

Investigating POLG-driven modulation of cancer stemness: a pilot study in breast cancer cells

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ABSTRACT

Mitochondria have emerged as key regulators of breast cancer stem cell (CSC) biology. Mitochondrial metabolic pathways, including oxidative phosphorylation (OXPHOS) and mitochondrial biogenesis, are frequently altered during tumorigenesis, highlighting their role in breast cancer pathogenesis. Since breast CSCs are highly dependent on mitochondrial metabolism, targeting mitochondrial DNA (mtDNA) replication may represent a strategy to impair CSC maintenance. Mitochondrial DNA polymerase- γ (POLG), composed of a catalytic subunit encoded by POLG1 and an accessory subunit encoded by POLG2, is essential for mtDNA replication and repair.

In this pilot study, we investigated whether targeting POLG could modulate breast CSC activity. Genetic knockdown of POLG1 and POLG2 in MCF-7 cells resulted in mtDNA depletion, reduced mitochondrial protein expression, impaired energy production, and loss of stemness-related features. To pharmacologically target POLG, we tested Alovudine, a nucleoside reverse transcriptase inhibitor known to act as an off-target POLG inhibitor. In MCF-7 cells, Alovudine reduced clonogenic potential, decreased mitochondrial DNA-encoded protein levels, and lowered oxygen consumption.

To further validate these findings, we employed the independent POLG inhibitor Zalcitabine (ddC) in additional breast cancer models. ddC impaired mitochondrial respiration, reduced mammosphere formation, and modulated SOX2 and NANOG expression. Preliminary Kaplan–Meier analyses in a cohort of 458 breast cancer patients showed that high POLG1 expression correlates with worse clinical outcomes, including relapse-free and overall survival.

Taken together, these findings suggest that POLG supports mitochondrial function and CSC maintenance, highlighting its potential as a therapeutic target and prognostic biomarker in breast cancer.

INTRODUCTION

Originally, mitochondria evolved from bacteria which were engulfed by eukaryotic cells and ultimately developed into distinctive and functional organelles [1, 2]. Mitochondria fulfill several important roles, such as sustaining cellular homeostasis, determining cellular differentiation as well as apoptosis. One of their most important roles is the generation of the majority of cellular ATP, through the process of OXPHOS, using the electron transport chain (ETC) [1, 2]. The mitochondrial genome consists of double-stranded, circular DNA situated in distinct nucleoids within the mitochondrial matrix, inside the inner membrane. Mitochondrial DNA size is different between species; in humans, mtDNA is approximately 17,000 base pairs and it encodes 13 proteins of the OXPHOS complexes, as well as for 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) which are required for mitochondrial biosynthetic pathways [3–5]. The mitochondrial genome exhibits a higher mutational rate and poor repair mechanisms compared to the nuclear genome, with frequent abnormalities such as changes in mtDNA copy number and epigenetic alterations, which have been associated with several cancers, including breast cancer [6–10]. For mitochondrial biogenesis to be executed correctly, mtDNA replication function must be maintained [11, 12]. The mammalian mtDNA replication is achieved by a group of nuclear-encoded proteins, including the mitochondrial single-stranded DNA binding protein (MTSSB), the mtDNA helicase twinkle protein, and a dedicated polymerase called mtDNA polymerase γ [13]. POLG, which is the exclusive DNA polymerase present in the mammalian mitochondria, has a pivotal role in mtDNA replication, maintenance and repair [14]. Structurally, POLG is a heterotrimer which presents a catalytic subunit (p140) encoded by POLG1 gene on chromosome 15, and two identical accessory subunits (p55) that form a dimer, encoded by POLG2 gene on chromosome 17. The human POLG1 catalytic subunit presents the 3'→5' exonuclease and 5'dRP lyase activities, acting to proofread the newly synthesized DNA strand. The human POLG2 accessory subunit has the function to enhance the binding of polymerase γ to mtDNA, to promote the processivity of DNA synthesis and to stabilize the catalytic subunit [15]. The POLG gene is frequently mutated in mitochondrial disorders, resulting in mtDNA mutations, mtDNA instability and alterations of mtDNA content [13–16]. As mitochondrial DNA polymerase γ is associated with mtDNA in the mitochondrial matrix, in proximity to the electron transport chain complexes, this enzyme is susceptible to oxidation by hydrogen peroxide and iron ions [14], resulting in increased mtDNA mutations. Although mutations in the POLG genes are related to several

mitochondrial diseases, it is still unclear whether these mutations could have a role in breast cancer and particularly in breast cancer stem cell propagation. Breast CSCs represent a subset of tumor cells (around 2%) within breast tumors, which possess stemness properties, such as the ability of self-renewal and multilineage differentiation capacity, as well as migration and invasion [17–19]. Breast CSCs may activate or deregulate crucial pathways which confer a more aggressive tumor phenotype, leading to metastatic dissemination and therapy resistance [20]. Importantly, recent studies have shown that breast CSCs display elevated mitochondrial biogenesis and metabolism [21, 22]. Since breast CSCs are highly dependent on mitochondrial oxidative metabolism and mitochondrial biogenesis, targeting mtDNA replication may represent a novel strategy to selectively impair CSC maintenance and propagation [23, 24]. In this context, POLG is particularly attractive because it represents the only DNA polymerase responsible for mtDNA replication and repair in mammalian mitochondria, making it a unique regulator of mitochondrial genome integrity and mitochondrial function [25]. Despite its central role in maintaining mitochondrial homeostasis, the contribution of POLG-driven mtDNA maintenance to breast CSC biology remains poorly understood [26]. Here, we aimed to investigate the role of polymerase γ in breast CSC propagation, using a genetic and a pharmacological approach. As POLG subunits are encoded by nuclear DNA and are crucial for the integrity of the mitochondrial function, we hypothesized that POLG may represent a nodal protein in the crosstalk between the nucleus and mitochondria and that this crosstalk may become unbalanced in breast cancer. In this way, polymerase γ could be identified as a potential mitochondrial target for halting breast CSCs. To investigate this hypothesis, we initially employed the high affinity POLG inhibitor Alovudine (3'-deoxy-3'-fluorothymidine), a nucleoside reverse transcriptase inhibitor originally developed for the treatment of HIV, and subsequently validated our findings using the independent POLG inhibitor ddC (2'-3'dideoxycytidine) across multiple breast cancer cell models. These approaches allowed us to evaluate the impact of POLG inhibition on mitochondrial metabolism and CSC-associated phenotypes in breast cancer cells.

RESULTS

POLG1 knockdown reduces mitochondrial DNA content

Since elevated mitochondrial biogenesis and metabolism represent key features of breast cancer stem cells, here we aimed to inhibit mitochondrial DNA replication using POLG genes as targets. In animal

cells, mtDNA is replicated by the mitochondrial DNA polymerase γ protein, which is composed by a single catalytic subunit encoded by the nuclear gene POLG1 and a dimeric subunit encoded by the nuclear gene POLG2 [14]. *In silico* analysis of protein expression datasets (Supplementary Figure 1) revealed that POLG1 levels are highest in the MCF-7 cell line compared to other breast cancer models, supporting the selection of this cell line for functional studies. Firstly, we focused on the mtDNA polymerase γ catalytic subunit, POLG1. To investigate the role of POLG1 subunit in influencing the phenotype and the heterogeneity of breast CSCs, an shRNA approach was used. Briefly, the Estrogen Receptor (ER) positive MCF-7 breast cancer cells were stably transduced with lentiviral vectors encoding shRNA which target POLG1 gene (MCF-7 sh-POLG1). In parallel, MCF-7 cells were also transduced with a shRNA scramble that was used as a control (MCF-7 sh-CTRL). The plasmids also contained a puromycin-resistant cassette for antibiotic selection and a GFP (Green Fluorescent Protein) gene: once the vector is transduced, it emits a green fluorescence. GFP was thus monitored using microscopy, through EVOS cell imaging system, as shown in Figure 1A, indicating that MCF-7 cells were efficiently transduced.

Next, the expression of endogenous POLG1 protein was validated, after transduction, by immunoblotting analysis. As shown in the Figure 1B, the protein is completely absent in sh-POLG1 cells as compared to the sh-CTRL counterparts, confirming that the transduction was efficient and POLG1 silencing was successful.

Next, given the importance of POLG1 subunit in influencing mtDNA copy number, the impact of POLG1 knockdown on mtDNA content was assessed, using qPCR. The relative mtDNA copy number changes were evaluated using two different set of primers: the mtDNA primer set which amplifies specifically one of the most conserved regions on human mtDNA (without amplifying any sequences on nuclear genomic DNA), and the single copy reference (SCR) primer set which recognizes and amplifies a specific region on human chromosome 17 and was used for the normalization. As shown in Figure 1C, the knockdown of POLG1 subunit induces approximately an 80% reduction of mtDNA content compared to the sh-CTRL cells. These data confirm the importance of POLG1 subunit in influencing mtDNA copy number in breast cancer cells.

POLG1 knockdown inhibits mitochondrial function and metabolism

Mitochondrial function is essential for generating ATP which is mainly produced in the mitochondria through

OXPHOS system [27]. To test if POLG1 knockdown may interfere with mitochondrial function, the Mitochondrial Cytochrome C Oxidase subunit II (MTCO2) protein levels were evaluated by immunoblotting analysis. MTCO2 is one of the three mtDNA encoded subunits of complex IV (Cytochrome C oxidase), which is the last complex involved in the mitochondrial electron transport chain and represents the regulatory center of oxidative phosphorylation [28]. As expected, MTCO2 protein levels are decreased in MCF-7 sh-POLG1 cells, compared to the control cells (Figure 2A).

Interestingly, the knockdown of POLG1 subunit has no effect on TOMM20 protein levels, a well-established mitochondrial mass marker, suggesting that POLG1 silenced cells and control cells have a similar mitochondrial mass, although with distinct functional features (Figure 2B).

Thereafter, the mitochondrial membrane potential, a metabolic parameter which allows to measure the electrochemical proton gradient, was measured using FACS analysis. As shown in Figure 2C, the knockdown of POLG1 subunit results in a nearly 20% decrease in mitochondrial membrane potential compared to sh-CTRL cells. To determine if the knockdown of POLG1 contributes to the generation of superoxide anion in mitochondria, flow cytometry analysis was performed using MitoSOX Red probe. Interestingly, the levels of mitochondrial superoxide anions in POLG1 silenced MCF-7 cells were significantly higher than the control cell levels, as shown in Figure 2D.

Thus, the knockdown of POLG1 causes mitochondrial stress with an increase in the mitochondrial ROS levels, suggesting that it may interfere with breast cancer cells mitochondrial metabolism. To test this hypothesis, metabolic flux analysis was performed, using the Seahorse XFe96 analyzer. Interestingly, consistent with MTCO2 protein levels reductions, POLG1 silenced cell monolayers showed that oxygen consumption rate (OCR) was decreased compared to controls, with ~50% decrease in basal respiration, maximal respiration, and ATP production (Figure 3A). Next, the extracellular acidification rate (ECAR) was evaluated in POLG1 silenced cells compared to controls. MCF-7 sh-POLG1 cells showed no differences in glycolysis, but significant reductions in glycolytic capacity and glycolytic reserve, as compared to sh-CTRL cells (Figure 3B).

POLG1 knockdown inhibits CSCs activity

Next, we aimed to test the hypothesis that mtDNA replication is a key determinant in influencing the

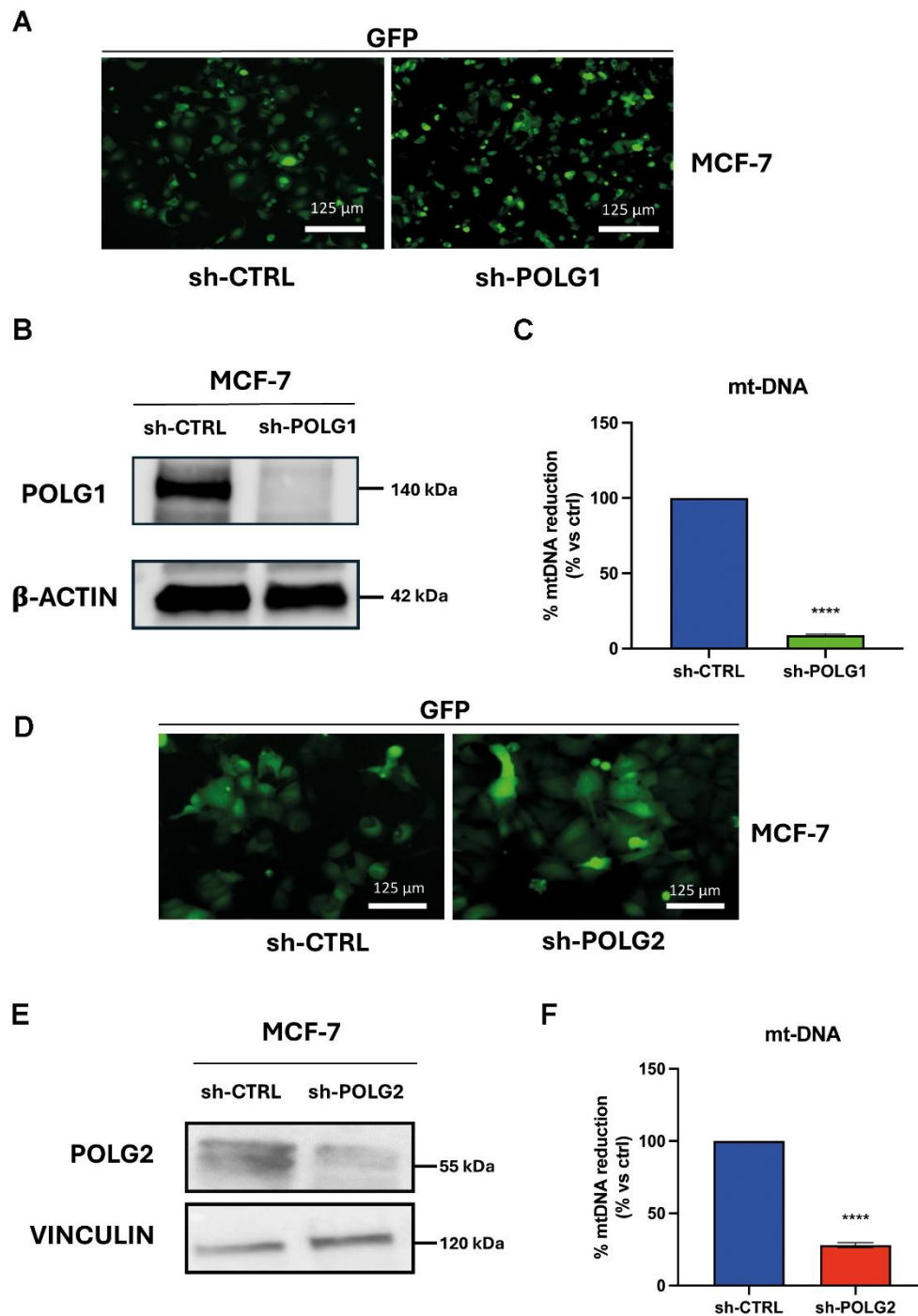


Figure 1. Knockdown of the nuclear gene encoding for the POLG1 and POLG2 subunit of the mitochondrial DNA polymerase γ . (A) MCF-7 breast cancer cells were transduced with shRNA vectors targeting POLG1 gene and the scrambled control vector (sh-CTRL); both vectors contained GFP signal that was detected using EVOS cell imaging system. (B) Protein expression of POLG1 performed by immunoblot analysis on MCF-7 POLG1 silenced cells compared to control cells, to verify POLG1 knockdown. (C) Relative mtDNA copy number of sh-POLG1 cells compared to the scrambled control cells was assessed by qPCR. Three biological repeats with three technical replicates for each condition were analyzed. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): **** p < 0.0001, evaluated by Student's t test, two-tailed test. (D) MCF-7 breast cancer cells were transduced with shRNA vectors targeting POLG2 gene (sh-POLG2) and the scrambled control vector (sh-CTRL); GFP signal was detected using the EVOS imaging system. (E) Protein expression of POLG2 was evaluated by immunoblot analysis on MCF-7 POLG2 silenced cells compared to control cells, to verify POLG2 knockdown. (F) Relative mtDNA copy number of sh-POLG2 cells compared to the scrambled control cells was assessed by qPCR. Three biological repeats with three technical replicates for each condition were analyzed. Error bars represent mean $\pm$ SEM. Statistical significance (in relation to control): **** p < 0.0001, evaluated by Student's t test, two-tailed test.

condition. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): * $p < 0.05$, evaluated by Student's *t* test, two-tailed test. (I) The total ROS levels were measured by flow cytometry using CellRox dye (5 μ M). Data are shown as mean $\pm$ SEM obtained from three biological repeats with three technical replicates for each condition. Statistical significance (related to control): ns not statistically significant, evaluated by Student's *t* test, two-tailed test.

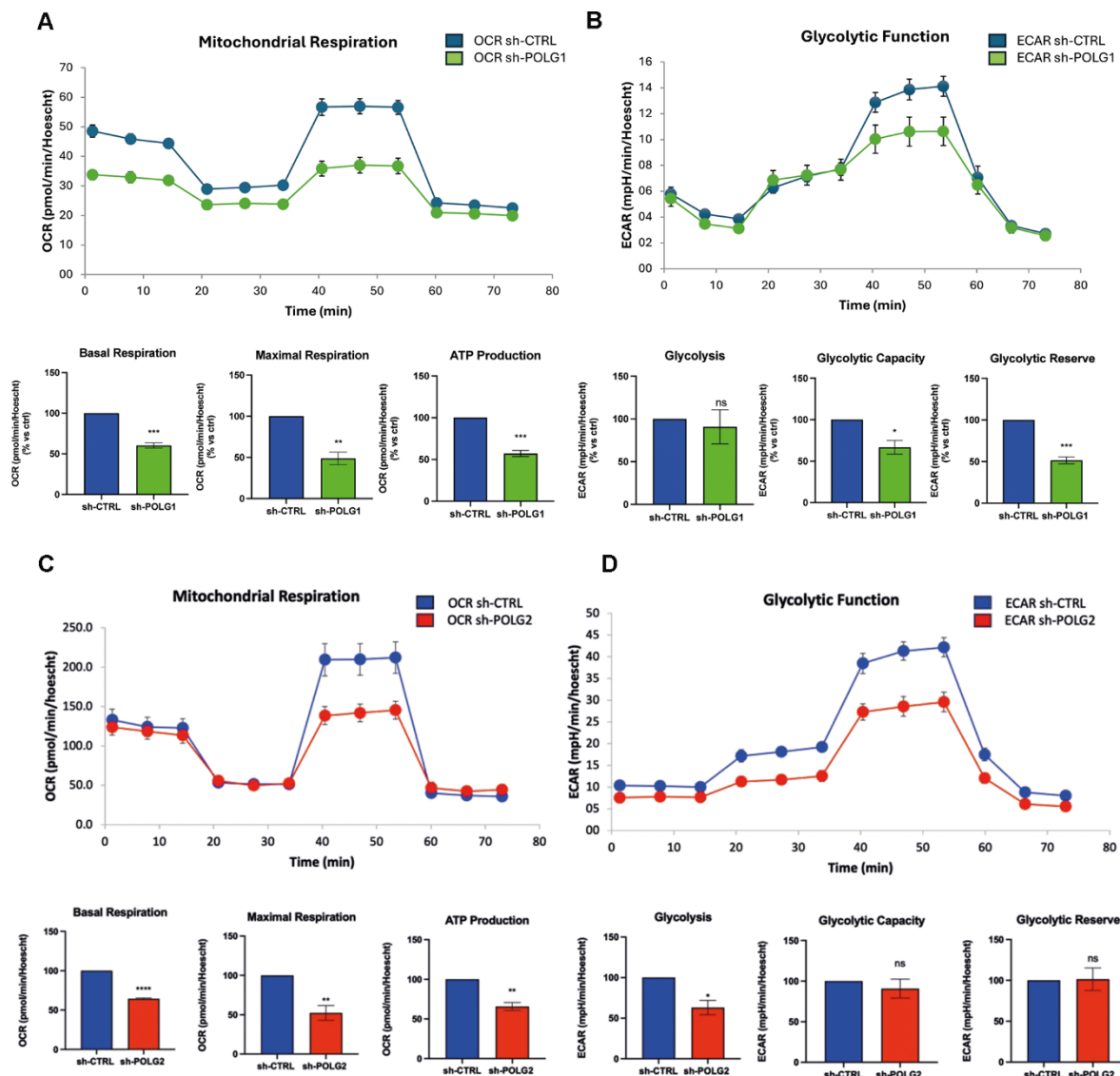


Figure 3. Mitochondrial respiration is significantly reduced in POLG1 and POLG2 silenced MCF-7 cells. The Seahorse XFe96 analyzer was employed to determine the mitochondrial and glycolytic function of MCF-7 POLG1 and POLG2 silenced cells and control cells. (A) In the top panel, a representative tracing of OCR (oxygen consumption rate) is shown. The lower panel shows basal respiration, maximal respiration, and ATP production results obtained from OCR analysis. Data are shown as mean $\pm$ SEM obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): ** $p < 0.01$, *** $p < 0.001$, evaluated by Student's *t* test, two-tailed test. (B) In the top panel, a representative tracing of ECAR (extracellular acidification rate) is shown. In the lower panel, glycolysis, glycolytic capacity, and glycolytic reserve data obtained from the ECAR are shown. Data are shown as mean $\pm$ SEM obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): * $p < 0.05$, *** $p < 0.001$, ns: not statistically significant, evaluated by Student's *t* test, two-tailed test. (C) In the top panel, a representative tracing of OCR (oxygen consumption rate) is shown. In the lower panel, basal respiration, maximal respiration, and ATP production results obtained from the OCR analysis are shown. Data are shown as mean $\pm$ SEM obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): ** $p < 0.01$, *** $p < 0.0001$, evaluated by Student's *t* test, two-tailed test. (D) In the top panel, a representative tracing of ECAR (extracellular acidification rate) is shown. In the lower panel, glycolysis, glycolytic capacity, and glycolytic reserve data obtained from the ECAR are shown. Data are shown as mean $\pm$ SEM obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): * $p < 0.001$, ns not statistically significant, evaluated by Student's *t* test, two-tailed test.

growth of CSCs. First, sh-POLG1 cells grown in monolayers were analyzed, compared to sh-CTRL cells (Figure 4A). Cells were grown and subjected to SRB assay after 3 days, to measure the protein content which represent a surrogate marker of cell growth. As shown in Figure 4A, the knockdown of POLG1 does not affect MCF-7 cells viability in 2D monolayer. A similar pattern was observed by cell cycle analysis: no significant changes were identified in the cell cycle phases between the POLG1 silenced and control cells (Figure 4B).

Next, we analyzed the stemness characteristics of POLG1 silenced cells compared to control cells. Specifically, in order to determine whether a loss of POLG1, depleting the mtDNA content, is associated with reduction of stemness, the well-established embryonic pluripotency marker OCT4 was used. Figure 4C shows that the knockdown of POLG1 subunit results in a decrease in the stemness marker OCT4 compared to control cells, measured by FACS analysis. This is consistent with a study showing that OCT4 levels were reduced after knockdown of POLG gene in murine embryonic stem cells [29]. To further characterize the stemness phenotype of POLG1 silenced cells, mammosphere assay, which is a functional read-out for breast CSCs activity and propagation, was performed. Interestingly, the knockdown of POLG1 resulted in a 70% decrease in mammosphere formation in MCF-7 cells (Figure 4D), indicating a reduced ability of 3D anchorage-independent growth. Importantly, it is well established that CSCs, but not bulk cancer cells, survive and proliferate in 3D anchorage-independent conditions.

Finally, we next employed colony formation assay, which is another functional assay to investigate stemness characteristics of cancer cells and to measure clonal expansion of single cells. Consistent with the other results, POLG1 silencing leads to a markedly reduced ability of MCF-7 to grow into colonies (Figure 4E).

POLG2 knockdown reduces mitochondrial DNA content

POLG2 is an accessory subunit of polymerase γ , which promotes the processivity of DNA synthesis and enhances the binding of POLG to mtDNA [30]. Recent data have shown that POLG2 does not only have these functions, but it is also involved in mtDNA organization, and it represents a key determinant of mtDNA copy number [31]. In order to determine the functional role of POLG2 in the metabolic and stemness phenotype of MCF-7 breast cancer cells, and to validate it as a potential therapeutic target, stable POLG2-knockdown cell lines were generated.

We used an shRNA approach to down-regulate the POLG2 subunit, using a lentiviral vector containing a shRNA which targets the POLG2 gene. In parallel, also the shRNA scramble control MCF-7 cells were generated. After transduction, since the constructs contain also a GFP gene, MCF-7 cells emit a green fluorescence that was evaluated using EVOS Imaging system (Figure 1D). Next, POLG2 protein expression was assessed by western blot analysis: Figure 1E shows that POLG2 protein is not expressed in MCF-7 POLG2 silenced cells, indicating that the silencing was successful. To further validate the phenotype of sh-POLG2 MCF-7 cells, the mtDNA content was measured using qPCR. The knockdown of POLG2 gene causes approximately a 70% reduction in levels of mtDNA in MCF-7 cells, compared to control cells (Figure 1F).

POLG2 knockdown inhibits mitochondrial function

Consistent with reductions in mtDNA content, the protein levels of the mtDNA encoded respiratory chain complex IV subunit II (MTCO2) are reduced in POLG2 silenced cells, compared to the control (Figure 2E). In contrast, mitochondrial mass was not changed in POLG2 silenced cells compared to control cells, as assessed using the MitoTracker Deep Red probe by FACS analysis (Figure 2F). Next, cell monolayers were subjected to FACS analysis with MitoNIR probe, for measuring mitochondrial membrane potential. As shown in Figure 2G, mitochondrial membrane potential was reduced by 30% in MCF-7 sh-POLG2 cells compared to sh-CTRL cells. Furthermore, as shown in Figure 2H, MCF-7 sh-POLG2 cells displayed increased mitochondrial superoxide anions levels, relative to control cells. No significant changes were observed in the total ROS levels in POLG2 silenced cells, as measured by flow cytometry using the CellRox dye (Figure 2I).

To further assess the role of POLG2 knockdown in causing mitochondrial stress, the metabolic phenotype was measured, using the Seahorse XFe96 analyzer. As shown in Figure 3C, OCR (oxygen consumption rate) was decreased in MCF-7 sh-POLG2 cells compared to control, with decreases in basal respiration, maximal respiration, and ATP production. Extracellular acidification rates (ECAR) were measured in MCF-7 POLG2 silenced cells and in control cells: glycolysis was significantly reduced while there were no significant changes in both glycolytic capacity and glycolytic reserve (Figure 3D). These results suggest that POLG2 gene knockdown affects the metabolic flux of MCF-7 breast cancer cells, determining an impairment of oxidative phosphorylation.

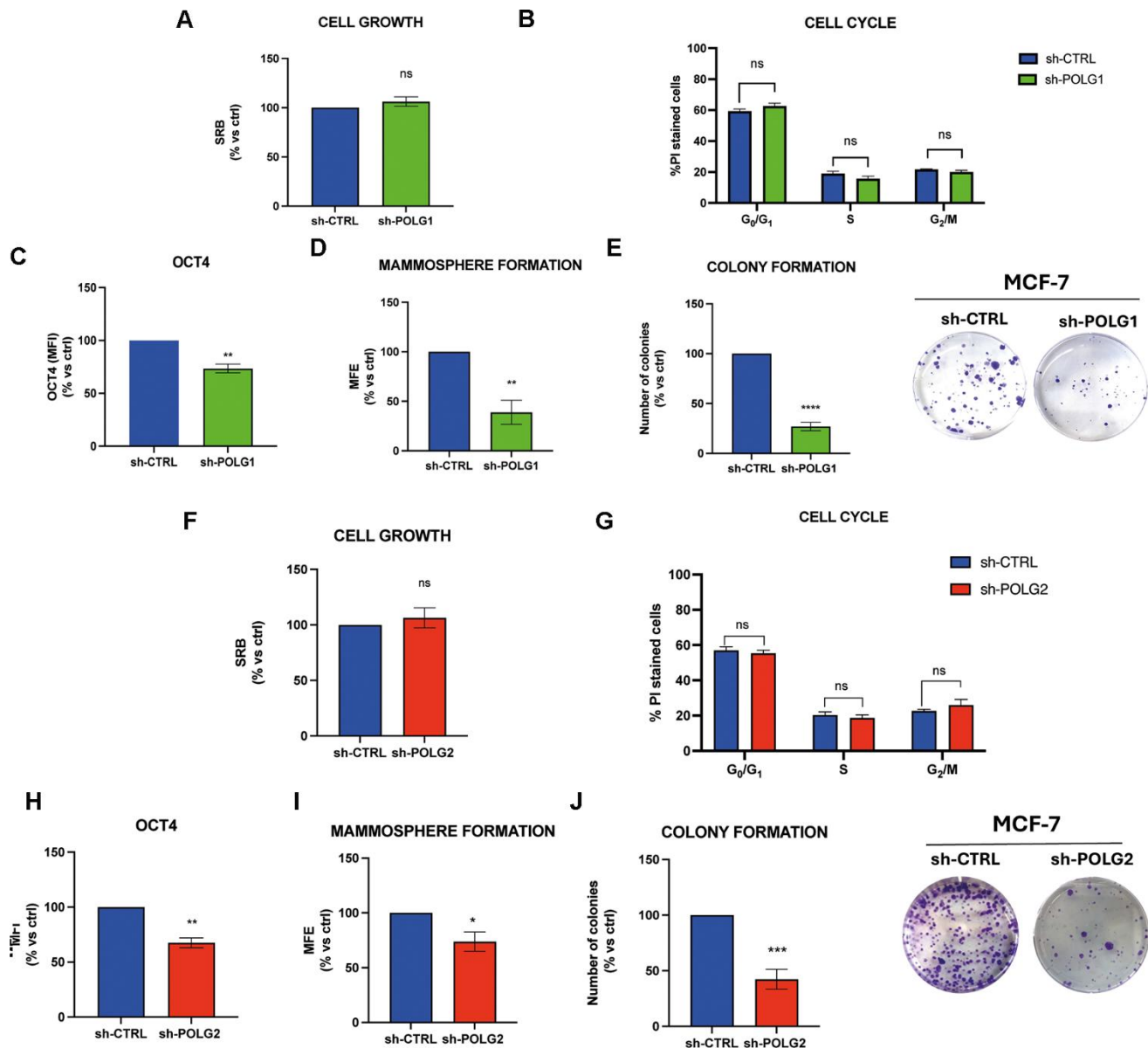


Figure 4. POLG1 and POLG2 knockdown has no effect on 2D cell growth, but it decreases the stemness features of MCF-7 cells. (A) MCF-7 POLG1 silenced, and control cells were grown for 72 hours, and then assessed for cell viability, using the SRB assay. Experiments were repeated three times with ten technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): ns not statistically significant, evaluated by Student's t test, two-tailed test. (B) Cell cycle analysis of MCF-7 POLG1 silenced cells and control cells was evaluated with propidium iodide (PI) by flow cytometry. The graph represents the percentage of cells distributed in G₀/G₁, S and G₂/M phases. Experiments were repeated three times independently with three technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): ns not statistically significant, evaluated by Student's t test, two-tailed test. (C) OCT4 levels were analyzed using OCT4-PE antibody by flow cytometry. Three biological repeats with three technical replicates were performed for each condition. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): ** p < 0.01, evaluated by Student's t test, two-tailed test. (D) MCF-7 POLG1 silenced cells and control cells were plated in low-attachment conditions for mammosphere assay and analyzed after five days. Experiments were repeated three times independently with 3 technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance: ** p < 0.01, evaluated by Student's t test, two-tailed test. (E) MCF-7 transduced cells were seeded at a density of 500 cells/well in a 6 well plate. After 14 days, cells were fixed, stained with Crystal Violet and colonies were counted manually. Representative images are shown on the right. Experiments were repeated three times independently with 3 technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance: **** p < 0.0001, evaluated by Student's t test, two-tailed test. (F) MCF-7 transduced cells were grown in 2D, and cell viability was assessed after 72 hours, using the SRB assay. Experiments were repeated three times with ten technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical

significance (relative to control): ns not statistically significant, evaluated by Student's *t* test, two-tailed test. (G) Cell cycle analysis of MCF-7 POLG2 silenced cells and control cells, evaluated with propidium iodide (PI) by flow cytometry. The graph represents the percentage of cells distributed in G0/G1, S and G2/M phases. Experiments were repeated three times independently with three technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): ns not statistically significant, evaluated by Student's *t* test, two-tailed test. (H) OCT4 levels were analyzed using OCT4-PE antibody by flow cytometry. Three biological repeats with three technical replicates were performed for each condition. Error bars represent mean $\pm$ SEM. Statistical significance (relative to control): ***p* < 0.01, evaluated by Student's *t* test, two-tailed test. (I) MCF-7 POLG2 silenced cells and control cells were plated in low-attachment conditions for mammosphere assay and analyzed after five days. Experiments were repeated three times independently with 3 technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance: **p* < 0.05, evaluated by Student's *t* test, two-tailed test. (J) MCF-7 transduced cells were seeded at a density of 500 cells/well in a 6 well plate. After 15 days, cells were stained with Crystal Violet and colonies were counted manually. Experiments were repeated three times independently with 3 technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance: ****p* < 0.001, evaluated by Student's *t* test, two-tailed test.

POLG2 knockdown inhibits CSCs activity

The effect of POLG2 knockdown on cell growth and cell cycle was tested next. To evaluate the effect of POLG2 knockdown on cell growth, the SRB assay was used. As shown in Figure 4F, POLG2 knockdown does not determine significant changes in MCF-7 cells viability. Next, the proliferative capacity of MCF-7 sh-POLG2 cells was further evaluated by measuring cell cycle progression using flow cytometry analysis. The knockdown of POLG2 does not affect cell cycle progression of MCF-7 cells (Figure 4G). Altogether these data indicate that POLG2 silencing in breast cancer cells does not change 2D cell growth. Since mitochondrial biogenesis is a crucial process in maintaining mitochondrial homeostasis and it is required for the anchorage independent survival and propagation of CSCs [22], we next investigated whether the knockdown of POLG2, which is involved in the synthesis of new mtDNA, might affect CSCs propagation. Thus, the expression of the CSCs marker OCT4 was analyzed using FACS analysis. The downregulation of POLG2 significantly reduced OCT4 levels as shown in Figure 4H. Additionally, the ability of POLG2 knockdown cells to grow in an anchorage independent manner was tested, by mammosphere formation assay. Figure 4I shows that POLG2 knockdown results in a significant decrease in mammosphere formation. Next, we investigated whether the knockdown of POLG2 could influence the capability of single MCF-7 cells to self-renew into colonies of 50 or more cells, using the colony formation assay. Interestingly, POLG2 knockdown decreases the ability of MCF-7 to grow into a colony (Figure 4J). Altogether, these results indicate that POLG2 plays a direct role in CSCs maintenance and propagation.

The nucleoside analog Alovudine inhibits CSCs activity and reduces mitochondrial energy production in breast cancer cells

Based on the data shown above, the knockdown of mtDNA polymerase γ (POLG) subunits determined an impairment of mitochondrial function, a decrease in

mitochondrial energy production and a reduction in stemness features of MCF-7 breast cancer cells. Next, we focused on identifying a pharmacological approach to inhibit mtDNA replication using POLG gene as a new therapeutic target for CSCs eradication. To this end, we focused on Nucleoside Reverse Transcriptase Inhibitors (NRTIs), originally developed for targeting viruses such as HIV, because of their off-target effects as POLG inhibitors.

Alovudine (3'-deoxy-3' fluorothymidine) is a nucleoside analogue of thymidine and was first developed as a NRTI for the treatment of drug-resistant HIV infections. Alovudine inhibits mtDNA polymerase γ activity through its 5'-triphosphate metabolite. A previous study demonstrated that Alovudine exerts anti-cancer activity in Acute Myeloid Leukemia (AML) cells, decreasing mtDNA content and mitochondrial respiration [32]. However, its effect on MCF-7 breast cancer stem cells has not been previously elucidated. Firstly, to validate the hypothesis that Alovudine targets breast CSCs, mammosphere formation assay was performed. As shown in Figure 5A, Alovudine inhibits mammosphere formation by 50%, at a concentration of 25 μ M. Next, the ability of Alovudine to inhibit the capability of MCF-7 to grow into colonies was tested. Interestingly, Alovudine has a potent effect on MCF-7 self-renewal and stemness features, inhibiting colony formation by 50% at very low concentrations of 40 nM (Figure 5B). The effect of Alovudine on cell viability on MCF-7 monolayers and normal human fibroblasts (hTERT-BJ1 cells) was tested. MCF-7 cells and hTERT-BJ1 fibroblasts were treated with increasing concentrations of Alovudine (from 100 nM to 10 μ M) for 72 hours. Interestingly, Alovudine treatment had no toxic effect on cell viability on both MCF-7 and hTERT-BJ1 monolayers, suggesting a specific effect of the compound on 3D models (Figure 5C, 5D). These results suggest that the POLG inhibitor Alovudine preferentially target CSCs, rather than "bulk" cancer cells and normal fibroblasts. To further investigate whether Alovudine treatment affected mitochondrial respiratory chain integrity, the expression levels of the mtDNA

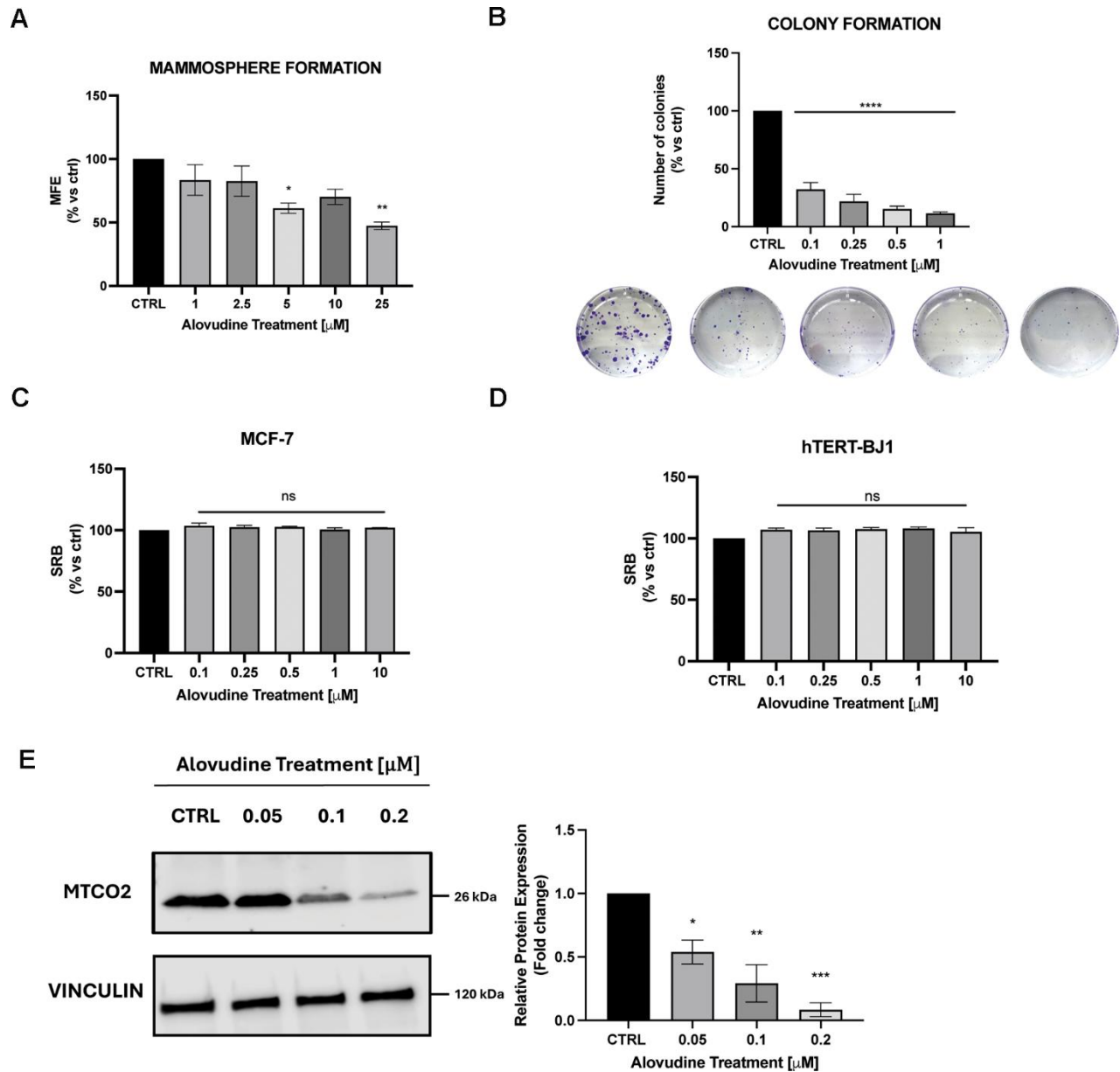


Figure 5. Alovudine treatment decreases mammosphere formation, colony formation, the protein levels of MTCO2, and has no effect on cell viability of MCF-7 cells. (A) Inhibition of mammosphere-forming ability of MCF-7 cells, after Alovudine treatment. Cells were seeded in low-attachment conditions and treated for 5 days in mammosphere media with increasing concentrations of Alovudine (1, 2.5, 5, 10 and 25 μ M) or DMSO control. Data shown are the mean $\pm$ SEM normalized to vehicle-treated control, of three biological repeats with three technical replicates per condition. Statistical significance (in relation to control): * p <0.05, ** p <0.01, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. (B) Colony formation assay of MCF-7 cells treated for 14 days with Alovudine. Cells were seeded at low-density (500 cells/well) in a 6-well plate and treated with Alovudine (at 0.1, 0.25, 0.5 and 1 μ M concentration). After 14 days, colonies were stained with Crystal Violet and counted. The graph shows the number of colonies as mean $\pm$ SEM normalized to vehicle-treated (DMSO) control. Representative pictures are shown. Experiments were repeated three times with three technical replicates per condition. Statistical significance (relative to control): **** p <0.0001, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. (C, D) The cell viability of MCF-7 cells and hTERT-BJ1 fibroblasts was measured using SRB assay after 72 hours of treatment with Alovudine (0.1, 0.25, 0.5, 1, 10 μ M). Experiments were repeated three times independently with six technical replicates for each condition. Error bars represent mean $\pm$ SEM. Statistical significance (in relation to control): ns not statistically significant, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. (E) MCF-7 cells were treated with increasing concentrations of Alovudine (0.05, 0.1, 0.2 μ M). After 6 days, cells were lysed and analysed by immunoblotting with antibodies against MTCO2. The graph shows the densitometric quantification of the bands, using Licor Empiria Studio software, normalized to vinculin that was used as loading control. Data are shown as mean $\pm$ SEM of three independent biological replicates. Statistical significance (in relation to control): * p <0.05, ** p <0.01, *** p <0.001, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test.

encoded respiratory chain protein MTCO2 were evaluated by western blot analysis following 6 days of treatment in MCF-7 cells. Interestingly, MTCO2 protein levels were considerably and dose-dependently reduced following Alovudine exposure (Figure 5E), supporting the hypothesis that pharmacological inhibition of POLG impairs mitochondrial respiratory chain maintenance.

Thereafter, the Seahorse XFe96 analyzer was used to evaluate the metabolic profiles in terms of mitochondrial energy production and glycolytic function of MCF-7 cells treated with increasing concentrations of Alovudine for 6 days. Interestingly, the treatment with Alovudine (from 0.05 to 0.2 μ M) results in a significant decrease in oxygen consumption rate (OCR) already at 0.1 μ M concentration, resulting in a reduction of both maximal respiration and ATP generation, with no significant changes in basal respiration (Figure 6A).

The glycolytic function of MCF-7 cells treated with Alovudine for 6 days was assessed, evaluating the ECAR (extracellular acidification rate). Interestingly, glycolysis was slightly increased already at 0.05 μ M concentration, while the glycolytic reserve was significantly reduced, and no changes were observed in the glycolytic capacity of treated cells compared to control cells (Figure 6B).

Taken together, these findings suggest that Alovudine may impair mitochondrial function and CSC-associated phenotypes in breast cancer cells through mechanisms consistent with POLG inhibition, phenocopying several effects observed following POLG1 and POLG2 silencing. Although the previous clinical development of Alovudine as an antiviral agent was limited by hematological toxicities, these results support its utility as a proof-of-concept mitochondrial-targeting compound to investigate the biological consequences of POLG inhibition in breast cancer models.

The nucleoside analog Zalcitabine (ddC) inhibits CSCs activity and reduces mitochondrial energy production in breast cancer cells

To further validate the hypothesis that pharmacological inhibition of mitochondrial DNA polymerase γ may impair CSC-associated phenotypes in breast cancer cells, we next employed the independent POLG inhibitor ddC (2'-3'dideoxycytidine), an NRTI originally used for the treatment of HIV/AIDS [21, 32]. Following intracellular phosphorylation to its active metabolite ddCTP, the compound interferes with mtDNA replication and mitochondrial oxidative phosphorylation [33]. Previous studies demonstrated

that ddC selectively impairs mitochondrial metabolism and proliferation in acute myeloid leukemia (AML) cells, supporting its potential as a mitochondrial-targeting agent in cancer models [21, 34]. Therefore, considering the clinical limitations previously associated with Alovudine, ddC was employed as an additional pharmacological approach to further investigate the relationship between POLG-dependent mitochondrial dysfunction and CSC-associated phenotypes in breast cancer cells. Initially, the effect of ddC treatment on the ability of MCF-7 breast cancer cells to grow under anchorage-independent conditions was evaluated using the mammosphere formation assay. Interestingly, ddC treatment impaired mammosphere formation in a dose-dependent manner, reaching approximately 50% inhibition at a concentration of 50 μ M (Figure 7A), further supporting the role of POLG-dependent mitochondrial metabolism in sustaining CSC-associated properties. Next, the effect of ddC on clonogenic potential was assessed using the colony formation assay. Consistent with the mammosphere results, prolonged ddC treatment markedly reduced the ability of MCF-7 cells to form colonies, with approximately 50% inhibition already observed at 0.1 μ M concentration (Figure 7B), suggesting that ddC strongly impairs CSC-associated self-renewal capacity. Next, the effect of ddC on 2D monolayer viability was evaluated in MCF-7 breast cancer cells and in non-transformed hTERT-BJ1 fibroblasts using the SRB assay. Cells were treated with increasing concentrations of ddC for 72 hours. Interestingly, ddC treatment did not significantly affect the viability of either MCF-7 cells or hTERT-BJ1 fibroblasts under 2D culture conditions (Figure 7C, 7D). These findings suggest that ddC preferentially impairs CSC-associated phenotypes and anchorage-independent growth, rather than short-term monolayer viability. To further investigate whether ddC treatment affected mitochondrial respiratory chain integrity, the expression of the mtDNA encoded respiratory chain protein MTCO2 was evaluated by western blot analysis. Interestingly, ddC treatment induced a dose-dependent reduction in MTCO2 protein expression in MCF-7 cells (Figure 7E), further indicating that POLG inhibition impairs mitochondrial function through disruption of mtDNA maintenance. Next, to further characterize the metabolic consequences of ddC treatment, the bioenergetic profile of MCF-7 breast cancer cells was evaluated following 6 days of exposure to Zalcitabine using the Seahorse XFe96 metabolic analyzer. Consistent with the observed reduction in MTCO2 expression, ddC treatment markedly reduced mitochondrial respiration, as evidenced by a significant decrease in oxygen consumption rate (OCR) at 2.5 μ M concentration compared to untreated control cells (Figure 8A). In contrast, glycolytic function was not significantly

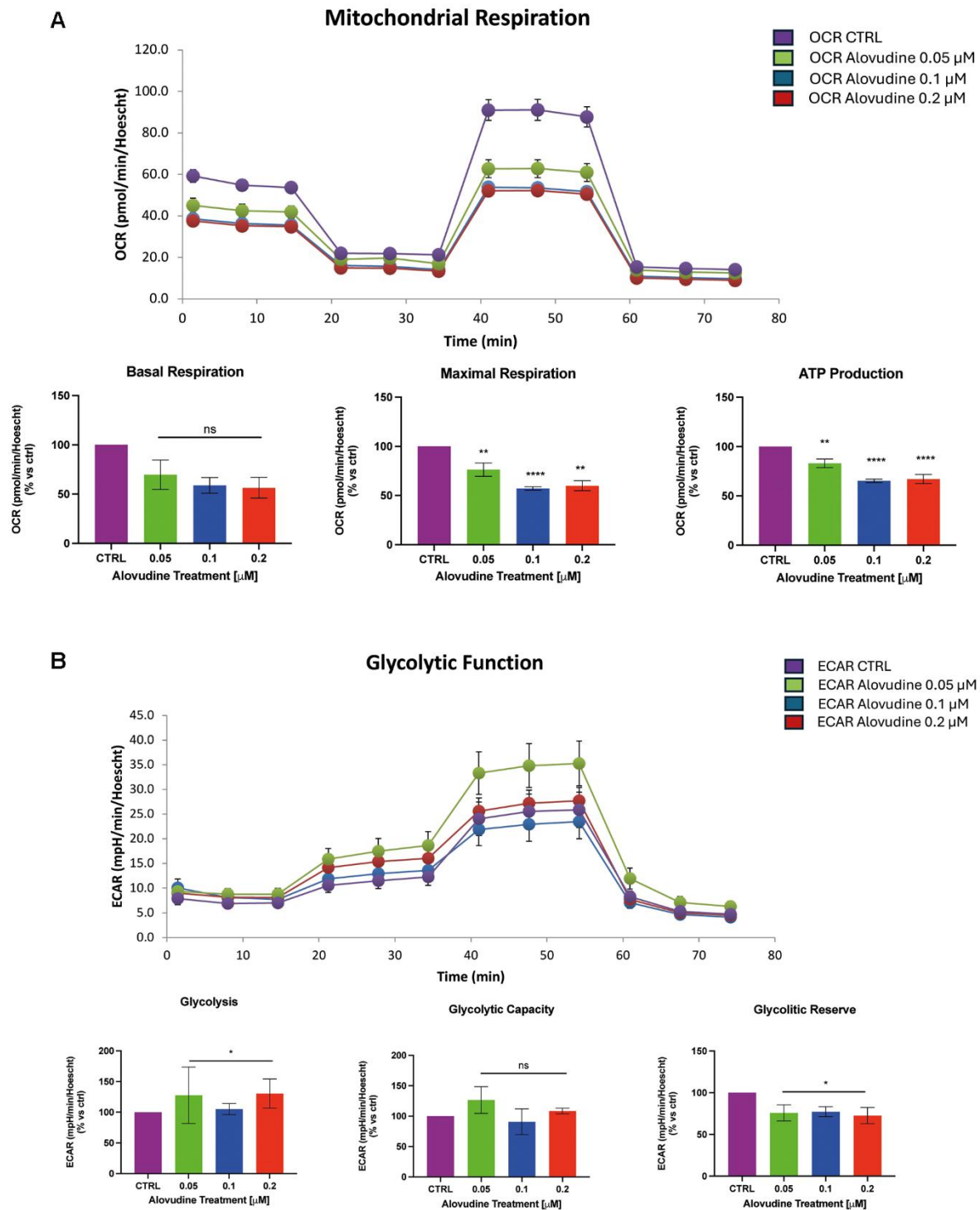


Figure 6. Effect of Alovudine on mitochondrial respiration and glycolysis of MCF-7 cells. The Seahorse XFe96 analyzer was employed to determine the mitochondrial function of MCF-7 treated with Alovudine (0.05, 0.1, 0.2 μ M) for six days. **(A)** In the top panel, a representative tracing of OCR (oxygen consumption rate) is shown. In the lower panel, basal respiration, maximal respiration, and ATP production results obtained from the OCR analysis are shown. Data are shown as mean $\pm$ SEM, obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): ** $p < 0.01$, **** $p < 0.0001$, ns not statistically significant, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. **(B)** In the top panel, a representative tracing of ECAR (extracellular acidification rate) is shown. In the lower panel, glycolysis, glycolytic capacity, and glycolytic reserve data obtained from the ECAR data are shown. Data are shown as mean $\pm$ SEM obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): * $p < 0.05$, ns not statistically significant, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test.

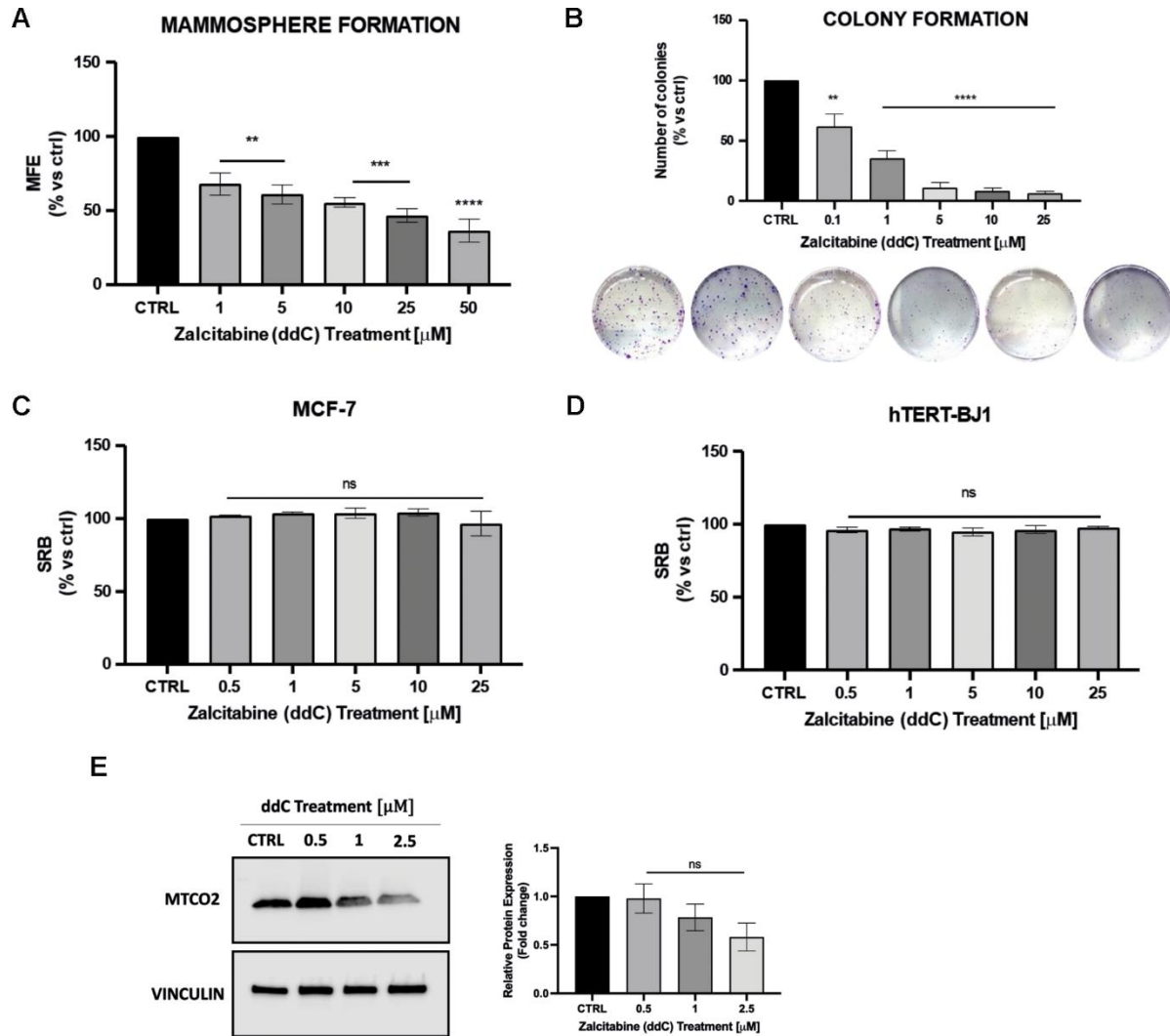


Figure 7. ddC treatment decreases mammosphere formation, colony formation, the protein levels of MTCO2, and has no effect on cell viability of MCF-7 cells. (A) Effect of Zalcitabine (ddC) treatment on mammosphere formation efficiency in MCF-7 breast cancer stem-like cells. MCF-7 cells were cultured under low-attachment conditions in mammosphere medium and exposed to increasing concentrations of ddC (1–50 μ M) or vehicle control (DMSO) for 5 days. Mammospheres were quantified and results are presented as percentage relative to vehicle-treated controls. Data shown are the mean $\pm$ SEM normalized to vehicle-treated control, of three biological repeats with three technical replicates per condition. Statistical significance (in relation to control): ** p <0.01, *** p <0.001, **** p <0.0001, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. (B) Colony formation assay of MCF-7 cells treated for 14 days with ddC. Cells were seeded at low-density (500 cells/well) in a 6-well plate and treated with ddC (at 0.1, 1, 5, 10 and 25 μ M concentration). After 14 days, colonies were stained with Crystal Violet and counted. The graph shows the number of colonies as mean $\pm$ SEM normalized to vehicle-treated (DMSO) control. Representative pictures are shown. Experiments were repeated three times with three technical replicates per condition. Statistical significance (relative to control): ** p <0.01, **** p <0.0001, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. (C, D) Effect of ddC treatment on cell viability in MCF-7 breast cancer cells and non-transformed hTERT-BJ1 fibroblasts. Cells were exposed to increasing concentrations of ddC (0.5–25 μ M) for 72 hours and cell viability was subsequently evaluated by SRB assay. Results are expressed relative to vehicle-treated control cells (DMSO) and represent mean $\pm$ SEM from three independent experiments performed with six technical replicates for each condition. Statistical analysis was carried out using ordinary one-way ANOVA followed by Bonferroni's multiple comparisons test. ns, not statistically significant versus control. (E) MCF-7 cells were exposed to increasing concentrations of Zalcitabine (ddC; 0.5, 1 and 2.5 μ M) for 6 days. Subsequently, cells were harvested and protein lysates were subjected to immunoblot analysis to evaluate the expression of the mtDNA encoded respiratory chain protein MTCO2. Densitometric quantification of western blot bands was performed using Licor Empiria Studio software and normalized to Vinculin, used as loading control. Results are presented as mean $\pm$ SEM from three independent biological experiments. Statistical analysis was performed using ordinary one-way ANOVA followed by Bonferroni's multiple comparisons test. ns, not statistically significant versus control.

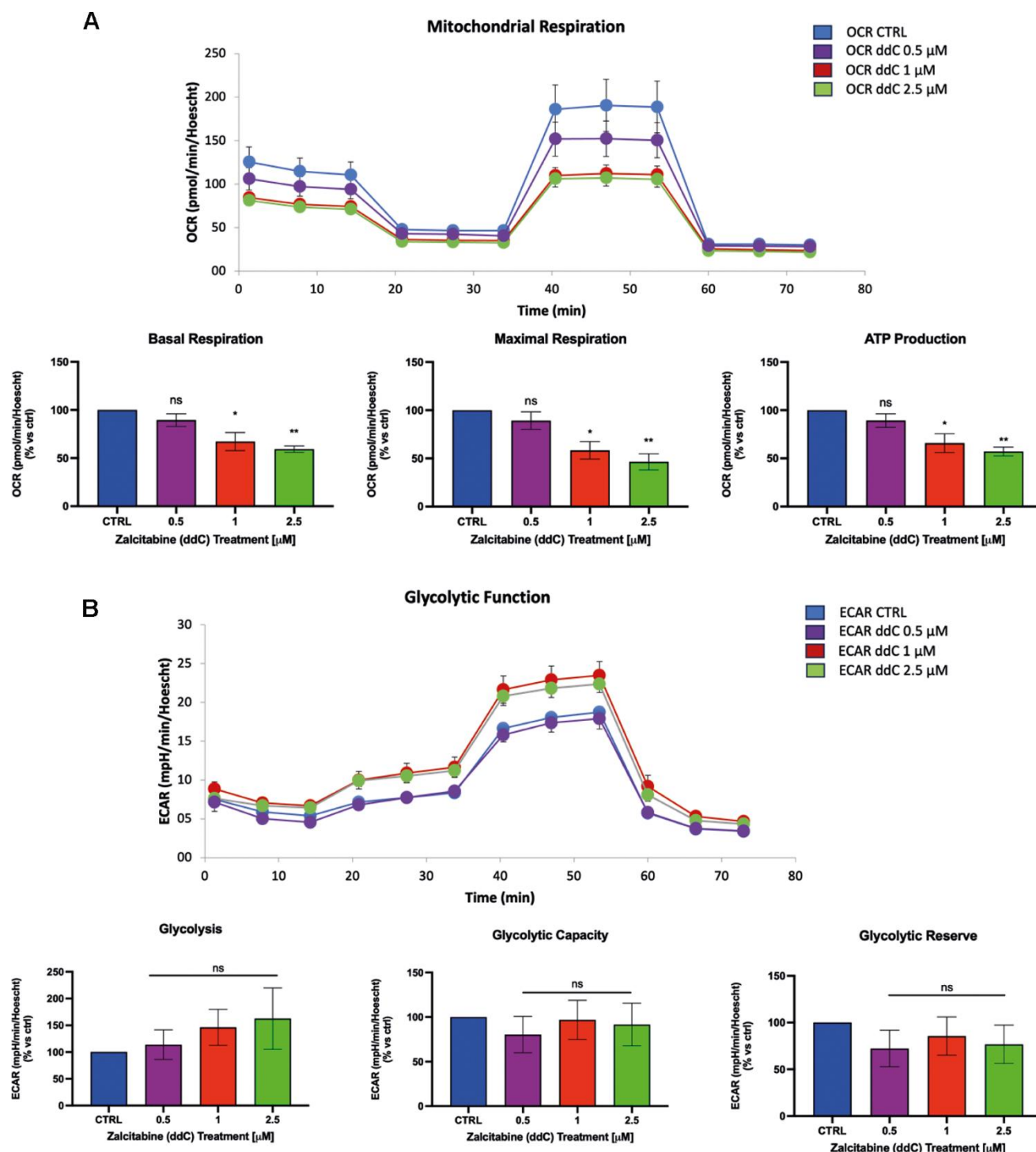


Figure 8. Effect of ddC on mitochondrial respiration and glycolysis of MCF-7 cells. The Seahorse XFe96 analyzer was employed to determine the mitochondrial function of MCF-7 treated with ddC (0.5, 1, 2.5 μ M) for six days. (A) In the top panel a representative tracing of metabolic flux (OCR- oxygen consumption rate) is shown. In the lower panel, basal respiration, maximal respiration, and ATP production results obtained from OCR analysis are shown. Data are shown as mean $\pm$ SEM, obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): * p <0.05, ** p <0.01, ns not statistically significant, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test. (B) In the top panel, a representative tracing of metabolic flux (ECAR- extracellular acidification rate) is shown. In the lower panel, glycolysis, glycolytic capacity, and glycolytic reserve data obtained from the ECAR are shown. Data are shown as mean $\pm$ SEM obtained from three biological repeats with six technical replicates for each condition. Statistical significance (related to control): ns not statistically significant, evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test.

altered following ddC treatment at either 1 μM or 2.5 μM concentrations (Figure 8B), suggesting that inhibition of mtDNA replication primarily affects mitochondrial energy metabolism in MCF-7 cells.

Accordingly, to strengthen the translational relevance of our findings and address the limitation of relying predominantly on MCF-7 cells, we extended our analyses to additional breast cancer cell lines, including T47D, MDA-MB-231, MDA-MB-436 and MDA-MB-453 cells and MCF10A non-tumoral mammary epithelial cells. First, the effect of ddC treatment on 2D monolayer viability was evaluated using the SRB assay. Interestingly, ddC treatment exerted heterogeneous effects across different breast cancer models. Importantly, MCF10A non-tumoral mammary epithelial cells were largely unaffected by ddC treatment across all tested concentrations, showing no significant reduction in cell viability (Figure 9A). In T47D cells, ddC treatment reduced cell viability in a dose-dependent manner, reaching approximately 50% inhibition at concentrations between 5 μM and 25 μM (Figure 9B). Similarly, MDA-MB-231 cells displayed a marked reduction in viability, with approximately 40% inhibition observed following treatment with 10 μM ddC (Figure 9C). In contrast, MDA-MB-436 cells showed only modest sensitivity to ddC treatment, while MDA-MB-453 cells were largely unaffected under 2D culture conditions even at the highest tested

concentration (25 μM) (Figure 9D, 9E). Collectively, these findings indicate that ddC exerts variable anti-proliferative effects across different breast cancer subtypes while showing limited toxicity toward non-tumoral mammary epithelial cells.

Next, the effect of ddC on CSC-associated phenotypes was evaluated using the mammosphere formation assay. Remarkably, ddC treatment consistently impaired mammosphere formation across all tested breast cancer cell lines in a dose-dependent manner. T47D cells displayed nearly 70–80% reduction in mammosphere formation at 25 μM ddC (Figure 10A). Similarly, MDA-MB-231 cells showed a progressive impairment in mammosphere formation, reaching approximately 70% inhibition at 25 μM concentration (Figure 10B). In MDA-MB-436 cells, ddC treatment reduced mammosphere formation by approximately 50% already at 5 μM concentration, with a more pronounced inhibitory effect observed at 10–25 μM (Figure 10C). Notably, MDA-MB-453 cells exhibited one of the strongest responses, with mammosphere formation reduced by nearly 75–80% following treatment with 25 μM ddC, despite minimal effects on short-term monolayer viability (Figure 10D). Collectively, these findings further support the hypothesis that pharmacological inhibition of POLG-dependent mitochondrial function preferentially targets CSC-associated properties rather than general cell proliferation.

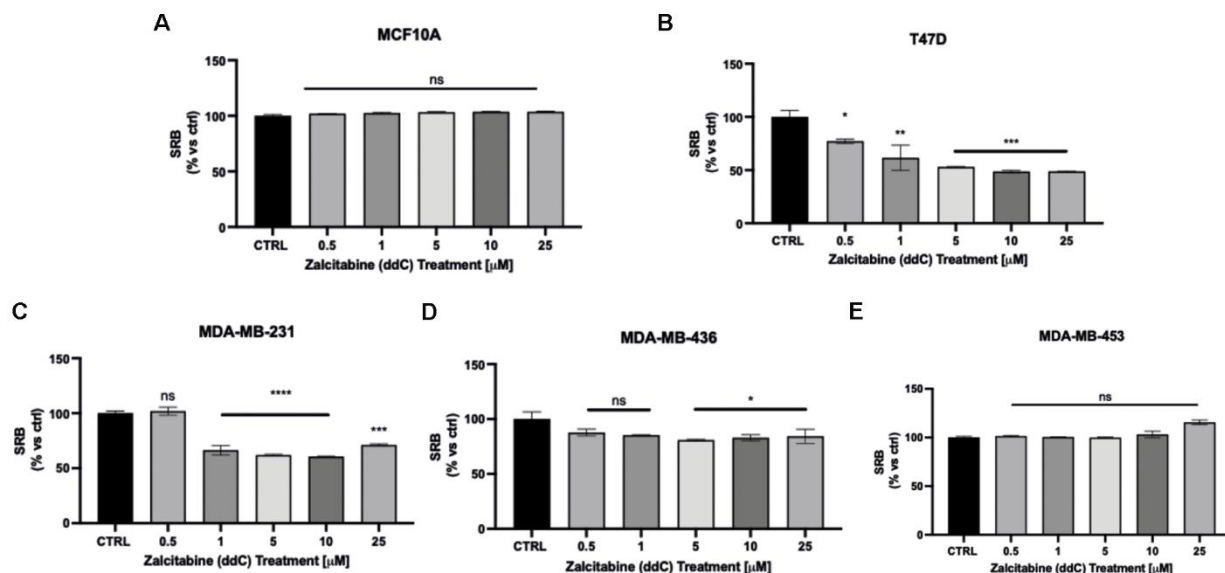


Figure 9. Effect of ddC treatment on monolayer viability across breast cancer cell lines and non-tumoral mammary epithelial cells. (A–E) MCF10A, T47D, MDA-MB-231, MDA-MB-436 and MDA-MB-453 cells were treated with increasing concentrations of Zalcitabine (ddC) (0.5, 1, 5, 10 and 25 μM) for 72 hours and cell viability was subsequently evaluated by SRB assay. Results are expressed as percentage relative to vehicle-treated control cells (DMSO). Data shown represent mean $\pm$ SEM from three independent biological experiments, each performed with six technical replicates per condition. Statistical analysis was performed using ordinary one-way ANOVA followed by Bonferroni's multiple comparisons test. Statistical significance (relative to control): ns, not statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Moreover, to further investigate the impact of POLG inhibition on stemness-associated transcriptional programs, MCF-7 and MDA-MB-453 cells were selected for gene expression analyses based on their responsiveness to ddC treatment in the mammosphere assay and to represent distinct breast cancer subtypes, including luminal (MCF-7) and triple-negative/HER2-positive-like (MDA-MB-453) models. The expression

levels of the pluripotency-associated genes SOX2 and NANOG were then evaluated following 72 hours of ddC exposure. Interestingly, treatment of MCF-7 cells with 2.5 μ M ddC resulted in a marked reduction of both SOX2 and NANOG mRNA expression, with transcript levels decreased by approximately 80–85% compared to untreated control cells (Figure 11A). MDA-MB-453 cells also exhibited a reduction

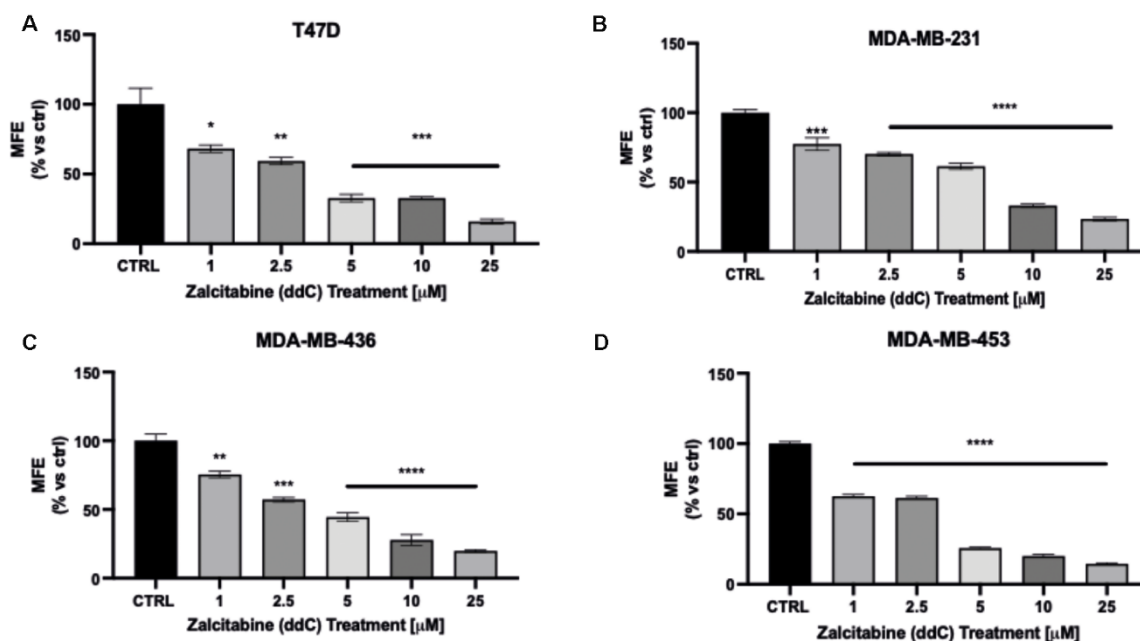


Figure 10. Effect of ddC treatment on mammosphere formation across multiple breast cancer cell lines and non-tumoral mammary epithelial cells. (A–D) Inhibition of mammosphere-forming ability of T47D, MDA-MB-231, MDA-MB-436, MDA-MB-453 after ddC treatment. Cells were seeded in low-attachment conditions and treated for 5 days in mammosphere media with increasing concentrations of ddC (0.5, 1, 5, 10 and 25 μ M) or DMSO control. Data shown are the mean $\pm$ SEM normalized to vehicle-treated control, of three biological repeats with three technical replicates per condition. Statistical significance (in relation to control): * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 evaluated by ordinary one-way ANOVA, Bonferroni's multiple comparisons test.

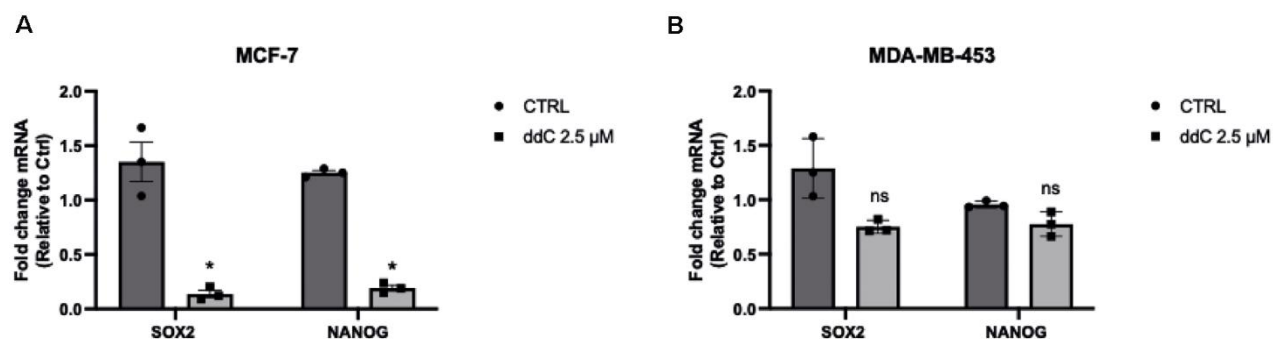


Figure 11. ddC treatment modulates the expression of stemness-associated genes SOX2 and NANOG in breast cancer cells. (A, B) MCF-7 and MDA-MB-453 cells were treated with ddC (2.5 μ M) for 72 hours and the expression levels of SOX2 and NANOG were evaluated by quantitative real-time PCR analysis. Relative mRNA expression levels were normalized to 18S rRNA and calculated relative to vehicle-treated control cells using the $\Delta\Delta$ Ct method. Data are presented as mean $\pm$ SEM from three independent biological replicates. Statistical significance was evaluated using unpaired two-tailed Student's t-test. Statistical significance (relative to control): ns, not statistically significant; * p <0.05.

in SOX2 expression following ddC treatment, whereas NANOG levels appeared only modestly affected under the same experimental conditions (Figure 11B).

These findings suggest that pharmacological inhibition of POLG-dependent mitochondrial function may differentially modulate stemness-associated transcriptional programs across distinct breast cancer subtypes, while further supporting a functional link between mitochondrial homeostasis and CSC maintenance. Finally, to investigate whether pharmacological inhibition of POLG-dependent mitochondrial function was associated with oxidative stress, mitochondrial and total ROS levels were evaluated in MCF-7 and MDA-MB-453 cells following ddC treatment using MitoSOX Red and DCFH-DA probes, respectively. Interestingly, ddC treatment did not induce significant changes in either mitochondrial superoxide levels or total intracellular ROS production in both MCF-7 and MDA-MB-453 cells at 5 μ M concentration (Supplementary Figure 2). Specifically, a modest increase in MitoSOX fluorescence was observed following ddC treatment, consistent with the increase in mitochondrial ROS previously detected following POLG1 and POLG2 silencing (Supplementary Figure 2A), whereas DCFH-DA staining remained substantially unchanged compared to untreated controls (Supplementary Figure 2B). These findings suggest that pharmacological inhibition of POLG-dependent mitochondrial function may induce mild mitochondrial oxidative stress without causing a substantial increase in overall cellular ROS levels under the tested experimental conditions.

Establishing the clinical relevance of POLG1 gene in breast cancer patient population

Next, we aimed to investigate the potential clinical relevance of POLG1 expression in breast cancer. To this end, we performed a Kaplan-Meier survival analysis to assess the association between POLG1 expression levels and patient outcomes in a cohort of breast cancer patients (n=458). Specifically, the analysis included estrogen receptor-positive (ER+), lymph node-positive patients diagnosed with luminal A subtype tumors, as defined in the selected dataset from a publicly available online survival analysis tool. We examined relapse-free survival (RFS), overall survival (OS), distant metastasis-free survival (DMFS), and post-progression survival (PPS). Preliminary results revealed that high POLG1 expression was associated with significantly poorer overall survival (HR = 1.34, p = 0.0024; Figure 12B). A similar trend was observed for PPS, where high POLG1 expression correlated with a worse prognosis (HR = 1.58), indicating a potential negative prognostic role (Figure 12D). Notably, patients with lower POLG1 expression exhibited prolonged PPS. In addition, high POLG1 expression was linked to shorter RFS and DMFS (Figure 12A, 12C), further supporting its potential as a negative prognostic indicator. Collectively, these data suggest that POLG1 expression may serve as a clinically relevant biomarker in breast cancer, although further validation is needed.

DISCUSSION

Previous studies have shown that elevated mitochondrial biogenesis and metabolism represent key

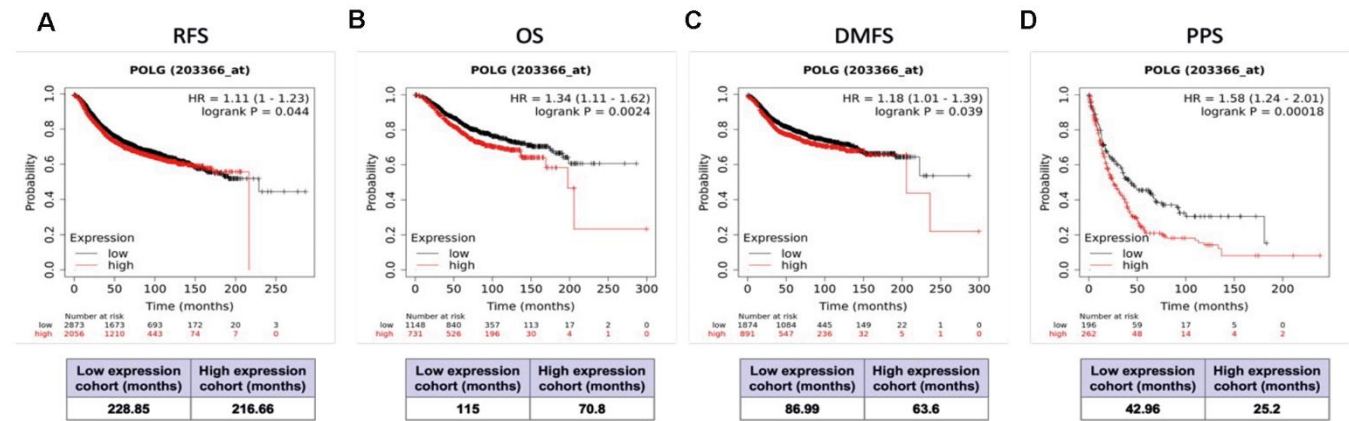


Figure 12. POLG1 expression could predict clinical outcome in human breast cancers. Kaplan-Meier analysis was performed in breast cancer patients (n=458) with high and low expression of the POLG1 gene (A–D), as described in the Materials and Methods section. The analysis was conducted using the Kaplan-Meier Plotter online tool and included high-risk, ER-positive, lymph node-positive patients with luminal A subtype. Kaplan-Meier survival analysis calculated the hazard ratio (HR) and logrank P-value.

features of breast cancer stem cells. In this pilot study, we aimed to explore whether inhibition of mitochondrial DNA replication through genetic silencing of POLG and pharmacological inhibition through the use of POLG inhibitors, could represent a potential strategy to impair CSC-related functions.

Targeting tumor heterogeneity presents significant therapeutic opportunities for many cancer types, including breast cancer [17, 35]. Cancer cells with stem-like properties (CSCs) have been identified in several tumors, including breast cancer, and are now believed to be the main drivers of tumor progression and therapy failure [2, 19, 36]. In this scenario, mitochondria, which are key organelles for ATP production, emerge as critical regulators of CSC maintenance, supporting tumor growth, metastasis formation and drug resistance [37–39]. It was recently shown that CSCs display distinct metabolic characteristics, including increased OXPHOS, and elevated mitochondrial biogenesis and mass [19]. In line with these observations, our preliminary results show that impaired mitochondrial metabolism in POLG1 and POLG2 silenced cells is associated with a reduction of stemness properties, as assessed by decreased anchorage-independent growth and clonogenic potentials, as well as reduction in the expression of the stemness marker OCT4. Although short-term viability was only modestly affected, the marked reduction observed in long-term clonogenic and mammosphere assays suggests that POLG inhibition may preferentially impair long-term proliferative capacity and CSC-associated functions rather than early bulk cell viability. These observations further support the hypothesis that mitochondrial dysfunction induced by POLG targeting primarily affects stemness-associated phenotypes and anchorage-independent growth.

It is becoming increasingly evident that also alterations in the mtDNA levels lead to breast tumorigenesis [40]. In this context, mitochondrial DNA polymerase γ , which is encoded by the nuclear genes POLG and is the only DNA polymerase present in mammalian mitochondria, is a crucial protein in mtDNA replication and in maintaining mitochondrial homeostasis [16]. Mitochondrial DNA polymerase γ is a heterotrimer, composed by a catalytic subunit encoded by POLG1 gene and a dimeric accessory subunit encoded by POLG2 gene [14]. Both POLG1 and POLG2 genes are encoded by the nuclear DNA. POLG1 and POLG2 have distinct but complementary roles within the POLG holoenzyme [41]. POLG1 encodes the catalytic subunit responsible for DNA polymerase activity, proofreading and mtDNA synthesis, whereas POLG2 encodes the accessory subunit that enhances DNA binding, processivity and stability of the catalytic subunit [15,

42]. Therefore, targeting POLG1 may directly impair polymerase catalytic activity, while targeting POLG2 may destabilize the functional POLG complex and reduce mtDNA replication efficiency [43, 44]. In our pilot study, we hypothesized that reduced expression of POLG genes in MCF-7 breast cancer cells, could induce mtDNA depletion as well as mitochondrial dysfunction, thereby supporting POLG as a potential therapeutic target for breast cancer. To test our hypothesis, we have transduced MCF-7 cells with shRNA vectors targeting POLG1 and POLG2 genes. Firstly, we presented evidence that the knockdown of both subunits of the mitochondrial DNA polymerase γ induces a substantial depletion of mtDNA content. This result is consistent with the idea that mitochondrial DNA polymerase γ subunits are crucial for mtDNA maintenance, confirming recent findings which showed that the knockout in mice of POLG1 [45] and of POLG2 [44] causes mitochondrial depletion and is embryonic lethal. Despite the role of OCT4 in cancer is well-established, the importance of mtDNA in cancer development has been elucidated only recently. Indeed, it has been demonstrated that the cancer cells with reduced respiratory function show reduced capability to form tumors, and that entirely glycolytic ρ^0 cancer cells are not able to form tumors unless they acquired mtDNA from the tumor microenvironment, through the process of mtDNA transfer [46]. The correlation between breast cancer and mtDNA content is still being debated, although recent study found high mtDNA content in the blood of breast cancer patients and it was correlated with high breast cancer risk [8].

Other studies have shown that mtDNA alterations may destabilize OXPHOS, since the OXPHOS complexes contain 13 proteins encoded by mitochondrial DNA [5]. In accordance with these data, we confirm that alterations in the balance between mitochondrial and nuclear genes can affect mitochondrial function and determine metabolic reprogramming. Specifically, the knockdown of POLG1 and POLG2 subunits of mtDNA polymerase γ suppresses mitochondrial respiration and results in ATP depletion and decreased expression of the mtDNA encoded respiratory chain complex IV subunit MTCO2. Interestingly, high MTCO2 has been recently demonstrated to be a marker of poor prognosis and tumor progression in breast cancer [47]. Despite substantial mtDNA depletion following POLG1 and POLG2 knockdown, POLG-silenced MCF-7 cells maintained residual mitochondrial respiration and membrane potential. This phenomenon is consistent with findings in mtDNA-depleted “ ρ^0 cells,” where mitochondrial membrane potential and residual respiration are maintained despite impairment of the electron transport chain. Specifically, under conditions of electron transport chain dysfunction, cells depleted of

mtDNA can preserve mitochondrial membrane potential by reversing the activity of the mitochondrial ATP synthase (Complex V) [48, 49]. Indeed, instead of producing ATP, the enzyme hydrolyzes the glycolytic ATP, through the F1 domain, to pump proton across the inner mitochondrial membrane, further maintaining the proton gradient. Additionally, the adenine nucleotide translocator (ANT) helps preserve this gradient by exchanging cytosolic ATP, for mitochondrial ADP, in an electrogenic manner [49]. Although energetically costly, this ATP driven mechanism enables mitochondria to import nuclear encoded proteins and support modest oxygen consumption, consistent with the residual OCR observed in our POLG silenced cells. Variability in absolute OCR values between independent experiments is likely attributable to biological and technical differences between stable cell populations, whereas the overall reduction in mitochondrial respiration remained reproducibly associated with POLG inhibition. Collectively these findings highlight that breast cancer cells can maintain basic mitochondrial energetics via non-canonical, ATP-dependent mechanisms, even when mtDNA encoded respiratory complexes are severely compromised.

Our data also confirm previous evidence showing that mitochondrial DNA polymerase γ plays an essential role in preserving mitochondrial DNA from oxidative damage [14]. Oxidative phosphorylation is coupled with electron leakage, which is known to generate reactive oxygen species (ROS) and other free radicals. We hypothesized that energy deprivation and alteration of electron transport chain (ETC), due to POLG1 and POLG2 silencing, could generate increased mitochondrial ROS. Indeed, we showed that POLG1 and POLG2 silencing leads to an increase in mitochondrial superoxide anions in MCF-7 breast cancer cells. Consistent with these data, it was recently demonstrated that the pharmacological inhibition of mitochondrial DNA polymerase γ , using Congo Red (CR) (a novel POLG inhibitor), causes increased mitochondrial ROS production and oxidative mtDNA damage [50]. In addition, CSC maintenance is controlled by several developmental pathways, including Wnt/ β -catenin, Notch and Hedgehog signaling, which are known to regulate self-renewal, therapy resistance and tumor recurrence [51, 52]. Since mitochondrial dysfunction and ROS production can influence redox-sensitive signaling networks, our data suggest a potential correlation between POLG-dependent mitochondrial impairment, oxidative stress and dysregulation of CSC-associated signaling pathways. However, the direct involvement of Wnt/ β -catenin, Notch and Hedgehog pathways was not experimentally addressed in the present study and represents an important area for future investigation. Our data indicate that knockdown of either subunit is

sufficient to induce mtDNA depletion, mitochondrial dysfunction and reduction of CSC-associated features, suggesting that both subunits are functionally relevant for maintaining mitochondrial homeostasis in breast cancer cells. Interestingly, as a validation of this pilot study, previously published experiments employing a genetic model in MDA-MB-231 breast cancer cells demonstrated that POLG1 silencing resulted in mtDNA depletion, impaired mitochondrial respiration, and reduced mammosphere-forming capacity, findings that are consistent with our preliminary results [53]. In this pilot study, in search of new mitochondrial targets for halting breast cancer stem cells dissemination, we found that POLG genes knockdown determines mtDNA depletion, mitochondrial energy deprivation and a consequent reduction of stemness properties of MCF-7 breast cancer cells. Thus, we aimed to identify a pharmacological approach which could mimic the effect of POLG gene silencing on MCF-7 cells. Interestingly, it has been demonstrated that a family of compounds called NRTIs present an off-target effect on POLG. NRTIs were the first drugs used for the treatment of HIV infection; their mechanism of action is to act on the viral polymerase blocking viral DNA replication. However, NRTIs cross react with the mitochondrial DNA polymerase γ and inhibit mtDNA replication, causing mitochondrial toxicity. The NRTIs-induced mitochondrial toxicity theory is commonly known as “mitochondrial DNA polymerase γ theory” [54]. Thus, the mitochondrial DNA polymerase γ sensitivity to NRTIs could represent a unique opportunity to target cancer cell mtDNA replication, since certain cancers rely heavily on mitochondrial function [34]. Alovudine is a nucleoside analog of thymidine and is one of the first NRTIs to be investigated for the treatment of HIV infection; however its clinical development was discontinued after phase II clinical trial due to toxicity-related adverse effects [55]. Nonetheless, recent findings have reignited interest in Alovudine as a mitochondrial-targeting agent in oncology. Yehudai et al. demonstrated that Alovudine selectively impairs mitochondrial function in acute myeloid leukemia (AML) cells by inhibiting POLG, leading to mtDNA depletion, suppression of oxidative phosphorylation, and down-regulation of mitochondrial-encoded respiratory proteins [32]. Notably, these effects occurred without significant nuclear toxicity and were associated with a reduction in leukemic stem cell activity, thus reinforcing the therapeutic potential of POLG inhibition in metabolically active cancer cells [32]. Once inside the cell, Alovudine is phosphorylated to its active form, 3'-fluoro-3'-deoxythymidine triphosphate (FLT-TP), which can be incorporated by POLG into nascent mtDNA strands. Due to the absence of a 3'-OH group, FLT-TP acts as a chain terminator, halting mtDNA elongation and ultimately leading to mitochondrial genome

depletion and impaired oxidative phosphorylation [56]. Weising et al. extensively characterized this mechanism, demonstrating that FLT-TP can interact not only with HIV reverse transcriptase but also with host cellular DNA polymerases, including mitochondrial DNA polymerase γ (POLG). To enhance FLT's bioavailability and selectivity in specific cellular contexts, the authors developed modified FLT-TP derivatives designed to bypass the need for intracellular phosphorylation and minimize off-target effects [56]. Nevertheless, the parent nucleoside Alovudine retains its ability to interact with POLG, particularly in metabolically active or transformed cells where nucleoside uptake and activation are enhanced [56]. In our model, treatment with Alovudine in MCF-7 breast cancer cells led to mitochondrial dysfunction and a reduction in stem-like properties, phenocopying the effects observed upon POLG1 and POLG2 knockdown, and supporting a functional role for POLG in sustaining mitochondrial metabolism in breast cancer stem-like cells. Particularly, we demonstrated that Alovudine potently decreased colony and mammosphere formation, suggesting that the compound may preferentially target CSCs. Interestingly, the preferential effect of Alovudine on CSC-associated phenotypes may reflect the distinct metabolic requirements of breast CSCs compared to bulk tumor cells or non-transformed cells. Breast CSCs are known to rely heavily on mitochondrial OXPHOS, mitochondrial biogenesis and metabolic plasticity to sustain self-renewal and anchorage-independent growth [22, 23]. Therefore, pharmacological inhibition of mtDNA replication may preferentially impair CSC propagation even when short-term viability is only modestly affected. In this context, the inhibitory effects of Alovudine on mammosphere and clonogenic formation may reflect the increased dependence of CSC-enriched populations on mitochondrial integrity and OXPHOS-driven metabolism. We also observed that six days exposure to Alovudine significantly decreases mitochondrial function, determining a slight shift to the glycolytic pathway in MCF-7 cells. Furthermore, this results in a concomitant reduction in mtDNA encoded MTCO2 protein, which represents an essential component of mitochondrial respiratory chain. Interestingly, Alovudine does not affect the viability of normal hTERT-BJ1 fibroblasts. Although our findings support the involvement of POLG inhibition in mediating the biological effects of Alovudine, further mechanistic studies combining pharmacological POLG inhibition with POLG-silenced breast cancer cells will be important to better clarify the specificity of these mitochondrial-targeting effects and to further exclude potential off-target mechanisms. However, several observations support a POLG-dependent mechanism, including mtDNA depletion, reduced mitochondrial respiration, decreased MTCO2 expression and

impairment of CSC-associated phenotypes. Despite its promising mitochondrial-targeting properties, the clinical development of Alovudine as an antiviral agent was previously limited by hematological toxicities, including reversible anemia, leukopenia and myelosuppression [32, 55, 57]. These adverse effects are consistent with the mitochondrial toxicity associated with several NRTIs and their interference with mitochondrial DNA polymerase γ [34]. Nevertheless, the increased dependence of CSCs on mitochondrial metabolism may still represent a selective metabolic vulnerability. In this context, we further expanded our study by employing the alternative POLG inhibitor ddC in additional breast cancer models, further reinforcing the idea that pharmacological disruption of POLG-dependent mitochondrial function may impair breast CSC-associated phenotypes. ddC is a nucleoside analog previously developed as an antiviral agent for HIV treatment and is also recognized as an inhibitor of mitochondrial DNA polymerase γ [58]. Previous evidence demonstrated that prolonged ddC exposure selectively impairs mtDNA replication and mitochondrial oxidative phosphorylation in cancer cells, supporting its potential as a mitochondrial-targeting agent [21]. Moreover, Yehudai et al. demonstrated that ddC, similarly to Alovudine, reduces mtDNA content and mitochondrial-encoded respiratory proteins while promoting comparable biological effects in acute myeloid leukemia (AML) models, further supporting the role of POLG inhibition in targeting mitochondrial metabolism in cancer cells [32]. In our pilot study, ddC treatment in MCF-7 breast cancer cells reduced clonogenic and mammosphere-forming capacity, consistent with the role of POLG-dependent mitochondrial metabolism in maintaining CSC-associated properties. Moreover, prolonged ddC exposure impaired mitochondrial respiration and decreased the expression of the mtDNA encoded respiratory chain protein MTCO2 in MCF-7 cells, further reinforcing the link between POLG inhibition, mitochondrial dysfunction and reduced stemness phenotypes. While MTCO2 was selected in the present study as a representative mitochondrially encoded respiratory chain subunit directly dependent on mtDNA integrity [47], future studies investigating additional OXPHOS complex components and mitochondrial respiratory chain subunits will be important to further characterize the broader impact of POLG inhibition on mitochondrial function and respiratory chain organization in breast cancer cells. Collectively, ddC treatment in MCF-7 cells reproduced several phenotypes previously observed following POLG1/POLG2 silencing and Alovudine treatment, including altered mitochondrial respiration and reduced oxygen consumption. Accordingly, to strengthen the translational relevance of our findings and address the limitation of relying predominantly on MCF-

7 cells, we extended our analyses to additional breast cancer cell lines, including T47D, MDA-MB-231, MDA-MB-436, MDA-MB-453 cells, and MCF10A non-tumoral mammary epithelial cells as non-tumoral controls. ddC treatment consistently impaired CSC-associated phenotypes across multiple breast cancer subtypes, particularly mammosphere formation, supporting the broader role of POLG-dependent mitochondrial maintenance in breast CSC biology. Importantly, ddC treatment exerted limited effects on the viability of non-tumoral MCF10A mammary epithelial cells, further supporting a potential therapeutic window for POLG-targeting strategies. Furthermore, preliminary analyses of NANOG and SOX2 expression following ddC treatment further support a functional connection between POLG-dependent mitochondrial homeostasis and the regulation of CSC-associated transcriptional programs. In MCF-7 cells, ddC exposure markedly reduced the expression of both SOX2 and NANOG, suggesting that mitochondrial perturbation induced by POLG inhibition may directly affect stemness-associated pathways in luminal breast cancer cells. Under the same experimental conditions, MDA-MB-453 cells also displayed a reduction in SOX2 expression, whereas NANOG levels appeared only modestly affected, indicating that the transcriptional response to POLG inhibition may vary according to the molecular and metabolic background of different breast cancer subtypes. Finally, the evaluation of mitochondrial and intracellular ROS levels following ddC treatment provided additional insights into the biological consequences of POLG inhibition. Consistent with the results obtained following POLG1 and POLG2 silencing, ddC exposure induced a modest increase in mitochondrial superoxide levels, as assessed by MitoSOX Red staining, suggesting that impairment of mtDNA maintenance and mitochondrial respiration may contribute to mild mitochondrial oxidative stress. However, total intracellular ROS levels measured using the DCFH-DA probe remained substantially unchanged under the same experimental conditions in both MCF-7 and MDA-MB-453 cells. These findings suggest that POLG inhibition primarily affects mitochondrial redox homeostasis without inducing a generalized oxidative stress response and further support the hypothesis that mitochondrial dysfunction, rather than extensive ROS accumulation, may contribute to the impairment of CSC-associated phenotypes observed following ddC treatment. Collectively, these additional findings obtained using an independent POLG inhibitor and multiple breast cancer models substantially reinforce the broader biological relevance of POLG inhibition as a potential mitochondrial-targeting strategy in breast cancer. In parallel, we performed a Kaplan-Meier survival analysis using the Kaplan-Meier Plotter tool on a cohort of 458 breast cancer patients, specifically high-

risk, ER-positive, lymph node-positive cases with luminal A subtype, revealing that high POLG1 expression significantly correlates with poorer prognosis, including lower overall survival (HR = 1.34, $p = 0.0024$), relapse-free survival, distant metastasis-free survival, and post-progression survival (HR = 1.58), supporting POLG1 as a potential prognostic biomarker in breast cancer. Notably, the association between elevated POLG1 expression and worse clinical outcome is consistent with our *in vitro* findings, suggesting that POLG1 may promote tumor progression through enhanced mitochondrial function and metabolic adaptation, thereby contributing to a more aggressive breast cancer phenotype. However, these findings should be further validated in independent clinical cohorts using multivariate Cox regression analyses including established clinicopathological variables, while extending survival analyses to POLG2 expression may provide additional insight into the clinical relevance of the POLG holoenzyme in breast cancer. Although the observed phenotypes were consistent across POLG1 and POLG2 silencing and were further supported by pharmacological inhibition, potential shRNA-related off-target effects cannot be completely excluded. Future studies employing additional independent shRNAs, rescue experiments, or CRISPR-based approaches will therefore be important to confirm the specificity of POLG1 and POLG2 targeting. Moreover, while our *in vitro* data support a role for POLG inhibition in reducing mitochondrial function and CSC-associated phenotypes, *in vivo* validation using xenograft or orthotopic breast cancer models will be necessary to determine whether POLG inhibition can effectively impair tumor initiation, tumor growth, CSC frequency, and therapeutic resistance. Such studies will also be essential to assess potential systemic toxicity, particularly in tissues characterized by high mitochondrial activity or proliferative rates. Taken together, these preliminary findings suggest that POLG may represent an important regulator in maintaining mitochondrial metabolism and CSC-associated properties in breast cancer, highlighting its potential as a therapeutic target. Nevertheless, further validation in *in vivo* models and larger clinical cohorts will be required to better define the translational relevance of POLG inhibition and to determine whether optimized dosing strategies, alternative POLG inhibitors, or combinatorial therapeutic approaches may improve the clinical applicability of mitochondrial-targeting therapies.

MATERIALS AND METHODS

Cell lines and other reagents

MCF-7, T47D, MDA-MB-231, MDA-MB-436, MDA-MB-453 and MCF10A breast cancer cell lines were

acquired from ATCC (American Type Culture Collection, USA). Human immortalized fibroblasts (hTERT-BJ1) were originally purchased from Clontech, Inc., USA. MCF-7, T47D, MDA-MB-453 and hTERT-BJ1 were maintained in culture in high glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma, Poole, UK), supplemented with 10% HI FBS (Heat-Inactivated Fetal Bovine Serum) (Gibco, Paisley, UK), 1% Glutamax (100X, Gibco, cat. Number: 35050061), and 1% Penicillin-Streptomycin (Gibco). MDA-MB-436, MDA-MB-231 and MCF10A cells were cultured in DMEM/F12 medium supplemented with 10% HI FBS, 1% Glutamax and 1% Penicillin-Streptomycin. Cells were grown in a humidified incubator at 37° C and 5% CO₂.

Alovudine (3'-deoxy-3' fluorothymidine) (CS-0013305) was purchased from Chemscone (Monmouth Junction, NJ, USA). The compound was dissolved in DMSO at 50 mM stock concentration. Zalcitabine (2',3'-dideoxycytidine, ddC) was purchased from MedChemExpress (MCE, Monmouth Junction, NJ, USA). The compound was dissolved in DMSO at 10 mM stock concentration.

Silencing of POLG1/POLG2 genes

POLG1 and POLG2 genes were silenced by using the Lenti-PacTM HIV Expression Packaging Kit (GeneCopoeia, Rockville, MD, USA), following the manufacturer's instructions. Forty-eight hours after seeding, 1.5 x 10⁶ 293Ta packaging cells (human embryonic kidney cells) were plated in T75 flasks and when they reached 70-80 % of confluence, they were transfected with the shRNA vectors targeting POLG1 (CS-HSH063505-LVRU6GP-01), POLG2 (HSH091588-LVRU6GP) and the scrambled controls (CSHCTR001-LVRU6GP) (GeneCopoeia). The vector contained also a GFP reporter gene. The DNA-Endofectin lentiviral mix was prepared using 2.5 µg of lentiviral ORF/RNA plasmid, 5 µl of Lenti-Pac HIV mix and 15 µl of EndoFectin Lenti reagent (GeneCopoeia), which were diluted in 200 µl of Opti-MEM (Gibco). After 8-14 hours of incubation in a CO₂ incubator at 37° C overnight, the complete medium was replaced with 10 ml of medium supplemented with 5 % of heat-inactivated FBS and 20 µl of TiterBoost reagent (GeneCopoeia). After 48 hours, the pseudovirus-containing culture medium was collected using a 0.45 µm filter and stored at -80° C or used immediately. 48 hours before transduction, 0.5 x 10⁶ MCF-7 cells were seeded in T25 flasks. When they reached a 70-80 % confluence, 3 ml of virus suspension diluted in 3 ml of medium supplemented with polybrene (final concentration 7.5 µg/ml; Santa Cruz), was added to the cells. Cells were incubated overnight; next day, the medium was replaced with fresh medium, and cells were cultured until they

reached 80-90% confluence. Since the plasmids carry the gene coding for puromycin resistance, the transduced cells were selected using puromycin at 1 µg/ml concentration, while the mock control cells died because they do not carry the resistance. The shRNA POLG1 or POLG2 MCF-7 cells and the shRNA control cells were maintained in culture with normal media supplemented with 50 µg/ml Uridine (Sigma-Aldrich, St. Louis, MO, USA).

Western blotting

Cells were collected and lysed for proteins extraction in RIPA lysis buffer (Sigma-Aldrich, #R0278) containing protease and phosphatase inhibitors (Roche Applied Science, #04906845001 #05892970001). Briefly, cell lysates were centrifuged (10 min, 13,000 rpm), and protein concentration was measured using PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, #23225). Next, the Laemmli sample buffer was added to the samples (Invitrogen, #NP0007) and the samples were boiled for 10 min at 99° C. Proteins were loaded into 4–20% Mini-PROTEAN[®] TGXTM Precast Protein Gels (Bio-Rad, #4561094) and after the gel electrophoresis, they were transferred to 0.2 µm nitrocellulose membranes, using the Trans-Blot Turbo Transfer System (Bio-Rad, Inc., USA). After 1 hour of blocking at room temperature with 5% BSA in PBS-T (PBS 1%, 0.5% Tween 20, Sigma-Aldrich), membranes were incubated overnight at 4° C with the primary antibodies against POLG1 (1:1,000, Abcam ab97661), POLG2 (1:1,000, Proteintech 10997-2-AP), MTCO2 (1:500, Abcam ab109739), Vinculin (1:2,000, Santa Cruz sc-73614), β-Actin (1:3000, Sigma- Aldrich), and TOMM20 (1:1000, Santa Cruz sc-17764). Next, the membranes incubated with HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse IgG, 1:2,000, Cell Signalling) diluted in 5% BSA at room temperature for 1 hour. Then, the membranes were developed using SuperSignal West Pico chemi-luminescent substrate (Thermo Fisher Scientific, USA) and pictures were obtained using a Syngene G:BOX imaging system or a LI-COR imaging system. Densitometric analyses were performed with ImageJ software or LI-COR Empiria Studio software.

Quantitative PCR analysis

DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions for direct cell lysis of cultured cells. Relative mitochondrial DNA copy number was obtained using a Relative Human Mitochondrial DNA Copy Number Quantification qPCR Assay Kit (ScienCell, USA). Briefly, 10 µl reaction contained: 5 µl Reaction GoldNStart TaqGreen qPCR master mix, 1 µl primers (10 µM, Forward and Reverse mixed), 1 µl of

DNA (5 ng), 0.2 µl ROX dye and 2.7 µl water. qPCR reactions were performed in triplicate, and three biological repeats were analyzed for each condition. qPCRs were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems), using the following conditions: 95° C for 10 min, followed by 32 cycles of 95° C for 20 s, 52° C for 20 s, and 72° C for 45 s. The relative expression levels were calculated using $\Delta\Delta C_T$ method presented as the fold change of target gene expression (mtDNA), in a target sample (MCF-7 transduced cells), relative to a reference sample (controls) and normalized to a reference gene (gDNA) [59].

For gene expression analysis, total cellular RNA was extracted from MCF-7 and MDA-MB-453 breast cancer cells treated with ddC for 72 hours using TRIZOL reagent (Sigma-Aldrich, USA), according to the manufacturer's instructions. RNA purity and integrity were assessed by spectrophotometric analysis. Complementary DNA (cDNA) synthesis was performed using the OneScript® Plus cDNA Synthesis Kit (Applied Biological Materials Inc., Canada), according to the manufacturer's instructions. Quantitative real-time PCR analysis of SOX2 and NANOG expression was performed using the QuantStudio™ 3 Real-Time PCR System platform (Life Technologies, Waltham, MA, USA) and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). Reactions were performed in triplicate, and gene expression levels were normalized to 18S control gene expression. Relative mRNA levels were calculated using the $\Delta\Delta C_T$ method [59].

MitoNIR analysis

NIR Mitochondrial Membrane Potential Assay kit (Abcam, ab112149) was used for evaluating mitochondrial membrane potential. Cells were trypsinized, washed with PBS and counted (5×10^5 cells/sample). 5 µl of 200X MitoNIR Dye solution were diluted in 1 ml of cell suspensions. After incubation for 15-30 min at room temperature, cells were centrifuged at 1000 rpm for 4 min, resuspended in 1 ml of assay buffer and analyzed by FACS (Attune™ NxT Flow Cytometer, Thermo Fisher Scientific). Results were evaluated by GraphPad Prism, considering the mean fluorescent intensity (MFI), to compare mitochondrial membrane potential across samples.

MitoSOX analysis

MitoSOX Red probe (M36008, Invitrogen™) is a derivative of Ethidium Bromide and was used for evaluating mitochondrial superoxide anion, in live cells. Firstly, to make a stock solution of 5 mM, 50 µg of MitoSOX mitochondrial superoxide indicator were

dissolved in 13 µl of DMSO. The stock solution was diluted to a final concentration of 5 µM. For drug treatment experiments, MCF-7 and MDA-MB-453 cells were treated with ddC for 72 hours prior to analysis. Following treatment, cells were incubated with MitoSOX Red dye (5 µM) for 30 minutes at 37° C and subsequently washed with PBS. MitoSOX fluorescence was measured using the GloMax® Discover Microplate Reader (Promega, Madison, WI, USA) at 510 nm excitation and 580 nm emission wavelengths. Fluorescence values were normalized to cell viability assessed by MTT assay [60]. For flow cytometry experiments, cells were trypsinized, washed with PBS and counted (5×10^5 cells/sample). After, cells were incubated with MitoSOX Red Dye (5 µM) for 30 minutes at 37° C. After the incubation, samples were centrifuged (1,200 rpm, 5 min) and washed with PBS. Next, samples were resuspended in 400 µl of PBS and analyzed by FACS (Attune™ NxT Flow Cytometer, Thermo Fisher Scientific). Results were evaluated by GraphPad Prism, considering the mean fluorescent intensity (MFI), to compare superoxide anion levels across samples.

Mitotracker deep red analysis

Mitochondrial mass was determined using MitoTracker Deep Red (Thermo Fisher Scientific #M22426). MitoTracker staining solution was prepared from the 1 mM stock solution diluting the dye in complete media to a final concentration of 50 nM. Cells were incubated with MitoTracker staining solutions for 20 minutes at 37° C. Then, cells were washed with PBS, collected, and resuspended in 500 µL of PBS. Samples were analyzed by FACS analysis using Attune™ NxT Flow Cytometer and results were analyzed by Graph Pad Prism software.

CellROX analysis

CellROX (C10422- Invitrogen™) is a fluorogenic probe for detecting oxidative stress in live cells. Firstly, the stock solution (2.5 mM) was diluted to a final concentration of 5 µM. Cells were trypsinized, washed with PBS and counted (5×10^5 cells/sample). After incubation with CellROX for 30 minutes at 37° C, samples were centrifuged (1,200 rpm for 5 min) and washed with PBS. Next, samples were resuspended in 400 µl of PBS and analyzed by FACS (Attune™ NxT Flow Cytometer, Thermo Fisher Scientific). Results were evaluated by GraphPad Prism, considering the mean fluorescent intensity (MFI), to compare total ROS levels across samples.

Seahorse XFe-96 metabolic flux analysis

Oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) were evaluated using the

Seahorse Extracellular Flux (XFe96) analyzer (Agilent). Briefly, ten thousand cells were seeded into XFe-96-well cell culture plates in normal media and incubated at 37° C with 5% CO₂, for 48 hours. Next, sensor cartridges were incubated with XF calibrant medium pH 7.4 (Agilent Seahorse XF) for 1 hour at 37° C, in an incubator without CO₂. Then, cells were maintained in 175 µl/well of XFe media at 37° C, in the incubator without CO₂ for 1 hour. After the calibration, the sensor cartridges were loaded with inhibitors: 25 µl of 80 mM glucose, 9 µM oligomycin and 1M 2-deoxyglucose for ECAR measurement and 25 µl of 10 µM oligomycin, 9 µM FCCP and 10 µM of rotenone and 10 µM antimycin for OCR measurement. Data were normalized to cell number with Hoechst (1:1000 Thermo Fisher Scientific), incubated for 30 min at 37° C with 5% CO₂. Data were analyzed with Wave software and GraphPad Prism software.

Sulforhodamine B (SRB) cell viability assay

For SRB assay, MCF-7, MDA-MB-231, MDA-MB-436, MDA-MB-453, T47D, hTERT-BJ1 and MCF10A cells were seeded at a density of 1×10^4 cells/well in 96-well plates. For growth assays, cells were incubated for 72 hours and then fixed. For drug treatment, cells were plated 24 hours before the treatment and incubated with Alovudine and ddC for three days. Then, cells were fixed using 10% Trichloroacetic acid (TCA) at 4° C for 1 hour, and then they were washed twice with PBS and dried overnight. Next day, cells were incubated with SRB solution for 15 min, washed with 1% acetic acid and dried. Finally, the SRB was dissolved in 10 mM pH 8.8 Tris-HCl buffer (Trizma Base, Sigma-Aldrich) solution and absorbance was measured at 565 nm using either a Varioskan™ LUX microplate reader (Thermo Fisher Scientific) or a GloMax® Discover Microplate Reader (Promega).

Cell cycle analysis

Cell cycle analysis was performed by flow cytometry using the Attune NxT Flow Cytometer. For the analysis, cells were stained with 200 µl Muse Cell Cycle Kit (Luminex, Austin, TX, USA), a buffer containing propidium iodide (PI) stain, which is a nuclear DNA stain, for 20 min at room temperature in the dark. The gated cells were manually categorized into cell-cycle stages. Data analysis was performed using the GraphPad Prism software.

Colony formation assay

For colony formation assay, 500 cells/well were seeded in 6-well plate in 2 ml of normal medium or in medium with increasing concentrations of Alovudine and ddC,

and incubated for 14 days. Next, cells were fixed for 20 minutes in 70% ethanol, after fixation cells were washed twice with PBS and stained with 0.2 % Crystal Violet (Sigma-Aldrich) for 15 minutes. The staining was removed with water and the plates were dried overnight. Finally, colony numbers were counted manually, considering that a single colony consists of at least 50 cells. The IC₅₀ of Alovudine for colony formation assay was calculated using GraphPad 9 software.

Mammosphere formation assay

In this assay, 6-well plates were coated with 2 mL of 2-hydroxyethyl-methacrylate (poly-HEMA) (12 g of poly-HEMA (Sigma) dissolved in 1 L of 95% ethanol) and incubated at 50° C for 72 hours. MCF-7, T47D, MDA-MB-231, MDA-MB-436 and MDA-MB-453 were then plated into poly-HEMA coated 6-well plates at a density of 5000 cells/well in mammosphere media (DMEM-F12 medium without phenol red (Gibco); B27 supplement (Gibco); 20-ng/ml EGF (Invitrogen); 1% Penicillin-Streptomycin (Gibco)) and maintained in the incubator, at 37° C and 5% CO₂. POLG1- and POLG2-silenced cells were cultured under the same experimental conditions. For pharmacological experiments, Alovudine and ddC treatments were added directly to the mammosphere medium at the time of cell seeding. After 5 days, spheres >50 µm were counted manually using an eye-piece graticule, and the percentage of cells plated which formed spheres was calculated. Data analysis was performed on GraphPad Prism.

OCT4 analysis

OCT4 (octamer-binding transcription factor 4) stemness marker was measured using OCT4-PE antibody (PE anti-OCT4, Sony. #3868520). Cells were fixed with 4% PFA, and permeabilized with 0.1% Triton and incubated with OCT4-PE antibody (1:50 dilution) for 90 min at room temperature. After the incubation, samples were centrifuged (1,200 rpm for 5 min) and washed with PBS. Next, samples were resuspended in 400 µl of PBS and analyzed by FACS (Attune™ NxT Flow Cytometer, Thermo Fisher Scientific).

DCFH-DA assay

DCFH-DA (2',7'-Dichlorofluorescein diacetate; Diacetyldichlorofluorescein; Sigma-Aldrich, USA) probe was used to evaluate total intracellular ROS levels. A 10 mM stock solution of DCFH-DA was diluted in PBS to obtain a final working concentration of 5 µM. For drug treatment experiments, MCF-7 and MDA-MB-453 cells were treated with ddC for 72 hours prior to analysis. Following treatment, cells were incubated with DCFH-DA probe (5 µM) for 30 minutes at 37° C and

subsequently washed with PBS. Fluorescence was measured using the GloMax® Discover Microplate Reader (Promega) at 485 nm excitation and 535 nm emission wavelengths. Fluorescence values were normalized to cell viability assessed by MTT assay [60]. Data were analyzed using GraphPad Prism software.

Statistical and survival analyses

Data analysis was performed with GraphPad Prism 9.0. Data were shown as mean $\pm$ SEM. Statistical analysis was performed only for experiments conducted three times independently. Student's t test and ANOVA test were used for calculating statistical differences. Kaplan-Meier analysis was performed using the Kaplan-Meier Plotter, an open-access online survival analysis tool that incorporates gene expression and clinical data from a large database of breast cancer tumors. For this analysis, a specific subset of patients was selected: high-risk, estrogen receptor-positive (ER+), lymph node-positive breast cancer patients diagnosed with luminal A subtype (n = 458). The hazard ratio (HR) and log-rank p-value were calculated to assess the prognostic significance of POLG1 expression levels.

Abbreviations

ATP: Adenosine Triphosphate; CSCs: Cancer Stem Cells; ddC: Zalcitabine; DNA: Deoxyribonucleic Acid; ECAR: Extracellular Acidification Rate; ECT: Electron Transport Chain; ER: Estrogen Receptor; HIV: Human Immunodeficiency Virus; MFI: Mean Fluorescent Intensity; mtDNA: Mitochondrial DNA; NRTIs: Nucleoside Reverse Transcriptase Inhibitors; OCR: Oxygen Consumption Rate; OCT4: Octamer-Binding Transcription Factor 4; OXPHOS: Oxidative Phosphorylation.

AUTHOR CONTRIBUTIONS

CC, MPL and FS designed the study. CC performed most of the experiments, analysed the data, generated the figures, and wrote the first draft of the manuscript. CJS contributed to the mtDNA qPCR experiments, while MF contributed to the analysis and interpretation of the Kaplan-Meier survival curves. ARC and MF contributed to the design of the additional revision experiments and manuscript revision. MPL and FS supervised the study and edited the final manuscript. All authors have read and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

M.P.L. and F.S. hold a minority interest in Lunella Biotech, Inc. The authors declare no conflicts of interest.

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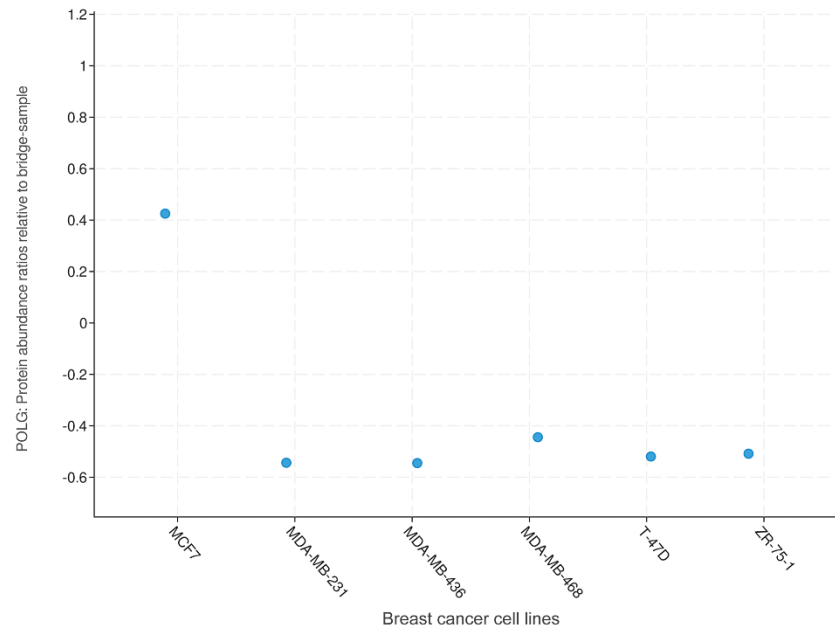
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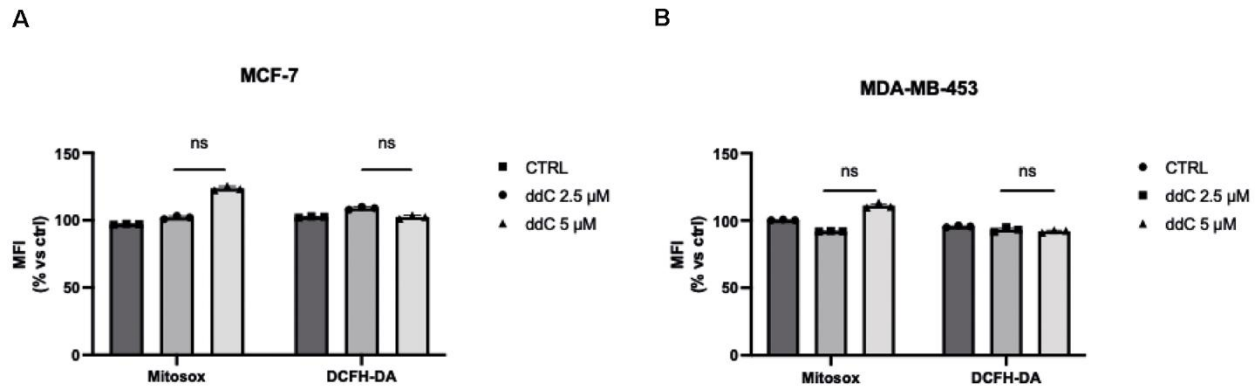
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SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. *In silico* analysis of protein abundance levels in breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-436, MDA-MB-468, T-47D, ZR-75-1). The analysis was conducted on cbiportal.org by using the Cancer Cell Line Encyclopedia (Broad, 2019) dataset.



Supplementary Figure 2. ddC treatment induces modest changes in mitochondrial ROS levels without affecting total intracellular ROS production in breast cancer cells. MCF-7 and MDA-MB-453 cells were treated with ddC (2.5 and 5 μ M) for 72 hours. (A, B) Mitochondrial ROS levels were evaluated using the MitoSOX probe, whereas total intracellular ROS levels were assessed using the DCFH-DA probe. Fluorescence intensity was measured and normalized to cell viability assessed by MTT assay. Results are presented as mean fluorescent intensity (MFI) relative to vehicle-treated control cells (DMSO). Data shown represent mean $\pm$ SEM from three independent biological replicates. Statistical analysis was performed using ordinary one-way ANOVA followed by Bonferroni's multiple comparisons test. Statistical significance (relative to control): ns, not statistically significant.