chemoresistance by treating TNBC cells with a single dose (IC50 concentration) of doxorubicin (DOXO), and collecting colonies that outgrew in the following weeks, which we called doxorubicin-resistant cells (DOXO-R). We hypothesized that such a single bolus treatment would provide more clinically relevant clues to the mechanisms underlying chemoresistance, which could be exploited therapeutically. Here, we present the results of our analysis of these DOXO-R cells, identifying a complex chemoresistance phenotype. We validated features of this phenotype in chemoresistant TNBCs from the Q-CROC-3 trial, in which the genomic profile of postchemotherapy residual tumors was compared with that of prechemotherapy tumors (10). For the cluster dendrogram, we used a hierarchical clustering algorithm based on the Ward method to cluster the samples (11). Bioconductor package “SVA” was used to remove batch effects. Gene expression clustering analysis was performed under ethics protocol (14–16).

Materials and Methods

Breast cancer cell line

MDA-MB-436 (parental cells) were plated (density \(1.6 \times 10^4\) cells/cm²) and treated the next day with doxorubicin (0.3 \(\mu\)mol/L) or paclitaxel (0.01 \(\mu\)mol/L) corresponding to their IC\(_{50}\). After 48 hours of drug exposure the media containing drug was removed and replaced with fresh medium. After 4 to 6 weeks, colonies that grew were isolated, expanded, and kept in culture in absence of drug in complete medium. Pictures of growing cells were taken at 20× magnification using an Olympus CKX41 Microscope (IMEB Inc.). Pictures were taken using Infinity Analyze Software (Microscope World).

Xenograft model

A total of 1 × 10⁷ DOXO-R and parental cells resuspended in 30 \(\mu\)L of PBS and mixed with 30 \(\mu\)L of Matrigel (Corning 354234) were injected into the mammary fat pads of 5-week-old athymic nude mice (Charles River Laboratory) using a tuberculin syringe 27 G1/2 \(\frac{1}{2}\) cc. Tumor growth was monitored twice a week and tumors were measured with electronic calipers. When tumors reached about 400 mm³, mice were treated intravenously once a week with doxorubicin (5 mg/kg). At the end of the study, mice were sacrificed and tumors collected and snap frozen or put in formalin for paraffin embedding. Five-micrometer section from formalin-fixed, paraffin-embedded blocks were cut and stained with hematoxylin and eosin or used for other IHC analysis. The studies were approved by the review board of the research center.

Gene expression clustering analysis

Expression values of three biologic RNA replicates for each probe in the expression array were analyzed. The dataset consisted of four arrays from two batches, 32 samples in total. Background correction was performed for each array. The data were then filtered on the basis of gIsWellAboveBG, a quality control metric included in the Agilent platform. Probes that scored 0 for gIsWell AboveBG in all samples were removed. Of the 42,405 probes on the Agilent array, 32,405 probes remained in our analyses. Quantile normalization was performed on the log-transformed data separately for each batch. The function “Combat” from the Bioconductor package “SVA” was used to remove batch effects. For the cluster dendrogram, we used a hierarchical clustering algorithm based on the Ward method to cluster the samples based on the 1,000 most variable probes. The gene expression data is available at GEO under accession number GSE138233.
Figure 1.
Isolation and characterization of DOXO-R cells in vitro. A, Schematic of the isolation of DOXO-R cells in vitro. B, Light microscopy picture (20×) of a PGCC in DOXO-R cells actively budding daughter cells. Arrow indicates the region filled with multiple small nuclei, arrow heads indicate cell protrusions, and stars indicate mononuclear daughter cells. C and D, Colonies selected with doxorubicin (C1 and C8) are more resistant to doxorubicin and not cross-resistant to paclitaxel compared with parental cells following 48 hours of treatment (n = 4 independent experiment, six replicates/experiment; ***, P < 0.001 vs. parental cells). E, Parental and DOXO-R cells (C8) were engrafted in nude mice mammary fat pads. Once tumors reached 400 mm3, mice were treated once per week for 3 weeks with doxorubicin (5 mg/kg) and tumor growth was measured weekly. Tumor growth (%) is represented relative to beginning of treatment (week 0; n = 6 per group; ***, P < 0.001; **, P < 0.01). F, Percent of BCSC markers CD44HighCD24low is markedly decreased in C1 and C8 DOXO-R colonies versus parental cells as measured by flow cytometry (n = 3 independent experiments, two replicates/experiment; ***, P < 0.001; ***, P < 0.001).

(Continued on the following page.)
Functional transcriptomic analyses
Genes up- or downregulated by at least 1.5-fold (or Log2 ≥ 0.6; P < 0.05) in DOXO-R cells C1 and C8 compared with parental cells were considered for further analyses. Gene set enrichment analysis (GSEA; http://www.broad.mit.edu/gsea/) was performed using GSEA software and the Molecular Signature Database (MsigDB) on genes with significant changes. Top 100 significant gene sets using the C5 module overlap analysis were considered significant with P value and FDR < 0.05. We acknowledge our use of the GSEA, GSEA software, and MSigDB [11].

ATP and lactate measurement
ATP measurement was performed according to the manufacturer’s protocol using the Cell Titer-Glo Assay (C7571, Promega). Cells were plated (625–5,000 cells per well, four replicates) in a 96-well plate in RPMI1640 medium with 10% FBS and 5 mMol/L glucose. The next day, medium was replaced with the same medium containing 12.5 μMol/L oligomycin or vehicle (DMSO) and incubated for 45 minutes [12]. Lysis buffer was then added to the wells and incubated for 10 minutes on a shaker and then transferred into a white bottom plate and luminescence was recorded using FLIIOstar Optima. For lactate measurement, 3 × 10^4 cells/well (6-well plate) were plated in RPMI1640 medium with 10% FBS and 5 mMol/L glucose in triplicate. The supernatants were collected and centrifuged 24 and 48 hours later. Adherent cells were also collected and counted at the same time of supernatant harvesting. Lactate measurement was performed using Lactate Reagent (735-10) and Lactate Standard Solution (735-11) from Trinity Biotech according to the manufacturer’s protocol. Data were presented by abs/cell.

Seahorse
Oxygen consumption rate (OCR) was analyzed using a XF96 Seahorse Metabolic Flux Analyzer (Agilent Seahorse Technologies). Cells were seeded at a density of 1 × 10^4 in each well of a 96-well Seahorse cell culture plate in complete media, with 12 replicates per experiment. Twenty-four hours later, cells were incubated in assay media (serum-free RPMI1640 media with 1 mMol/L glucose, 2 mMol/L glutamine, and 1 mMol/L pyruvate, pH 7.4) in a non-CO2 incubator for 1 hour prior to the metabolic flux assay. OCR was measured and oligomycin (1.0 μMol/L), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (1.0 μMol/L), and rotenone/antimycin A (0.5 μMol/L) were added sequentially as indicated. Upon completion of the Seahorse XF24 Flux analysis, cells were lysed to calculate the protein concentration using BCA assay (Thermo Fisher Scientific) and normalize the measurements to the total amount of protein per well.

Clonogenic assay
Cells were seeded at a density of 1,000 cells per well and grown in RPMI1640 supplemented with 10% FBS for 24 hours. Media was then replaced with 3 mL of drug. Cells were stained with a fixing solution containing crystal violet 14 to 21 days after the addition of the drug. Quantification was performed using the Gel Count software.

siRNA. Two PLIN4 and two PPARG siRNA plasmids were purchased from Qiagen: PLIN4 siRNAs: HS_KIAA1881_5 (SI04233421) and HS_KIAA1881_6 (SI04241762), PPARG: HS_PPARG 2 (SI00071680) and HS_PPARG 5 (SI02634275) and control siRNA (All Stars Neg Control siRNA, Qiagen) plasmid was also used. MDA-MB-436 cells were counted and plated at 4 × 10^3 cells/well in a 6-well plate, and immediately transfected with one of the three plasmids using RNAlipse Max Lipofectamine (Invitrogen).

After 24 hours, the transfection media was replaced with fresh RPMI plus 10% FBS (ATTC). Cells were replated 72 hours post-transfection at 2 × 10^5 cells/well for future lystate harvesting. Cell lysates were harvested 4 to 9 days after transfection.

Proteomics
Protein from cell pellets was extracted as described by Coman and colleagues [13] with minor modifications. Further details are described in the Supplementary Data. The proteomics data is available at PRIDE archive under the accession number: PXD013872.

KM analysis. These analyses were performed using the following website http://kmplot.com/analysis/ [14].

Statistical analysis
Data are expressed as mean ± SEM. Statistical analysis was performed with GraphPad Prism software program (version 6).

For some analyses, differences were determined by using the two-tailed Student t test. When indicated, we used two-way ANOVA with Dunnett or Sidak multiple comparison test. Details of these analyses are shown in Supplementary Table S18. Differences with values of P < 0.05 were considered to be significant.

Clinical samples and ethics
Tumor samples were collected prior to and after treatment from patients with TNBC participating in the Q-CROC-03 neoadjuvant study (NCT01276899; ref. 15). All patients provided informed consent and the study was approved by each institution’s research ethics board.

Results
Treatment with chemotherapy enriches for a genomically divergent subpopulation of chemoresistant TNBC cells
To isolate drug-resistant cells in vitro, we simulated the clinical conditions experienced by patients with TNBC undergoing neoadjuvant chemotherapy (NAC) with doxorubicin or paclitaxel...
tal cells and similar or decreased levels of ALDH classic surface markers for BCSCs (CD44High/CD24Low), as well as selected for breast cancer stem cells (BCSC), we measured the doxorubicin treatment (Fig. 1E). To determine whether we had cells in vivo 40 hours, C1 and C8 were isolated and grown individually without drug and then retested for chemosensitivity at different passages. Colonies C1 and C8 were DOXO-R but not cross-resistant to paclitaxel or cisplatin (Fig. 1C and D; Supplementary Fig. S1A and S1B). Importantly, mRNA levels of ABCB1 were unchanged in DOXO-R cells compared with parental cells (Supplementary Fig. S1C). These colonies were growing slightly slower than parental MDA-MB-436 cells (doubling time of parental cells = 40 hours, C1 = 43 hours, and C8 = 53 hours, Supplementary Fig. S1D). We further confirmed the chemoresistance of DOXO-R cells in vivo by showing that DOXO-R xenografts were resistant to doxorubicin treatment (Fig. 1E). To determine whether we had selected for breast cancer stem cells (BCSC), we measured the classic surface markers for BCSCs (CD44High/CD24Low), as well as the percent of ALDH + cells measured by the Aldefluor assay (8). Surprisingly, we found a relative decrease in the CD44High/CD24Low subpopulation in DOXO-R cells compared with parental cells and similar or decreased levels of ALDH + cells in DOXO-R cells C1 and C8 compared with parental cells (Supplementary Fig. S1E), indicating that our DOXO-R cells do not represent these BCSCs (Fig. 1F).

Interestingly, colonies selected with paclitaxel (CA, CD, and CF) were also resistant to doxorubicin on retesting, even though they shared the cellular phenotypic response described above (Fig. 1G; Supplementary Fig. S1F–S1H). Indeed, unsupervised clustering of gene expression profiles of different biological replicates show that paclitaxel-selected and doxorubicin-selected–resistant cells clustered together and separately from the parental MDA-MB-436 cells (Fig. 1H). Array comparative genomic hybridization (array CGH) of the different resistant colonies revealed that C1 and C8 DOXO-R cells shared almost all DNA copy changes in parental and DOXO-R tumor xenografts using hematoxylin as a primary antibody and fluorescent secondary antibodies (Fig. 3A, right; Supplementary Fig. S2). Quantification of PGCCs in parental and DOXO-R tumor xenografts using hematoxylin as a well as Ki67 IHC staining (Fig. 3B) confirmed that PGCCs were present in significantly higher quantities (4–5-fold) in DOXO-R compared with parental tumors (Fig. 3C) and that the proportion of cycling PGCCs was similar to that of mononuclear cells.

To complete the characterization of the morphologic phenotype, ultrastructural studies using electron microscopy were performed on both DOXO-R and parental cells. Electron microscopy revealed the presence of small mitochondria in the cytoplasm as well as a marked enrichment of lipid droplets in DOXO-R cells compared with parental cells (Fig. 3D), confirmed by BODIPY staining (Fig. 3E). Indeed, lipid droplet quantification by flow cytometry using BODIPY showed a 3-fold increase in DOXO-R compared with parental cells (Fig. 3F). Electron microscopy also revealed that lipid droplets were interacting with mitochondria (Fig. 3D, bottom, right), which, to our surprise, were twice as small and twice as numerous in DOXO-R cells compared with parental cells (Fig. 3G and H). We also found that mRNA and protein levels of peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), the key regulator of mitochondrial biogenesis (21), were increased in DOXO-R cells
(Fig. 3J–K) suggesting that increased mitochondrial biogenesis may contribute to the presence of numerous smaller mitochondria in DOXO-R cells. Taken together, the appearance of increased numbers and “budding” PGCCs, the increased numbers of lipid droplets and small mitochondria, as well as the development of neuron-like cellular projections in DOXO-R colonies represent a novel complex cellular phenotype associated with chemoresistant TNBC cells.
Figure 3.
PGCCs, lipid droplets, and mitochondria are characteristic of chemoresistant TNBC cells. A, Light microscopy (LM, top; magnification, 20×) and Phalloidin and DAPI staining (immunofluorescence (IF), bottom; magnification, 40×) of parental (left) and DOXO-R C8 cells showing two PGCCs with different morphologic features including large cytoplasmic size (middle) and the presence of long cell projections and minimal cytoplasm (right). B and C, Ki67 and hematoxylin staining by IHC for PGCC quantification in tumors from mice engrafted with parental and DOXO-R C8 cells following treatment with doxorubicin in vivo (see Fig. IE). Arrow heads indicate PGCCs (n=5; ***, P<0.001). D, Ultrastructural analysis of parental and DOXO-R cells using electron microscopy, low power (left), revealed increased lipid droplets (arrows) and smaller mitochondria (arrowheads) in DOXO-R compared with parental cells. Scale bar, 2 μm. Close contact of lipid droplets and mitochondria (stars) are shown in islets of each micrograph (right). Nuclei are labeled with the letter N. E and F, Increased lipid droplets in DOXO-R versus parental cells as visualized with Bodipy staining (IF green and nuclei are stained with DAPI) and quantified by flow cytometry (MFI, mean fluorescence intensity), n=3 independent experiments, two replicates/experiment; ****, P<0.0001. G and H, Quantification of mitochondria size and number using electron micrographs in parental and DOXO-R cells shows doubling of the number of mitochondria and halving of their size in DOXO-R versus parental cells (n=25 micrographs quantified/group; *** , P<0.0001; ***, P<0.0001). I, VDAC IHC shows increased VDAC staining in DOXO-R versus parental mouse tumor xenografts. Scale bar, 50 μm. J and K, Endpoint PCR shows increased PGC1-alpha mRNA levels and immunoblots of VDAC and PGC-1alpha showed increased protein levels in DOXO-R cells compared with parental cells, (n=5; ***, P<0.0001). Actin, RPS6, and β-tubulin are shown as loading control. Data represent the mean ± SEM and Student t test was used for C, F–H, and J analyses.
MRI cells have altered metabolism.

The increase in lipid droplets and the altered mitochondrial phenotype indicate that DOXO-R cells may have altered their metabolism relative to parental cells. First, we found that lactate secretion is significantly decreased in DOXO-R cells compared with parental cells, suggesting less glycolytic activity (Fig. 4A). We then found that DOXO-R cells are more sensitive to treatment with oligomycin, a mitochondrial ATPase inhibitor (Fig. 4B). Moreover, DOXO-R cells displayed higher baseline OCR than parental cells as measured by Seahorse (Fig. 4C and D). Finally, DOXO-R cells were more sensitive than parental cells to treatment with metformin, a drug targeting the electron transport chain of mitochondria (Fig. 4E). These results demonstrate that the smaller mitochondria are functional and are associated with a greater dependency on oxidative phosphorylation (OXPHOS) in DOXO-R cells.

The phenotypical and physical association of mitochondria with lipid droplets in DOXO-R cells led us to hypothesize that the fat stored in these lipid droplets could be an energy substrate for ATP production in mitochondria, rendering DOXO-R cells more reliant on fat as a source of energy. Addition of the unsaturated fatty acid oleate to the cell medium decreased the viability of parental but not DOXO-R cells after 3 and 6 days (Fig. 4F), suggesting a greater tolerance to extracellular fatty acids in these cells.
cells. Because stored fatty acids are transferred into mitochondria for beta-oxidation through the CPT1 transporter, we evaluated whether chemical inhibition of CPT1 using the drug etomoxir could affect DOXO-R cells. Indeed, we found that DOXO-R cells were more sensitive to etomoxir compared with parental cells as evaluated by cell viability and clonogenic assays (Fig. 4G–I). Thus, DOXO-R cells are more dependent on fatty acid transport into mitochondria.

"Omics" analysis supports the morphologic and metabolic phenotype of chemoresistant TNBC cells

We performed a transcriptomic analysis of DOXO-R C1 and C8 cells compared with parental cells, and identified 1,852 significantly upregulated genes common to both C1 and C8 (fold change ≥ 1.5, P ≤ 0.05). Pathway analysis of these genes was performed using the GSEA/MsigDB tool (11, 22, 23). The analysis using the gene ontology (GO) terms (C5 module, 5,917 gene sets) revealed that lipid metabolism, mitochondrion, neurogenesis/neuron, and cell projection gene sets were significantly enriched (among the top 100 most significant GO terms) in the DOXO-R cells (Supplementary Fig. S3A; Supplementary Tables S2 and S3), supporting the morphologic and metabolic phenotype observed in DOXO-R cells.

Second, we investigated the copy-number variation gains observed in the DOXO-R cells. Of the 17 regions of increased copy number in the DOXO-R cells (Fig. 1E), we selected 16 regions in which all genes contained within the regions also showed increased RNA levels compared with the parental cells. Using these criteria, we identified 407 genes in these 16 areas that showed both increased DNA copy number and RNA levels compared with parental cells (Supplementary Fig. S3A; Supplementary Tables S1 and S4). GSEA of these genes revealed that mitochondrion, lipid metabolism, and cell projection gene sets were enriched for in the DOXO-R cells using the C5 module, suggesting that the genomic features of DOXO-R cells marked these cells with an altered metabolic activity.

Third, to identify a putative upstream transcriptional regulator in DOXO-R cells, we performed Ingenuity Pathway Analysis of the genes up- and downregulated in these cells. Among the top 10 most significant upstream regulators associated with the DOXO-R transcriptomic changes, we identified peroxisome proliferator-activated receptor gamma (PPARG) and peroxisome proliferator-activated receptor alpha (PPARA), nuclear transcription factors known to regulate mitochondria and lipid metabolism (Supplementary Fig. S3B; ref. 24). PPARG mRNA levels were unchanged in DOXO-R versus parental cells, whereas both PPARG mRNA and protein levels were significantly increased in DOXO-R cells (Fig. 5A and B). Interestingly, we observed nuclear staining of PPARG suggestive of sustained PPARG activity in DOXO-R cells that were also positive for BODIPY staining (Fig. 5C). To assess the functional role of PPARG on the survival of DOXO-R cells we used a specific PPARG inhibitor, GW9662, and found that it significantly decreased the formation of DOXO-R colonies (Fig. 5D and E), confirming that PPARG itself is a potential upstream regulator in these cells.

To complete our analysis, we performed a comparative proteomic analysis of parental and DOXO-R cells (C1 and C8). Label-free quantification of 2,000 proteins revealed that 98 proteins were significantly increased in DOXO-R cells compared with parental cells. GSEA analysis of the genes encoding for these proteins using the C5 module revealed significant gene sets.
related to lipids and mitochondria (Supplementary Fig. S3A; Supplementary Tables S6 and S7). Taken together, the omics analysis of DOXO-R cells supports the morphologic and metabolic features of the DOXO-R phenotype.

The DOXO-R phenotype uncovers a subpopulation of chemoresistant TNBC tumors

To validate our observations related to the DOXO-R phenotype in clinical samples, we analyzed chemoresistant TNBCs from the...
PLIN4 is a novel vulnerability in chemoresistant TNBC cells. A and B, PLIN4 mRNA quantification by RT-PCR (n = 3 independent experiments; **, P < 0.01) and PLIN4 immunoblotting (n = 6) in parental and DOXO-R cells. C, Exclusive PLIN4 staining by immunofluorescence (green) in DOXO-R cells compared with parental cells. Magnification, 40x. D, PLIN4 protein is exclusively expressed in DOXO-R mouse tumor xenografts from Fig. 1E as shown by IHC PLIN4 staining. Scale bar, 100 μm. E, PLIN4 mRNA quantification by RT-PCR following PLIN4 and control (Ctrl) scrambled siRNA using two oligos (siRNA(5) and (6)) in DOXO-R C8 cells (n = 3 independent experiments; ***, P < 0.001). Note that PLIN4 mRNA and protein levels in parental cells are barely detectable (B) and so could not be measured in siRNA-transfected parental cells. F and G, Immunoblotting and quantification by densitometry of PLIN4 protein levels following siRNA treatment [ctrl, PLIN4(5), and PLIN4(6)] in DOXO-R cells, n = 3 independent experiments; ***, P < 0.001 compared with siRNA control. RPS6 is shown as loading control. PLIN4 siRNA decreased cell viability of DOXO-R cells but not parental cells as measured by Alamar blue (72 hours; n = 3 independent experiments, three replicates/experiment; ***, P < 0.001). H and I, clonogenic assays (14–21 days; n = 4 independent experiment, two replicates/experiment; ***, P < 0.0001; I and J). (Continued on the following page.)
Q-CROC-3 clinical trial (15). We collected pre- and post-NAC tumors in these patients and focused on 9 of 13 pairs of pre-post-chemotherapy tumors treated with doxorubicin and paclitaxel and for which we had RNAseq data. First, histologic quantification of PGCCs was performed in all 18 tumor samples using Ki67 and hematoxylin staining to identify PGCCs (Fig. 6A–D). Although we found no difference in mean % PGCC number when comparing all pre- versus all post-NAC samples (Fig. 6A), we did identify a subset of patients (4/9) with increased percent PGCCs in their chemoresistant residual tumors (NEO 24, 25, 27, and 31; Fig. 6B–D). Interestingly, in one of these patients (NEO25) in whom a metastatic liver lesion was later biopsied, the metastatic lesion contained a much higher proportion of PGCCs compared with the postchemotherapy primary breast tumor sample (Fig. 6D). These data are concordant with the enrichment of PGCCs observed in DOXO-R cells.

We then performed GSEA using the C5 gene sets modules on genes differentially expressed in the residual tumors of each of the 9 patients. Because of the great number of differentially expressed genes between post-NAC and pre-NAC tumor samples, we used more stringent criteria for this analysis (post/pre-NAC ≥ 2.5-fold, top 100 significant gene sets; Supplementary Tables S8–S17). Cross-checking C5 module GO terms associated with the DOXO-R transcriptome phenotype, we found that seven of nine tumors were significantly enriched with lipid, seven of nine with neuron, six of nine with cell projection, and one of nine with mitochondria and gene sets (Supplementary Fig. S3D). These results support the enrichment of the DOXO-R transcriptional phenotype in chemoresistant tumors.

We further looked for specific genes related to the metabolic and neuron-like changes characterizing the DOXO-R phenotype in each of the 9 chemoresistant patients (Supplementary Table S0). We found that PPARGC1A mRNA was overexpressed (post/pre-NAC ≥ 2-fold) in four post-NAC tumors (NEO25, 28, 35, and 44) and that PPARG mRNA levels were overexpressed in three post-NAC tumors (NEO25, 27, and 44). At the protein level, higher nuclear PPARG protein expression was observed by IHC in the post-NAC samples of patients NEO25 and NEO27, with the highest level of expression in the metastatic tumor of patient NEO25 (Fig. 6E–F). Similarly, MAPT mRNA was overexpressed in the residual tumors of NEO27 and NEO30 and protein expression was validated by IHC (Fig. 6F).

PLIN4 is a novel marker and vulnerability of the DOXO-R phenotype of chemoresistant TNBC cells

Considering the important role of PPARG in regulating the expression of genes involved in lipid and glucose metabolism, we looked at the most overexpressed genes to identify key genes that could be associated with the complex phenotype observed in DOXO-R cells and regulated by PPARG. Indeed, a perilipin-associated protein, PLIN4, was among the top five genes overexpressed in DOXO-R cells (Fig. 7A–D; Supplementary Fig. S3C, Supplementary Table S5). Perilipins are lipid droplet-associated proteins that play an important role in fat mobilization (25) and their expression is transcriptionally regulated by PPARG (26, 27). PLIN4 mRNA and protein levels were exclusively present and highly abundant in DOXO-R cells (including PGCCs; Fig. 7A–C) compared with parental cells, and PLIN4 proteins was only expressed in DOXO-R C8 mice tumor xenografts (Fig. 7D). We then validated the role of PPARG in PLIN4 expression regulation in DOXO-R cells. Transient and partial silencing of PPARG in DOXO-R cells resulted in a significant decrease in mRNA and protein levels of PLIN4 (Supplementary Fig. S4A–S4E) demonstrating that PPARG regulates the expression of PLIN4 in our model.

Looking at the clinical samples (Supplementary Table S0), we found that the mRNA levels of PLIN4 were upregulated in seven of nine resistant tumors (NEO02, 24, 25, 27, 28, 30, and 44). We then found that PLIN4 protein expression was increased in the post-NAC samples of 4 of 9 patients by IHC (NEO02, 25, 27, and 30), including NEO25 and NEO27, which also show increased numbers of PGCCs and expression of PPARG in their post-NAC samples (Fig. 6E–F). Interestingly, the residual tumor from patient NEO25 showed 100% tumoral PLIN4 staining with high PLIN4 protein expression also being observed in the metastatic tumor obtained from this patient (Fig. 6F), suggesting that high PLIN4 expression is maintained during disease progression in this tumor. In our small cohort, the tumors expressing PLIN4 in the postchemotherapy samples showed a worse overall survival (OS, \( P = 0.048 \)) than those without PLIN4 expression. In a larger dataset, high PLIN4 expression is a poor prognosis marker in ER– but not in ER+ breast cancer resulting in shorter OS in an independently available cohort of 626 patients with breast cancer (Fig. 6G; ref. 14). Interestingly, in the subgroup of patients in this cohort that received chemotherapy, the difference of outcomes of PLIN4 expressors and nonexpressors between ER– and ER+ breast cancer is also evident, with a \( P \) value of 0.00095 and a HR of 2.92 (\( n = 104 \)) favoring PLIN4 low expressors for ER+ patients and the absence of prognostic value (\( P = 0.79 \)) for ER– patients (\( n = 155 \); Supplementary Fig. S5A and S5B). These results validate our \( in \) \( vitro \) observations and further demonstrate that a lipid metabolic program alteration is a clinically relevant feature of a subset of chemoresistant TNBCs.

Accumulation of free fatty acids in the cells could be toxic and storage in lipid droplets not only serves to provide fatty acid for energy but also to protect cells from lipotoxic cell death. Considering that PLIN4 was among the top overexpressed genes in DOXO-R cells and is expressed in 78% of chemoresistant TNBC tumors treated with doxorubicin, we hypothesized that PLIN4 could represent a novel vulnerability in DOXO-R cells. To study the functional role of PLIN4 in the...
viability of DOXO-R cells, we silenced PLIN4 expression with siRNA in parental and DOXO-R cells (Fig. 7E–G). PLIN4 silencing resulted in a significant and marked decrease in cell viability in DOXO-R cells but not in parental cells (Fig. 7H). Similarly, silencing of PLIN4 almost totally abrogated the capacity of colony formation in DOXO-R cells compared with scrambled siRNA controls but had only a minor impact on parental cells (Fig. 7I–J).

We further validated the effect of PLIN4 knockdown on a conditionally reprogrammed cell line (CRC T786) generated in our laboratory from a residual drug-resistant TNBC tumor. Interestingly, like DOXO-R cells, CRC T786 cells also expressed high levels of PPARγ and PLIN4 proteins (Fig. 7K–M and P), T786 cells also contained PGCCs, positive for PLIN4 as shown by IHC and hematoxylin staining (Fig. 7M), many lipid droplets, and neuron-like cellular projections (Fig. 7N–O). We confirmed that CRC T786 cells were also resistant to doxorubicin in vitro (Supplementary Fig. S6A and S6B). We found that siRNA targeting of PLIN4 also resulted in significant decreased clonogenic growth in CRC T786 cells (Fig. 7Q–S). Therefore, PLIN4 plays an important role in the viability and growth of chemoresistant cells, marking it as a potential therapeutic vulnerability to target chemoresistant TNBC tumors.

**Discussion**

In the clinic, the most aggressive form of breast cancer, TNBC, is treated with chemotherapy alone, with clinical evidence of chemoresistance indicating very poor prognosis. Multiple therapeutic approaches have been attempted to solve the TNBC conundrum. Possible therapeutic targets have included the underlying genomic instability itself associated with defective DNA repair (e.g., PARP; ref. 28), epithelial-to-mesenchymal transition (EMT; ref. 30), EGFR (31), PLIN4, or polo-like kinase-1 dependence (ref. 29), among others. None of these, with the recent exception of PARP inhibitors in the 10% to 15% of TNBCs that are BRCA1/2 mutation carriers (34), have shown clinical utility, although some are being further investigated in early phase clinical trials (35).

Avoiding the limitations of prolonged high dose models of drug resistance, we developed a clinically relevant model of chemoresistance using short-term high dose doxorubicin or palmitate treatment in the mesenchymal MDA-MB-436 cell line, both drugs forming the backbone of chemotherapeutic regimens in TNBCs. Strikingly, treating the cells with either high dose doxorubicin or palmitate resulted in the emergence of cells with a common unique constellation of major morphologic changes including the appearance of PGCCs, neuron-like cellular projections, multiple small mitochondria, and a marked enrichment of lipid droplets, which we called the DOXO-R phenotype. The DOXO-R phenotype in vitro was permanent even with drug removal. We found molecular changes supporting these morphologic alterations, including increased levels of VDAC, PPARγC1A, MAPT/Tau, PPARγ, and PLIN4, the latter found to be a novel targetable vulnerability in chemoresistant TNBCs.

Although the importance of each of these features in the acquisition of resistance per se was not addressed in this study, the individual elements of our complex DOXO-R phenotype have been associated with chemoresistance by others. The DOXO-R cells appear to be “budding” from PGCCs, similar to several recent reports, which describe the presence of multinucleated giant cells from which mononuclear cells bud off via asymmetrical division in the context of drug resistance in different cancers (36–47). Indeed, Liu and colleagues wrote of an “evolutionary conserved archaic embryonic program in somatic cells that can be repressed for oncogenesis” (36). Like us, Fei and colleagues (37) also noted the appearance of PGCCs in metastatic lesions. These PGCCs have stem cell–like features and have been associated with EMT (37). However, our DOXO-R cells were not analogous to the cancer stem cells as described by Al-Hajj and colleagues (8), but appear to represent a different differentiation program likely originating from preexisting PGCCs in TNBCs. This may be because the parental cells are already “mesenchymal”, and thus cannot undergo EMT. Indeed, DOXO-R cells appear to have undergone a type of mesenchymal-to-neuronal differentiation potentially associated with MAPT expression. This neuron-like morphology was described in the differentiation of PGCCs in previous reports as well (36, 39), although without providing molecular factors associated with it, that is, expression of MAPT. Hence, MAPT expression has been associated with resistance to chemotherapy in patients with breast cancer (48).

Abnormal or reprogrammed fatty acid or lipid metabolism has also been associated with drug resistance (49–54) and lipid droplets have been observed in both cancer stem cells and chemoresistant cancer cells (54–56). Alterations in mitochondria dynamics have also been reported to be associated with chemoresistance due to their critical role in the cellular response to oxidative stress (57–60). Interestingly, Rambold and colleagues recently reported a link between mitochondrial dysfunction and lipid droplet lipolysis in starving cells (61). Furthermore, the key upstream regulator in our DOXO-R phenotype, PPARγ, is also known to play a critical role in mitochondrial biogenesis through its interaction with PGC-1α (62). PPARγ has also been linked to a role as a stress response regulator in neurons (63). In summary, by observing the presence of these different processes simultaneously active in our DOXO-R phenotype, our model provides a unique opportunity to study the multifaceted aspects of chemoresistance together.

The above findings point to a severe metabolic disturbance and suggest a metabolic reprogramming in chemoresistant TNBCs. Indeed, despite these mitochondrial alterations, the metabolism of DOXO-R cells reflects a greater reliance on mitochondrial respiration, OXPHOS, with an increased sensitivity to metformin. The importance of OXPHOS in drug resistance in cancer has been appreciated more recently, with OXPHOS inhibitors now being tested increasingly in clinical trials (64). Besides metformin, DOXO-R cells were more sensitive to GW9662, a PPARγ inhibitor, and Etomoxir, an inhibitor of the mitochondrial fatty acid transporter CPT1, indicative of lipid metabolic reprogramming underlying the emergence of these cells. However, the most significant vulnerability was the dependence of DOXO-R cells on lipid packaging proteins that protect the intracellular environment from excess free lipids. There are five different perilipins, which are critical to the integrity of lipid droplets in different tissues (65). Silencing of the perilipin most expressed in DOXO-R cells (and in chemoresistant TNBC tumors), PLIN4, resulted in marked cell death in DOXO-R cells, indicating a novel lipid-related vulnerability in chemoresistant TNBCs, which may have implications for other chemoresistant cancers. Although lipid droplets have been associated with chemoresistance and stemness, their reliable detection in clinical samples has been
cumbersome (57), leading to a clinical under-appreciation of their critical role in maintaining the survival of chemoresistant tumors. Our use of a protein-based biomarker for lipid droplets (PLIN4) will enable the further study of the role of lipid droplets in the clinical context of chemoresistant tumors.

Finally, not only do we report for the first time a constellation of these various changes occurring together in chemoresistant TNBC cells, but we also show that our observations are consistent with what is observed in clinical specimens. In fact, similar morphologic and molecular changes are present in a distinct primary cell line model derived from a chemoresistant TNBC tumor and more importantly, similar morphologic and/or molecular features were observed in a subset of chemoresistant clinical tumor TNBC samples. Although we cannot perform ultrastructural studies on our patient tumor samples, about two of three TNBC tumors resistant to doxorubicin acquired some or most of the molecular changes observed in DOXO-R cells. RNAseq of these chemoresistant tumors revealed enrichment of gene sets related to lipid metabolism, mitochondria, neuron biology, and cell projection, which were also enriched for in DOXO-R cells. Increased numbers of PGCCs and PLIN4 expression were easily observed in some of these postchemotherapy samples. In particular, one of our chemoresistant TNBC tumor samples (NEO25) and the corresponding metastatic lesion of this patient showed most of the molecular features and morphologic changes of the DOXO-R phenotype. The clinical implications are that metabolic vulnerabilities, including increased OXPHOS and dependence on lipid metabolism, could be targeted in a subset of chemoresistant TNBCs. Furthermore, the observation of increased PLIN4 protein expression and PGCCs (identified by KI67 and hematoyxlin) provides a biomarker phenotype that can readily identify this specific subset of metabolically vulnerable chemoresistant TNBCs by IHC. In summary, our work has revealed a unique, coordinated portrait of chemoresistant TNBCs that is potentially targetable and that may account for at least some of the poor results obtained in this most aggressive form of breast cancer.

Disclosure of Potential Conflicts of Interest

C.H. Borchers is a CSO at MRG Proteomics, is a staff member at MRG Proteomics, is a CTO at Molecular You, is a founder and CSO at Creative Molecules, and has ownership interest (including patents) in MRG Proteomics, Molecular You, and Creative Molecules. P. Tonellato is a professor and director of Bioinformatics at the University of Missouri. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: I. Sirois, A. Aguilar-Mahecha, H.U. Saragovi, C.H. Borchers, M. Basik
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Sirois, A. Aguilar-Mahecha, M. Buchanan, A. Ramanathan, S. Légaré, C. Lan, C.H. Borchers, M. Basik
Study supervision: I. Sirois, A. Aguilar-Mahecha, C. Mihalcioiu, J. Majewski, M. Basik
Other (performed scientific assays and collected and analyzed data): M. Scriver
Other (contributed in sample preparation, acquisition, data analysis, and text pertaining to proteomics experiments): V.R. Richard
Other (supervised aspects of computational analysis and bioinformatics): P. Tonellato

Acknowledgments

The breast biobank infrastructure is supported by the FRQS Réseau Recherche cancer arc sein and Quebec Breast Cancer Foundation. The QCROC-3 project was supported by Genome Quebec. The project was also supported by the Canadian Research Society (Elucidating novel mechanism of drug resistance in triple negative breast cancer, #21439). Establishment of PDXs and patient-derived cell lines is supported from the Canadian Institutes of Health Research (Innovative preclinical models to overcome drug resistance in triple-negative breast cancer, #377429). McPeak Sirois Consortium, JCH Foundation, McGill University, and private donations. I. Sirois was supported by Bourse de recherche en cancer du sein (Profil moléculaire de la double résistance du cancer du sein triple négatif aux agents anti-tumeur, #38W-127602) in partnership with Eileen Iwanicki Foundation, Canadian Institutes of Health Research, and the Breast Cancer Society of Canada (North Sarnia, Ontario, Canada). I. Sirois and V. Vu were also supported from The McGill Integrated Cancer Research Training Program. A. Ramanathan was supported by Biotalent Canada and E. Fowler was supported by the Lady Davis Institute TD Stuudentship award. V.R. Richard, R.P. Zahedi, and C.H. Borchers are grateful to Genome Canada GTP platform funding for operations and technology development: 264PRO. C.H. Borchers is supported by the Segal McGill Chair in Molecular Oncology at McGill University (Montreal, Quebec, Canada) and the Warren Y. Soper Charitable Trust and the Alvin Segal Family Foundation to the Jewish General Hospital (Montreal, Quebec, Canada). Special thanks to: J(B)M, ZMS, SL, MD, JD, ML, MHF, RP, MS, PJ, Jack Iwanicki, and Johanne Ouellette for help with electron microscopy, Christian Young for flow cytometry, and Mireille Khacho for her help with metabolic assays. We thank all the patients for their generous contribution to science.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


Metabolic Reprogramming and Chemoresistance in Breast Cancer
Sirois et al.


A Unique Morphological Phenotype in Chemoresistant Triple-Negative Breast Cancer Reveals Metabolic Reprogramming and PLIN4 Expression as a Molecular Vulnerability

Isabelle Sirois, Adriana Aguilar-Mahecha, Josiane Lafleur, et al.