Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Unraveling the Role of PCDH9 in Breast Cancer and Identifying Therapeutic Strategies for PCDH9-Deficient Tumors

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Introduction: Protocadherin 9 (PCDH9), a member of the cadherin superfamily of transmembrane proteins, plays a role in cell adhesion and neural development. Recent studies suggest that PCDH9 may function as a tumor suppressor in certain cancers, though its specific role in breast cancer remains unclear.

Methods: UALCAN database to retrieve information on PCDH9 expression in breast cancer tissues compared with that in normal tissues. The biological effects of PCDH9 in breast cancer cells were analyzed using the DepMap database. Stable knockdown or overexpression of PCDH9 in breast cancer cell lines and subsequently assessed tumor cell proliferation and migration. Synthetic lethal screening was conducted for breast cancer cells with low PCDH9 expression or deficiency.

Results: In this study, we observed significant downregulation of PCDH9 in breast cancer tissues, with its expression negatively correlated with progression-free survival. Further investigations revealed that decreased PCDH9 expression promotes breast cancer cell proliferation and migration, while overexpression of PCDH9 has the opposite effect. Subsequently, we identified the TAS-102, an approved drug for metastatic colorectal cancer, exhibited selective cytotoxicity against breast cancer cells with low PCDH9 expression.

Conclusion and discussion: In summary, our study identified PCDH9 as a tumor suppressor in breast cancer and highlighted TAS-102 as a potential therapeutic option for tumors with low PCDH9 expression or deficiency. The specific interaction between TAS-102 and PCDH9 warrants further exploration, providing deeper insights into its mode of action in treating PCDH9-deficient breast cancer.

Keywords: breast cancer, PCDH9, synthetic lethality, TAS-102

Introduction

Breast cancer is the most common malignant tumor in women, posing a serious threat to their health and lives.[1–3] According to recent epidemiological data, breast cancer has the highest incidence and mortality rate among female malignancies.[2] Breast cancer pathogenesis is closely related to various gene expression abnormalities. Currently, known genes associated with breast cancer include BRCA1 and BRCA2, and mutations or deletions in these genes lead to impaired DNA repair in tumor cells, thereby increasing the risk of breast cancer.[4] Additionally, overexpression of human epidermal growth factor receptor 2 (HER2),[5] estrogen receptor (ER),[6] and progesterone receptor[7] is common in breast cancer. Furthermore, p53 mutations have been observed in breast cancer, disrupting cell apoptosis and DNA repair, thereby promoting tumor progression.[8] Another example of gene alteration in breast cancer is the PIK3CA mutation, which frequently occurs within the PI3K/Akt/mTOR signaling pathway, thus promoting tumor cell proliferation and survival.[9,10] Abnormalities in gene expression play a crucial role in guiding breast cancer treatments. Targeted therapies and individualized treatment strategies can be employed based on specific gene expression profiles. For instance, in HER2-overexpressing breast cancer, HER2 inhibitors (such as trastuzumab) suppress tumor growth and metastasis by inhibiting the HER2-mediated signaling pathway.[11] In hormone receptor-positive breast cancer, endocrine therapy effectively controls disease progression by inhibiting estrogen synthesis or by blocking estrogen receptor functions.[12]

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However, breast cancer is highly individualized, and patient responses to treatment can vary significantly.[13,14] The discovery of new genes associated with tumorigenesis and the development of corresponding therapeutic options offers more effective treatment options.

Cadherin is a calcium-dependent adhesion protein belonging to a large family of cell adhesion molecules. Cadherins can be categorized into three groups: classical, desmosomal, and protocadherins (PCDHs).[15] PCDHs are predominantly expressed in the nervous system and play a crucial role in maintaining brain functions.[16] However, recent studies have revealed that members of the PCDH family may have additional functions, including suppression some of cancer.[17,18] Recent research has highlighted the potential tumor-suppressive role of Protocadherin-9 (PCDH9) in various cancers. In liver cancer, PCDH9 expression levels are generally reduced compared to normal tissues, and its expression is negatively correlated with tumor proliferation, metastasis, and poor prognosis.[19] However, the specific roles and underlying mechanisms of action of PCDH9 in breast cancer remain unclear.

Drug repurposing, also known as drug repositioning or reprofiling, involves identifying new clinical applications for approved or investigational drugs beyond their originally intended uses.[20] This strategy has gained prominence, especially after the Food and Drug Administration (FDA) granted emergency authorization for several repurposed drugs for the treatment of Covid-19.[21] The significance of drug repurposing lies in its ability to accelerate drug development, discover new indications, improve drug utilization efficiency, shorten clinical research timelines, reduce development risks, and promote drug reuse. These repurposed drugs have already undergone clinical validation, and their safety profiles and pharmacokinetic data are well established. In this study, we employed a drug repositioning strategy to specifically screen drugs for potential use in clinical scenarios involving low PCDH9 expression or PCDH9 deficiency in breast cancer. This combination screening not only allows for the targeted identification of effective drugs against PCDH9 but also benefits from the fact that the screened drugs are already approved and available on the market. Consequently, this approach significantly shortens the drug development timeline, facilitating its rapid application in patients with breast cancer with low PCDH9 expression or PCDH9 deficiency.

In summary, we investigated the regulatory role of PCDH9 in breast cancer and evaluated its potential as a drug target. We meticulously analyzed PCDH9 expression patterns, studied its impact on breast cancer cell proliferation and migration, and found that PCDH9 is significantly downregulated in breast cancer. Moreover, it exerts a negative regulatory effect on breast cancer growth and metastasis. Furthermore, we explored the therapeutic potential of TAS-102 by specifically targeting the PCDH9-deficient breast cancer cells. These findings not only establish PCDH9 as a tumor suppressor gene in breast cancer but also identify a novel therapeutic option for breast cancer patients with low or absent PCDH9 expression.

Materials and Methods

Cell Lines, Culture and Reagents

Human breast cancer cell lines MCF-7 and T47D were purchased from the Cell Bank of the Chinese Academy of Sciences. MCF-7 cells were cultured in Eagle's Minimum Essential Medium supplemented with 0.01 mg/mL human recombinant insulin, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. T47D cells were cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All the cell lines were maintained in 5% CO2 at 37 °C. The primary antibodies of PCDH9 (Cat no: 25090-1-AP) and GAPDH (Cat no: 60004-1-Ig) were purchased from Proteintech. TAS-102 was purchased from Topscience (cat. T3658).

Cell Viability Assay

SRB assay was used to evaluate the effects of the drugs on tumor cell proliferation. Briefly, 3000–5000 tumor cells were seeded in 96-plate, after overnight incubation, different drug concentrations were added to the cells, followed by staining with SRB dye and measuring the absorbance at 515 nm. The inhibitory effect of the drug was assessed by plotting drug concentration against the cell proliferation inhibition rate curve (IC_{50}).

Transwell Assay

The transwell assay was performed using a transwell chamber with an 8-micrometer pore. 3000–5000 cells were seeded in the upper chamber along with the indicated concentrations of drugs in serum-free medium, and serum-containing medium containing the indicated drugs was added to each well. After 12 or 20 h of incubation, non-migrated cells in the upper chambers were removed. The cells that migrated to the lower chambers were fixed with 5% paraformaldehyde and stained with 0.2% crystal violet. Images were captured using an inverted microscope (Olympus), and the number of invading cells was quantified manually.

PCDH9 Stable Knockdown and Ectopic Expression

Silencing PCDH9 using short hairpin RNAs (shRNAs), the shRNAs for silencing PCDH9 were as follows, PCDH9-1#: 5′-GCGGTATATGACAACCAATAT-3'; shPCDH9-2#: 5′-CCCAAGTTTACTCATAATCAT-3′; shNC: 5′-CAACAAGATGAAGAGCACC-3'. shRNAs were inserted into the pLKO.1 vector. For ectopic expression of PCDH9, the cDNA of PCDH9 was obtained from GenScript and cloned into the pLVX-puro-expressing vector (Takara). The pLKO.1 vector containing shRNAs or pLVX-puro-PCDH9 was co-transfected into 293T cells along with packaging plasmids (pMD2G and pXPAX2). Lipofectamine 2000 (Thermo Fisher Scientific) was used as transfection reagent. After 72 h of transfection, lentiviruses were harvested. The harvested lentiviruses were used to infect cancer cell lines. To obtain stable PCDH9 knockdown cells, puromycin was added. Only cells expressing short hairpin RNAs survived the puromycin resistance. Finally, the knock-down efficiency was determined by real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting.

Western Blot

Tumor cells were collected and lysed with RIPA buffer (Cat. P0013B), followed by the BCA method to determine the protein concentration. The proteins were separated by SDS-PAGE, and transferred proteins to PVDF membrane. Non-specific binding proteins were blocked with 5% BSA. The primary antibodies were incubated with the membrane overnight and the secondary antibodies were incubated at room temperature for 1h. Visualizing and analyzing Protein imaging was performed using an LI-COR Odyssey infrared imaging system (LI-COR Biotechnology, Lincoln, NE, USA).

Real-Time Quantitative PCR(RT-qPCR)

Total RNA was isolated using TRIzol reagent (Takara, Japan). RNA was used to generate single-stranded cDNA according to the manufacturer's protocol using a cDNA Reverse Transcription Kit (Takara, Japan), followed by polymerase chain reaction (PCR) using SYBR Green Master Mix (Thermo Fisher Scientific). The PCDH9 primers used for RT-qPCR were as follows: F: 5'-CTGCTCTGATTGCCTGTTTAAGG-3'; R: 5'-ACCAGTCTGTAGACAAGGCTG-3.'

Screening Procedure

T47D and MCF-7 PCDH9 shRNA cells were grown in the presence of 2 μg/mL puromycin for two days and plated in 96-well plates (4000 cells/well). For the first-round drug screening, after tumor cells were seeded the next day, they were treated with test compounds (at a concentration of 10 μM) from our internal approved drug library. After 72 h, the SRB assay was performed to determine cell viability. For the second-round screening, T47D and MCF-7 PCDH9 shRNA cells were employed for subsequent tests, and compounds from the library that selectively suppressed the growth of PCDH9-defective cells but had only modest effects on PCDH9-proficient cells were selected.

Statistical Analysis

Experiments were carried out with two or more replicates. Statistical analyses were done by Student's *T* test. P values < 0.05 were considered significant. The differences between control and experimental groups were determined by one-way ANOVA. Since treatment and time course were investigated, two-way ANOVA followed by post hoc test was also applied. All analyses were performed using Microsoft Excel 2010 and GraphPad Prism 7 software.

Results

Down-Expression of PCDH9 in Breast Cancer Tissue

To clarify the potential function of PCDH9 in breast cancer, we first utilized the UALCAN database[22] to retrieve information on PCDH9 expression in breast cancer tissues compared with that in normal tissues. The results indicated that PCDH9 was significantly downregulated in breast cancer tissues compared to normal tissues (Figure 1A). Next, we analyzed the correlation between PCDH9 expression and breast cancer survival rates using GEPIA2.[23] We observed that patients with low PCDH9 expression had longer progression-free survival compared to those with high PCDH9 expression (Figure 1B and C). This suggests that PCDH9 may be involved in breast cancer progression through its tumor-suppressive functions. To further investigate this, we used the DepMap database, which is widely used to study gene-tumor relationships. We examined the biological effects of the PCDH9 knockout in breast cancer cells. Among the 46 human breast cancer cell lines in DepMap, only seven showed inhibited tumor cell growth upon PCDH9 knockout (Figure 1D). Conversely, the knockout of PCDH9 in 39 other breast cancer cell lines promoted tumor cell proliferation (Figure 1D). These findings further support the notion that PCDH9 regulates breast cancer progression by acting as a tumor suppressor. In summary, our study revealed that PCDH9 is significantly downregulated in breast cancer and may be closely associated with progression-free survival. Additionally, DepMap data demonstrated that PCDH9 knockout

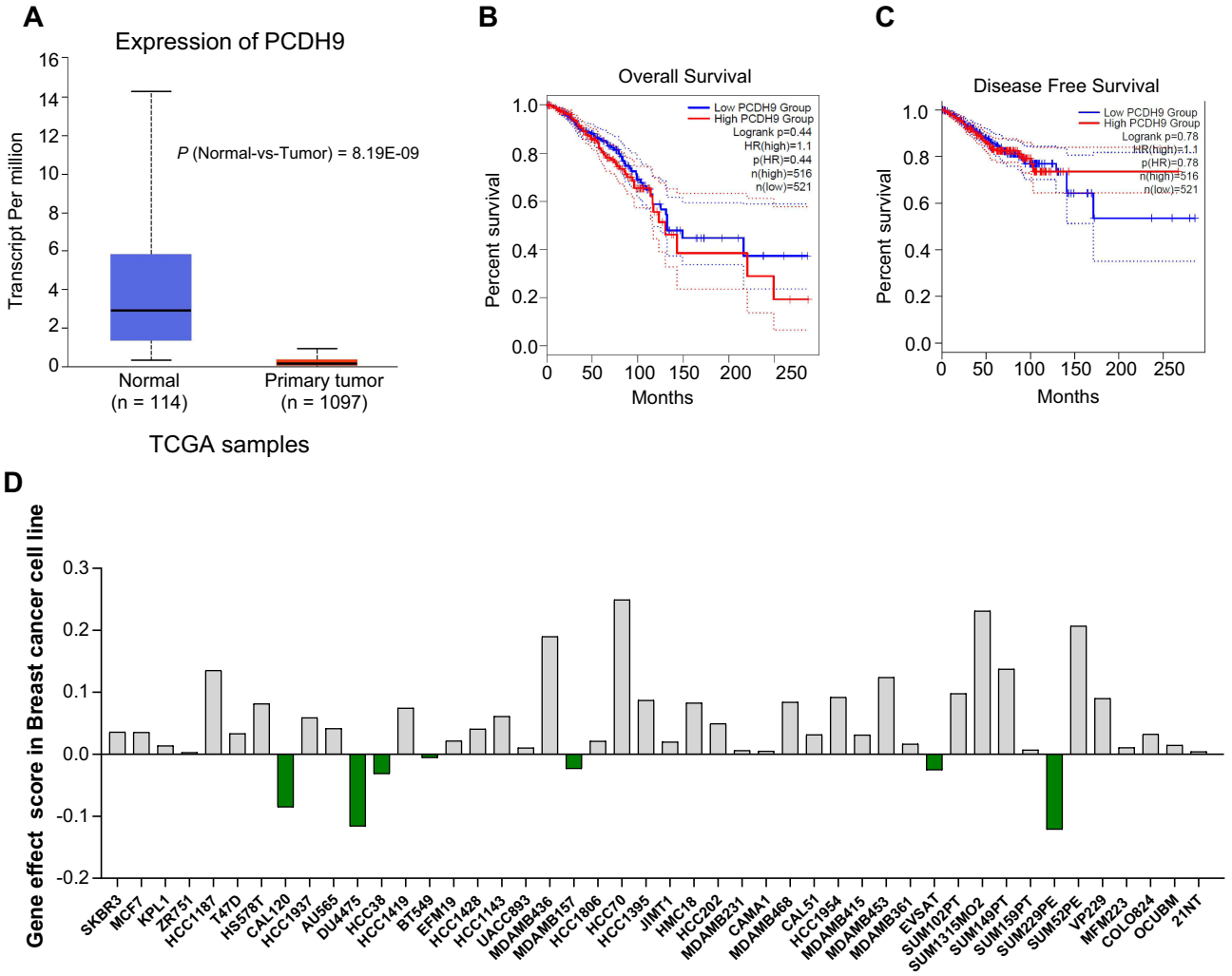

Figure 1 Down-expression of PCDH9 in breast cancer tissue. (**A**) The expression of PCDH9 in breast cancer tissue and normal tissue. The data analyzed by UALCAN database. (**B**) The correlation of PCDH9 expression with breast cancer patient overall survival were analyzed by GEPIA2. (**C**) The correlation of PCDH9 expression with breast cancer patient disease-free survival were analyzed by GEPIA2. (**D**) The dependency of PCDH9 in human breast cancer cell lines was analyzed by DepMap database, the gene effect score was shown.

promoted tumor cell proliferation in most breast cancer cell lines, highlighting PCDH9's role as a tumor suppressor in breast cancer.

Knock-Down PCDH9 Promotes Breast Cancer Cells Growth and Migration

To further investigate whether PCDH9 regulates breast cancer proliferation and migration through its tumor-suppressive functions, we first stably knocked down PCDH9 in two human breast cancer cell lines, MCF-7 and T47D (Figure 2A and B). Subsequently, we assessed the effect of PCDH9 knockdown on the proliferation of breast cancer cells. The results revealed that Stable PCDH9 knockdown significantly enhanced the growth of both MCF-7 and T47D breast cancer cells (Figure 2C and D). Furthermore, through Transwell experiments, we confirmed that stable PCDH9 knockdown promoted the migration of these two breast cancer cell lines (Figure 2E). In summary, our findings validate that stable PCDH9 knockdown significantly promotes both proliferation and migration of breast cancer cells, highlighting PCDH9 as a critical tumor suppressor in breast cancer.

Over-Expression PCDH9 Inhibits Breast Cancer Cell Proliferation and Migration

Upon observing that stable PCDH9 knockdown enhanced breast cancer cell proliferation and migration, we investigated whether overexpression of PCDH9 in breast cancer cells might yield opposite results. To explore this, we stably overexpressed PCDH9 in two human breast cancer cell lines by using lentiviral constructs (Figure 3A–D). Subsequently, we investigated the effect of PCDH9 overexpression on the proliferation and migration of breast cancer cells. Stable PCDH9 overexpression significantly inhibited cell proliferation and migration (Figure 3E–G). This further validates PCDH9's role as a critical tumor suppressor that regulates breast cancer growth and migration. In summary, PCDH9 appears to play a pivotal role in breast cancer pathogenesis by acting as a tumor suppressor, regardless of whether it is downregulated or overexpressed.

TAS-102 Selectively Kills Breast Tumor Cells with PCDH9-Defective

Targeting tumor suppressor genes remains a significant challenge. A notable example is the development of drugs targeting P53 tumor suppressor genes. These drugs include P53 activators and inhibitors of MDM2, which is a ubiquitin ligase that mediates P53 degradation.[24] Several of these inhibitors are currently in clinical research and have shown promising results, demonstrating the feasibility of drug development to target cancer genes.[24] Our results suggest that PCDH9 is a potential tumor suppressor gene in breast cancer. Considering this, we wondered whether we could follow an approach similar to of P53 drug development to investigate potential drugs for breast cancer cells with low PCDH9 expression or loss. To expedite drug development, we used approved drugs for repurposing. The advantage of repurposing existing drugs lies in their established safety profiles as they have already undergone toxicology studies. Our initial screening involved testing compounds from a compound library against T47D breast cancer cells with reduced PCDH9 expression (T47D shPCDH9 1#) (Figure 4A). We identified 37 compounds that exhibited inhibitory effects in these cells. In the second round of screening, we combined these compounds with control cells (T47D shRNA control) and PCDH9-knockdown cells (T47D shPCDH9 1#) (Figure 4A). The goal was to identify compounds selectively toxic to PCDH9-knockdown cells, while sparing control cells. Among these compounds, compound 208 (TAS-102) exhibited the highest selectivity, suggesting that it could selectively kill PCDH9-knockdown cells (Figure 4A). TAS-102, an oral fluoropyrimidine drug, has been approved for the treatment of metastatic colorectal cancer with wild-type RAS.[25] TAS-102 consists of two components: trifluridine (FTD), which directly binds to tumor cell DNA and inhibits its growth and proliferation, and tipiracil hydrochloride (TPI), which protects FTD from degradation in vivo.[26] Further validation revealed that TAS-102 was more sensitive to stable PCDH9-knockdown T47D and MCF-7 cells than to the control cells (Figure 4B and C). This specificity highlights TAS-102's potential of to selectively target breast cancer cells with low or no PCDH9 expression. Additionally, the overexpression of PCDH9 reduced TAS-102 sensitivity, reinforcing its selective action (Figure 4D and E). In summary, we identified TAS-102 as a potential candidate drug that specifically targeted breast cancer cells with low PCDH9 expression or loss.

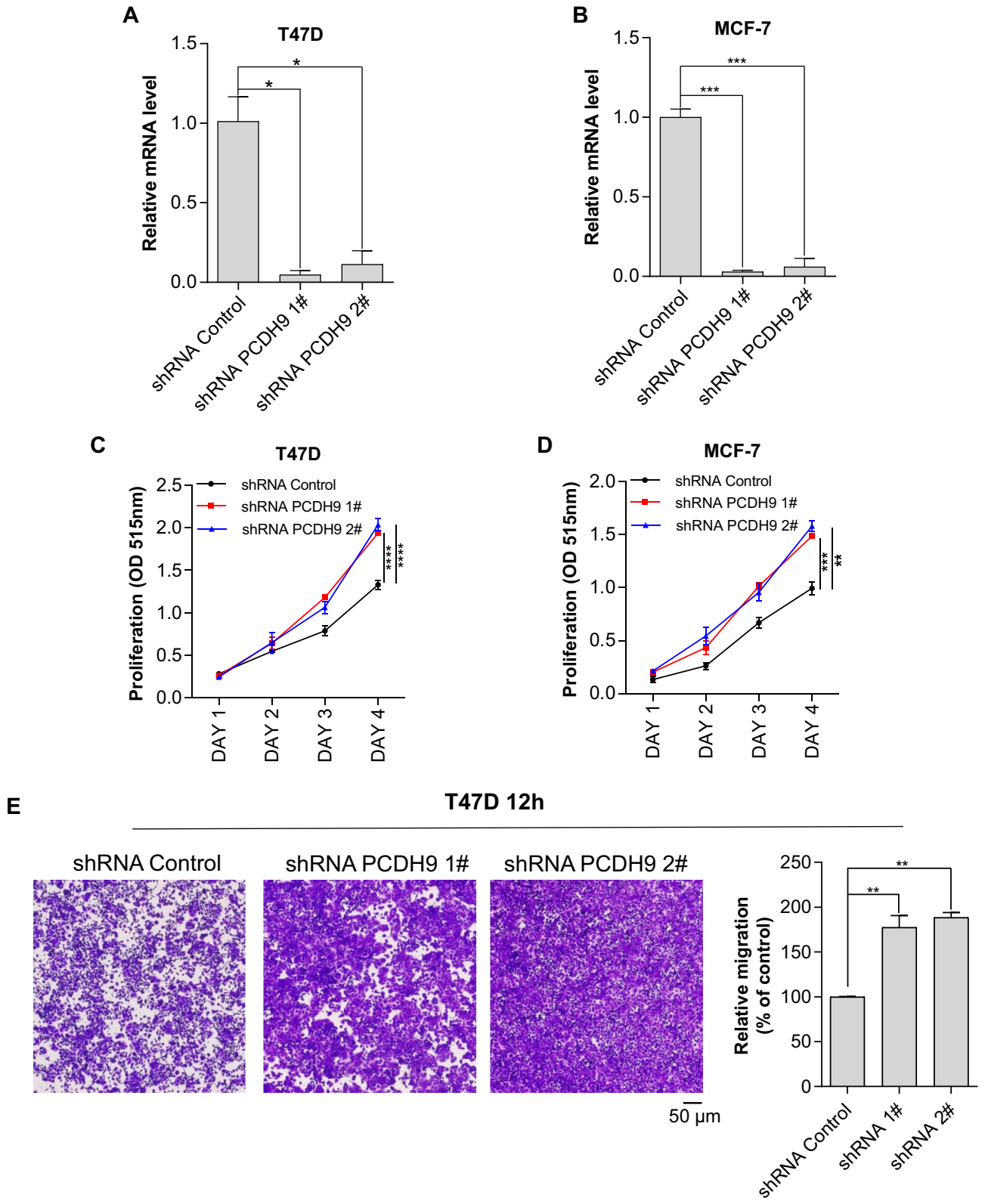

Figure 2 Knock-down PCDH9 promotes breast cancer cells growth and migration. (**A, B**) The knock-down efficiency by shRNA in T47D (**A**) and MCF-7 (**B**) was measured by RT-qPCR. Data were expressed as mean ± s.d.; One-way ANOVA were performed; *P<0.05 (n = 2). (**C, D**) Knock-down PCDH9 promoted T47D (**C**) and MCF-7 (**D**) cells growth. Data were expressed as mean ± s.d.; Two-way ANOVA were performed; **P<0.01, ***P<0.001, ****P < 0.0001 (n = 3) (**E**) Knock-down PCDH9 induced T47D cells migration. Data were expressed as mean ± s.d.; One-way ANOVA followed by Dunnet multiple analysis were performed; **P<0.01 (n = 2). Scale bar, 50 μm.

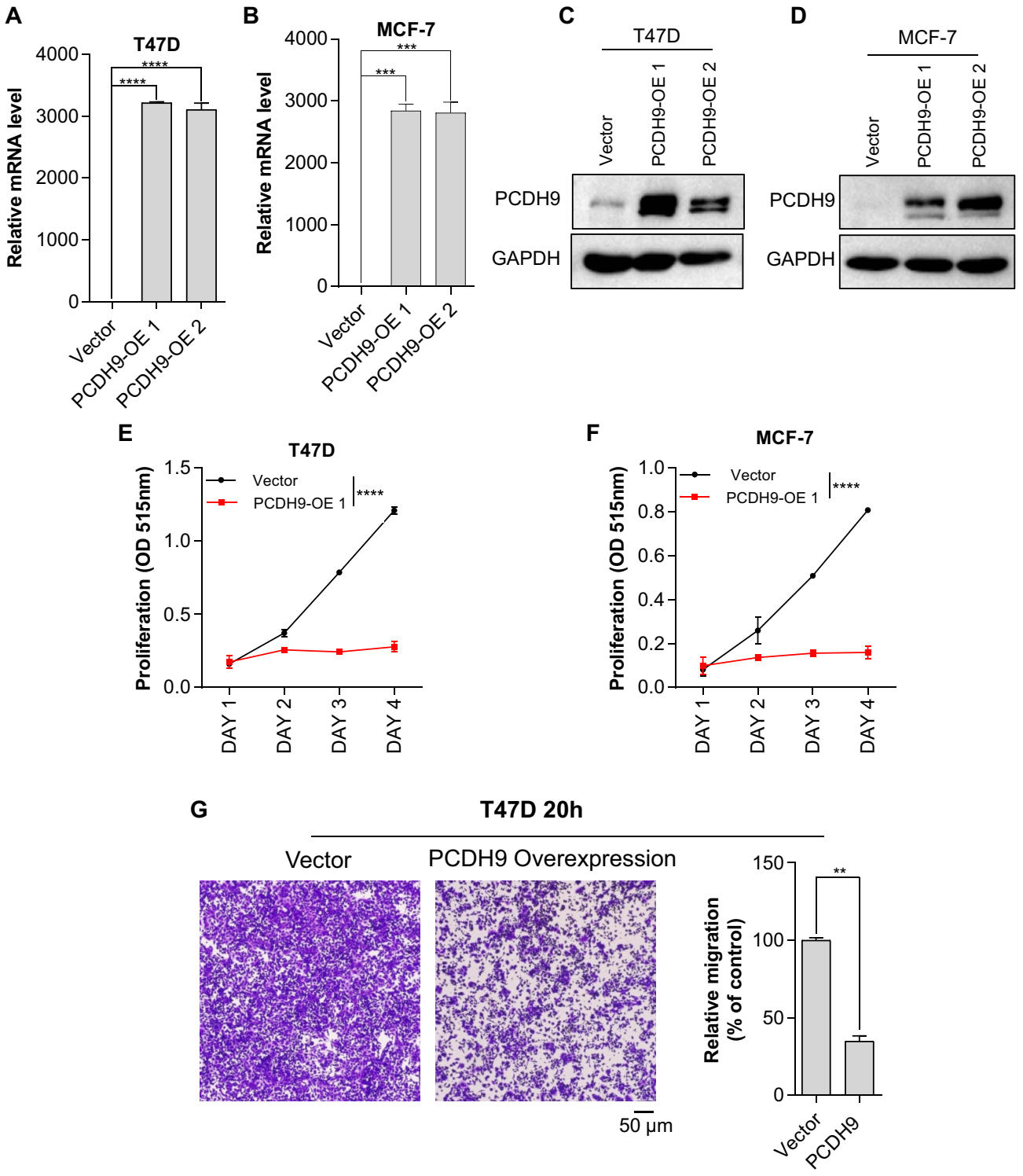

Figure 3 Over-expression PCDH9 inhibits breast cancer cell proliferation and migration. (**A, B**) The ectopic expression of PCDH9 in T47D (**A**) and MCF-7 (**B**) was determined by RT-qPCR. Data were expressed as mean ± s.d.; One-way ANOVA followed by Dunnet multiple analysis were performed; ***P<0.001, ****P < 0.0001 (n = 2). (**C, D**) The ectopic expression of PCDH9 in T47D (**C**) and MCF-7 (**D**) was determined by Western blot. (**E, F**) Over-expression of PCDH9 inhibited T47D (**E**) and MCF-7 (**F**) cells proliferation. Data were expressed as mean ± s.d.; Two-way ANOVA were performed; ****P < 0.0001 (n = 3) (**G**) Over-expression of PCDH9 inhibited T47D cells migration. Data were expressed as mean ± s.d; Student's t-tests were performed; **P<0.01. Scale bar, 50 μm.

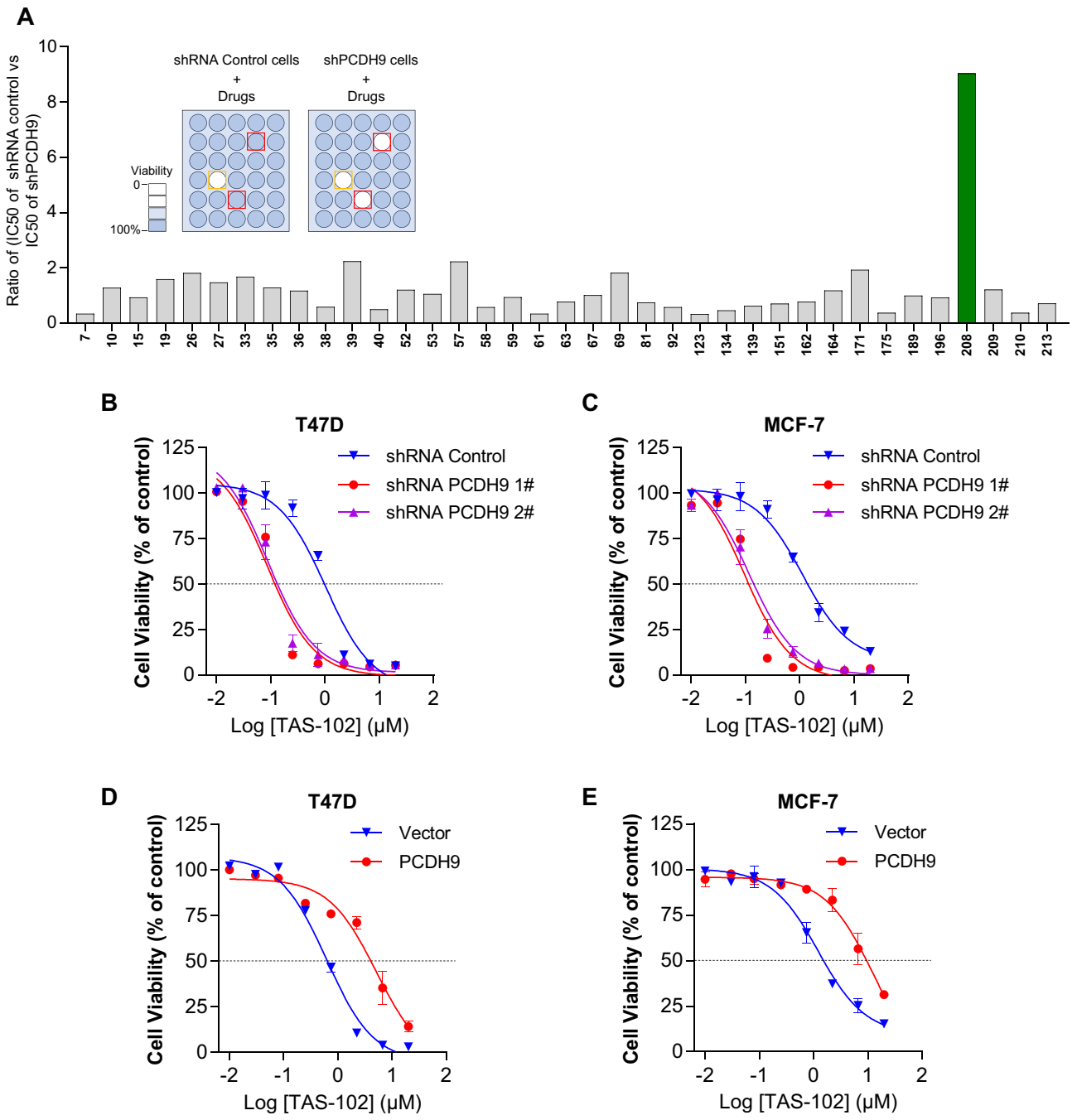

Figure 4 Tas-102 selectively kills breast tumor cells with low PCDH9-defective. (**A**) T47D shRNA control cells and T47D shPCDH9-1# cells were seeded in 96-plate, then the indicated drugs were treated for 72h, the cell viabilities were detected with SRB assay, the ration (IC_{50} of shRNA control vs IC_{50} of shPCDH9 1#) was calculated. The compound 208 was TAS-102. (**B, C**) Knock-down PCDH9 induced selective cytotoxicity of TAS-102 to T47D (**B**) and MCF-7 (**C**) cells. (**D, E**) Over-expression of PCDH-9 reduced sensitivity of TAS-102 to T47D (**D**) and MCF-7 (**E**) cells.

Discussion

This study's results have several important implications. First, we identified PCDH9 as being downregulated in breast cancer and demonstrated its role as a tumor suppressor in breast cancer. Second, our functional research revealed that PCDH9 negatively regulated breast cancer cell proliferation and migration. Finally, using a compound combination screening approach, we identified that TAS-102 has potential for treating PCDH9-deficient breast cancer. This compound

may exert its effects through synergistic cytotoxicity, targeting both PCDH9-low expression and PCDH9-deficient patients with breast cancer.

However, our study warrants further investigation of the underlying mechanisms. PCDH9, a crucial cell adhesion molecule, likely plays a key regulatory role in breast cancer development owing to its low expression. However, the specific regulatory mechanisms of PCDH9 require further investigation. Our findings indicated that PCDH9 inhibits the migration and invasion of breast cancer cells. Previous studies have suggested that PCDH9 deficiency may lead to tumor cells losing their normal cell adhesion capabilities, thereby promoting tumor metastasis and spread.[27] Further research is needed to explore the molecular mechanisms by which PCDH9 regulates breast cancer metastasis, including its interaction with cell adhesion-related signaling pathways. Additionally, PCDH9 expression negatively correlated with breast cancer cell proliferation. Previous studies have indicated that low PCDH9 expression may disrupt cell cycle regulation, resulting in abnormal tumor cell proliferation.[19] Therefore, investigating PCDH9's role in cell cycle regulation mechanisms, such as its impact on cell cycle-related genes, will help uncover the molecular basis of its inhibitory effect on breast tumor growth. Lastly, TAS-102 (Trifluridine/tipiracil) is an orally administered fluoropyrimidine drug that is currently approved for the treatment of metastatic colon cancer. Its primary mechanism involves the inhibition of thymidine phosphorylase, which increases cancer cell exposure to trifluridine and is then incorporated into DNA, leading to tumor cell death.[26] In breast cancer research, TAS-102 has been investigated in several prospective preclinical and clinical studies as a monotherapy or in combination therapies. For instance, in p53-mutant breast cancer, there is a significant dysregulation of base excision repair (BER), leading to the accumulation of DNA damage following treatment with nucleoside analogs like TAS-102. Based on this, the study explored combination therapy for p53-mutant breast cancer, specifically TAS-102 combined with PARP inhibitors (PARPi) for TP53-mutant breast cancer. The results showed that this combination therapy was more effective than using either drug alone, demonstrating the potential of TAS-102 combined with PARP inhibitors for future treatment of TP53-mutant breast cancer.[28] Additionally, in another prospective clinical study, stable disease was observed in nine patients with rectal cancer, small cell lung cancer, breast cancer, thymic cancer, duodenal cancer, and prostate cancer during repeated use of TAS-102.[29] Of note, further research and exploration of TAS-102 in breast cancer, especially in precision types such as PCDH9-deficient types, warrants further exploration.

Conclusion

In summary, our study identified PCDH9 as a potential tumor suppressor in breast cancer, which negatively regulates both breast cancer growth and metastasis. Furthermore, drug screening identified TAS-102 as a potential therapeutic option for breast cancer patients with low PCDH9 expression or PCDH9 deficiency. Our study offers novel treatment strategies for specific breast cancer patients and provides new options for their management.

Abbreviations

PCDH9, Protocadherin 9; HER2, Human epidermal growth factor receptor 2; ER, Estrogen receptor; BRCA1, BReast CAncer gene 1; BRCA2, BReast CAncer gene 2; PI3K, Phosphoinositide 3-kinase; mTOR, The mammalian target of rapamycin; TAS-102, Trifluridine/tipiracil; FBS, Fetal bovine serum; SRB, Sulforhodamine B.

Data Sharing Statement

The authors declare that the data supporting this study are available within the paper. All other data are available from the authors upon reasonable request.

Ethical Statement

Ethics approval for this study was obtained from The IRB of Third Xiangya Hospital, Central South University (No: 2023-S032). This study followed the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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