Article

Combined CDK2 and CDK4/6 Inhibition Overcomes Palbociclib Resistance in Breast Cancer by Enhancing Senescence

Kamal Pandey [1,3]**, Nahee Park** [1]**, Kyung-Soon Park** [3]**, Jin Hur** [1,3]**, Yong Bin Cho** [1]**, Minsil Kang** [1]
**Hee-Jung An** [2]**, Sewha Kim** [2]**, Sohyun Hwang** [2,3] **and Yong Wha Moon** [1,]*****

[1] Hematology and Oncology, Department of Internal Medicine, CHA Bundang Medical Center, CHA University, Seongnam,13488, Korea; pkamal@chauniv.ac.kr (K.P.); skgml0413@naver.com (N.P.); hurjinz@naver.com (J.H.); ybyoungbin@naver.com (Y.B.C.); rbtnr1@naver.com (M.K.)
[2] Department of Pathology, CHA Bundang Medical Center, CHA University, Seongnam,13488, Korea; hjahn@cha.ac.kr (H.-J.A.); sewhakim@chamc.co.kr (S.K.); blissfulwin@cha.ac.kr (S.H.)
[3] Department of Biomedical Science, CHA University, Seongnam,13488, Korea; kspark@cha.ac.kr
* Correspondence: ymoon@cha.ac.kr; Tel.: +82-10-2825-6357; Fax: +82-31-780-3929

Supplementary figures:

(A)

(B)

(C)

(D)
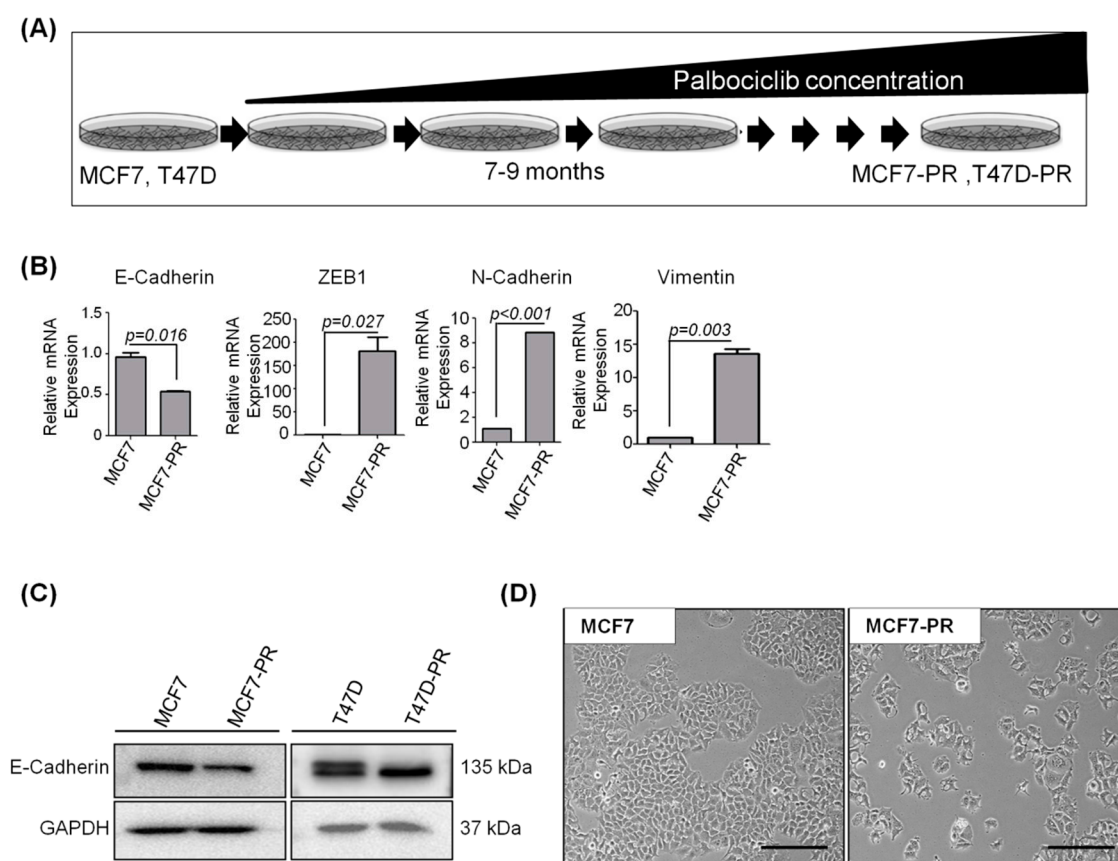
Supplementary Figure S1. Derivation and confirmation of HR-positive cells resistant to CDK4/6 inhibitors **(A)** Schematic diagram showing the generation of palbociclib-resistant cells. MCF7 and T47D cells were continuously treated with a gradually increasing concentration of palbociclib for 7-9 months and named as MCF7-PR and T47D-PR, respectively. **(B)** Palbociclib-sensitive and resistant cells were compared for the expression of EMT markers. qRT-PCR analysis of the indicated genes

was performed. *P*-values were calculated by student's *t*-test. Data are presented as the mean ± S.E.M. of triplicate experiments. **(C)** Western blot analysis showing the decrease of the epithelial marker E-cadherin in the palbociclib-resistant cells compared with their sensitive counterparts. **(D)** Images showing the morphological changes of MCF7-PR (mesenchymal) compared with MCF7. Scale bars = 100 μm. Full length blots **(C)** are presented in Figure S5.
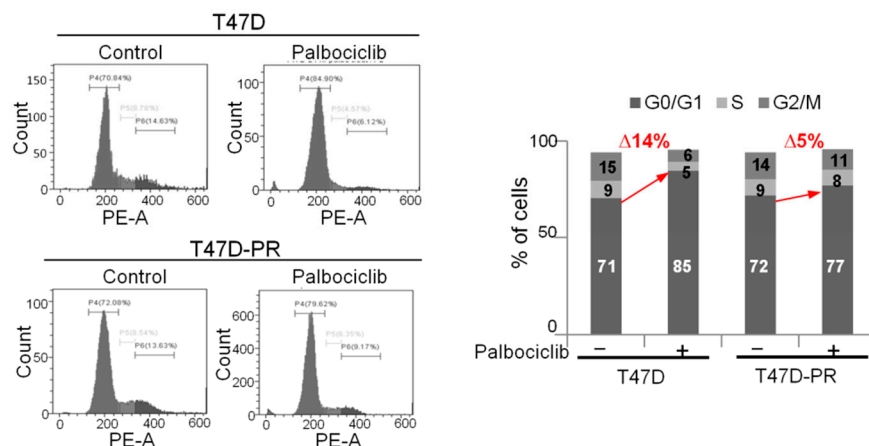
Supplementary Figure S2. Alteration of cell cycle and protein expression in palbociclib-resistant cells. Similarly to MCF7 and MCF7-PR, T47D cells were significantly arrested at the G1 phase by palbociclib, however, T47D-PR cells were not blocked at G1 by palbociclib (260 nM).
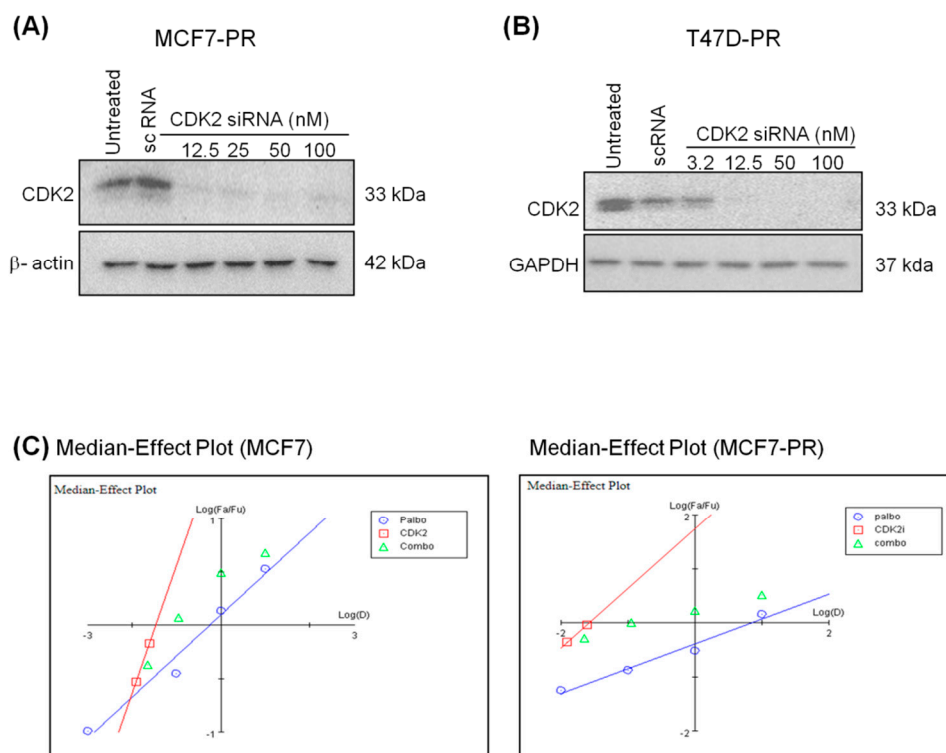
Supplementary Figure S3. CDK2 siRNA effectively inhibits CDK2. **(A)** MCF7-PR and **(B)** T47D-PR cells were treated with various concentrations of CDK2 siRNA for 48 hours. The complete inhibition of CDK2 was observed with 12.5 nM and higher concentrations. **(C)** Median-effect plot of MCF7 and MCF7-PR. Full length blots **(A, B)** are presented in Figure S5.
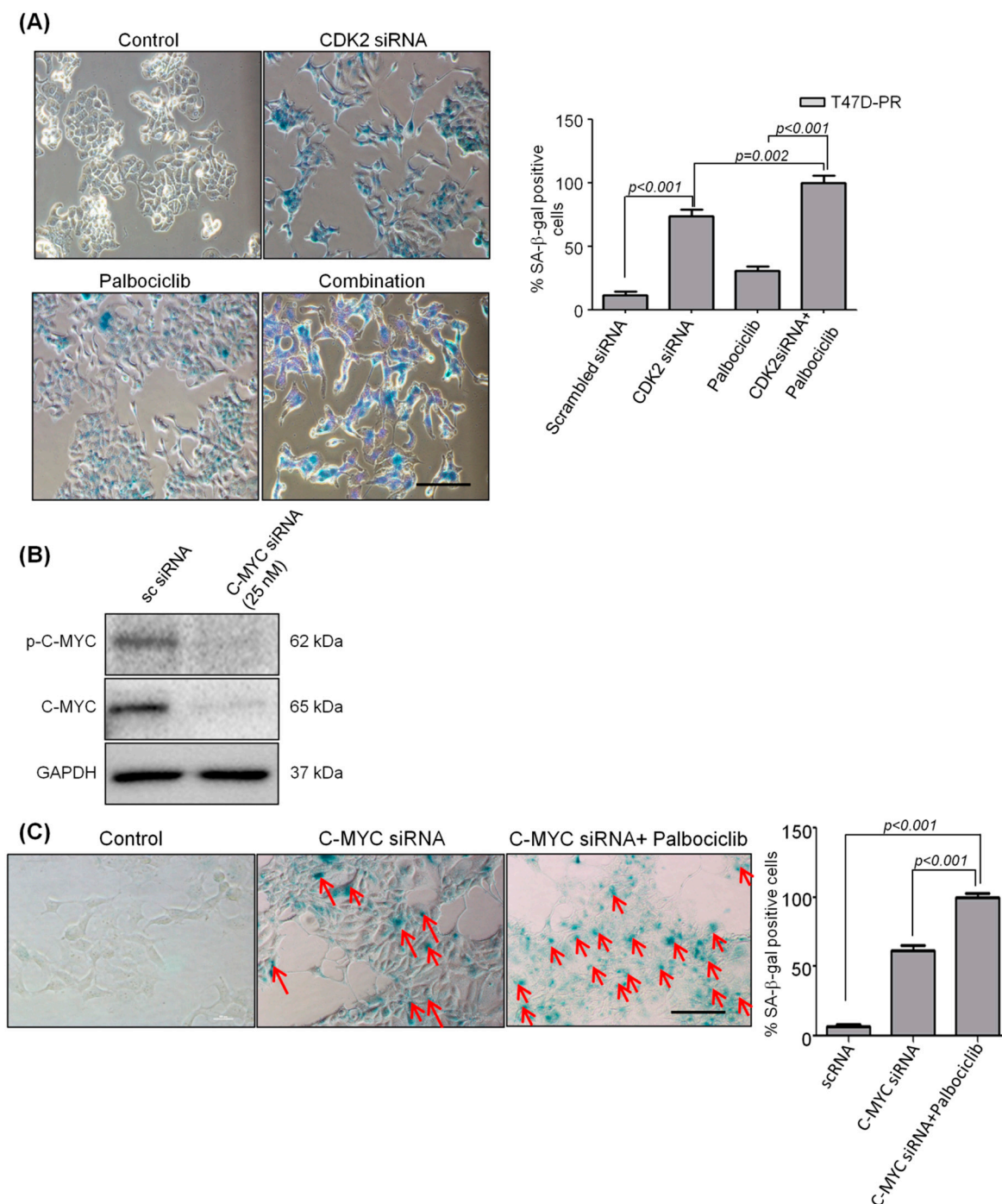
Supplementary Figure S4. SA-β-gal staining in T47D-PR cells. (A) In T47D-PR cells, SA-β-gal-positive cells were increased by CDK2 siRNA (12.5 nM) or palbociclib (260 nM) and further increased by combination treatment, which was similar to MCF7-PR cells. Green indicates the nuclear staining of β-galactosidase. *P* values were calculated by *t*-test after counting positive cells in 10 randomly chosen, non-overlapping fields. Data are presented as the mean ± S.E.M. Scale bar = 100 μm. (B) MCF7-PR cells were treated with C-MYC siRNA for 48 hours. The complete inhibition of phospho-C-MYC (ser62) and C-MYC was observed with 25 nM concentration. (C) In MCF7-PR cells, SA-β-gal-positive cells were increased by C-MYC siRNA (25 nM) and further increased by combination treatment with palbociclib. Green indicates the nuclear staining of β-galactosidase. *P* values were calculated by *t*-test after counting positive cells in 10 randomly chosen, non-overlapping fields. Data are presented as the mean ± S.E.M. Scale bar = 100 μm. Full length blots **(B)** are presented in Figure S5.

Supplementary Methods

Drugs

Palbociclib (PD-332991) was provided by Pfizer Inc. (North Peapack, NJ 07977, USA). Abemaciclib and ribociclib were purchased from ChemScene (NJ 08852, USA). Palbociclib was dissolved in water, whereas all other drugs were reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

Cell culture

All breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in a humidified atmosphere with 5% CO2 at 37°C. MCF7 and T47D cells were cultured in RPMI 1640 medium (Welgene Inc., Deagu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (cat# S 001-01; FBS, Welgene Inc.) and 1% 100X penicillin/streptomycin solution (Welgene Inc.).

Cell viability assay

The MTT assay was used to access the viability of the cells. Cells growing in a logarithmic phase were seeded at the density of 500-1000 cells per well in 96-well plates. Once the cells adhered to the dish 24 hours after plating, different concentrations of palbociclib and a fixed concentration of CDK2 siRNA were added to the cells and incubated for 24, 48, or 72 hours or for up to 6 days at 37°C. MTT solution (5 mg/ml) was added into each well, and 4 hours later, the medium was discarded. DMSO (100 μl) was added into the wells to dissolve the formazan product from the metabolism of MTT. Finally, the optical density was measured at 540 nm using a Microplate Reader (Multiskan GO Microplate Spectrophotometer). IC_{50} and Combination Index (CI) values were analyzed using CompuSyn software (ComboSyn, Inc., NJ, USA).

Western blot and co-immunoprecipitation

Cells were lysed in RIPA lysis buffer (cat# # 89901, Thermo Scientific) supplemented with a protease inhibitor cocktail (cat# 11873580001, Roche) and phosphatase inhibitor (cat# 1862495, Thermo Scientific). Total protein lysates were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 hour in Tris-buffered saline (TBS) containing 5% milk and 0.1% Tween 20 at room temperature. All primary antibodies were incubated overnight at 4°C, followed by incubation with a secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature. Detection was performed with a Super Signal Chemiluminescent reagent according to the manufacturers' protocol (cat#32209, ThermoFisher Scientific, Waltham, MA USA).

For the co-immunoprecipitation assay, MCF7-PR cells were lysed in ice-cold NETN lysis buffer [250 mM NaCl, pH 8.0 5 mM Tris-HCL, 5 mM EDTA, and 0.5% NP-40 (IGEPAL CA-630, Sigma-Aldrich) supplemented with protease and phosphatase inhibitor cocktails]. Total protein lysates (500 μg) were then separately immunoprecipitated with anti-CDK2 (1:50) and anti-cyclin E (2 μg) antibodies overnight at 4°C in the presence of 50 μL of protein A/G agarose beads (cat#20421, ThermoFisher Scientific, Waltham, MA USA). Normal mouse IgG (cat# sc-2025, Santa Cruz Biotechnology) and rabbit IgG isotypes (cat#3900, Cell Signalling Technology) were used as internal

controls. Immunoblotting detection was completed, as described above. A list of antibodies is given in Supplementary Table S1.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol (Life Technologies). Total RNA was reverse transcribed to complementary DNA (cDNA) using the Takara prime script 1st strand cDNA synthesis kit (cat# 6110A, Takara Bio Inc). The quantitative real-time polymerase chain reaction was performed using an ABI StepOne Real-time PCR System (Applied Biosystems, Warrington, UK) and a Power-up SYBR Green Master Mix (cat#A25741, ThermoFisher scientific). Double-stranded DNA dissociation was assessed by melting curve analysis, and β-actin was used as the endogenous control. Gene expression levels were calculated and analyzed using the $2^{-\Delta\Delta ct}$ method. Primers were obtained from Macrogen (Macrogen, Inc. Korea). A list of primers is given in Supplementary Table S2**.**

siRNA transfection

Cells were seeded at a density of 3×10^5 cells/well were in six-well plates. Lipofectamine RNAiMAX Reagent (cat# 13778-030, Invitrogen) was used for siRNA transfections according to the manufacturers' instructions. siRNAs and lipofectamine were separately diluted in Opti-MEM® I Reduced-Serum Medium (cat#31985070, ThermoFisher Scientific) according to the manufacturers' procedure. Subsequently, the mixtures were added to the wells containing cells. Furthermore, the cells treated with only lipofectamine and scrambled siRNA were used as the siRNA blank control. The cells were then incubated for 24 to 72 hours at 37°C in a humidified CO_2 incubator. After transfection, qRT-PCR and western blot analyses were performed to assess the effect of siRNA on gene silencing.

Gene expression microarray analyses

RNA samples were extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The Affymetrix Whole Transcript Expression array process was executed according to the manufacturers' protocol (GeneChip Whole Transcript PLUS reagent Kit). For the quality control, RNA purity and integrity were evaluated by the OD 260/280 ratio and analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). cDNA was synthesized using the GeneChip WT (Whole Transcript) Amplification kit, as described by the manufacturer. The sense cDNA was then fragmented and biotin labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal labeling kit. Approximately 5.5 µg of labeled DNA target was hybridized to the Affymetrix GeneChip Human 2.0 ST Array at 45°C for 16 hours. Hybridized arrays were washed, stained on a GeneChip Fluidics Station 450, and scanned on a GCS3000 Scanner (Affymetrix). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. Only genes that were upregulated or downregulated by 2-fold were considered meaningful.

Fluorescence-activated cell sorting analysis (FACS) for cell cycle

MCF7 and MCF7-PR cells (3×10^5) were grown in 6 well plates in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C. Cells were treated with the IC_{50} concentration of palbociclib for 48 hours. Cells were harvested and fixed overnight in 70% ethanol at -20°C. The cells were washed twice with ice-cold PBS, rehydrated, re-suspended in RNase A (100 µg/ml), and stained with propidium iodide (50 µg/ml) in PBS. The stained cells were incubated in the dark at room temperature for 20 minutes, and analyzed by flow cytometry (Beckman Coulter Cytoflex, Indianapolis, USA). Approximately 10,000 cells/sample were analyzed, and the percentage of cells in each phase of the cell cycle was determined.

Immunohistochemistry of cell blocks

MCF7 and MCF7-PR cells were collected by trypsinization. Following centrifugation, the pellets were kept at the center of the plate and surrounded with 1.5% agarose gel. When the gel solidified, the area of gel containing the cell pellet was cut, incubated in 4% PFA for fixation, and sent to the histology lab to prepare paraffin blocks.

For immunohistochemical staining, cell blocks were sectioned with a microtome to generate 5 µM sections, which were placed on slides. After deparaffinization, citrate-based antigen retrieval was performed, followed by blocking with a protein block serum-free medium (cat# X0909; DAKO, Carpinteria, CA) for 30 min at room temperature. The slides were then incubated with anti-RB (cat# 9309, cell signaling technology) or anti-cyclin E (cat# sc-248, Santa Cruz Biotechnology, Inc) primary antibodies overnight at 4°C in a humid chamber. The slides were washed 3 times with PBS and incubated with a secondary antibody for 1 hour at room temperature. Antigen-antibody complexes were detected by the avidin-biotin-peroxidase method, using 3, 3'-diaminobenzidine as a chromogenic substrate (cat# 1855920, Thermo Scientific). Finally, the slides were counterstained with hematoxylin (cat# S3309, DAKO, Carpinteria, CA). Coverslips were mounted after dehydration of the sections using permanent mounting medium (cat# C1795, Sigma-Aldrich) and then examined under a light microscope.

Animal studies

All mice were housed in a specific pathogen-free animal facility with a controlled temperature of 24 ± 3°C and a light/dark cycle of 12 hours with unlimited access to food and water at CHA University (Seongnam, Korea). Animals were acclimatized to the environment for at least 1 week prior to the experiment. All animal procedures were performed according to the approved protocol by the Institutional Animal Care and Use Committee (IACUC) of CHA University. A total of 1×10^7 MCF7-PR cells in 0.1 ml of PBS containing a 50%(V/V) Matrigel solution (corning, NY, USA) were inoculated subcutaneously into the mammary fat pad of the mouse. Estrogen valerate (3 µg/mouse) was subcutaneously injected every week as an estrogen supplement. When the tumor volume reached 100–150 mm³, the animals were randomized into four groups. Each treatment group consisted of five or six mice. Mice were treated with control siRNA (group 1), palbociclib 100 mg/kg (group 2), 5 µg of hCDK2-siRNA (group 3), hCDK2- siRNA with palbociclib (group 4). siRNAs were formulated with *in vivo*-jetPEI (cat# 201-10G, Polyplus Transfection) according to the manufacturers' instructions using an N/P ratio of 6 and were injected intratumorally every 3 days for 6 times. Palbociclib (100 mg/kg) was injected by oral gavage once daily for 21 days. Tumor sizes

were measured using a Vernier caliper three times a week and calculated by the formula tumor length^2 × tumor width × 0.5. Additionally, bodyweight was assessed three times a week. After 23 days, all mice were sacrificed according to the animal experimental guidelines, and the xenografted tumors were excised and preserved for further analysis.

Statistical analysis

Student's *t*-test was performed to compare two groups in qRT–PCR, cell viability assay and SA-β-galactosidase assay whereas, western blot data were analyzed using paired *t*-test. The correlations between gene expression and palbociclib sensitivity in the CCLE data were analyzed using the Pearson correlation coefficient. Distant recurrence-free survival (DRFS) was defined as the time from curative surgery to recurrence in distant organs or the last date at which the patient was known to be free of distant recurrence (censoring time). DRFS was measured using the Kaplan-Meier estimator. In the public gene expression profiling data sets, to divide patients into two groups of high or low CCLE1 mRNA expression, an optimal cut-off score was selected as the quartile with the minimum log rank *P*-value in DRFS analysis. The statistical significance of comparisons of survival curves were calculated by log-rank test. Multivariate analysis for prognostic factors was performed by the Cox proportional hazards regression model. All statistical analyses were performed using SPSS version 19.0 (IBM SPSS statistics 19.0, NY, USA), except for Pearson correlation coefficient, which was performed using GraphPad Prism (version 5.01; GraphPad Software, Inc.). All *P*-values were two-sided, and *P*-values of less than 0.05 were considered significant

Supplementary Tables

Supplementary Table S1. Primary antibodies used for western blot or immunohistochemistry

Antibody	Host species	Dilution	Company (Catalog#)
RB	Mouse	1:1000	9309, cell signalling
Phospho-RB	Rabbit	1:1000	D59B7, cell signalling
CDK2	Rabbit	1:1000	2546,cell signalling
E-cadherin	Rabbit	1:1000	3195, cell signalling
C-MYC	Rabbit	1:1000	5605, cell signalling
Phospho-C-MYC	Rabbit	1:1000	13748,cell signalling
Cyclin E	Mouse	1:1000	Sc-248,santa cruz
Caspase-3	Rabbit	1:1000	9662, cell signaling
Beta actin	Rabbit	1:5000	LF-PA0207,AbFrontier
GAPDH	Rabbit	1:5000	LF-PA0212,AbFrontier
Anti-Rabbit HRP	Goat	1:5000	AP132P,Millipore
Anti-Mouse HRP	Goat	1:5000	GTX213111-01, GeneTex

Supplementary Table S2. Primers used for qRT-PCR

Primer name	Orientation	Sequence
E-Cadherin	Sense	5′-GGT CGA CAA AGG ACA GCC TA-3′
	Anti-sense	5′-GCG TGA CTT TGG TGG AAA AC-3′
N-Cahderin	Sense	5′-CAA CCC CAT CTC GGG TCA GC-3′
	Anti-sense	5′-GGG CAT TGG GAT CGT CAG CA-3′
ZEB1	Sense	5′-CAG CAT CAC CAG GCA GTC CC-3′
	Anti-sense	5′-CAA CAG CTT GCA CCA TGC CC-3′
Vimentin	Sense	5′-CCC TCA CCT GTG AAG TGG AT-3′
	Anti-sense	5′-TCC AGC AGC TTC CTG TAG GT-3′
CDK2	Sense	5′-AGA CTG AGG GTG TGC CCA GT-3′
	Anti-sense	5′-AAG GTC TCG GTG AGG ACC CC-3′
MYC	Sense	5′-AGA GAA GCT GGC CTC CTA CC-3′
	Anti-sense	5′-CGT CGA GGA GAG CAG AGA AT-3′
TERT	Sense	5′-TGT CAA GGT GGA TGT GAC G-3′
	Anti-sense	5′-CTG GAG GTC TGT CAA GGT A-3′
Beta -actin	Sense	5′-AGA GCT ACG AGC TGC CTG AC-3′
	Anti-sense	5′-AGC ACT GTG TTG GCG TAC AG-3′

Supplementary Table S3. A list of 38 breast cancer cell lines in CCLE data base

BT-20 ,HCC70, BT-474, Hs 578T, BT-549, KPL-1, CAL-51,MCF7, CAMA-1, MDA-MB-134-VI,

DU4475, MDA-MB-157, EFM-192A, MDA-MB-175-VII, EFM-19, MDA-MB-231, HCC1143,

MDA-MB-361, HCC1187, MDA-MB-415, HCC1395, MDA-MB-436, HCC1419, MDA-MB-453,

HCC1500, MDA-MB-468, HCC1569, SK-BR-3, HCC1806, T47D, HCC1937, UACC-812, HCC1954,

UACC-893, HCC202, ZR-75-1, HCC2218, ZR-75-30

Supplementary Table S4. Patient characteristics in four public mRNA expression data sets

Data set (patients no.)	GSE6532 (N=261)	GSE26971 (N=210)	GSE2034 (N=209)	GSE113863 (N=181)
HR				
Positive	261	210	209	181
Negative	0	0	0	0
pTstage				
T1	122	82	NA	NA
T2	139	122	NA	NA
T3	0	6		
pNstage				
N0	NA	126	NA	NA
N1	NA	46	NA	NA
N2	NA	18	NA	NA
N3	NA	20	NA	NA
LN metastasis				
Yes	79	126	209	NA
No	182	84	0	NA
Distant recurrence				
Yes	59	44	80	14
No	202	166	129	167
Median F/U (yr)	5.91	6.21	7.17	5.58
HR, Hormone Receptor; LN, Lymph Node; NA, Non-Available; F/U, Follow Up; yr, year; Staging is based on American Joint Committee on Cancer staging revised in 2002.				

Supplementary Table S5. Multivariate analysis of prognostic factors for distant recurrence-free survival in hormone receptor-positive breast cancer

Data set	Variables		DRFS	
			HR (95% CI)	*p*
GSE6532				
	CCNE1	(low vs high)	1.93(1.13-3.32)	**0.017**
	pTstage	(T1 vs T2)	2.90(1.61-5.21)	**<0.001**
	LN metastasis	(no vs yes)	1.75(1.03-2.99)	**0.039**
GSE26971				
	CCNE1	(low vs high)	2.48(1.33-4.62)	**0.004**
	pTstage	(T1 vs T2)	1.52(0.74-3.12)	0.249
		(T1 vs T3)	3.53(0.92-13.60)	0.066
	pNstage	(N0 vs N1)	0.69(0.28-1.69)	0.686
		(N0 vs N1)	1.28(0.47-3.44)	0.632
		(N0 vs N1)	3.11(1.31-7.38)	**0.010**
DRFS, Distant Recurrence-Free Survival; HR, hazard ratio; CI, confidence interval; Criteria of CCNE 'low vs high' are 'median' in the GSE6532 and '75 percentile' in the GSE26971; Staging is based on American Joint Committee on Cancer staging revised in 2002.				