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CRYβB2 alters cell adhesion to promote invasion in a triple-negative breast cancer cell line

Amr A. Waly[1], London Harper[1], Jodie M. Fleming[1,2] and Lindsey M. Costantini[1*]

Abstract

Objective African American women with breast cancer experience disproportionately poor survival outcomes, primarily due to the high prevalence of the deadliest subtype; triple-negative breast cancer (TNBC). The CRYβB2 gene is upregulated in tumors from African American patients across all breast cancer subtypes, including TNBC, and is associated with worse survival rates. This study investigated the effect of CRYβB2 on the invasion of TNBC cells and the underlying mechanisms contributing to this phenotype.

Results We utilized the SUM159 cells with stable CRYβB2 overexpression in a 3D-culture tumor spheroids model in our investigation. A quantitative 3D invasion assay demonstrated that CRYβB2 overexpression significantly enhanced invasion (median invasion %; SUM159 = 0.14 and SUM159 + CRYβB2 = 0.33). RNA sequencing analysis indicated that CRYβB2 overexpression modulated cell-cell adhesion and extracellular matrix organization pathways, which are critical to invasion of cancer cells. Specifically, CRYβB2 suppressed the expression of key cell-cell adhesion genes known as clustered protocadherins and promoted the expression of PCDH7, a nonclustered protocadherin with known oncogenic roles in various cancers. Notably, the knockout of PCDH7 diminished the invasive capacity induced by CRYβB2 (median invasion %; SUM159 = 0.093, SUM159 + CRYβB2 = 0.184 and SUM159 + CRYβB2/PCDH7$^{-/-}$=0.082). These findings provide a novel link between a previously identified differentially expressed gene, CRYβB2, in driving breast cancer phenotypes by modulating a class of adhesion proteins.

Keywords Cancer disparities, Triple negative breast cancer, CRYβB2, Invasion, Protocadherins

Introduction

Breast cancer (BC) is the most prevalent type of cancer among women; however, African American (AA) BC patients experience higher mortality rates compared to their European American (EA) counterparts. Despite advancements in therapeutic options and improved survival rates for both races, this survival disparity persists [1, 2]. In addition to socioeconomic factors, the higher incidence of the most lethal BC subtype—triple-negative breast cancer (TNBC)—among premenopausal AA patients significantly contributes to these varied survival outcomes [3, 4]. Tumors of this subtype are characterized

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*Correspondence:
Lindsey M. Costantini
lmcostantini@nccu.edu
[1]Biological and Biomedical Sciences Department, University of North Carolina Central University, Durham, NC 27707, USA
[2]Present address: National Institutes of Health, Bethesda, MD 20892, USA

by the highest rates of growth and metastasis, and patients diagnosed with TNBC face a limited set of effective targeted therapeutic options [5, 6].

In this context, β-crystallin B2 (CRYβB2) is a gene that has garnered particular interest due to its elevated expression levels in tumors from AA patients and its association with poor overall survival, not only in BC but also in other malignancies including colorectal, renal cell carcinoma, glioblastoma, and prostate cancer [7–12]. Consequently, CRYβB2 has been linked to disparate survival outcomes among AA individuals with cancer; however, its cellular and molecular mechanisms driving tumor progression remained poorly understood. In our previous study, we utilized TNBC cell models with stable CRYβB2 overexpression and discovered, for the first time, that CRYβB2 significantly promoted proliferation, invasion, and metastasis [13]. In the present study, we employed 3D cultured spheroids as a model that mimics in vivo characteristics of tumor microenvironments [14] to further investigate CRYβB2 and its role in altering gene expression to drive invasion in TNBC.

Methods

Cell lines and generation of expression models

TNBC SUM159 cell line was obtained from Asterand. Cells were authenticated using short tandem repeat (STR) profiling and tested for mycoplasma and culture medium for this cell line was prepared according to supplier's instructions. Procedures to generate knock-in models were previously described [13]. More information on culturing conditions and PCDH7 knockout generation are detailed in the 'supplemental methods'.

Tumor spheroids 3D cultures

Tumor spheroids were grown on Matrigel® (Corning) according to manufacturer's instructions. To retrieve spheroids from Matrigel® for subsequent analyses, Cell Recovery Solution (Corning) was used according to manufacturer's instructions. For spheroid invasion assay, grown spheres were imaged at day 9 at 10x magnification using Nikon Eclipse Ti2 Inverted Microscope, sCMOS pco.edge camera and Nikon NIS Elements software (Nikon). The invasive properties of single spheroids were quantified using ImageJ software [15]. More details on spheroid culture, image analysis, extraction and downstream processing are detailed in the 'supplemental methods'.

Transcriptome RNA sequencing and pathway analyses

Total RNA from tumor spheroids was then extracted using the RNeasy Plus kit (Qiagen) according to manufacturer's instructions. RNA concentrations were measured using nanodrop and RNA quality (RNA integrity Number ≥ 7) was validated using the Bioanalyzer 2100 system (Agilent). Libraries were made using the Illumina Truseq Ribo-Zero Gold protocol and sequenced at 40 million reads per sample on an Illumina HiSeq4000 with paired end 100 bp reads. Identification of differentially expressed genes (DEGs) and pathway analyses were done using Dr. Tom software (BGI Genomics).

RNA extraction and quantitative real-time PCR (RT-qPCR)

For mRNA quantification, High-Capacity cDNA Reverse Transcription Kit (Thermofisher) was used to prepare cDNA from RNA samples according to manufacturer's instructions. The SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used to prepare qPCR assays which were loaded to the QuantStudio™ 3 system (Applied Biosystems) for thermal cycling and signal detection. Primer sequences are listed in Supplementary Table S1.

Western blotting

MPER buffer (Thermofisher) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermofisher) was used to prepare lysates. Protein concentration of whole cell lysates was determined by BSA assay using the Coomassie Plus Protein Assay Reagent (Thermofisher). Equal amounts of protein samples were run using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membranes using Trans-Blot Turbo Transfer System (Bio-Rad). Total protein signals were detected using the Revert700 Total Protein stain (Licor) following manufacturer's instructions. Information about antibodies, dilutions, incubation conditions and imaging are detailed in the 'supplemental methods'.

Cell-flipping assay

The schematic in Fig. 2D summarizes the broad steps for the cell-flipping assay [16]. More information on cell counts, fixation, staining, imaging and data analysis are detailed in the 'supplemental methods'.

Results

CRYβB2 overexpression induced invasion of 3D cultured TNBC spheroids

In our previous study, we reported that CRYβB2 induced a notable invasive phenotype in 3D cultured SUM159 spheroids on Matrigel [13]. This study aims to confirm and quantitatively advance our earlier findings. Initially, 3D spheroids were collected and the overexpression of the CRYβB2 protein in SUM159 spheroids (SUM159 + C) was confirmed using western blot analysis (Fig. 1A). Prior to collection, spheroids were imaged (Fig. 1B) for subsequent invasion analysis. The detection of core and invaded areas, viewed as spindle-like protrusions away from the spheroid's core, allowed for the calculation of invasion for each spheroid. The overall results

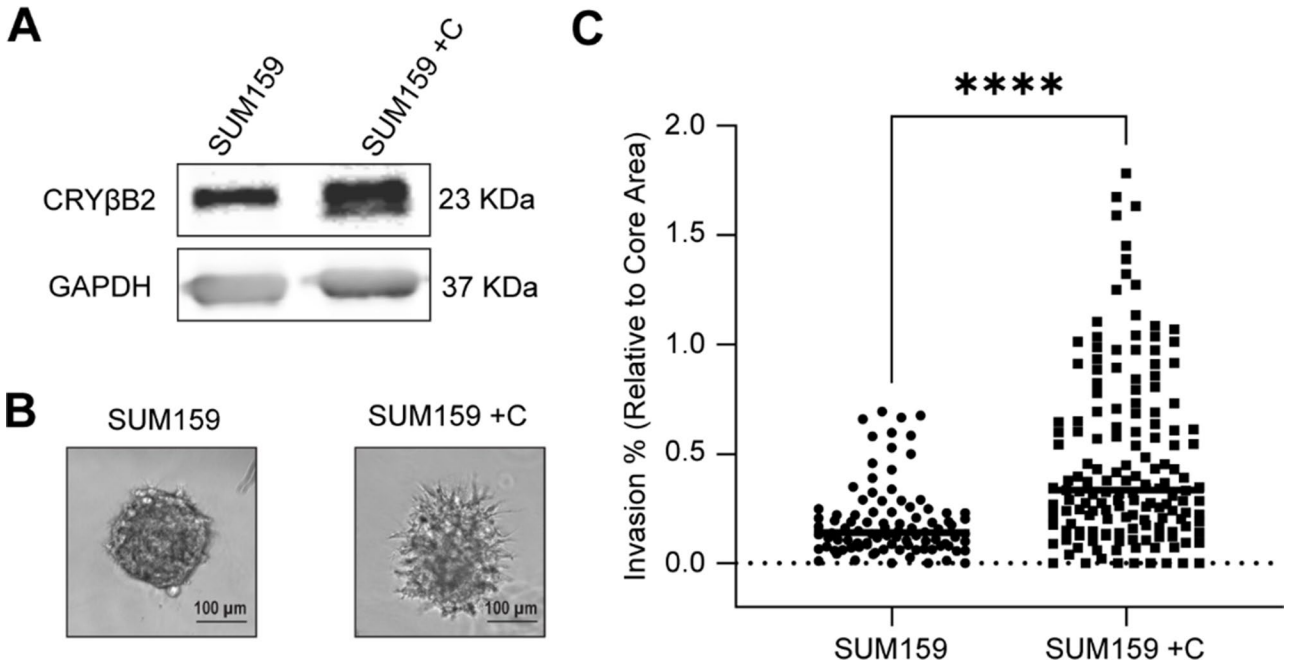

Fig. 1 CRYβB2 overexpression induced invasion in SUM159 spheroids. **(A)** Western blot analysis confirming the overexpression of CRYβB2 in SUM159 3D cultured spheroids (SUM159 + C). Each representative blot is cropped to show an $n = 1$ for each cell line from their respective full-length blots. Full length unmodified blots can be found in the 'Supplementary Information' file. **(B)** Representative images of tumor spheroids grown on Matrigel from SUM159 and SUM159 + C cells. **(C)** Quantification of invasion induced by CRYβB2 in SUM159 ($n = 94$, median = 0.14) and SUM159 + C ($n = 156$, median = 0.33) spheroids. Each dot on the graph indicates quantification of one spheroid. Scale bar = 100 microns. Statistical significance was determined using the Mann-Whitney U test. For all panels, +C refers to cells with CRYβB2 overexpression. ****$p < 0.0001$

demonstrated that CRYβB2 overexpression significantly increased invasion in spheroids derived from SUM159 cells (Fig. 1C and Fig. S1).

CRYβB2 suppressed the expression of protocadherins (PCDHs)

To investigate the impact of CRYβB2 overexpression on gene expression, RNA extracted from SUM159 spheroids was subjected to RNA-seq transcriptome analysis. The findings indicated that CRYβB2 overexpression led to differential expression of 1,973 genes (Table S2). Further examination of these differentially expressed genes (DEGs) through pathway enrichment analysis revealed that 'cell adhesion' was the most significantly affected process by CRYβB2 (Fig. 2A and Table S3), with PCDHs constituting a major group of adhesion-related genes influenced by CRYβB2 (Table S4). Specifically, CRYβB2 overexpression resulted in a general downregulation of clustered PCDH (cPCDH) genes, alongside an upregulation of the nonclustered PCDH (ncPCDH) gene PCDH7 (Fig. 2B). RT-qPCR analysis validated the downregulation of alpha and gamma PCDH (αPCDHs and γPCDHs) gene clusters by CRYβB2 in 3D spheroids and 2D monolayers (Fig. 2C and Fig. S2A). A cell-flipping assay confirmed that CRYβB2 overexpression significantly reduced cell-cell adhesion in SUM159 cells (Fig. 2D). Analysis of The Cancer Genome Atlas (TCGA_BRCA) patient data demonstrated an overall downregulation of cPCDHs in TNBC and highlighted the impact of several PCDH genes on overall survival in these patients (Fig. S2B and Table S5).

Knockout of PCDH7 attenuated CRYβB2-iduced invasion

PCDH7 is a ncPCDH gene that has been previously demonstrated to enhance migration, invasion, and metastasis in TNBC cell lines [17, 18]. RT-qPCR analysis indicated that CRYβB2 overexpression led to a significant upregulation of PCDH7 mRNA in SUM159 + C 3D spheroids (Fig. 3A), and to a lesser extent in 2D monolayers (Fig. S3A). Western blot analysis confirmed the upregulation of PCDH7 protein levels in SUM159 + C 3D spheroids (Fig. 3B). To investigate the role of PCDH7 in CRYβB2-mediated invasion, we performed a knockout (KO) of PCDH7 in SUM159 + C cells and confirmed the absence of PCDH7 protein in both SUM159 + C 2D cells and 3D spheroids (Fig. 3C). Altogether, the KO of PCDH7 resulted in a reduction in the CRYβB2 protein (Fig. 3C), suggesting a potential regulation and/or interaction between both proteins. Of note, in the SUM159 + C 3D spheroids, PCDH7 protein showed a light band at the expected size (116 KDa) and a more prominent band at ~140 KDa suggesting possible post-translational

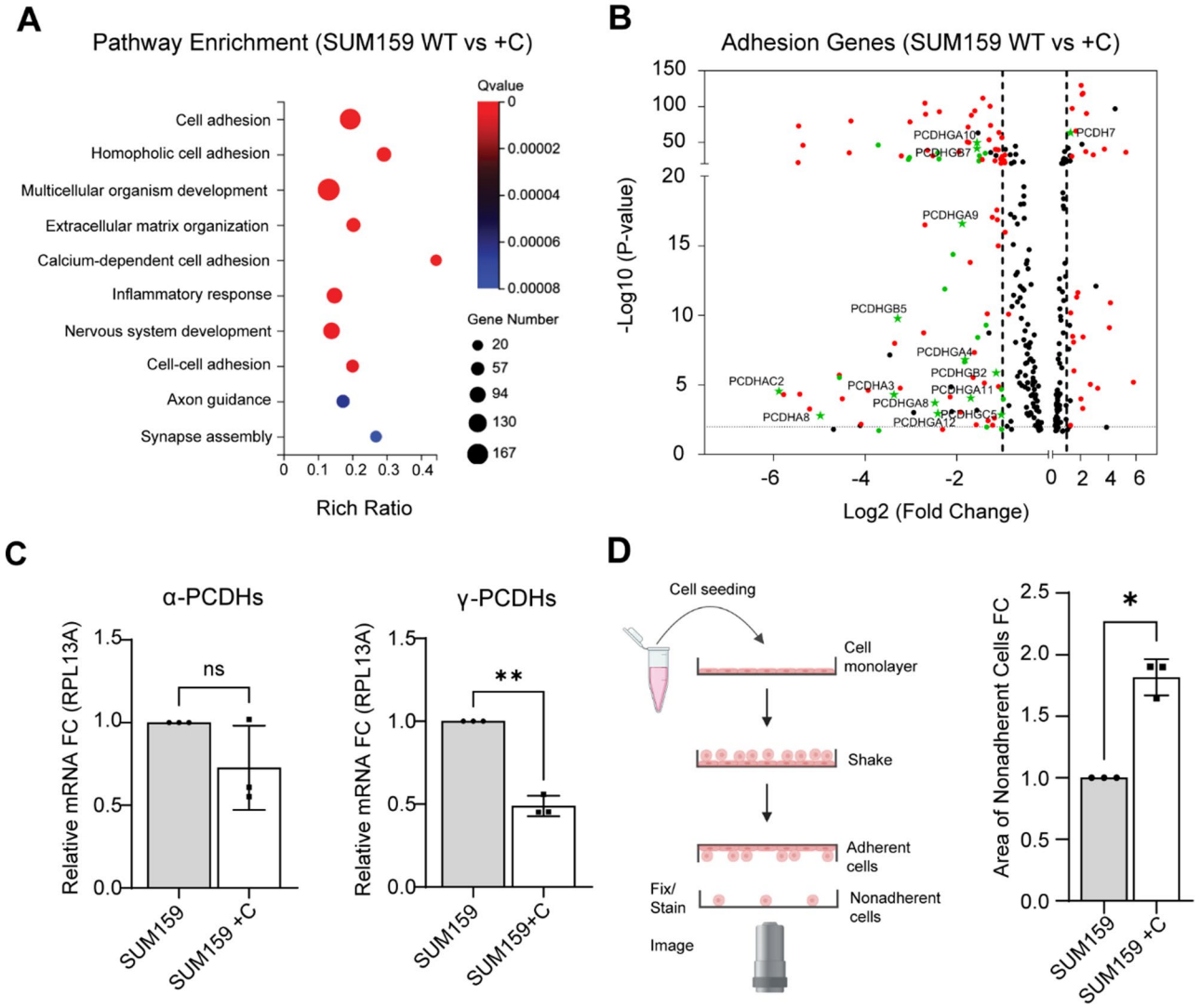

Fig. 2 CRYβB2 suppressed the expression of protocadherins (PCDHs). **(A)** Enrichment analysis plot of the most significantly impacted pathways by CRYβB2 in SUM159 spheroids. Stratification of genes was done according to the Gene Ontology pathways (GO_P) where dot size indicated the number of genes in each pathway, and dot color indicates significance of Q-value. Pathways are ranked based on both number of regulated genes (indicated by sphere size) and Q-value (indicated by sphere color). **(B)** Volcano plot of adhesion genes regulated by CRYβB2 as shown by RNA-seq analysis (black = non-DEGs, red = DEGs, and green = PCDH genes and green stars = labelled PCDH genes). **(C)** RT-qPCR using pan-α-PCDH and pan-γ-PCDH primers to quantitate the effect of CRYβB2 on α-PCDH and γ-PCDH genes in SUM159 3D spheroids. **(D)** Left: a schematic showing the stepwise procedures for the cell flipping assay used to measure the effect of CRYβB2 on cell-cell adhesion; Right: a quantification of the area occupied by nonadherent cells which is indicative of lower cell-cell adhesion. Statistical significance was determined using Student T test. For all panels, +C refers to cells with CRYβB2 overexpression, ns = nonsignificant, $*p < 0.05$, $**p < 0.01$

modifications (PTMs), degradation or variant expression of PCDH7 protein. Subsequently, 3D invasion assay showed that the KO of PCDH7 diminished the invasive capabilities of SUM159 + C 3D spheroids to levels comparable to wild-type spheroids (Fig. 3D and Fig. S3B).

Discussion

In this study, we used the physiologically relevant 3D culture system to further validate and quantitate the effect of CRYβB2 on invasion and investigate how overexpression of CRYβB2 reprograms gene expression to increase the invasive capacity of TNBC cells.

The CRYβB2 protein is predominantly associated with the structural integrity and functionality of the ocular lens. Within the lens, CRYβB2 is instrumental in preserving lens transparency and refractive characteristics [19]. Similar to the ethnic disparities observed in BC, polymorphisms impacting CRYβB2's protein structure induces ocular disease (e.g., cataracts) disparities in certain ethnicities [20, 21]. Nevertheless, CRYβB2 has functional relevance beyond the eye lens by aiding in

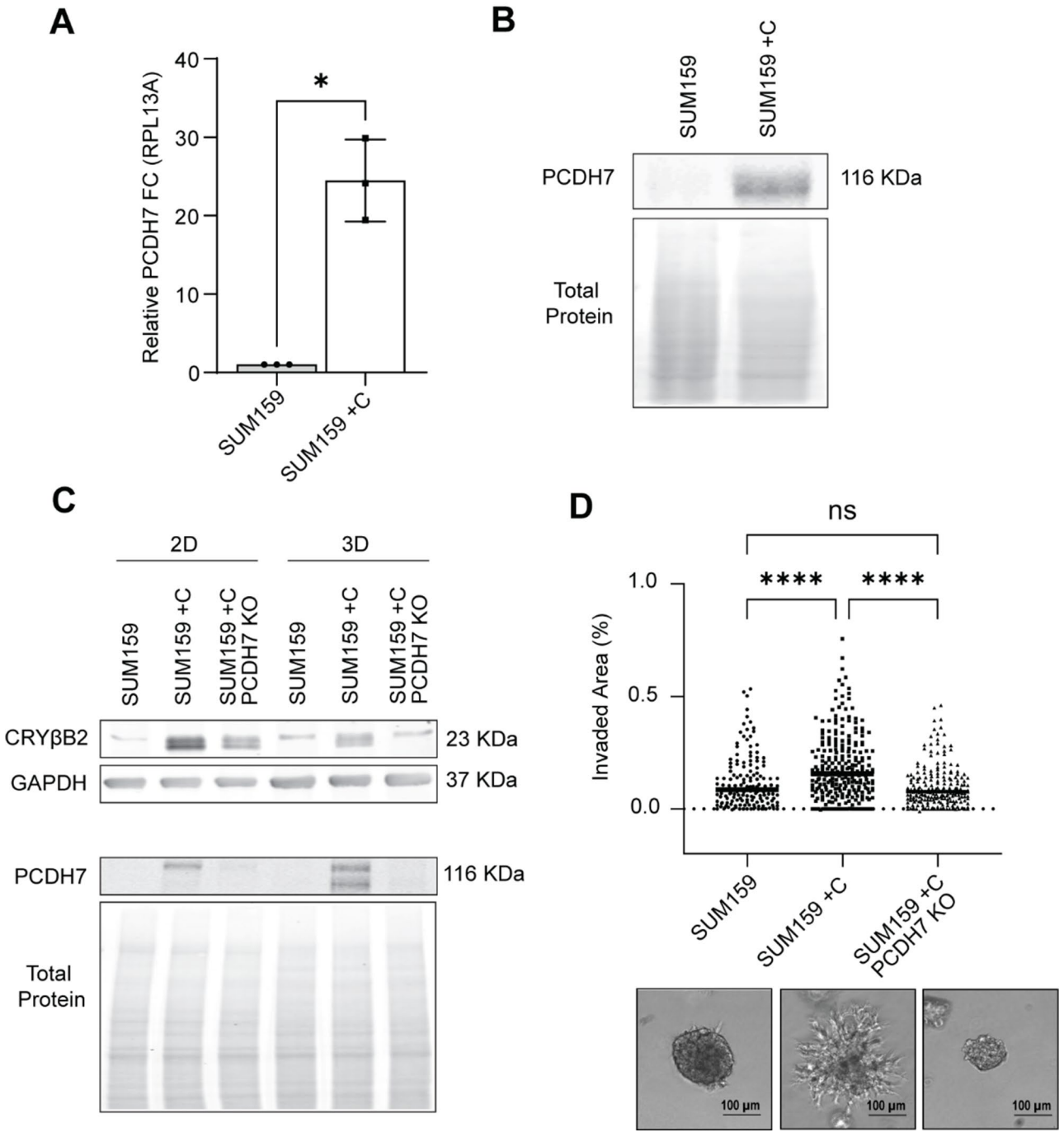

Fig. 3 Knockout of PCDH7 attenuated CRYβB2-iduced invasion. **A.** RT-qPCR analysis of the effect of CRYβB2 on the expression of PCDH7 in the SUM159 3D spheroids. Statistical significance was determined using Student T test. **B.** Western blot analysis of the effect of CRYβB2 on the expression of PCDH7 in the SUM159 spheroids. Each representative blot is cropped to show an $n = 1$ for each cell line from their respective full-length blots. Full length unmodified blots can be found in the 'Supplementary Information' file. **C.** Western blot analysis to confirm the successful knockout of PCDH7 in SUM159 + C spheroids, in addition to the status of CRYβB2 protein expression in each of the conditions both in 2D and 3D culture conditions. **D**. Quantification of invasion induced by CRYβB2 in SUM159 ($n = 150$, median = 0.093), SUM159 + C ($n = 259$, median = 0.184) and SUM159 + C/PCDH7 KO ($n = 214$, median = 0.082) spheroids. Below the graph are representative images for each condition. Scale bar = 100 microns. Each dot on the graph indicates quantification of one spheroid. Statistical significance was determined using the Kruskal–Wallis test. For all panels, +C refers to cells with CRYβB2 overexpression. ns = nonsignificant , *$p < 0.05$ and **** $p < 0.0001$

regenerating the optic nerve, inducing optimal neuronal signaling and branching and maintaining cellular homeostasis in male and female reproductive organs [22–28]. CRYβB2 emerged as a potential culprit for BC racial disparities due to its upregulation in AA patients across all BC subtypes [29, 30], and multiple other cancers [8, 10, 11] and its association with survival outcomes [31]. Until recently, the role of CRYβB2 in cancer development and its mechanism(s) of action were obscure. We and others studied CRYβB2 in TNBC cells and showed that it promoted tumor progression by increasing proliferation of cells in vitro as well as increasing tumor volume and metastasis in vivo [13, 32]. Additionally, we previously reported the novel observation that CRYβB2 induced a distinctive invasive phenotype when cells were cultured in 3D [13].

TNBC has the highest metastatic rate and, thus, highest mortality compared to all other BC subtypes [33]. Notably, premenopausal AA women have a significantly higher incidence of TNBC compared to other races, which may contribute to their disparate survival outcomes [34]. But even among TNBC patients, incidence rate of distant metastasis among AAs is higher than in EA counterparts [35]. AAs have worse survival compared to EA patients after controlling for socioeconomic factors and delay of treatment, suggesting that biological factors play a role in this survival disparity [3, 36]. In this context, we show that CRYβB2 significantly increased invasion in the 3D spheroids derived from SUM159 cells (Fig. 1B-C). Invasion represents a critical early step in the process of metastasis involving changes in gene expression, signaling and tumor microenvironment. Furthermore, CRYβB2 drives TNBC proliferation [13, 32], which likely leads to higher growth capacity at metastatic sites to form secondary tumors. These finding along with previous reports showing higher CRYβB2 in AA tumors and linking it to enhanced metastasis [7, 32] could, therefore, explain a role of CRYβB2 in TNBC disparities.

Our next aim was to understand the changes in gene expression that are induced by CRYβB2 to drive invasion. It is well-established that the processes of invasion and metastasis are orchestrated by a complex network of genes [37], therefore, it was anticipated that CRYβB2 modulated the expression of a wide array of genes, including growth factors, adhesion genes and inflammatory markers among others, as our transcriptome analysis confirmed (Table S2). Pathway analysis revealed 'cell adhesion' as the most significantly altered process by CRYβB2 in addition to others such as 'extracellular matrix (ECM) organization' (Fig. 2A and Table S3). These pathways are crucial for tumor progression and provide a mechanistic explanation to the observed effect of CRYβB2 on invasion. The invasive behavior of cancer cells is driven by changes in cell adhesion, increased motility, and the production of enzymes like matrix metalloproteinases (MMPs), which degrade the ECM to provide open channels for the invading cells [37]. Of note, our RNA-seq analysis shows several MMPs to be upregulated in response to CRYβB2 overexpression (Table S6), including MMP1, a well-studied collagenase whose upregulation is observed in multiple solid tumors to induce metastasis and tumor progression [38]. Of note, our data showed that CRYβB2 regulated genes involved in 'nervous system development' (Fig. 2A). The CRYβB2 transcript is detectable during postnatal development and throughout adolescence in the olfactory bulb, hippocampus, cerebral cortex, and cerebellum [39]. Functional studies on mouse models demonstrated that the release of CRYβB2 via exosomes facilitated neuronal repair, axonal elongation and dendritic growth through autocrine and paracrine pathways by inducing proteins such as CNTF and TMSB4X [22, 26, 40]. These neuronal processes are driven by invasion and proliferation which are likely to be conserved functions of CRYβB2 between neurons and cancer cells. Furthermore, the neurotrophic functions of CRYβB2 may partially explain its oncogenic roles, as high intratumoral nerve density correlates with increased metastatic rates and poor prognosis across various solid tumors [41]. Whether CRYβB2 can work in a similar fashion to induce invasion of neighboring cells, modulate immune cells or condition distant organs for metastatic spread is to be addressed in future studies.

One of the key hallmarks of cancer is the activation of invasion and metastasis, which heavily relies on altered cell-cell adhesion enabling cancer cells to detach from the primary tumor, invade surrounding tissues, and metastasize to distant sites [37]. One mode of cell-cell interaction is through adhesive contact mediated by cadherin proteins [42]. The ~70 PCDH genes constitute the largest group within the broader cadherin superfamily of cell adhesion molecules, which also includes the canonical classical cadherins (such as N-cadherin and E-cadherin). cPCDHs are organized in gene clusters (alpha, beta, and gamma) on a single chromosome, allowing for complex combinatorial expression [43–46]. The unique genomic organization of cPCDHs and the strong involvement of epigenetic mechanisms in their regulation make them particularly subject to hypermethylation which has been shown to cause collective suppression of cPCDHs expression in breast, colorectal and Wilm's tumors [47–49]. Similarly, our RNA-seq analysis revealed a broad suppression of cPCDHs expression in the CRYβB2-overexpressing 3D spheroids (Fig. 2B-C). Notable examples of the suppressed cPCDHs include PCDHA3, PCDHGA5, PCDHGB4, PCDHGB5 and PCDHGB6 whose downregulation is shown to be associated with lower overall survival in TNBC patients in the TCGA cohort (Table S5). Our findings align with

previous studies that had shown PCDHA3 to inhibit proliferation, invasion and migration in lung cancer by suppressing several EMT markers such as N-cadherin, fibronectin and vimentin [50]. Furthermore, the overall downregulation of the γPCDHs in lung cancer has been linked to EMT, migration and invasion [51]. The link between cPCDHs and EMT complements previous findings by us and others showing the stimulatory effect of CRYβB2 on EMT. For example, we previously showed that CRYβB2 overexpression suppressed the EMT key marker E-cadherin leading to higher metastasis to the liver [13]. Likewise, another study using premalignant TNBC cells showed that CRYβB2 activated the PI3K/AKT signaling to induce the expression of the EMT transcription factors ZEB1 and Snail, resulting in increased lung and bone metastases [32]. Whether the downregulation of cPCDHs in the CRYβB2-overexpressing spheroids is part of a broader EMT mechanism in TNBC needs further investigation.

Our results also show that CRYβB2 induced the expression of the PCDH7 protein (Fig. 3A-B). PCDH7 belongs to the ncPCDHs which, unlike cPCDHs, are scattered throughout the genome and are more structurally diverse, often functioning in broader contexts of cell adhesion and signaling outside of neurons [52]. PCDH7 is frequently overexpressed in breast, lung and prostate cancers [17, 53, 54]. In TNBC, PCDH7 was reported to promote brain and bone metastases [17, 18]. While the mechanism of action of PCDH7 in BC is unknown, PCDH7 is known to synergize with KRAS mutations to activate MAPK signaling in lung cancer [53, 55]. In colon cancer, PCDH7 has been found to activate ERK/cFOS signaling to induce tumor proliferation and metastasis to the liver [56]. Our results indicated that the PCDH7 protein may be subject to PTMs given our observation of a prominent larger band in the cells overexpressing CRYβB2. According to GeneCards, PCDH7 has seven glycosylation sites. Cadherin family proteins are known to undergo glycosylation which affects their stability, activity and cell adhesion properties. For example, glycosylation of E-cadherin was found to inhibit its cell-cell adhesion function and alter cytoskeletal organization [57]. Whether the glycosylation of PCDH7 has functional consequences on invasion and metastasis is yet to be studied. Alternatively, the two bands of PCDH7 protein may be the result of different isoforms, as PCDH7, according to the ensemble database, has multiple protein variants ranging in size from 13 to 138 KDa. Our finding that the KO of PCDH7 diminished the invasion of the SUM159 + C spheroids (Fig. 3D and Fig. S3B) confirmed its functional impact on invasion and metastatic spread. However, future work is warranted to delineate the precise mechanism(s) of action of PCDH7 in our CRYβB2-overexpressing model and in TNBC overall.

Conclusion

We used a biologically relevant 3D culture system to quantitatively confirm that CRYβB2 promoted invasion in a TNBC cell line. Our data provides a novel mechanistic understanding in which CRYβB2 reprograms gene expression to suppress key adhesion genes (cPCDHs) to facilitate the loss of cell-cell adhesion which is crucial for initiation of invasion and metastasis. We further provided a direct link between PCDH7, a known oncogene, and the invasive capacity caused by CRYβB2. CRYβB2 may facilitate the identification of individuals at elevated risk for more aggressive forms of the disease and, therefore, contribute to mitigating the survival disparities observed between AA and EA TNBC patients.

Limitations

Despite the advantages of 3D cultures, disadvantages include high complexity and, in some cases, weak reproducibility [14]. Accordingly, we sought to confirm some of our gene expression data in 2D monolayers that are known for their higher reproducibility [14]. While the α and γPCDHs did not show a significant difference in their pattern of expression between 2D and 3D systems (Fig. 2C and S2A), the expression of PCDH7 showed a significantly higher magnitude of increase in SUM159 3D spheroids compared to 2D cells. Accordingly, future studies to investigate the cellular signaling and mechanisms that control the expression of PCDH7 are warranted.

The quantification of cPCDH genes on the protein level proved to be challenging due to the scarcity of commercially available antibodies that work efficiently for western blot or immunofluorescence assays. We observed that KO of PCDH7 resulted in downregulation of the CRYβB2 protein (Fig. 3C) which was unexpected. We were unable, due to lack of resources, to follow up on this observation and investigate the potential for a feedback loop between CRYβB2 and PCDH7.

Abbreviations

AA	African American
αPCDHs	Alpha protocadherins
BC	Breast cancer
cPCDH	Clustered protocadherin
CRYβB2	Crystallin β B2
γPCDHs	Gamma protocadherins
DEG	Differentially expressed genes
EA	European American
EMT	Epithelial-to-mesenchymal transition
MMP	Matrix metalloproteinase
ncPCDH	Nonclustered protocadherin
RT-qPCR	Quantitative real-time PCR
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TCGA	The cancer genome atlas
TNBC	Triple-negative breast cancer

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-025-07090-w.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions
AW: Conceptualization, methodology, validation, investigation, writing – original draft, writing-review & editing, visualization, and funding acquisition. LH: Data analysis. JF: Conceptualization, writing – review & editing, supervision, project administration, and funding acquisition. LC: Conceptualization, writing – review & editing, supervision, project administration, and funding acquisition. The authors read and approved the final manuscript.

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Data availability
The datasets used and/or analyzed during the current study are available in the National Genomics Data center (NGDC) OMIX repository (https://ngdc.cncb.ac.cn/omix/submitList) (ID: OMIX004766). Clinical data is available from the cBioPortal repository (https://www.cbioportal.org/), specifically, the Breast Invasive Carcinoma TCGA, PanCancer Atlas (https://www.cbioportal.org/study/summary?id=brca_tcga_pan_can_atlas_2018).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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