

Supplementary Materials and Methods

1. Cell culture and *in vitro* studies

Stable shRNA knockdown, shRNA rescue and cDNA transduction

Knockdown of PCDH7 with a validated hairpin was achieved by PCDH7 shRNA (Santa Cruz Biotechnology). Two independent shRNAs (shRNA1 5'-GATCCCCATTGAAATCCGCAAGATTTCAAGAGAATCTTGCGGATTTCAATGGTTTTT-3' and shRNA2 5'-GATCCCAGGACAGTAGTAGCTACATTCAAGA GATGTAGCTACTACTGTCCTGTTTTT-3') were used to generate two PCDH7 knockdown cell lines. Transfections were performed using Lipofectamine2000 (Invitrogen). Puromycin (2µg/ml) was used to select for stable cell lines.

Rescue the PCDH7shRNA knockdown was achieved by introducing a silent mutated PCDH7 cDNA into the PCDH7-knockdown cell lines. Primers were designed using QuikChange Primer Design software (Stratagene) to introduce six or seven nucleotide substitutions within the PCDH7 shRNA hybridizing sequence, while retaining the amino acid identity of the wild-type protein. We introduced these silent mutations in a DDK-Myc-tagged PCDH7 cDNA clone (Origene). Mutagenesis reaction was carried out using the Stratagene Multisite-Directed Mutagenesis kit (Agilent Technologies). DNAs from five colonies were isolated using Wizard Plus SV DNA Miniprep kit (Promega) and sequenced to verify the presence of the designed mutations.

Overexpression of full-length *PCDH7* gene (FL-PCDH7) or only extracellular domain (EC1-3) of *PCDH7* gene (E-PCDH7) was achieved by transfection of cDNA for PCDH7 (OriGene Technologies), which clones the open reading frame (ORF) of this gene into the pCMV6 Entry vector. TurboFectin 8.0 (OriGene Technologies) was applied to perform transient transfection. The efficiency of the knockdown and transduction was confirmed by quantitative real-time PCR, and western immunoblotting analysis. Only cell lines with a transduction or knockdown rate over 80% were used for further studies.

Tumor cell and astrocyte co-culture assay

Normal murine primary astrocytes, normal human astrocytes (NHA) or NIH 3T3 fibroblasts were used to co-culture with the target tumor cells, as described previously (1). Tumor cell/astrocyte ratio was 1:1. Specific antibodies which were described in supplemental material and methods were used for immune-staining analysis.

Time-lapse Asante Calcium Red imaging

Tumor cell lines co-cultured with NHA were flow-sorted based on GFP and incubated for 45 min at room temperature with 10 µM Asante Calcium Red (AM) (Teflabs Inc, TX) solution in medium. The cells were then washed and allowed to stand for an additional 45 min to ensure complete hydrolysis of the AM ester. After then, the tumor cells were imaged on a FV1000 Olympus Live laser scanning confocal microscope. With 488-nm excitation, fluorescence images were

acquired at 525 nm and 650 nm at 10 frames per second. The background fluorescence for each image was defined as the mean intensity value in a region-of-interest located near the cell but outside the cell contour. The 650/525 fluorescence ratio as functions of time are plotted to indicate the calcium signal activity.

Tumorsphere differentiation assay

Disaggregated brain metastasis-derived tumorspheres were seeded on glass coverslips in mammosphere medium supplemented with 5% fetal bovine serum (FBS). Cells were allowed to adhere and differentiate for 10 days before fixing and staining for nestin, cytokeratin 5 (CK5), CK18, human Keratin19, FITC-CD44, PE-CD24, and PE-CD133 (Supplementary Methods).

Coimmunoprecipitation and Western Blotting

Lysates were prepared from cells in culture by washing them twice in PBS, followed by incubation for 30 minutes on ice with NP-40 lysis buffer (20 mmol/L Tris-HCl pH 8.0, 100 mmol/L NaCl, 10% glycerol, 1% Nonidet P40, and 2 mmol/L EDTA). Lysates were centrifuged at $13,000 \times g$ for 15 minutes. Protein concentration of the supernatants was measured using BCA assay. Dynabeads Protein-A/-G (Invitrogen) were incubated 10 minutes with primary antibody (anti- β -catenin) and 30 minutes with 1 mg of protein lysates, suspended in lysis buffer on a rotating mixer at room temperature. The beads were washed three times with PBS and proteins were eluted by boiling with NuPage loading buffer and sample reducing agent (Invitrogen). The supernatant was collected and separated using SDS-PAGE and processed as a Western blot analysis for PCDH7.

Proliferation assay

Tumor cells were seeded into 96-well plates at a density of 4,000 cells per well, in a fixed volume of 200 μ L of growth media, and kept under 5% CO₂ at 37°C. The seeding density depended on the growth characteristics of the cells and was chosen to avoid a 100% confluency of untreated cells. After 24 h, adherent cells were examined under microscopy before treating them with various concentrations of compound 10. After 48 h of treatment, MTS cell proliferation assay reagents (Promega) were used to quantify cell viability following the manufacturer's protocol. Proliferation of shRNA-knockdown or over-expressed cell lines were measured by MTS assay.

Antibodies

In Western blot assays, proteins were probed with antibodies PCDH7 (1:500, OriGene Technologies), GAPDH (1:5000, Abcam), E-cadherin (1:200, Cell Signaling), vimentin (1:200, Cell Signaling), PLCB1 (1:200, Cell Signaling), pPLCB1 (1:500, Cell Signaling), S100A4 (1:200, Cell Signaling), CaMKII (1:400, Cell Signaling), pCaMKII (1:500, Cell Signaling), Bcl-2 (1:200, Cell Signaling), Cyclin E (1:200, Cell Signaling) or Cyclin D (1:200, Cell Signaling). For the immunofluorescent staining, cell lines and tissue slides were label with GFAP (1:500, Cell Signaling), β -catenin (1:400, Cell Signaling). Immunohistochemistry for PCDH7 (1:100, Sigma), GFAP (1:200, Sigma), pPLCB (1:200, Cell Signaling), CD44 (1:100, DAKO), CD31 (1:200, Abcam) and Ki-67 (1:200, Abcam) were carried on the tissue sections.

2. Other animal procedures

Orthotopic tumor growth was measured by injecting 1×10^6 viable single cells in a 1:1 mixture of PBS and growth-factor-reduced Matrigel (BD Biosciences) into mammary gland 4 in a total volume of 50 μ l as previously described (3). Primary tumor growth rates were analyzed by measuring tumor length (L) and width (W), and calculating tumor volume on the basis of the formula $\pi LW^2/6$. For experimental lung metastasis assays, 2×10^5 cells were resuspended in 0.1 ml PBS and injected into the lateral tail vein. Lung metastatic progression was monitored and quantified using non-invasive bioluminescence as previously described (3).

Immunohistochemistry and Immunofluorescence analysis on clinical and mouse samples

Clinical specimens were obtained from 34 primary breast tumors and 29 brain metastatic tumors of breast cancer patients treated at our institution, following IRB-approved protocols. The tissue was fixed with 4% PFA, and embedded in paraffin for H&E, immunohistochemistry, or immunofluorescence staining. Anti-human PCDH7 (1:100, Sigma) was used as primary antibody. An H score was calculated by multiplying the fraction of positively stained tumor (percentage) by staining intensity (0, 1+, 2+, or 3+). Membranous immunoreactivity was scored (0 and 1+ indicates negative; 2+, indeterminate; and 3+, positive for overexpression), and the percentage of tumor cells staining positive was visually estimated.

Mouse brain tissue was harvested, embedded in paraffin for H&E, immunohistochemistry, or immunofluorescence staining. The specific antibodies were described as supplemental material and methods. Images were acquired with Olympus IX61 microscope equipped with a digital camera. Immunofluorescent images were acquired by Olympus FV1000 laser scan confocal microscope under a 40 \times objective.