Molecular Therapy
Oncology
Brief Report

Primary murine high-grade glioma cells derived from RCAS/tv-a diffuse glioma model reprogram naive T cells into immunosuppressive regulatory T lymphocytes

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High-grade gliomas (HGGs) and glioblastomas (GBMs) are the most aggressive and lethal brain tumors. The current standard of care (SOC) includes gross safe surgical resection followed by chemoradiotherapy. The main chemotherapeutic agents are the DNA-alkylating agent temozolomide (TMZ) and adjuvants. Due to the outdated therapeutic protocols and lack of specific treatments, there is an urgent and rising need to improve our understanding of tumor biology and design more effective therapeutic strategies. *In vitro* models are essential for investigating glioma biology and testing novel therapeutic approaches. While using commercially available and patient-derived glioma cell lines for *in vitro* studies is common practice, they exhibit several limitations, including failing to maintain the genetic and phenotypic diversity of primary tumors, undergo genetic drift over time, and often lacking the invasive and stem-like characteristics of patient tumors. These limitations can lead to inconsistent and non-reproducible results, hampering translational research progress. In this study, we established a novel primary murine HGG cell line, isolated from an immunocompetent HGG-bearing RCAS/T-va mouse. We characterized the transcriptome and phenotype to ensure that this cell line resembles the nature of HGGs and retains the ability to reprogram primary murine T lymphocytes.

INTRODUCTION

In the United States, approximately 13,000 new cases of malignant brain tumors are diagnosed every year, with an incidence rate of 3.21 per 100,000 population and a high mortality rate.[1] Among brain tumors, high-grade gliomas (HGGs) and glioblastomas (GBMs) are the most aggressive and lethal, classified as WHO grades III and IV and with a median survival of only 1–3 years. The current standard of care (SOC) includes gross safe surgical resection followed by chemoradiotherapy. The main chemotherapeutic agents are the DNA-alkylating agent temozolomide (TMZ)[2] and adjuvants.[3] Due to the outdated therapeutic protocols and lack of specific treatments, there is an urgent and rising need to improve our understanding of tumor biology and design more effective therapeutic strategies.

In vitro models represent the first step for the investigation of tumor biology and conducting preliminary experiments on novel therapeutic approaches. The use of commercially available and patient-derived glioma cell lines for *in vitro* investigation is a common practice. However, these cell lines have several limitations, such as failing to accurately maintain the genetic and phenotypic diversity of primary tumors, undergoing genetic drift over time, and often lacking the invasive and stem-like characteristics of patient tumors. These limitations can result in inconsistent and non-reproducible results, hampering translational research progress.[4,5]

In this study, we established a novel primary murine HGG cell line, isolated from an immunocompetent HGG RCAS/T-va mouse that had progressed from a lower-grade glioma.[6] We characterized the transcriptome and phenotype to ensure that this cell line is consistent with HGG characteristics[7] and retains the ability to reprogram primary murine T lymphocytes.[8,9]

This novel primary murine cell line may represent a valuable resource for advancing our understanding of glioma cellular biology while also serving as a valuable experimental tool.

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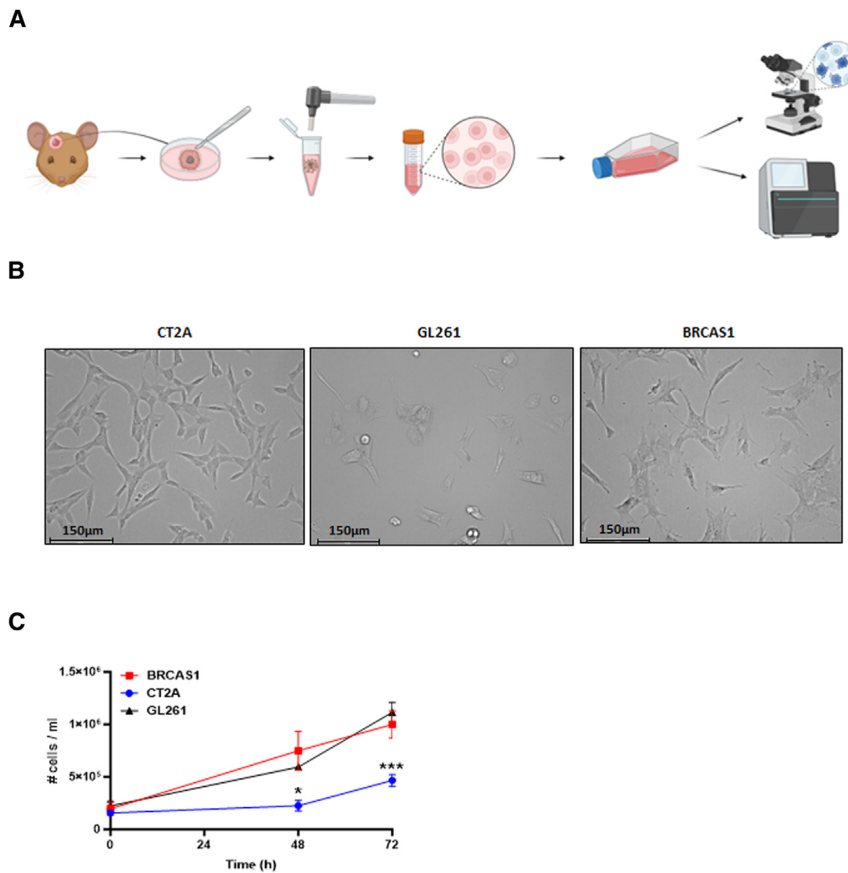


Figure 1. Establishment of the primary murine high-grade glioma BRCAS1 cell line

(A) Schematic of the project pipeline (biorender.com). (B) Bright-field imaging of CT2A, GL261, and BRCAS1 cells in culture. 20× magnification; scale bar: 150 μm (n = 3). (C) Assessment of the cellular proliferation of BRCAS1, GL261, and CT2A cells by cell count at 48 and 72 h (n = 3). Unpaired two-tailed Student's t test calculated the statistical significance. *$p < 0.05$ and ***$p < 0.001$.

RESULTS

Establishment of the primary murine HGG BRCAS1 cell line

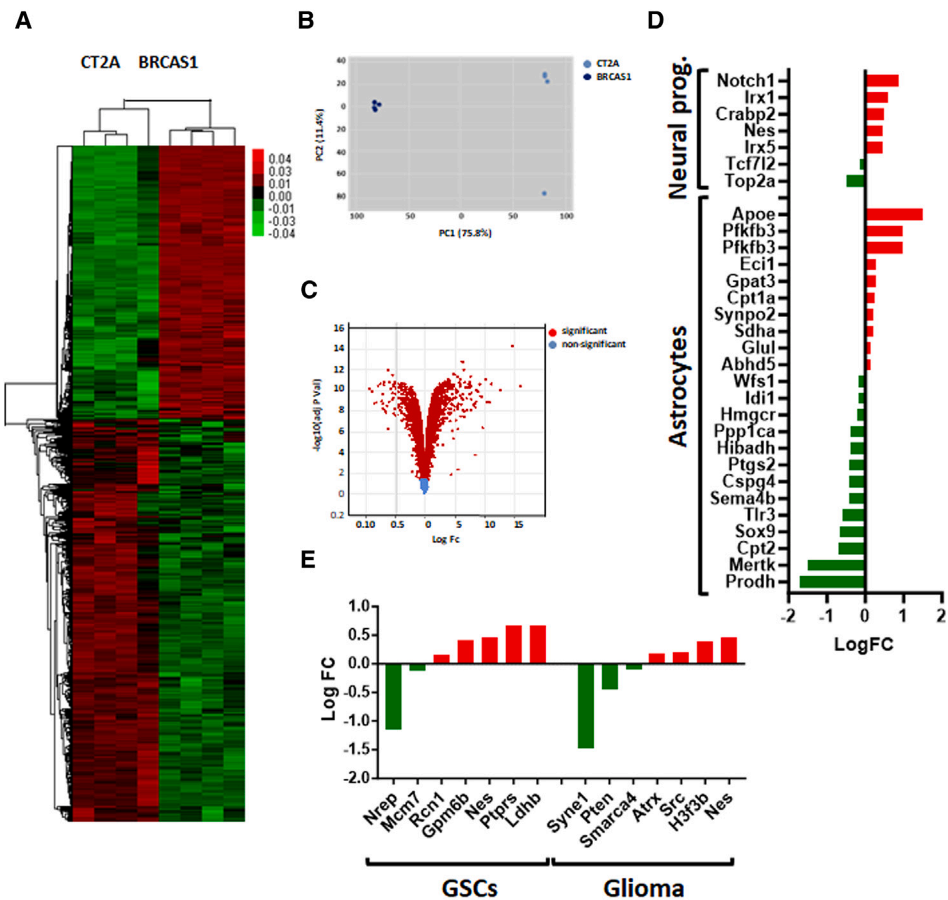
We isolated the primary HGG cancer cell line BRCAS1 from the tumor mass in the brain of an HGG RCAS mouse at week 7 post-gliomagenesis[10] (Figure 1A). For comparative morphological and phenotypical assessment, we used the commercially available murine HGG cell lines CT2A and GL261, and human HGG cell lines U87 and U251. BRCAS1, CT2A, GL261, U87, and U251 cell lines grow in adhesion and form monolayers (Figures 1B and S1A). Microscopic evaluation confirmed that CT2A, U87, and U251 exhibit a spindle shape, while BRCAS1 and GL261 cells display polygonal and amorphous shapes (Figures 1B and S1A). Interestingly, while BRCAS1 demonstrated comparable proliferation to GL261, it showed significantly faster proliferation *in vitro* compared with CT2A, U87, and U251 (Figures 1C and S1B). To validate the newly established HGG cell line, we performed total RNA sequencing (RNA-seq) on CT2A and BRCAS1 cells. We identified a total of 5,516 significantly and differentially expressed genes (Figures 2A–2E; Table S2). 2,229 genes were upregulated, and 3,287 were downregulated (GEO: GSE274163). Gliomas are cancer cells originating from astrocytes and neural progenitors.[11] Confirming the glioma-like phenotype of the new HGG cell line, the RNA-seq signature in BRCAS1 cells, compared with CT2A cells, showed increased expression of genes known to be associated with glioma,[12,13] glioma stem cells (GSCs),[14] and neural progenitors.[15,16] However, CT2A demonstrated increased expression of genes associated with astrocytic markers[17,18] (Figures 2D, 2E, and S2A).

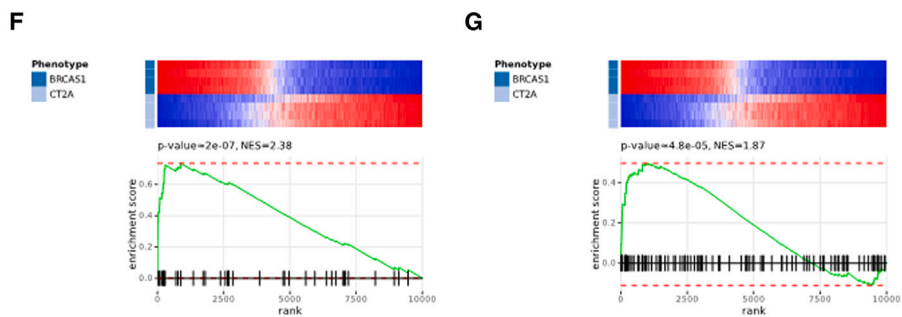
In vitro characterization of BRCAS1 transcriptome and phenotype

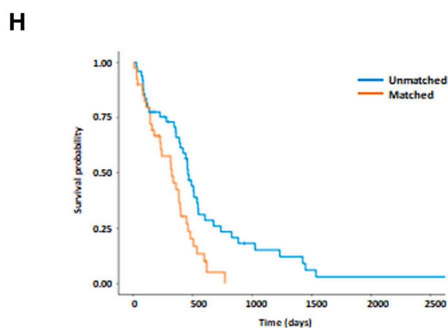
Gene enrichment analysis of BRCAS1 RNA samples compared to CT2A showed a significant upregulation of genes associated with the cellular growth pathway (Figure 2F) and the Extracellular signal-Regulated Kinase 1/2 (ERK1/2) cascade in BRCAS1 cells (Figure 2G). This pathway is crucial for migration, invasion, and proliferation in glioma.[19] Next, we aimed to align murine model properties with phenotypic effects observed in human HGG tumors. Notably, analysis of survival data from the TCGA GBM dataset indicated that genes identified as differentially expressed in the murine cell lines are associated with significantly poorer prognosis compared to patients with unmatched signatures (Figure 2H, p = 0.012, hazard ratio [HR] = 1.4, HR SD = 1.07–1.8) (Figure 2I).

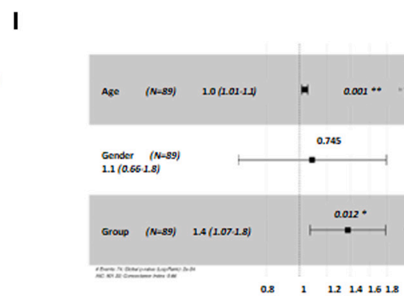
To confirm the transcriptomic results for BRCAS1 and CT2A, we performed a colony formation assay. Our results show that BRCAS1 forms colonies *in vitro* faster and more efficiently than CT2A (Figure 3A). Additionally, we performed a proliferation assay at 72 hours post-treatment with TMZ, the main chemotherapeutic agent for the treatment of glioma.[2] Our results showed greater sensitivity to TMZ in CT2A and BRCAS1 cells compared to the GL261, U87, and U251 cell lines (Figures 3B, S1C, and S1D).

Glioma cells are known to reprogram immune cells in the tumor microenvironment (TME), enhancing the activation of CD4+ regulatory T lymphocytes (Tregs) and reprogramming resident and circulating pro-inflammatory myeloid cells to become immunosuppressive. The immunosuppressive phenotype of tumor-infiltrating Tregs and myeloid cells is a major factor in the inactivation of cytotoxic T and natural killer (NK) cells.[8,20,21] To evaluate the capacity of the new HGG cell line to reprogram immune cells, we co-cultured BRCAS1 and CT2A cells with primary murine T cells (CD4+ and









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CD8+) isolated from the spleens of animals sharing the same genetic background as those used to generate BRCAS1 (ratio: 1:1). Interestingly, the analysis of the transcriptome of T cells harvested after 24 hours of co-culture with HGG cells showed increased expression of genes (FoxP3, CD39, interleukin [IL]-2, interferon [IFN]γ, Prf1, CTLA4, PD-1) associated with the activation of immunosuppressive Tregs (Figures 3C and S2B).[22,23]

DISCUSSION

In vitro cancer models are important tools for investigating malignant glioma cell biology and screening novel therapies. The establishment of primary murine glioma cell lines is crucial not only for neuro-oncology research but also for ensuring that we improve our knowledge on the intrinsic tumor biology within the glioma microenvironment. Moreover, these glioma cell lines can be genetically manipulated, allowing researchers to investigate the role of specific genes in gliomagenesis, tumor progression, and drug resistance. To create the BRCAS1 HGG cell line, the first step involved the isolation of glioma cells from HGG primary tumors. These tumors were induced in transgenic RCAS mice with partial loss of PTEN, p14, p16, and ectopic expression of the oncogenic driver platelet-derived growth factor b (PDGFb) in neural progenitors.[6,24–26] Of note, the primary cell line was isolated directly from glioma tissues harvested from an immunocompetent strain, thus providing a more relevant model compared to immortalized cell lines or human xenografts in immunodeficient models.[27,28] The HGG BRCAS1 cell line we isolated and characterized preserves numerous characteristics of the original tumors, including genetic mutations, morphological and phenotypical features, and TME interactions. BRCAS1 is a highly proliferative cancer cell line, with polygonal and amorphous shapes and a transcriptome that denotes the upregulation of glioma, GSCs, and neural progenitor-associated genes, confirming the genesis of the primary cancer cell line. Additionally, the newly established cell line showed upregulation of the ERK cascade and faster colony formation. Notably, patients with HGG who have a gene signature matching the differentially expressed genes between BRCAS1 and CT2A cells have significantly worse overall survival.

The immunosuppressive TME is essential for immune escape mechanisms and glioma progression. Co-culturing primary murine T cells with BRCAS1 demonstrated the ability of the HGG cell line to drive T cell differentiation into immunosuppressive Tregs, which is one of the initial steps in the remodeling of the immune cells in the TME.[8,29] Replicating the characteristics of the parent tumor enhances the relevance of BRCAS1 cells in *in vitro* experiments, especially in the context of drug screening and analysis of cancer-related biological processes. The main challenges in establishing these cell lines are maintaining sterility, cellular stability, and tumorigenic properties over several passages and avoiding contamination from other types of cells. Moreover, isolating and establishing low-grade glioma murine primary cell lines is more difficult and challenging. Monitoring genetic and phenotypic characteristics over time and optimizing cell culture conditions are mandatory requirements to guarantee their reliability. In the study, we did not investigate genetic drift over time, assess the capacity of the cells to establish HGG tumors *in vivo*, fully elucidate the stem-like properties of the BRCAS1 cells, or characterize the cellular phenotype when cultivated in serum-free media. Therefore, these limitations will need to be addressed in additional studies to fully elucidate the phenotype of this primary murine HGG cell line. To that end, the diffuse gliomas derived from the RCAS/tv-a system, such as BRCAS1, may harbor distinct cellular properties and serve as a unique *in vitro* modeling platform. In this study, we focused on demonstrating that these distinct BRCAS1 primary cell lines promote the differentiation of naive T lymphocytes into immunosuppressive T regulatory cells, underscoring various potential secreted factors that may play a role in driving the immunosuppressive reprogramming of adaptive cells. Given the relatively small number of murine glioma cell lines that are widely accepted in the field, the BRCAS1 cell line is an experimental tool that may help advance our understanding of tumor-intrinsic properties within the glioma microenvironment and the cellular crosstalk mechanisms that drive immunosuppression.

MATERIALS AND METHODS

Additional methods are detailed in the supplemental information.[26,30]

Mouse model

Gliomagenesis *in vivo* was induced in NTV-a; *Ink4a*$^{+/-}$*Arf*$^{+/-}$; *PTEN*$^{+/fl}$;LSL-Luc model using the RCAS system (Replication-Competent ALV Slice acceptor (RCAS) viral vectors) as previously described.[6,24–26] Pups 0–2 days old were injected with 1 μL DPBS (Gibco) containing the DF1-PDGFB and DF1-CRE[25] cell lines (100,000 cells/kind). The RCAS retrovirus particles, released into the right hemisphere in the brains of pups, targeted and induced transduction of neural stem cells expressing the T-va receptor. Animal studies were approved by the Institutional Animal Care and Use Committee of the Nationwide Children's Hospital of Columbus, Ohio (protocol #AR19-00146).

Figure 2. Analysis of the BRCAS1 transcriptome

(A) Dendrogram of the unsupervised hierarchical clustering analysis of total RNA sequencing of BRCAS1 cells compared with CT2A cells (n = 4). (B) Principal-component analysis highlighting differences between BRCAS1 and CT2A mouse models. (C) Volcano plot for differentially expressed genes among top 10,000 highly expressed genes. (D) Histograms generated using significant and differentially expressed markers (Table S2) associated with neural progenitors and astrocytes. (E) Histograms generated using significant and differentially expressed markers (Table S2) associated with glioma stem cells (GSCs) and glioma cells. (F) Gene set enrichment analysis (GSEA) showing upregulation of genes responsible for cell growth regulation. (G) GSEA showing upregulation of genes responsible for positive regulation of ERK1 and ERK2 cascade. (H) Survival difference between TCGA high-grade glioma samples with expression pattern matching the one observed in murine models. (I) Forest plot illustrating hazard ratios and significance of survival analysis.

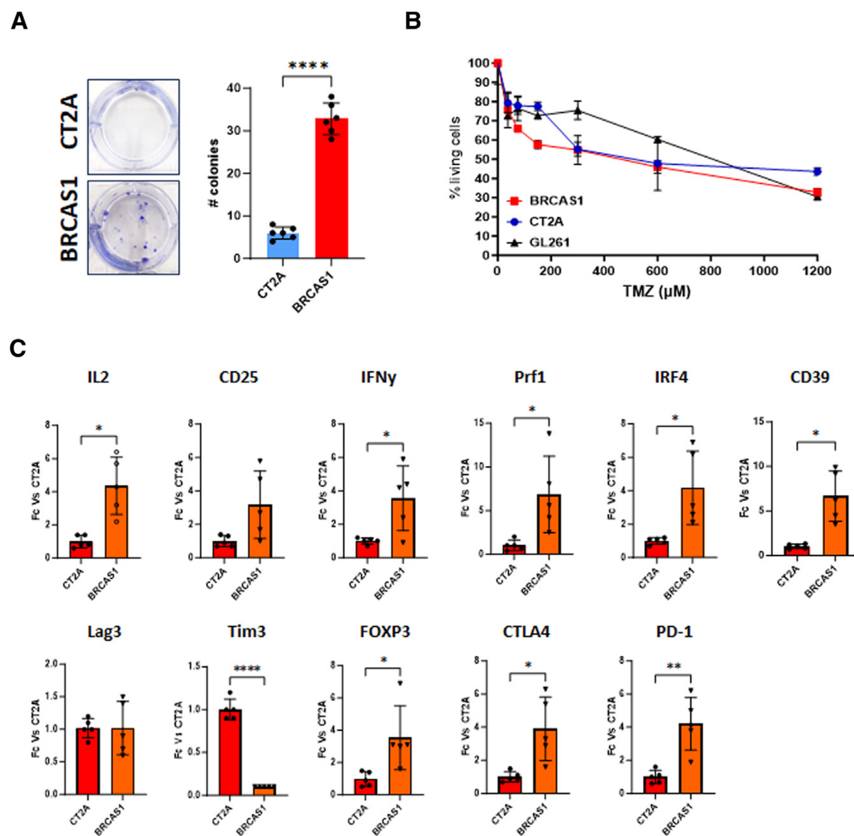

Figure 3. Analysis of the BRACS1 phenotype

(A) BRCAS1 and CT2A cells were seeded in 6-well plates (1,000 cells/well), and the colony formation was assessed after 96 h. The number of colonies/well was measured with an EVOS microscope (n = 6). (B) BRCAS1, GL2611, and CT2A cells were seeded in 96-well plate (15,000 cells/well in triplicate) and treated with different doses of temozolomide. Proliferation was assessed after 72 h by WST-1 staining and plotted as a percentage of proliferating cells under treatment compared with untreated cells (n = 3). (C) Co-culture of primary murine T cells with BRCAS1 or CT2A for 24 h (ratio: 1:1). RT-qPCR was used to measure the expression of genes associated with T cell activation and exhaustion (n = 5). Unpaired two-tailed Student's t test calculated the statistical significance. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Establishment of the novel primary-derived HGG cell line BRCAS1

To establish the primary murine HGG BRCAS1 cell line, HGG animals at week 7 post-induction of gliomagenesis were sacrificed, and the brains were dissected under sterile condition in a BSL2 biohazard hood. Tumor mass was removed from the brain tissue and transferred to a 10 cm Petri dish containing DMEM with 10% FBS (Gibco) and antibiotics/antifungal agents (100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B) (Corning). The tumor was minced using a sterile scalpel and scissored into 1 mm^3 pieces. The single-cell suspension was then further mechanically homogenized (15–20 s) using a Qiagen TissueRuptor and a sterile probe (Qiagen) and then seeded in a T25 tissue culture flask containing DMEM with 10% FBS and antibiotics. The flask was incubated at 37°C for 48 h. The proliferating and adherent cancer cell were washed, resuspended in the same medium, and kept for 3 passages before the antibiotics were removed. Phenotypical and morphological characterization of the cancer cells was performed on cells below 8 passages in culture. To confirm the HGG nature of the novel BRCAS1 murine cell line, CT2A, GL261, U87, and U251 cells were used as control (ATCC).

Quantification and statistical analysis

The results were generated by at least 3 independent observations. To assess the statistical significance ($p < 0.05$), unpaired two-tailed Student's t tests were calculated using GraphPad (Prism). Statistical significance was indicated as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

DATA AND CODE AVAILABILITY

Upon request, the corresponding author will grant access to all data for the scientific community.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

K.C., P.R. and A.C. conceptualized the project. K.C., U.S., and S.R. isolated and established the BRCAS1 cell line. A.C. designed and performed the morphological and phenotypical experiments and wrote the manuscript. A.C. and M.A. interpreted the data. M.A. and A.U. analyzed RNA-seq and human data. All the authors were involved in the final editing, and P.R. approved the final manuscript.

DECLARATION OF INTERESTS

P.R. and K.C. are associate editors of *Molecular Therapy Oncology*.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200861.

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