

# **$\beta$ -catenin blockers enhance the effect of CDK4/6 inhibitors on stemness and proliferation suppression in endocrine-resistant breast cancer cells**

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**Abstract.** Wnt/ $\beta$ -catenin signaling is involved in endocrine resistance and stem cell-like properties of hormone receptor-positive breast cancer cells. Palbociclib is a well-known inhibitor of cyclin-dependent kinase 4 and 6 (CDK4/6 inhibitor) that downregulates the activation of retinoblastoma protein, thereby inhibiting the cell cycle in breast cancer cells. The inhibitory effects of a combination of palbociclib and ICG-001, a  $\beta$ -catenin small-molecule inhibitor, were investigated in tamoxifen-resistant breast cancer cell lines. Tamoxifen-resistant MCF-7 (TamR) cells were established by continuously exposing MCF-7 cells to tamoxifen. The characteristics associated with the stem cell-like property of cancer were assessed using western blotting, cell cycle analysis, and the mammosphere assay. The effects of the combination of palbociclib and ICG-001 were evaluated in control MCF-7 and TamR cell lines. Compared with control cells, TamR cells exhibited elevated levels of Nanog, Sox2, ALDH1, and p-STAT3, indicating stem cell-like characteristics, and elevated  $\beta$ -catenin activity. TamR cells also showed significantly higher mammosphere-forming efficiency. Several markers of stem cell-like nature of TamR cells showed reduced levels upon treatment of cells with the drug combination; there was a greater reduction in the levels of these markers when the

cells were treated with the combination than in the case where cells were treated with one of the drugs individually (combination index value for 25  $\mu$ M palbociclib and 50  $\mu$ M ICG-001 was  $1.1 \pm 0.02$ ). TamR cells treated with the palbociclib and ICG-001 combination demonstrated significantly reduced cell proliferation and mammosphere-forming efficiency compared with the cells treated with one of these drugs. The combination of the drugs could additively inhibit proliferation and suppress stem cell-like characteristics. These results suggest that  $\beta$ -catenin plays a role in endocrine-resistant breast cancer; the inhibition of  $\beta$ -catenin and CDK4/6 together can overcome endocrine resistance in breast cancer cells.

## **Introduction**

Breast cancer is a heterogeneous disease categorized into several subtypes according to the expression of estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). ER-positive (ER<sup>+</sup>) breast cancer is present in approximately 70% of all breast cancer patients (1,2). Tamoxifen is the most effective primary drug compared with other selective estrogen receptor modulators. However, approximately 20% of patients administered tamoxifen experience tumor recurrence, and those with advanced breast cancer who respond to tamoxifen at the beginning of treatment also eventually experience disease progression (1,3,4). To date, well-known theories explaining the mechanisms of resistance to endocrine therapy include loss of ER expression, mutations in genes encoding ER, overactivation of growth factor signaling pathways, and difficulty in regulation of cell cycle signaling pathways (3,5,6). Among these, mutations in proteins involved in the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway are frequently observed in ER<sup>+</sup> breast cancer and have been studied extensively as one of the mediators of resistance to endocrine therapy. Several clinical trials targeting this pathway have also been conducted (7,8). However, the mechanism underlying endocrine resistance is complex, and recent studies have shown that characteristics of cancer stem cell-like properties and Wnt/ $\beta$ -catenin signaling are associated with drug resistance and poor prognosis (9-13). Transcription factors

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*Abbreviations:* TamR, tamoxifen-resistant MCF-7; CI, combination index; ER, estrogen receptor; CBP, CREB-binding protein

*Key words:* breast cancer, stemness, ICG-001,  $\beta$ -catenin, cyclin-dependent kinase 4 and 6 inhibitor, signal transducer and activator of transcription 3, endocrine resistance

that regulate cell proliferation and survival, such as signal transducer and activator of transcription 3 (STAT3), have also been reported to be associated with the development of stem cell-like properties in breast cancer (14).

Wnt/ $\beta$ -catenin signaling plays an important role in cell growth, proliferation, and differentiation and is associated with the pathogenesis of various types of cancer (15-20). In breast cancer, activation of  $\beta$ -catenin signaling is associated with poor outcomes in basal-like or triple-negative breast cancer subtypes lacking ER expression (21,22). Moreover, the number of breast cancer stem cells, the ability to initiate tumors, and metastasis have been demonstrated to be related to the Wnt/ $\beta$ -catenin signaling pathway (23). In a previous study by the authors, it was reported that  $\beta$ -catenin is associated with endocrine resistance in breast cancer and that inhibition of  $\beta$ -catenin can overcome endocrine resistance (5). ICG-001, a  $\beta$ -catenin small-molecule inhibitor, selectively binds to the CREB-binding protein (CBP) in the nucleus and is proposed to prevent  $\beta$ -catenin from performing CBP-dependent transcription of genes in the canonical Wnt/ $\beta$ -catenin signaling pathway that are related to stem cell-like properties (24). The inhibitory effects of ICG-001 have recently been demonstrated in several carcinomas associated with Wnt/ $\beta$ -catenin signaling, including pancreatic ductal adenocarcinoma (25), gastric cancer (19), and head and neck cancer (26). However, to the best of our knowledge, studies examining the effects of ICG-001 on endocrine-resistant breast cancer have been scarce, and its mechanism underlying the effects has not been established.

Cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors were introduced as primary drugs to inhibit the cell cycle pathway, overcome endocrine resistance, and increase the effectiveness of endocrine therapy. Moreover, several treatment combinations are being studied to increase the potency of CDK4/6 inhibitors in an endocrine-resistant setting; these include the combination of two different CDK4/6 inhibitors and a CDK4/6 inhibitor with fulvestrant (27-29). Considering the role of  $\beta$ -catenin in endocrine resistance and the acquisition of cancer stem cell-like properties, in the present study, the effects of a combination of palbociclib (a CDK4/6 inhibitor) and ICG-001 (a  $\beta$ -catenin small molecule inhibitor), as well as of ICG-001 on MCF-7 and an endocrine-resistant cell line, were explored with the aim of developing novel therapeutic strategies.

## Materials and methods

**Cell lines and culture.** MCF-7 (KCBL no. 30022), an ER<sup>+</sup> human breast cancer cell line, was obtained from the Korean Cell Line Bank. These cells were cultured in phenol-red-free RPMI-1640 medium containing 10% fetal bovine serum and antibiotics (1% penicillin/streptomycin; all Invitrogen; Thermo Fisher Scientific, Inc.). The medium was changed twice a week during culture. Tamoxifen-resistant MCF-7 (TamR) cells were prepared via sequential exposure of MCF-7 cells to increasing concentrations (from 0.05 to 3  $\mu$ M) of 4-hydroxy-tamoxifen at 37°C over a period of 9 months. Although the cell growth rate was not quantitated, TamR cells generally tended to show slow growth when exposed to the drug.

**Cell viability and mammosphere assay.** Cell viability was determined at different doses of the drugs using the Cell

Counting Kit-8 (CCK-8; Enzo Life Sciences, Farmingdale, Inc.). Briefly, MCF-7 and TamR cells were seeded in a 96-well plate at a density of  $5 \times 10^3$  cells/well. The concentrations of palbociclib (0.1, 1, 10, 25, 50 and 100  $\mu$ M; also known as PD-0332991; Sigma-Aldrich; Merck KGaA), ICG-001 (0, 12.5, 25, 50 and 100  $\mu$ M; Selleck Chemicals) and S3I-201 (3, 6.25, 12.5, 50 and 100  $\mu$ M; cat. no. S1155; Selleck Chemicals) were added to the wells and the plate was incubated for 24 h at 37°C. The optical density at 450 nm was measured using a spectrophotometer (VersaMax; Molecular Devices, LLC). The mammosphere assay was performed using the MammoCult<sup>TM</sup> Human Media kit (cat. no. 05620; STEMCELL Technologies, Inc.) according to the manufacturer's protocol. Cells were seeded at  $3.5 \times 10^4$  cells/2 ml culture medium in 6-well ultra-low attachment plates. Following incubation for 7 days in a 37°C CO<sub>2</sub> incubator, spheres with a diameter of 60  $\mu$ m or more were counted.

**Western blot analysis.** Protein extracts were prepared by lysing the cells in RIPA buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS] containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). The protein concentration was determined using the Bradford assay. Total protein lysates (40  $\mu$ g) were loaded into each lane, size-fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in Tris-buffered saline (TBS)-0.1% Tween-20 containing 5% skim milk for 1 h at room temperature, and then incubated with primary antibodies against ER $\alpha$  (D8H8; rabbit mAb; product no. 8644; dilution 1:1,000), epidermal growth factor (EGFR; C74B9; rabbit mAb; product no. 2646; dilution 1:1,000), HER2 (29D8; rabbit mAb; product no. 2165; dilution 1:1,000), Nanog (D73G4; rabbit mAb; product no. 4903; dilution 1:1,000), Sox2 (D6D9; rabbit mAb; product no. 3579; dilution 1:1,000), Oct4 (product no. 2750; dilution 1:1,000), ALDH1 (D4R9V; rabbit mAb; product no. 12035; dilution 1:1,000), phosphorylated (p)-STAT3 (D3A7; rabbit mAb; product no. 9145; dilution 1:1,000), STAT3 (124H6; mouse mAb; product no. 9139; dilution 1:1,000), mTOR (7C10; rabbit mAb; product no. 2983; dilution 1:1,000), p-mTOR (D9C2; rabbit mAb; product no. 5536; dilution 1:1,000), NOTCH1 (D1E11; rabbit mAb; product no. 3608; dilution 1:1,000),  $\beta$ -catenin (D10A8; rabbit mAb; product no. 8480; dilution 1:1,000), active  $\beta$ -catenin (D13A1; rabbit mAb; product no. 8814; dilution 1:1,000), E-cadherin (24E10; rabbit mAb; product no. 3195; dilution 1:1,000), N-cadherin (product no. 4061),  $\alpha$ -tubulin (11H10; rabbit mAb; product no. 2125; dilution 1:1,000), or GAPDH (D16H11; rabbit mAb; product no. 5174; dilution 1:1,000) (all the antibodies were procured from Cell Signaling Technology, Inc.) overnight at 4°C. Following washing with TBS-0.1% Tween-20 three times, the membranes were incubated for 1 h at room temperature with goat anti-rabbit (product no. 7074; dilution 1:1,000) or horse anti-mouse IgG HRP-conjugated secondary antibodies (product no. 7076; dilution 1:1,000; both from Cell Signaling Technology, Inc.). Western blot images were visualized with enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific, Inc.) and recorded using a LAS-4000 Mini camera (Fujifilm).

Band intensities were quantified using the ImageJ software (version 1.53k; <https://imagej.nih.gov/ij/>). Protein phosphorylation data were further expressed as the p-STAT3/STAT3 or p-mTOR/mTOR ratio.

**Luciferase assay.** Luciferase reporter assays were performed using the luciferase assay system (Promega Corporation). MCF-7 and TamR cells were plated in 24-well plates, a day before transfection, at a density of  $4 \times 10^4$  cells/well. The cells were co-transfected with the pGL4.49[luc2P/TCF-LEF RE/Hygro] and pRL-TK constructs (Promega Corporation) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The pRL-TK vector provides constitutive expression of *Renilla* luciferase as a control reporter vector. The cells were incubated for 24 h after transfection, and then treated with palbociclib (25  $\mu$ M) or ICG-001 (50  $\mu$ M) or S3I-201 (100  $\mu$ M) for 24 h at 37°C in an incubator. The cells were subsequently processed using the DUAL-Glo Luciferase Reporter Assay System (Promega Corporation), and luciferase activity was measured using a luminometer (Veritas Microplate Luminometer; Turner BioSystems, Inc.). The ratio of firefly to *Renilla* luciferase activity was representative of the transcriptional activity of  $\beta$ -catenin.

**Flow cytometric analysis of the cell cycle.** Cells ( $1 \times 10^6$ /ml; 30–40%) were treated with palbociclib (25  $\mu$ M) and ICG-001 (50  $\mu$ M) for 24 h for cell cycle analysis. The cells were detached with TrypLE (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc.) and pelleted by centrifugation at  $500 \times g$  for 5 min at 4°C. The cell pellets were fixed with 66% ethanol, overnight at -20°C, followed by washing with phosphate-buffered saline [PBS; 0.137 M sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate (dibasic, anhydrous), 1.4 mM potassium phosphate (monobasic, anhydrous)], and stained with a propidium iodide solution for 30 min at room temperature. Cell cycle analysis was carried out using the Propidium Iodide Flow Cytometry Kit (Abcam) and performed on a NovoCyte Flow Cytometer (ACEA Biosciences, Inc.; Agilent Technologies, Inc.). The data were analyzed using NoveExpress software (version 1.2.5; ACEA Biosciences, Inc.; Agilent Technologies, Inc.).

**Immunofluorescence.** Cells ( $1 \times 10^6$ /ml; 30–40%) were rinsed with PBS, fixed for 15 min at room temperature with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS (PBS-0.1% Tween-20), and blocked with 1% BSA in PBS-Tween-20 for 10 min at room temperature. The slides with fixed cells were incubated with primary antibodies against  $\beta$ -catenin (D10A8; rabbit mAb; product no. 8480) (Cell Signaling Technology, Inc.) at 4°C overnight. Fluorescence-labeled secondary antibody, goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor™ 488 (cat. no. A11008; Invitrogen; Thermo Fisher Scientific, Inc.) was applied for 1 h at room temperature. The cell nuclei were counterstained with 10  $\mu$ l DAPI (stock solution 1:1, DAPI:glycerol). All images were recorded using an LSM5 microscope (Carl Zeiss AG).

**siRNA transfection.** Cells ( $1 \times 10^6$ /ml; 30–40%) were transfected with small interfering (si)RNAs targeting STAT3 (siSTAT3)

and a non-targeting siRNA (both from Bioneer Corporation). AccuTarget™ Negative control siRNA (cat. no. SN-1002) was used as the negative control (siNC). The siSTAT3 sequences were as follows: 5'-UGUAGGAAACUUUUUGCUG-3' (sense) and 5'-CAGCAAAAAGUUUCCUACA-3' (antisense). Transfection experiments were performed using the jetPRIME reagent (Polyplus-transfection). Briefly, cells in the exponential growth phase were seeded in a 6-well plate, grown for 24 h, transfected with 200 pmol of the siRNA for 10 min at room temperature, and subsequently cultured for 3 days.

**Statistical analysis.** The statistical significance of differences between the groups was determined using unpaired Student's t-test and one-way ANOVA. The post hoc test employed the Tukey HSD method after one-way ANOVA. Data were expressed as the mean  $\pm$  standard deviation (SD). All data were analyzed for statistical significance using SPSS (version 25; IBM Corp.) and GraphPad Prism 7.0 for Windows (GraphPad Software, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**TamR cells have enhanced self-renewal properties of stem-like cells.** The characteristics of TamR and MCF-7 cells were compared by determining the expression of selected proteins using western blot analysis. TamR cells exhibited reduced expression of ER $\alpha$  and increased expression of EGFR and HER2 compared with MCF-7 cells (Fig. 1A). In addition, the expression of Nanog, Sox2, and ALDH1 was upregulated in TamR cells, indicating the characteristics of stem-like cells, and p-STAT3, which is associated with cell signaling related to stem-like cell characteristics, was also overexpressed compared with that in MCF-7 cells (Fig. 1B). The self-renewal properties of TamR cells were visualized using the mammosphere assay (Fig. 1C and D). The number of microspheres in TamR cells (average 569 spheres/well) was significantly increased (more than twice) compared with that in MCF-7 cells (average 273 spheres/well). Increased active  $\beta$ -catenin levels are shown in Fig. 2A and B. Western blot analysis revealed no significant difference in  $\beta$ -catenin levels between MCF-7 and TamR cells; however, the upregulation of active  $\beta$ -catenin was considered significant because  $\beta$ -catenin enters the nucleus and is activated. In addition, the results of the luciferase assay, which reflects the levels of only active  $\beta$ -catenin, revealed a significant increase in the levels of active  $\beta$ -catenin in TamR cells, consistent with the results of western blot analysis. These findings suggest that an increase in active  $\beta$ -catenin levels is associated with the acquisition of Wnt/ $\beta$ -catenin signals, which are known to be related to drug resistance. The localization of  $\beta$ -catenin in TamR cells was determined using immunofluorescence (Fig. 2C). Compared with that in the MCF-7 control group, the nuclear localization of  $\beta$ -catenin was high in TamR cells. These findings are in agreement with those of a previous study showing that  $\beta$ -catenin accumulates in the nucleus due to activation of the Wnt/ $\beta$ -catenin signaling pathway, leading to downstream signaling (30).

**Combination therapy with palbociclib and ICG-001 results in additive inhibition of growth in TamR cells.** To evaluate the

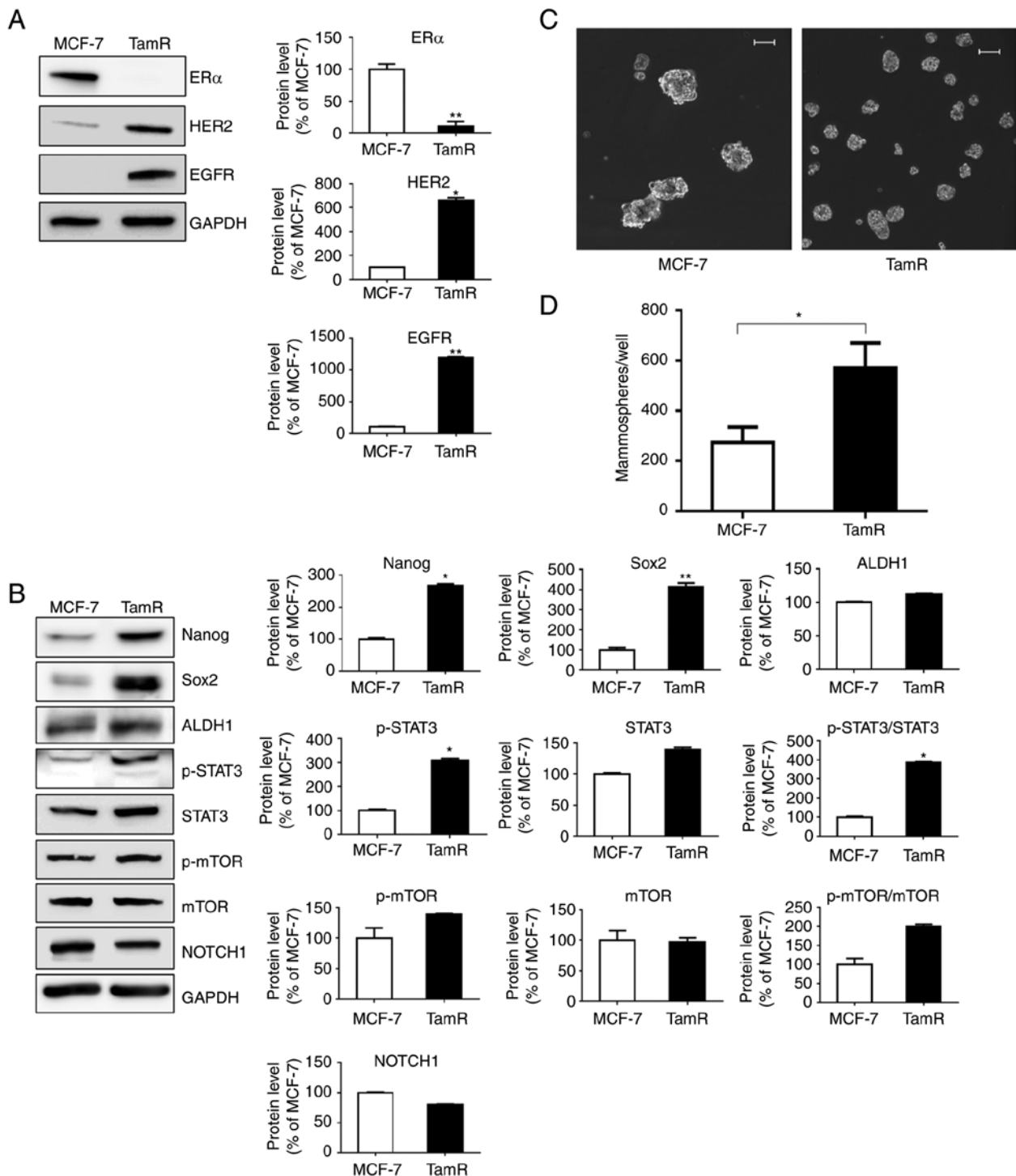


Figure 1. TamR cells have enhanced self-renewal properties of stem-like cells. (A) TamR cells exhibited decreased expression of ER $\alpha$  and increased expression of HER2 and EGFR. \* $P < 0.05$  and \*\* $P < 0.005$  vs. MCF-7 cells. (B) TamR cells exhibited overexpression of the markers of stem-like properties and cell signaling pathways compared with that in control MCF-7 cells. Conversely, among the markers related to cell signaling pathways, p-mTOR, mTOR, and NOTCH1 exhibited no difference between MCF-7 and TamR cells. Representative western blots are presented along with the densitometric analysis of the protein bands. (C) TamR cells exhibited a greater number of microspheres compared with that in MCF-7 cells. (D) The number of microspheres, with a diameter  $> 60 \mu$ m was counted. Scale bars, 100  $\mu$ m. Data are expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. The statistical hypothesis was examined using an unpaired Student's t-test. \* $P < 0.05$  and \*\* $P < 0.005$  vs. MCF-7 cells. TamR, tamoxifen-resistant MCF-7; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor; p-, phosphorylated.

combinatorial effect of palbociclib and ICG-001 on TamR cells, a cell viability assay was performed. First, the appropriate therapeutic dose of both drugs as the concentration of drug resulting in 50% inhibition of the viability (the inhibitory concentration 50 or IC<sub>50</sub> value) of naïve MCF-7 cells,

was determined. The IC<sub>50</sub> value of palbociclib was 25  $\mu$ M, whereas that of ICG-001 was 50  $\mu$ M (Fig. 3A and B). For palbociclib, no difference was observed between MCF-7 and TamR cells, and for ICG-001, TamR cells required relatively higher therapeutic concentrations than MCF-7 cells. Next, the

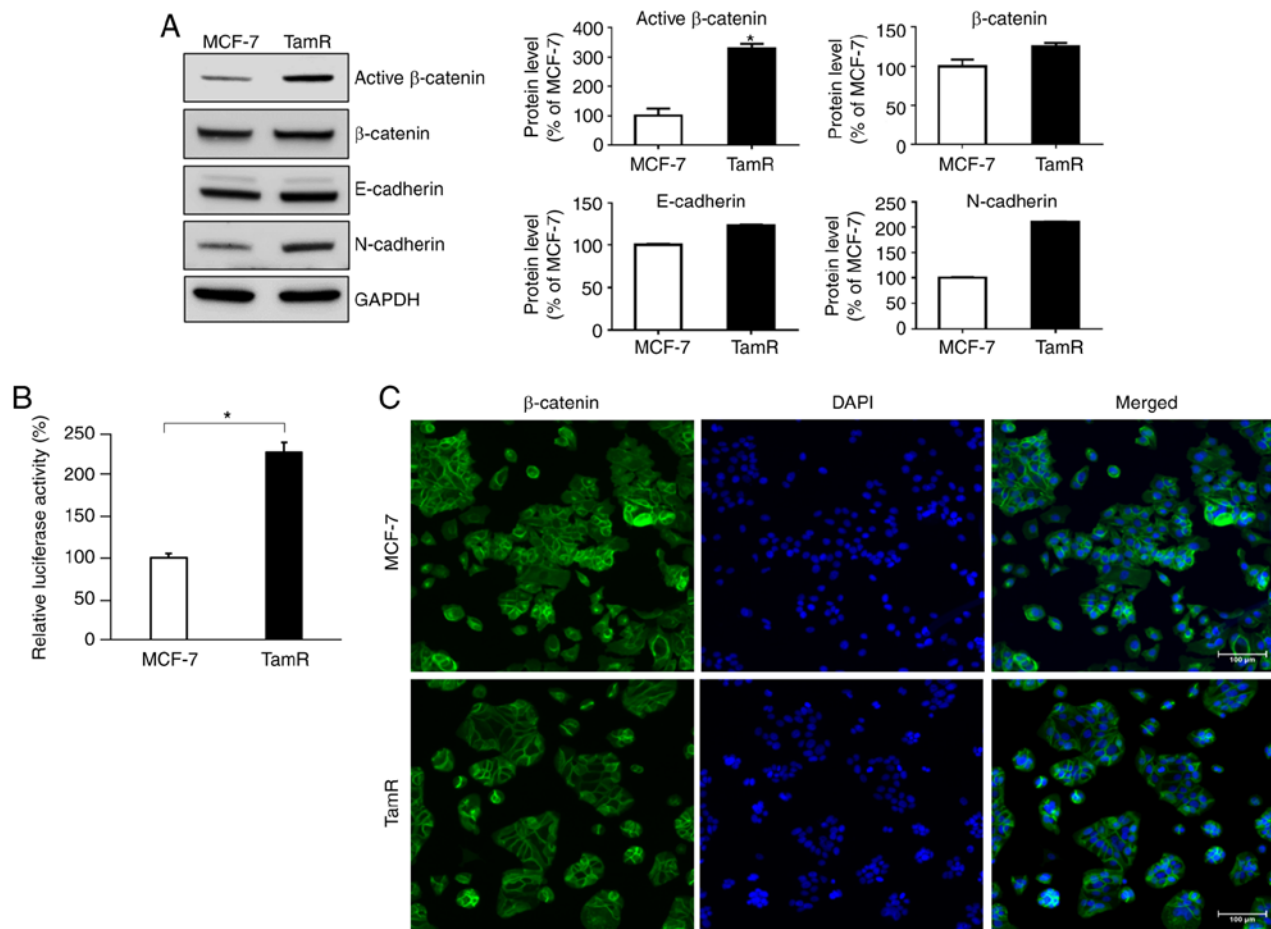


Figure 2. TamR cells have increased levels of active  $\beta$ -catenin. (A) Levels of active  $\beta$ -catenin were upregulated in TamR cells compared with that in MCF-7 cells. There was no difference in the expression of EMT markers, E-cadherin and N-cadherin. Representative western blots are presented along with densitometric data. (B) TamR cells exhibited an increase in the relative luciferase activity reflective of the increase in the level of active  $\beta$ -catenin. (C) Immunofluorescence images showing predominant distribution of  $\beta$ -catenin (green) in the nucleus of TamR cells. Nuclei were counterstained with DAPI (blue). Scale bars, 100  $\mu$ m. Data are expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. The statistical hypothesis was examined using an unpaired Student's t-test. \* $P < 0.05$  vs. MCF-7 cells. TamR, tamoxifen-resistant MCF-7; EMT, epithelial-mesenchymal transition; DAPI, 4',6-diamidino-2-phenylindole.

combination index (CI) values were determined to identify the synergistic effect of palbociclib and ICG-001. CI values  $< 0.8$ ,  $0.8-1.2$ , and  $> 1.2$  were defined as synergism, additive, and antagonism, respectively (31). The CI values of MCF-7 and TamR cells exposed to 25  $\mu$ M palbociclib and 50  $\mu$ M ICG-001 were  $1.1 \pm 0.04$  and  $1.1 \pm 0.02$ , respectively, representing additive effects (Fig. 3C). Likewise, the viability of TamR cells decreased by 29.8 and 57.5% upon treatment with palbociclib and ICG-001, respectively, and by 71% when treated concurrently with palbociclib and ICG-001.

Next, cell cycle analysis of MCF-7 and TamR cells treated with palbociclib and ICG-001 alone or in combination was performed (Figs. 3D and S1). Both the drugs suppressed the cell cycle progression in the two cell lines. Compared with vehicle-treated TamR cells, those treated with palbociclib and ICG-001 exhibited increased G0/G1 arrest. The percentage of cells exhibiting G0/G1 arrest was further increased in the combination treatment compared with that in the treatment with the two drugs individually.

*Combination therapy with palbociclib and ICG-001 inhibits stem cell-like properties.* A mammosphere assay was

conducted to visually confirm the effects of the two drugs (Fig. 4A and B). Treatment with palbociclib or ICG-001 significantly suppressed microsphere formation in TamR cells compared with that in the vehicle-treated cells. The suppression was greater in the combination treatment than in the treatment with the two drugs individually. However, there was no significant difference in P-values among the three treatment groups (palbociclib vs. ICG-001,  $P = 0.431$ ; palbociclib vs. combination,  $P = 0.900$ ; ICG-001 vs. combination,  $P = 0.225$ ).

The mechanism underlying the effect of the combination treatment was further elucidated. The expression levels of the markers of stem cell-like properties were determined (Fig. 4C). The expression levels of Nanog, Sox2, and ALDH1 were significantly reduced when TamR cells were treated with palbociclib (25  $\mu$ M) and ICG-001 (50  $\mu$ M) simultaneously, and the suppression of these proteins was similar in the combination therapy and palbociclib monotherapy.

*Active  $\beta$ -catenin and p-STAT3 are significantly associated with the combinatorial effect of palbociclib and ICG-001 on the reduction in stem cell-like properties.* To investigate the factors responsible for the reduction in stem cell-like

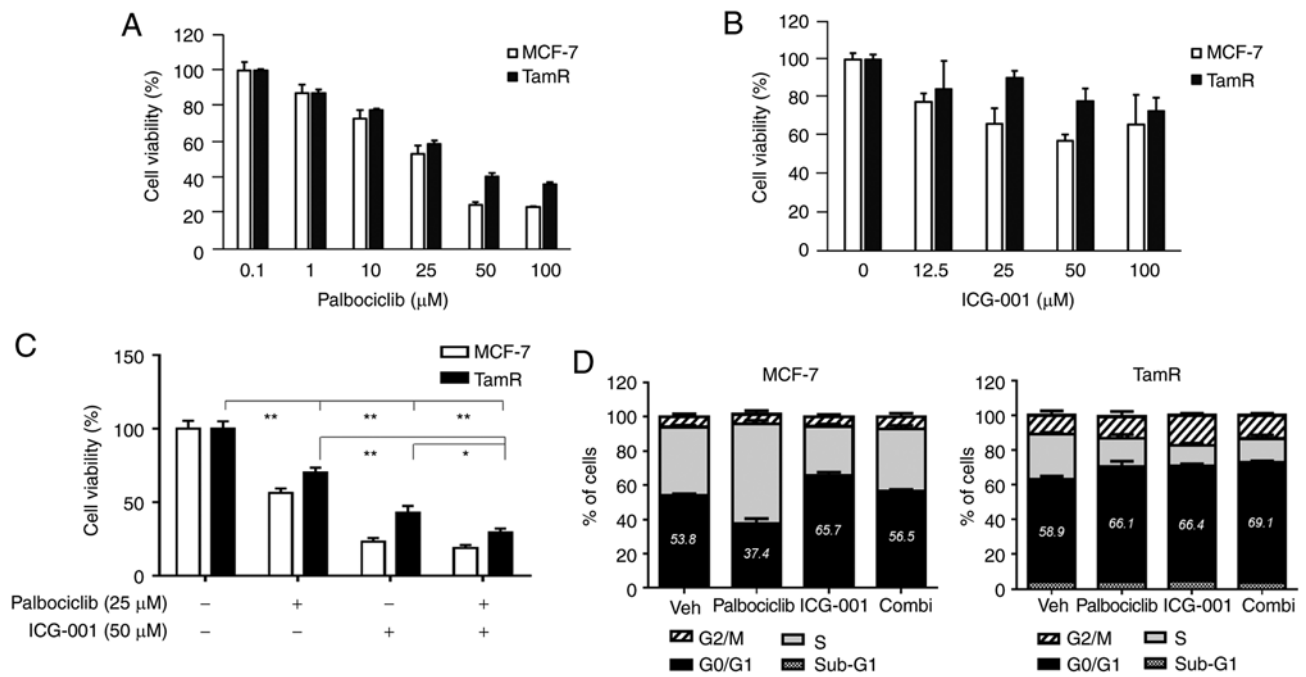


Figure 3. Combination therapy with palbociclib and ICG-001 results in additive cell growth inhibition in TamR cells. (A and B) Cell viability assays were performed to evaluate the effect of palbociclib and ICG-001 treatment on MCF-7 and TamR cells. (A) Palbociclib (25  $\mu$ M) reduced the viability of MCF-7 (46.9%) and TamR (41.4%) cells. (B) ICG-001 (50  $\mu$ M) reduced the viability of MCF-7 (42.4%) and TamR (21.8%) cells. (C) A statistical hypothesis was examined using a one-way ANOVA. Using 25  $\mu$ M palbociclib and 50  $\mu$ M ICG-001, the CI value was close to 1, confirming an additive effect. (D) The cell cycle was analyzed to confirm the mechanism of action of palbociclib and ICG-001 via flow cytometry. However, the effect was not statistically significant. Data are expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. \* $P$ <0.05 and \*\* $P$ <0.005. TamR, tamoxifen-resistant MCF-7; CI, combination index.

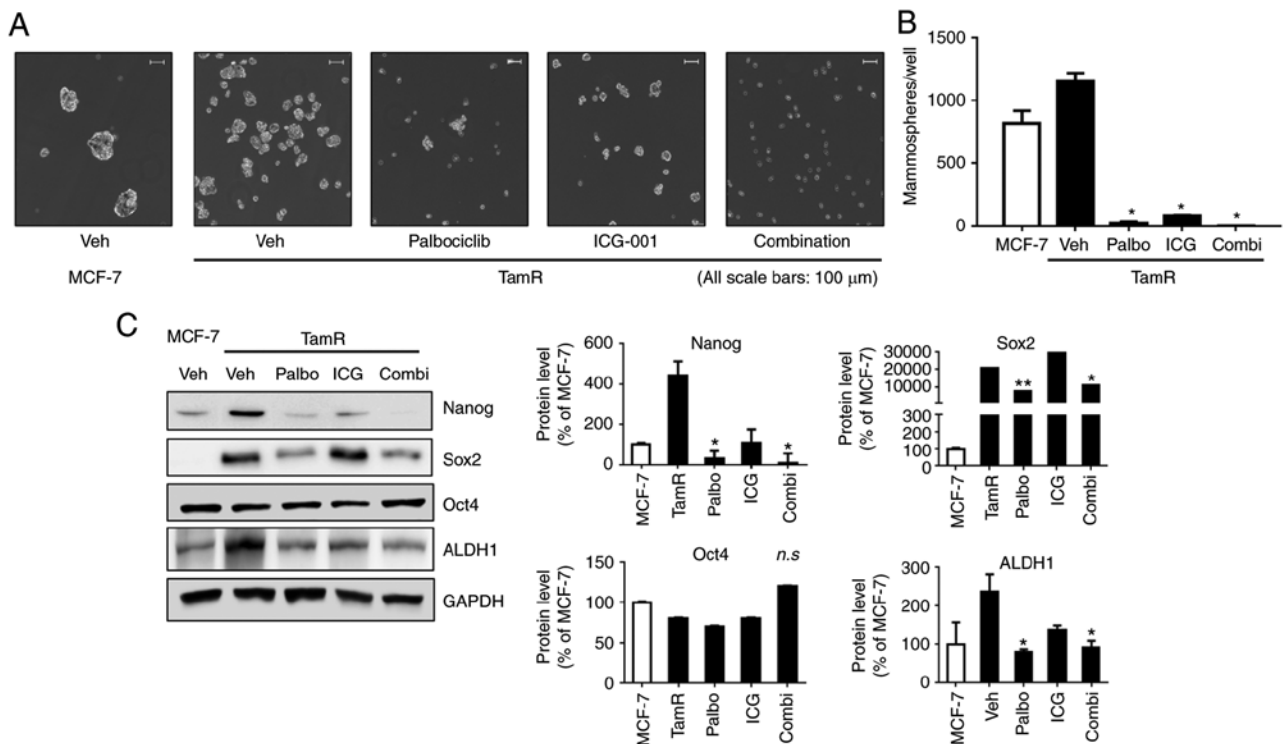


Figure 4. Combination therapy with palbociclib and ICG-001 inhibits stem cell-like properties. (A and B) For sphere formation, cells were seeded in 6-well ultra-low attachment plates, treated with palbociclib (25  $\mu$ M) and ICG-001 (50  $\mu$ M), and incubated for 7 days in a 37°C CO<sub>2</sub> incubator. The number of mammospheres, with a diameter >60  $\mu$ m, was counted. The experiment was performed in triplicate, and the average number of microspheres was as follows: TamR\_P (n=27.50), TamR\_I (n=83.50), and TamR\_C (n=5.00). TamR\_P, palbociclib; TamR\_I, ICG-001; TamR\_C, combination. (C) The expression of protein markers of stem cell-like properties in cells treated with the drugs was assessed. Nanog, Sox2, and ALDH1 were downregulated in TamR cells treated with palbociclib (25  $\mu$ M) and ICG-001 (50  $\mu$ M) in combination. Representative western blots are presented along with densitometric data. Data were expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. The statistical hypothesis was examined using a one-way ANOVA. \* $P$ <0.05 and \*\* $P$ <0.005 vs. TamR vehicle-treated cells. TamR, tamoxifen-resistant MCF-7; n.s., not significant.



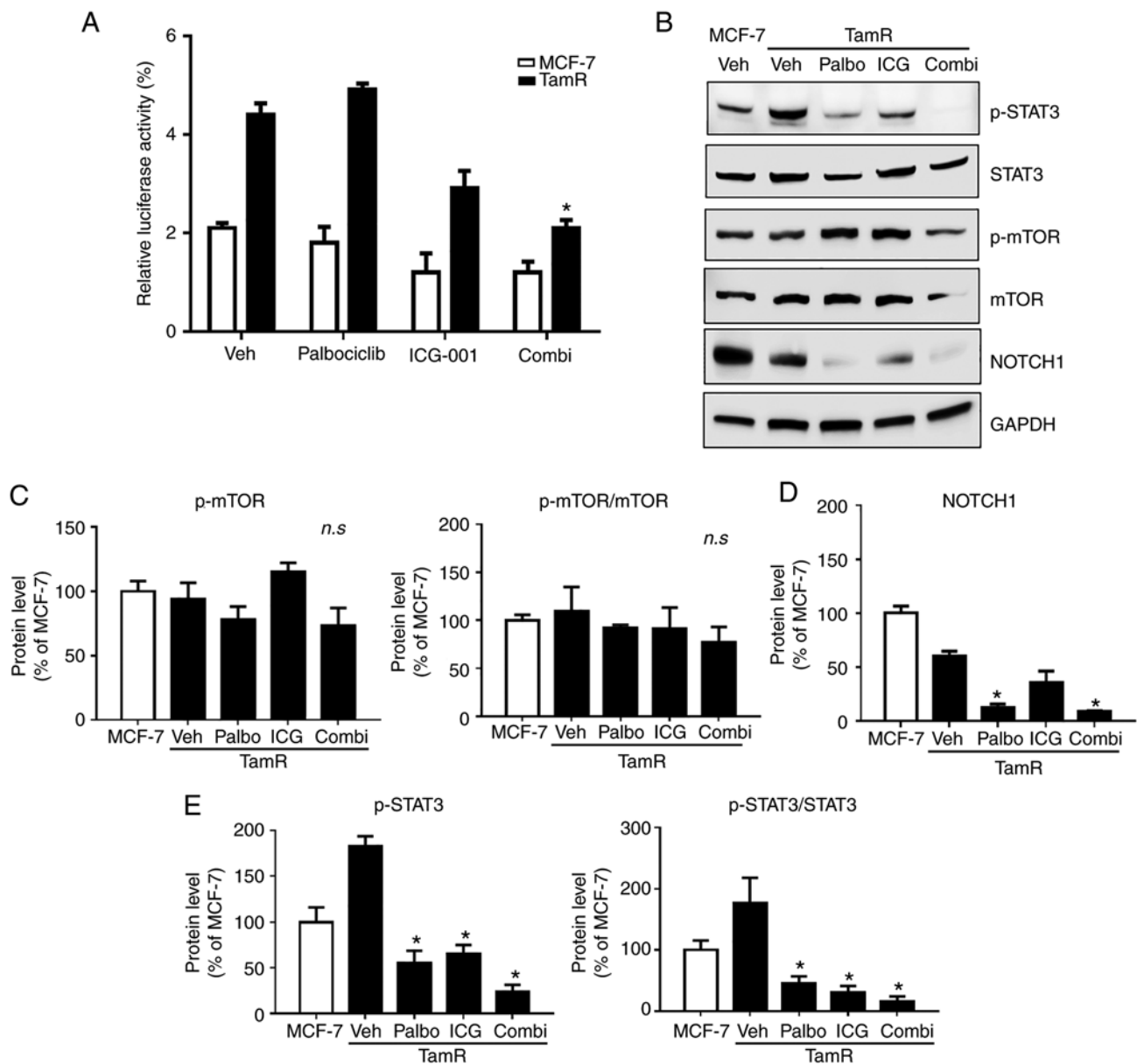


Figure 5. Active  $\beta$ -catenin and p-STAT3 are significantly associated with the combinatorial effect of palbociclib and ICG-001 on the reduction in the stem-like properties. (A) A luciferase assay was conducted to confirm the inhibitory effect of palbociclib and ICG-001 on active  $\beta$ -catenin. The levels of active  $\beta$ -catenin were reduced when cells were treated with ICG-001. Moreover, a combinatorial effect wherein the levels of active  $\beta$ -catenin were significantly reduced when TamR cells were treated with a combination of the drugs was observed. All experiments were conducted at least three times. (B-E) Expression of proteins related to cell signaling pathways was investigated in TamR cells treated with palbociclib (25  $\mu$ M) and ICG-001 (50  $\mu$ M). Representative western blots are presented along with densitometric data. The p-STAT3 protein expression was reduced when combination therapy was used. Data are expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. The statistical hypothesis was examined using a one-way ANOVA. \* $P < 0.05$  vs. TamR vehicle-treated cells. TamR, tamoxifen-resistant MCF-7; p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; n.s., not significant.

properties, the levels of active  $\beta$ -catenin and markers of cell signaling pathways were assessed. First, a luciferase assay was performed to evaluate the inhibitory effect of palbociclib and ICG-001 on active  $\beta$ -catenin levels (Fig. 5A). Compared with those in MCF-7 cells, the levels of active  $\beta$ -catenin levels were significantly increased in TamR cells. Treatment of TamR cells with ICG-001 downregulated the levels of active  $\beta$ -catenin compared with vehicle-treated TamR cells. Moreover, when TamR cells were simultaneously treated with the two drugs, the levels of active  $\beta$ -catenin were significantly reduced compared with vehicle-treated TamR cells, indicating a combinatorial effect. Second, using western blot

analysis, the expression of markers related to cell signaling pathways was determined (Fig. 5B-E). The level of p-STAT3 in TamR cells was reduced upon treatment with palbociclib and ICG-001 compared with those in the vehicle-treated cells. The levels of p-STAT3 were significantly reduced ( $P < 0.05$ ) in the combination treatment group compared with those in the vehicle-treated cells; however, the reduction was not significant when compared with the levels in the palbociclib and ICG-001 treatment groups ( $P = 0.990$  and  $P = 0.978$ , respectively). No significant change in the expression of p-mTOR was noted in the different treatment groups. The expression of NOTCH1 in vehicle-treated TamR cells was reduced compared with that

in MCF-7 cells; therefore, it was difficult to confirm whether this protein was involved in tamoxifen resistance before the cells were treated with the drugs. Based on the aforementioned results, it is strongly considered that the cell signaling pathway proteins, including p-STAT3, and the decrease in the levels of active  $\beta$ -catenin, are associated with the reduction in stem cell-like properties of endocrine-resistant TamR cells treated with the combination of palbociclib and ICG-001 compared with those of parental MCF-7 cells.

*Suppression of STAT3 does not affect the level of active  $\beta$ -catenin.* To investigate the crosstalk between  $\beta$ -catenin and STAT3 signaling, changes in the expression of STAT3 and  $\beta$ -catenin in cells treated with S3I-201, a targeted inhibitor of STAT3, were evaluated via western blot analysis and luciferase assay (Fig. 6A-C). The appropriate therapeutic dose of S3I-201 as the concentration of drug resulting in the  $IC_{50}$  of naïve MCF-7 cells was determined, and the  $IC_{50}$  value of S3I-201 was 100  $\mu$ M. When treated with S3I-201 (100  $\mu$ M), the expression of p-STAT3 and STAT3 was downregulated, indicating that S3I-201 worked, as expected. However, there was no change in the levels of active  $\beta$ -catenin in the luciferase assay and western blot analysis. Moreover, the expression of Sox2 was reduced after drug treatment, which shows its association with the STAT3 signaling pathway. The crosstalk between STAT3 and  $\beta$ -catenin was also confirmed using the siRNA transfection technique (Fig. 6D and E). Following siRNA transfection, reduction in the levels of p-STAT3 and STAT3 was confirmed using western blot analysis; however, the levels of  $\beta$ -catenin remained unchanged regardless of the STAT3 knockdown. Experiments with two different techniques demonstrated that STAT3 did not affect the levels of active  $\beta$ -catenin. Therefore, this additional mechanistic insight suggests that  $\beta$ -catenin may be involved in STAT3 signaling, leading to the theory that STAT3/Sox2 signaling may be involved in the reduction in the stem cell-like property.

## Discussion

Endocrine therapy has been the mainstay of ER<sup>+</sup> breast cancer treatment modalities over the last 60 years; however, endocrine resistance is inevitable (1). Recently, CDK4/6 inhibitors and PIK3CA pathway inhibitors have been used worldwide in clinical settings. Nevertheless, one-third of patients administered CDK4/6 inhibitors experienced recurrence within 2 years, and over 70% of patients treated with palbociclib experienced disease progression by 40 months in the PALOMA2 clinical trial (32). Thus, there is a need to develop additional therapeutic agents to treat patients with endocrine-resistant breast cancer.

In the present study, a model is proposed, in which the inhibition of  $\beta$ -catenin levels in combination with the administration of CDK4/6 inhibitors, which are, currently, the standard treatment for endocrine-resistant breast cancer, may be a reasonable treatment alternative. The levels of  $\beta$ -catenin increased in the tamoxifen-resistant breast cancer cell line, and inhibition of  $\beta$ -catenin in the nucleus suppressed stem cell-like properties. In particular, combining a  $\beta$ -catenin blocker with a conventional CDK4/6 inhibitor accelerated STAT3 suppression.

Contribution of the JAK/STAT3 signaling pathway to the hormone resistance mechanism in breast cancer has been reported in a previous study (33). In addition, STAT3 is a transcription factor that regulates cell proliferation and survival and is also involved in the growth of stem-like cells in breast cancer (14). In the present study, it was observed that STAT3, similarly to  $\beta$ -catenin, was upregulated in TamR cells and was associated with tamoxifen resistance (Fig. 1B). In particular, STAT3 may influence the additive effects of palbociclib and ICG-001 by reducing the levels of p-STAT3 (Fig. 5B and E). The results demonstrated that p-STAT3 was the main reason for the combinatorial effect of ICG-001 and palbociclib; however, this was not statistically significant when compared with the effects of treatments with palbociclib ( $P=0.990$ ) and ICG-001 ( $P=0.978$ ) individually. Nevertheless, in terms of cell viability, a clear combinatorial effect was observed when compared with the effects of treatments with the drugs individually. In addition, ICG-001 sufficiently contributed to the reduction in the levels of active  $\beta$ -catenin. This suggests that the inhibition of active  $\beta$ -catenin by ICG-001 and the consequent underactivation of the STAT3 signaling pathway may have an additive effect on the role of palbociclib in terms of cell growth inhibition.

Although several studies have been conducted to elucidate the signaling pathway between  $\beta$ -catenin and STAT3, it remains unclear (34,35). Armanious *et al* reported that STAT3 upregulates the expression of  $\beta$ -catenin and its transcriptional activity in breast cancer cells. They found a binding sequence for STAT3 in the  $\beta$ -catenin gene promoter through DNA sequence analysis and confirmed the amplification product using chromatin immunoprecipitation (35). In contrast, Yan *et al* reported that  $\beta$ -catenin upregulates the expression of STAT3 in esophageal squamous cell carcinoma. These authors scanned the human STAT3 promoter and found matching sequences for five T-cell factor (TCF)-binding protein elements; it was confirmed that TCF4 binds to the human STAT3 promoter. They performed RT-PCR and western blot analysis of several esophageal squamous cell lines to demonstrate that overexpression of  $\beta$ -catenin upregulates the mRNA and protein levels of STAT3 (34). Recent studies further support the notion that  $\beta$ -catenin regulates STAT3. Huang *et al* reported that  $\beta$ -catenin binds to the predicted promoter region of STAT3, as assessed using the chromatin immunoprecipitation assay in prostate cancer, and that a specific inhibitor of  $\beta$ -catenin (XAV-939) partially reduces its binding activity (36). Kawasaki *et al* reported that LGR5 activates  $\beta$ -catenin in intrahepatic cholangiocarcinoma, and that activated  $\beta$ -catenin further activates STAT3 to enhance cancer stem-like properties and epithelial-mesenchymal transition (37). To elucidate the association between  $\beta$ -catenin and STAT3, western blot analysis was performed after treatment of cells with a STAT3 inhibitor or after subjecting them to STAT3 knockdown to identify the nuclear/cytoplasmic fraction of  $\beta$ -catenin; however, no changes in the nuclear translocation of  $\beta$ -catenin were noted (data not shown). Therefore, after treatment of cells with the STAT3 inhibitor or siSTAT3, the levels of active  $\beta$ -catenin level were additionally verified using luciferase assay and were found to be unaltered (Fig. 6). This indicates that STAT3 does not regulate  $\beta$ -catenin activity. The results of the present study showed a reduction



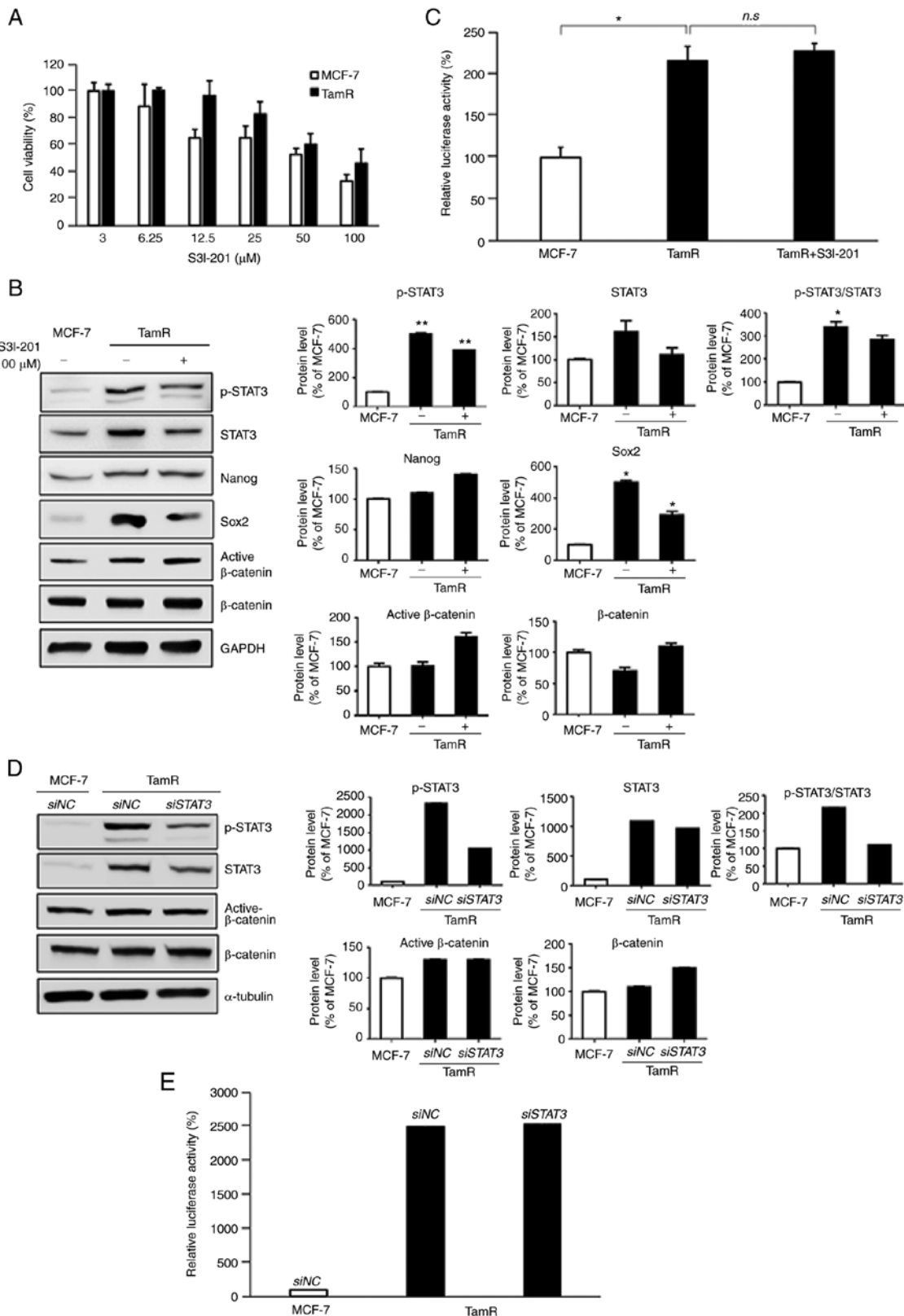


Figure 6. Suppression of STAT3 does not affect the levels of active  $\beta$ -catenin. (A) The cell viability assay of MCF-7 and TamR cells treated with S3I-201. Treatment with S3I-201 (100  $\mu$ M) reduced the viability of MCF-7 (66.7%) and TamR (53.5%) cells. The experiment was performed in triplicate. (B) Expression of proteins related to cell signaling pathways and markers of stem-like properties was investigated. Representative western blots are presented along with densitometric data. In cells treated with S3I-201 (100  $\mu$ M), the expression of Sox2, a marker of stem-like property, was reduced. Data are expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. The statistical hypothesis was examined using a one-way ANOVA. \* $P < 0.05$  and \*\* $P < 0.005$  vs. MCF-7 cells. (C) There was no inhibitory effect of S3I-201 (100  $\mu$ M) on the levels of active  $\beta$ -catenin, as assessed using the luciferase assay. Data are expressed as the mean  $\pm$  SD of values from three independent experiments under the same conditions. (D) Western blot analysis confirmed the knockdown of STAT3; however, siSTAT3 had no inhibitory effect on the levels of active  $\beta$ -catenin, as assessed using the western blot analysis. Representative western blots are presented along with densitometric data. (E) The results of a luciferase assay confirmed the change in the levels of active  $\beta$ -catenin with the use of siSTAT3; there was no inhibitory effect of siSTAT3 on active  $\beta$ -catenin levels. TamR, tamoxifen-resistant MCF-7; STAT3, signal transducer and activator of transcription 3; p-, phosphorylated; si, small interfering; siNC, siRNA negative control; n.s., not significant.

in the expression of p-STAT3 upon inhibition of  $\beta$ -catenin. Therefore, it is consistent with previous findings that  $\beta$ -catenin regulates STAT3 activity (34,36,37).

The present study has some limitations. First, in the experiments, the known targets were not considered. For example, the mechanism underlying the activation of cell cycle regulation by the upregulation of cyclin D1 through  $\beta$ -catenin/TCF signaling is already known and was, therefore, not verified in the present study. Comparison of the reduction in the stem-like properties and cell growth inhibition induced by  $\beta$ -catenin was focused on, to evaluate the effect of the combination treatment versus that of the existing CDK4/6 inhibitor. Second, the mechanism by which CDK4/6 inhibitors reduce the levels of STAT3 was not clearly elucidated. The IL-6/STAT3 pathway has been reported to be induced by a mechanism of acquired resistance to CDK4/6 inhibitors (38). However, the interaction with STAT3 during initial treatment with CDK4/6 inhibitors remains unclear. To the best of our knowledge, there are no reports of an association between treatment with CDK4/6 inhibitors and STAT3 levels. In this context, further experiments should be designed to evaluate this association.

In conclusion, it was revealed that  $\beta$ -catenin is activated in endocrine-resistant breast cancer and that the antitumor effects of conventional CDK4/6 inhibitors are further potentiated by  $\beta$ -catenin blockers. This suggests that  $\beta$ -catenin blockers may be a reasonable treatment alternative for endocrine-resistant breast cancer.

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

AH and KL contributed to the conception and design of the experiments. MC contributed to the acquisition of the experimental data. AH, MC, JJ, HW and KL contributed to the analysis of the data. AH drafted and revised the manuscript. HW and KL also revised the manuscript. AH and KL confirm the authenticity of all raw data. KL assisted with the funding. All authors critically reviewed, and have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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