Dinaciclib inhibits the stemness of two subtypes of human breast cancer cells by targeting the FoxM1 and Hedgehog signaling pathway

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Abstract. Cyclin-dependent kinase (CDK)4/6 inhibitors in combination with endocrine therapy are the current standard of care used in the first-line treatment of hormone receptor-positive/HER2-negative metastatic breast cancer (BC). Although CDK4/6 inhibitors mainly target the cell cycle, emerging evidence has indicated further potential roles of CDKs other than regulating cell cycle progression. The G_1 and G_2/M transition regulators, including cyclins D and E, as well as their catalytic partners, CDK2, CDK4 and CDK6, have been reported to play crucial roles in pluripotency maintenance and cell fate decisions of human pluripotent stem cells by controlling transcription factors, signaling pathways and epigenetic regulators. Dinaciclib, a CDK1/2/5/9 inhibitor, is currently being evaluated in clinical trials against various cancer types, including BC. However, the underlying molecular mechanisms of CDK1/2/5/9 inhibitors in regulating BC stemness remain poorly understood. The present study aimed to examine the stemness-inhibitory effects of dinaciclib in MCF-7 (luminal) and HCC-1806 (triple-negative) BC cells. We found that this drug not only effectively reduced the self-renewal abilities and other malignant properties, but also dose-dependently decreased the protein expression levels of three BC stem cell markers, CD44, aldehyde dehydrogenase 1 family member A1 (ALDH1A1) and BMI1 proto-oncogene, polycomb ring finger (Bmi1), as well as three embryonic stem cell markers, Oct4, Nanog and Sox2. Moreover, the dinaciclib-induced decrease of Oct4 and Nanog protein expression was able to be restored by co-treatment with MG-132, a proteasome inhibitor. Forkhead box M1 (FoxM1), both a stemness-stimulating transcription factor and a cell cycle regulator, along with the Hedgehog signaling pathway, were identified as the therapeutic targets of dinaciclib. Collectively, the present results demonstrated a novel role of dinaciclib in suppressing BC stemness and indicated its potential use for future cancer treatments.

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Introduction

Breast cancer (BC) is one of the most common cancer types and a leading cause of cancer-related mortality among women worldwide, and it can be classified clinically based on the expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 (1,2). In total, ~80% of patients with BC are hormone receptor-positive and usually respond well to hormone therapy (3). Patients who are $HER2^+$ can be treated more effectively with targeted anti-HER2 treatment, such as trastuzumab. On the other hand, patients with triple-negative BC (TNBC) respond poorly to hormone or targeted therapy, and chemotherapy remains the primary treatment option (4,5).

Molecularly, BCs are classified into four groups that largely overlap with the clinical subtypes (6). Luminal A and B are characterized by positive ER and/or PR expression, and generally have a more favorable prognosis (6). Luminal A usually has high ER/PR expression and is negative for HER2 expression, while luminal B has lower ER/PR expression and is associated with a slightly worse prognosis than luminal A (6). HER2-enriched cancer types exhibit amplification and upregulation of the HER2 gene (6). Basal cancer types are usually triple-negative, with minimal expression of ER/PR and no HER2 amplification, and they are associated with aggressive pathology and a poor prognosis (6).

Cancer stem cells (CSCs) are a small subpopulation accounting for 1-5% of cell types within the tumor, which have the potential to initiate clonal tumors to drive tumorigenesis and maintain the heterogeneity of the tumor through maintaining their self-renewal and differentiation abilities (7-9). Moreover, these cells contribute to treatment resistance and recurrence of tumors (7,10). Thus, identifying methods to eradicate CSCs may be the key to curing cancer. In this regard, $CD44^+/CD24^{-/low}$, aldehyde dehydrogenase 1 family member A1 (ALDH1) and BMI1 proto-oncogene, polycomb ring finger (Bmi1) were shown to be common

markers for breast (B)CSCs, and signaling pathways including Hedgehog, Wnt and Notch were found to be essential for their self-renewal (10).

Cyclin-dependent kinases (CDKs) are a group of serine/threonine kinases that require the binding of various cyclins to form different CDK/cyclin complexes to become activated (11), and most of them are known to play a role in cell cycle progression (12-15). Furthermore, CDK1 and CDK2 are also involved in the regulation of pluripotency and differentiation during embryonic development (16-18). Intriguingly, other CDKs play important roles in the self-renewal of SCs, neuronal functions and transcriptional regulation (8,19-22).

Malignant cells are defined by their uncontrolled proliferation, which often occurs as a result of malfunctioning cell cycle checkpoints (23). Accumulating evidence has indicated that a high expression of various CDKs and cyclins is correlated with poor prognosis, therapy resistance, tumor recurrence and CSC maintenance in BC (8,14). Several CDK4/6 inhibitors, including palbociclib, ribociclib and abemaciclib, have recently been approved by the US Food and Drug Administration for combined use with letrozole or other aromatase inhibitors in the first-line treatment of patients with hormone receptor-positive advanced BC (24-26). However, newly emerging evidence has suggested that ER-positive BC could develop resistance to these CDK4/6 inhibitors (27-29). Interestingly, drugs against other CDKs, such as dinaciclib (i.e., SCH727965), are being evaluated clinically for treating patients with advanced or metastatic TNBC (30,31) as CDK1 inhibition has been reported to trigger the apoptosis of human basal-like TNBC cells (32). Furthermore, CDK2 inhibition has been revealed to not only decrease the $CD44^{+}/CD24^{-/low}$ stem-like subpopulation, but also restore the chemosensitivity of SUM149PT TNBC cells (18).

The embryonic SC (ESC) markers Oct4, Nanog and Sox2 are transcription factors (TFs) that are not only highly expressed in ESCs, but they also work together to regulate a set of target genes that have important functions in ESC pluripotency (33). Interestingly, CDK2/cyclin E and CDK6/cyclin D3 complexes have been shown to phosphorylate these ESC markers, thereby promoting their interactions with peptidylprolyl *cis/trans* isomerase, NIMA-interacting 1 (Pin1), which protects them from polyubiquitination and proteasomal degradation (34). Forkhead box M1 (FoxM1) is another TF the activity of which has also been observed to be stimulated by various CDK/cyclin complexes in either a phosphorylation-dependent (35) or -independent (36) manner. More importantly, the aforementioned TFs have been shown to be involved in the activation of the Hedgehog and Wnt signaling pathways that are crucial for the maintenance of BCSCs (37-39).

Therefore, the present study aimed to investigate the cytotoxic and the stemness-inhibitory effects of dinaciclib in two human BC cell lines, MCF-7 (luminal A) and HCC-1806 (TNBC), as well as to elucidate the mechanisms underlying its stemness-suppressive effects.

Materials and methods

Cell culture. MCF-7 and HCC-1806 human BC cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM and RPMI-1640 medium, respectively, which were supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (1% PSA; Biological Industries, USA) at 37˚C with 5% CO_2. MDA-MB-231 cells purchased from the ATCC were maintained in Leibovitz's L-15 medium supplemented with 10% FBS and 1% PSA at 37˚C without CO_2.

MTT assay. MCF-7 ($2x10^3$) and HCC-1806 ($5x10^3$) cells were seeded into each well of 96-well plates the day before the indicated concentrations of dinaciclib were added into the wells. After a 72-h incubation, 1 mg/ml MTT reagent (Sigma-Aldrich; Merck KGaA) was added into each well for a 3-h incubation before the MTT medium was removed and replaced with DMSO to solubilize the formazan crystals. The optical density at 570 nm (OD_{570}) of each well was measured using an ELISA reader (Bio-Rad Laboratories, Inc.). Cells treated with 0.1% DMSO alone were considered as untreated cells and their viability was designated as 100%. The half maximal inhibitory concentration (IC_{50}) values of drug in each cell line were calculated using GraphPad Prism software version 4.0 (GraphPad Software, Inc.).

Western blotting. For total lysate preparation, the cells were washed with cold PBS and scraped into 1 ml cold PBS. After centrifugation, the cells were lysed in RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS and 1% Nonidet P-40 (NP-40); pH 7.4]. To prepare the nuclear fraction, cells were first incubated in a low-salt buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP-40; pH 7.4) and the nucleus was then pelleted via centrifugation. Nuclear proteins were subsequently extracted using a high-salt buffer (20 mM HEPES, 25% glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA; pH 7.9) on ice. Total lysates (20 μg) or nuclear proteins (40 μg) were separated via 10% SDS-PAGE and processed for blocking using 5% skim milk in 1X TBS-Tween 20 (TBST) for 1 h at room temperature. Next, immunoblotting was performed using primary antibodies against β-actin (1:13,000; cat. no. MAB1501; MilliporeSigma), FoxM1 (1:2,000; cat. no. GTX100276; GeneTex, Inc.), phosphorylated (p-)FoxM1 (Ser35; 1:1,000; cat. no. 14170; Cell Signaling Technology, Inc.), CD44 (1:2,000; cat. no. GTX102111; GeneTex, Inc.), ALDH1A1 (1:5,000; cat. no. GTX123973; GeneTex, Inc.), Bmi1 (1:2,000; cat. no. GTX114008; GeneTex, Inc.), Oct4 (1:1,000; cat. no. GTX101497; GeneTex, Inc.), Nanog (1:1,000; cat. no. GTX100863; GeneTex, Inc.), Sox2 (1:1,000; cat. no. GTX101506; GeneTex, Inc.), GLI family zinc finger 1 (GLI1; 1:1,000; cat. no. sc-20687; Santa Cruz Biotechnology, Inc.) and Lamin A/C (1:5,000; cat. no. E18-6056-2; EnoGene). After an overnight incubation at 4˚C, the blots were washed several times with 1X TBST before being probed with the HRP-conjugated secondary antibodies, including anti-mouse IgG or anti-rabbit IgG. Signals were detected using an ECL reagent (T-Pro Biotechnology) under a Luminescence Imaging system (FUJIFILM LAS-4000; FUJIFILM Wako Pure Chemical Corp.) and their intensities were semi-quantified via densitometry (ImageJ software version 1.50i; National Institutes of Health).

Sphere formation assay. MCF-7 ($1x10^4$) and HCC-1806 ($2x10^4$) cells were seeded on low attachment dishes containing DMEM and RPMI-1640 medium, respectively, supplemented with 2% B27 (cat. no. 17504044; Thermo Fisher Scientific, Inc.), 20 ng/ml EGF (PeproTech, Inc.), 10 ng/ml basic fibroblast growth factor (PeproTech, Inc.), insulin (5 μg/ml), 0.4% BSA and 1% PSA (referred to as defined media) in the absence or presence of various doses of dinaciclib. MCF-7 cells were cultured for 14 days, while HCC-1806 cells were cultured for 21 days before the spheres formed, following which cells were stained with 1 mg/ml MTT reagent at room temperature and counted using MetaMorph software version 7.8 (Molecular Devices LLC).

Sphere shrinkage assay. MCF-7 ($1x10^4$) and HCC-1806 ($2x10^4$) cells were seeded on low attachment dishes containing defined media for 8 and 10 days, respectively, before being treated without or with various doses of dinaciclib. After 10 days, the number of spheres was counted as described in the '*Sphere formation assay*'.

Soft agar colony formation assay. MCF-7 ($5x10^2$) and MDA-MB-231 ($5x10^3$) cells were resuspended in 2.7 ml DMEM and Leibovitz's L-15 medium, respectively, without or with varying doses of dinaciclib, before being mixed with 0.3 ml pre-warmed (55˚C) 3% agarose solution (in media). The cell suspension was then seeded into three different wells of a 6-well plate (1 ml/well) that were pre-coated with 2 ml 0.6% agarose in media. After 3 weeks of culture, during which the media were replenished every 3 days, the colonies were stained with MTT and counted using MetaMorph software version 7.8 (Molecular Devices LLC).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 μg RNA was reverse transcribed using a MMLV RT kit (Thermo Fisher Scientific, Inc.). SYBR Green-based quantitative PCR analysis was then carried using the CFX Connect™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) with primer sets designed to analyze the expression of specific genes including: *FOXM1* forward, 5'-CGTGGATTGAGGACCACTTT-3' and reverse, 5'-TCTGCTGTGATTCCAAGTGC-3'; *ACTB* (β-actin) forward, 5'-TGGCATTGCCGACAGGAT-3' and reverse, 5'-GCTCAG GAGGAGCAATGATCT-3'; *GLI1* forward, 5'-GTGCAAGTC AAGCCAGAACA-3' and reverse, 5'-ATAGGGGCCTGACTG GAGAT-3'; *PTCH1* forward, 5'-ACAAACTCCTGGTGCAAA CC-3' and reverse, 5'-CTTTGTCGTGGACCCATTCT-3'; and Importin-7 (*IPO7*) forward, 5'-TCTGAAGGCATTTGCTGT TG-3' and reverse, 5'-TGCCTTGTATATGGGGCTTC-3'. The reaction conditions were: Initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 30 sec, 65˚C for 30 sec and 72˚C for 30 sec. The relative quantity of target gene expression was calculated using the comparative Cq method (ΔΔCq), which was normalized to endogenous β-actin levels using CFX Manager version 3.1 (Bio-Rad Laboratories, Inc.).

Examination of the involvement of the proteasome in dinaciclib-induced decreases of the protein expression levels of FoxM1 and three ESC markers in MCF-7 cells. Total lysates (20 μg) prepared from MCF-7 cells, after they were treated without or with dinaciclib (10 nM) in the absence or presence of 10 μM MG-132 (cat. no. BML-PI102-0005; Enzo Life Sciences) for 24 h, were subjected to western blot analyses using primary antibodies against FoxM1, p-FoxM1, Oct4, Nanog and Sox2.

Establishment of the FoxM1-knockdown stable clones from MCF-7 and MDA-MB-231 cells

Preparation of the lentivirus. The day before transfection, $8x10^5$ 293 cells were seeded into 6-cm dishes in DMEM. A DNA mixture containing 0.25 μg pMDG, 2.25 μg pCMV-dR8.91 and 2.5 μg pLKO-FoxM1 short hairpin (sh)RNA (National RNAi Core Facility, Academia Sinica, Taipei, Taiwan; sh1/shA, 5'-GCCAATCGTTCTCTGACA GAA-3'; sh2/shB, 5'-AGGACCACTTTCCCTACTTTA-3') was added into serum-free DMEM to a final volume of 100 μl. Moreover, 7.5 μl PolyJet™ reagent (Signagen Laboratories LLC) was mixed with serum-free DMEM to a final volume of 100 μl. The diluted PolyJet™ reagent was then added to the diluted DNA mixture, which was placed at room temperature for 15 min to allow the formation of PolyJet/DNA complexes. Cells were then treated with the aforementioned complexes at 37˚C for 16 h before the medium was replaced with medium containing 1% BSA. Viruses presenting in the medium were collected respectively at 24 and 48 h post-medium change and were stored at -80˚C after being passed through a 0.22-μm filter (MilliporeSigma).

Selection of stable clones. To establish the FoxM1-knockdown stable clones from MCF-7 and MDA-MB-231 cell lines, cells were infected with the aforementioned viruses expressing shRNA against FoxM1 for 48 h, before being placed in a selection medium containing 1.5 μg/ml puromycin. Single clones (designated as sh1 and sh2) were selected and expanded, and the FoxM1 protein and mRNA expression levels of them were examined via western blotting and RT-qPCR analyses, respectively. The self-renewal abilities, as well as the protein levels of various stemness markers and GLI1, in these clones were analyzed as aforementioned.

Establishment of doxycycline-inducible FoxM1-expressing HCC-1806 cells. The day before transfection, $6x10^5$ HCC-1806 cells were seeded into 6-cm dishes containing RPMI-1640 medium. The plasmid pCW57.1-FOXM1b (Addgene, Inc.), which carries a *FOXM1* gene whose expression could be induced by doxycycline, was added into serum-free RPMI-1640 medium to reach a final volume of 100 μl. Furthermore, 7.5 μl PolyJet™ reagent was mixed with serum-free RPMI-1640 medium to reach a final volume of 100 μl. Both mixtures were left at room temperature for 15 min, before being mixed and added to cells at 37˚C for 16 h. The transfected cells were selected using selection medium containing 1.5 μg/ml puromycin and single clones were selected and expanded as aforementioned.

Doxycycline treatments. The aforementioned stable clones established from HCC-1806 cells were then treated with varying concentrations of doxycycline (0, 0.5, 1 and 2 μg/ml) at 37˚C for 72 h before western blotting was performed to determine

what clones and concentration of doxycycline should be used for the subsequent experiments. In total, 1 μg/ml doxycycline was selected for inducing FoxM1 expression in HCC-1806 cells to assess the effects of FoxM1 upregulation on the cytotoxicity and sphere-forming inhibition of dinaciclib in these cells.

Statistical analysis. Data are presented as the mean ± SD of three independent experiments. Significant differences between untreated or wild-type cells were determined by multiple comparison procedure using one-way ANOVA with Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Examination of the cytotoxic effects of dinaciclib on ER-positive MCF-7 and triple-negative HCC-1806 human BC cells. MTT assays were first performed to examine the cytotoxic effects of dinaciclib on MCF-7 human luminal A ($ER^+/PR^+/HER2^-$) and HCC-1806 human TNBC cells. As shown in Fig. S1, the IC_{50} values of this drug on MCF-7 and HCC-1806 cells were 8.3 and 29.0 nM, respectively.

Dinaciclib is effective in suppressing self-renewal and anchorage-independent growth abilities of MCF-7 and HCC-1806 cells. Having verified the cytotoxic effects of dinaciclib on two human BC cell lines, it was next examined whether this drug could reduce the self-renewal and anchorage-independent growth abilities in these cells. By performing a sphere formation assay, it was found that dinaciclib dose-dependently decreased the sphere-forming abilities of both cells, and the respective IC_{50} values of dinaciclib in suppressing the self-renewal abilities of MCF-7 (Fig. 1A) and HCC-1806 (Fig. 1B) cells were 2.3 and 1.3 nM. Subsequently, a sphere shrinkage assay was used to analyze whether dinaciclib could induce shrinkage of the pre-formed spheres. The results demonstrated that dinaciclib also dose-dependently induced the shrinkage of the spheres formed from both MCF-7 and HCC-1806 cells, with respective IC_{50} values of 4.9 (Fig. 1C) and 6.2 nM (Fig. 1D).

Moreover, as the anchorage-independent growth ability is considered as a hallmark of carcinogenesis, a soft agar colony formation assay was conducted to evaluate the effects of dinaciclib on this ability of MCF-7 cells, but not HCC-1806 cells, as they failed to form colonies in soft agar (data not shown). As expected, dinaciclib dose-dependently inhibited the anchorage-independent growth of MCF-7 cells, with an IC_{50} of 6.5 nM (Fig. 1E). These findings suggest that dinaciclib is capable of inhibiting crucial stemness properties of human BC cells.

Dinaciclib decreases the protein expression levels of various BCSC and ESC markers in MCF-7 and HCC-1806 cells. Next, the protein expression levels of three well-known BCSC markers (CD44, ALDH1 and Bmi1) and three ESC markers (Oct4, Nanog and Sox2) were examined via western blotting in MCF-7 and HCC-1806 cells treated without or with dinaciclib. In agreement with the aforementioned observations, dinaciclib decreased the expression levels of not only the three BCSC markers, but also the three ESC markers in a dose-dependent manner in MCF-7 (Fig. 2A) and HCC-1806 (Fig. 2B) cells. Since these BCSC and ESC markers are commonly used together for identifying BCSCs, the current results further indicated the potential of dinaciclib in diminishing the CSC populations in both BC lines.

FoxM1 expression and phosphorylation are decreased by dinaciclib in MCF-7 and HCC-1806 cells. Given that certain CDKs can regulate the transcriptional activity and stability of FoxM1 by phosphorylating various serine and threonine residues, including Ser35 (14), and the fact that FoxM1 plays a critical role in maintaining the stemness of a wide variety of cancers including BC (15), we hypothesized that some of the stemness-inhibitory effects of dinaciclib in MCF-7 and HCC-1806 cells may be due to its effects on the expression and/or stability of FoxM1. The RT-qPCR results demonstrated that the mRNA expression levels of FoxM1 were significantly decreased by dinaciclib treatment in both cell lines (Fig. 2C and E). Furthermore, the protein expression levels of total FoxM1 and p-FoxM1 (Ser35) in both cell lines were also markedly decreased by dinaciclib (Fig. 2D and F). Taken together, these data demonstrated that dinaciclib could not only downregulate the expression of FoxM1, but also decrease its phosphorylation at Ser35 in MCF-7 and HCC-1806 cells.

Co-treatment with MG-132 can prevent dinaciclib-induced reduction in the protein expression levels of FoxM1, Nanog and Sox2 in MCF-7 cells. Since the phosphorylation of FoxM1 and several ESC factors (e.g., Oct4, Nanog and Sox2) by various CDKs has been shown to increase their respective stabilities by inhibiting proteasome-mediated degradation (34), it was assessed whether the decreased protein expression levels of FoxM1 and three ESC markers in MCF-7 cells caused by dinaciclib treatment was due to proteasomal degradation. As shown in Fig. 3, co-treatment with MG-132, a proteasome inhibitor, completely prevented the decreases in the protein expression levels of total FoxM1, p-FoxM1 (Ser35) and Nanog, as well as partially hindered the reduction of Sox2 expression in cells induced by dinaciclib. However, no significant recovery of Oct4 expression was observed after this co-treatment. These findings suggested that dinaciclib decreased the stability of FoxM1, Nanog and Sox2 proteins by inducing their proteasomal degradation in MCF-7 cells.

Dinaciclib inhibits the Hedgehog pathway by decreasing GLI1 protein expression in MCF-7 cells. To determine the stemness-inhibitory mechanisms of dinaciclib in MCF-7 cells, the effects of this drug on Wnt, Notch and Hedgehog pathways, three of the main stemness-regulatory signaling pathways, were examined in these cells. After excluding the involvements of the first two pathways (data not shown), the current study focused on the Hedgehog pathway by analyzing the nuclear expression level of GLI1, the crucial mediator of this pathway, as well as the mRNA expression levels of its downstream target genes, including *GLI1* and *PTCH1*. The western blotting results showed dinaciclib dose-dependently decreased the nuclear expression level of GLI1 in MCF-7 cells (Fig. S2). In accordance with these findings, the mRNA expression levels of two downstream genes *GLI1* and *PTCH1* were also markedly diminished by this drug (Fig. 4A).

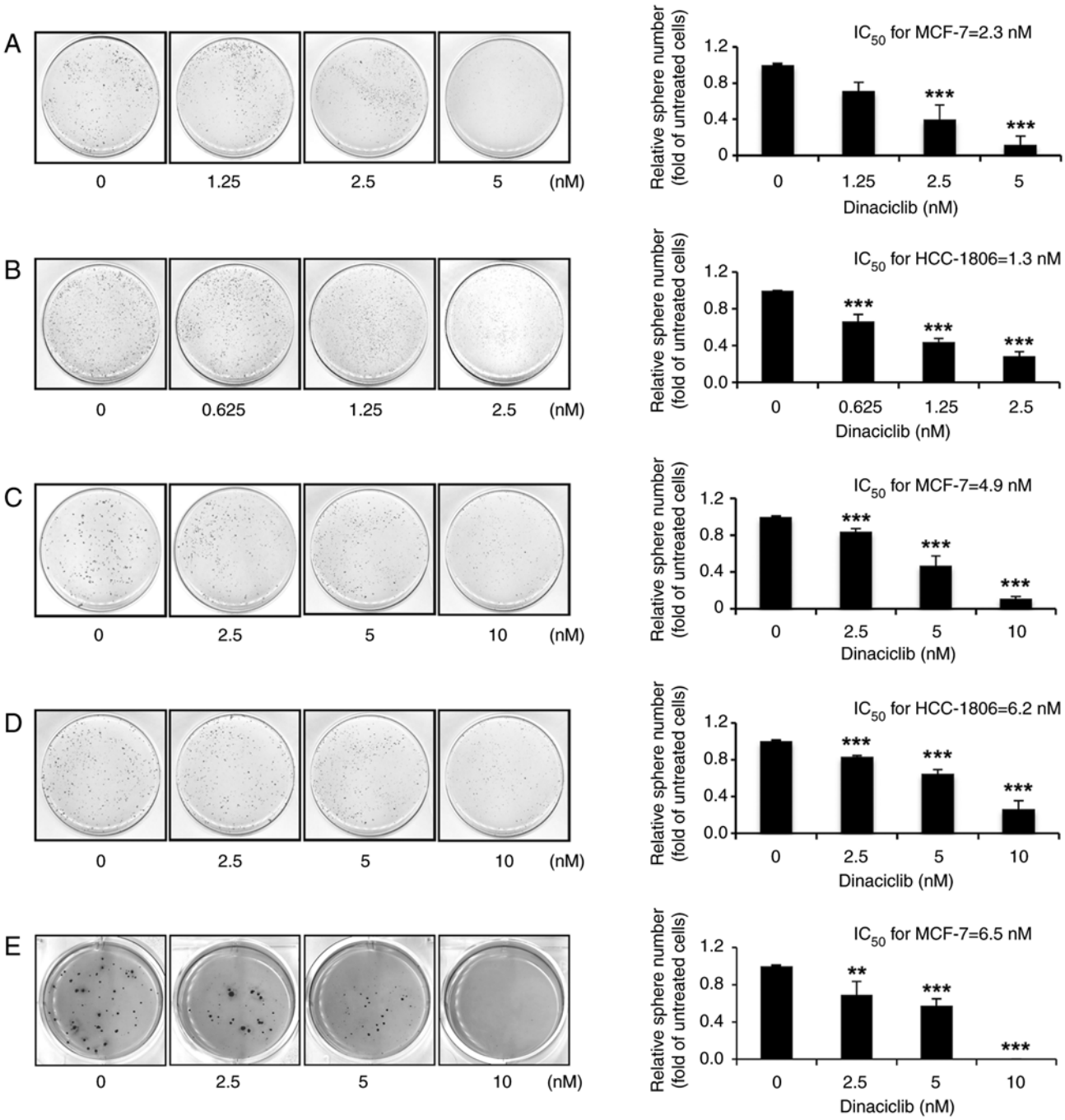

Figure 1. Inhibitory effects of dinaciclib on the stemness properties of MCF-7 and HCC-1806 cells. Sphere formation assays were conducted by seeding (A) $1x10^4$ MCF-7 and (B) $2x10^4$ HCC-1806 cells on the low-attachment dishes containing defined media, without or with indicated doses of dinaciclib for 14 days. Sphere shrinkage assays were performed by seeding (C) MCF-7 and (D) HCC-1806 cells on the low-attachment dishes containing defined medium for 8 days, before being treated without or with different doses of dinaciclib for a further 10 days. (E) A soft agar colony formation assay was conducted by seeding $5x10^2$ MCF-7 cells in 0.3% agar containing DMSO (0) or indicated doses of dinaciclib for 21 days. The numbers of spheres or colonies were counted using MetaMorph software after they were stained with MTT. Data are presented as the mean ± SD of three independent experiments. **P<0.01 and ***P<0.001 vs. untreated cells (using one-way ANOVA). The indicated IC_{50} values of dinaciclib in two breast cancer cell lines were calculated according to the bar graphs using Prism software.

Since the nuclear translocation of GLI1 has been shown to be enhanced by IPO7, whose promoter is able to be activated by FoxM1 (37), it was next examined whether dinaciclib affected the expression of *IPO7*. Indeed, the RT-qPCR results indicated that the mRNA expression levels of *IPO7* in MCF-7 cells were significantly downregulated after treatment with 10 nM dinaciclib (Fig. 4B). Collectively, these findings suggest that the stemness-inhibitory effect of dinaciclib in MCF-7 cells was attributed, at least in part, to its suppression of the Hedgehog pathway.

FoxM1 knockdown in MCF-7 cells diminishes their stemness properties and inhibits the expression of GLI1. Having demonstrated that dinaciclib could suppress both the expression and phosphorylation of FoxM1 in both cell lines (Fig. 2C-F), it was then assessed whether their stemness was also regulated by this

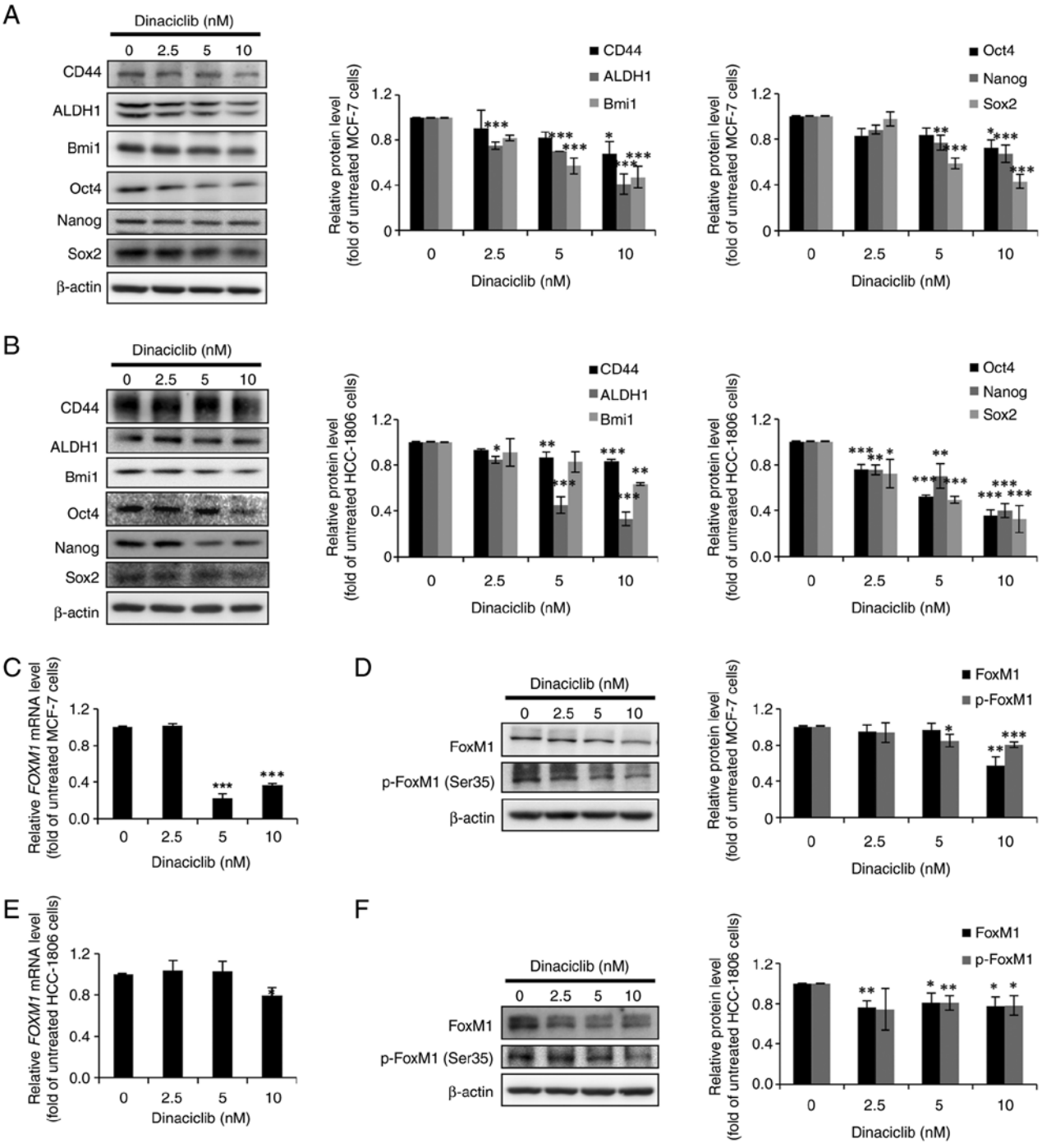

Figure 2. Dinaciclib reduces the protein expression levels of several breast cancer stem cell and embryonic stem cell markers, as well as those of FoxM1 in MCF-7 and HCC-1806 cells. Total lysates (20 μg) prepared from (A and D) MCF-7 and (B and F) HCC-1806 cells after being treated without or with indicated doses of dinaciclib for 24 h were subjected to western blot analysis using primary antibodies against CD44, ALDH1, Bmi1, Oct4, Nanog, Sox2, total FoxM1 and p-FoxM1 (Ser35). β-actin was used as a loading control. The semi-quantitative results were obtained via densitometry. Total RNAs (5 μg) extracted from (C) MCF-7 and (E) HCC-1806 cells after being treated without or with indicated doses of dinaciclib for 24 h were subjected to reverse transcription-quantiative PCR analysis to determine the mRNA expression levels of *FOXM1*. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. untreated cells (using one-way ANOVA). ALDH1, aldehyde dehydrogenase 1 family member A1; Bmi1, BMI1 proto-oncogene, polycomb ring finger; p-, phosphorylated; FoxM1, forkhead box M1.

TF. Thus, FoxM1-knockdown stable clones were established (designated as sh1 and sh2, or as shA and shB for MCF-7 and MDA-MB-231 cells, respectively), whose *FOXM1* mRNA (Fig. 5A and C) and protein (Fig. 5B and D) expression levels were significantly lower than their parental counterparts.

Sphere formation assays were then conducted to investigate the self-renewal abilities of FoxM1-knockdown MCF-7 clones. As expected, the sphere-forming abilities of the FoxM1-knockdown MCF-7 clones were markedly reduced (Fig. 5E). Moreover, the anchorage-independent growth abilities of the clones derived from MDA-MB-231 cells were diminished (Fig. 5F). In addition, the western blotting results demonstrated notable and significant decreases in the protein expression levels of three BCSC (CD44, ALDH1

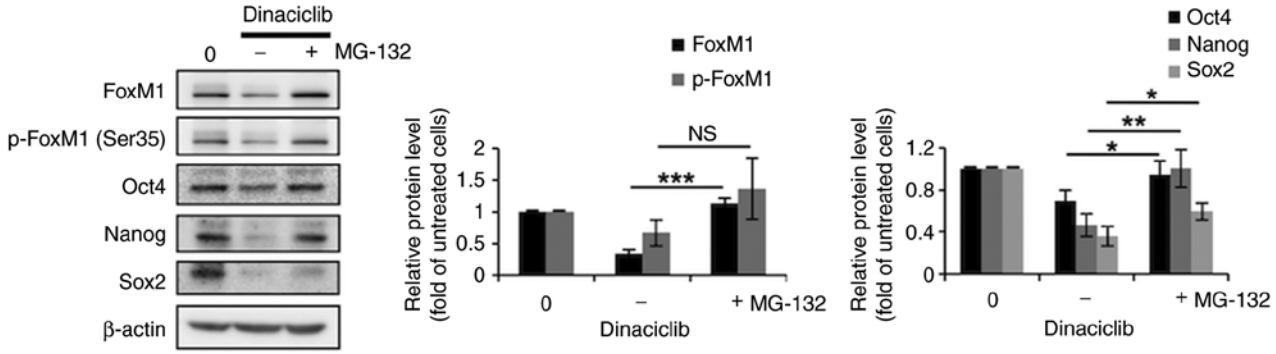

Figure 3. Comparison of the protein expression levels of FoxM1 and three embryonic stem cell markers in MCF-7 cells treated with dinaciclib in the absence or presence of MG-132, a proteasome inhibitor. Total lysates (20 μg) were prepared from MCF-7 cells after they were treated without or with dinaciclib (10 nM) in the absence or presence of MG-132 (10 μM) for 24 h, and they were subjected to western blot analysis, using primary antibodies against total FoxM1, p-FoxM1 (Ser35), Oct4, Nanog and Sox2. β-actin was used as a loading control. The semi-quantitative results were obtained via densitometry. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. cells treated with dinaciclib in the absence of MG-132 (using one-way ANOVA). p-, phosphorylated; FoxM1, forkhead box M1.

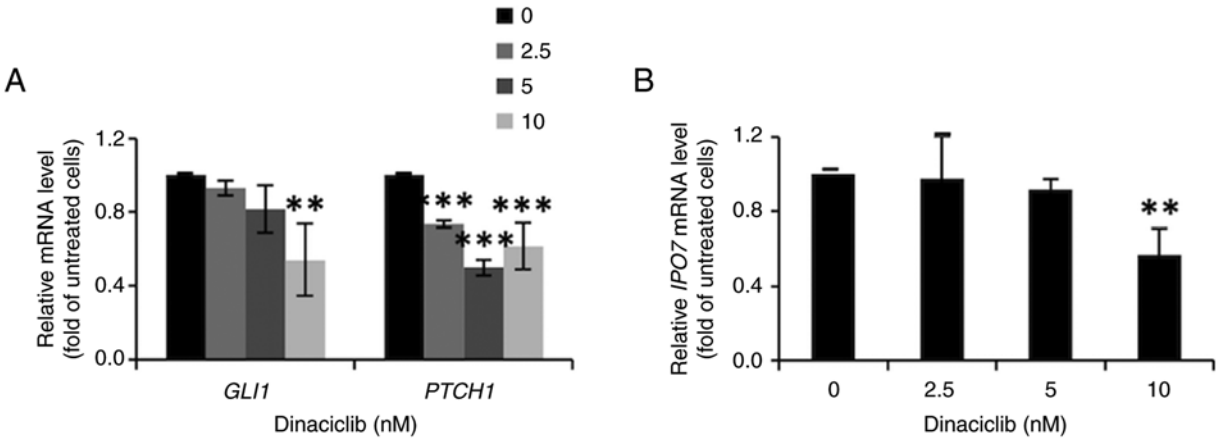

Figure 4. Effects of dinaciclib on the Hedgehog signaling pathway in MCF-7 cells. Total RNAs (5 μg) extracted from MCF-7 cells after they were treated without or with different doses of dinaciclib for 24 h were subjected to reverse transcription-quantitative PCR analysis to determine the mRNA expression levels of (A) *GLI1* and *PTCH1*, as well as (B) *IPO7*. Data are presented as the mean ± SD of three independent experiments. **P<0.01 and ***P<0.001 vs. untreated cells (using one-way ANOVA). *GLI1*, GLI family zinc finger 1; *PTCH1*, patched 1; *IPO7*, Importin-7.

and Bmi1) and three ESC (Oct4, Nanog, and Sox2) markers in the FoxM1-knockdown MCF-7 stable clones (Fig. 6A). The protein expression levels of CD44 and ALDH1 were also significantly decreased in the FoxM1-knockdown MDA-MB-231 clones (Fig. 6B). It was also assessed whether FoxM1 knockdown in MCF-7 cells could diminish total GLI1 expression as was observed with dinaciclib treatment. As expected, the mRNA and protein expression levels of GLI1 were significantly reduced in these clones (Fig. 6C and D). Collectively, these findings demonstrated an important role of FoxM1 in maintaining the stemness of two types of BC cells, and suggested that dinaciclib may inhibit their stemness properties by targeting two well-known stemness-regulatory TFs, FoxM1 and GLI1.

Upregulation of FoxM1 in HCC-1806 cells increases their resistance to the cytotoxic and sphere-forming inhibitory effects of dinaciclib. To further investigate whether the upregulation of FoxM1 in human BC cells could affect the effects of dinaciclib, a doxycycline-inducible FoxM1-expressing clone was established from HCC-1806 cells. The results demonstrated that the protein expression levels of FoxM1, as well as CD44 and ALDH1, two stemness markers, were dose-dependently elevated by doxycycline in this clone (Fig. S3A). MTT assays were then performed to examine whether the upregulation of FoxM1 in these cells affected their sensitivity to dinaciclib. As shown in Fig. S3B, FoxM1 upregulation markedly increased the resistance of doxycycline-inducible FoxM1-expressing HCC-1806 cells to the cytotoxic effects of dinaciclib. Finally, the results from a sphere formation assay further suggested that the self-renewal inhibitory effect of dinaciclib was notably diminished by the upregulation of FoxM1 in these clones (data not shown).

Discussion

Over the past few years, several cyclin-dependent kinase (CDK)4/6 inhibitors, including palbociclib, abemaciclib and ribociclib, have been approved by the US Food and Drug Administration, for use in combination with the aromatase inhibitor letrozole for treating patients with ER^{+}HER2^{-} metastatic BC (24-26). However, whether palbociclib can suppress

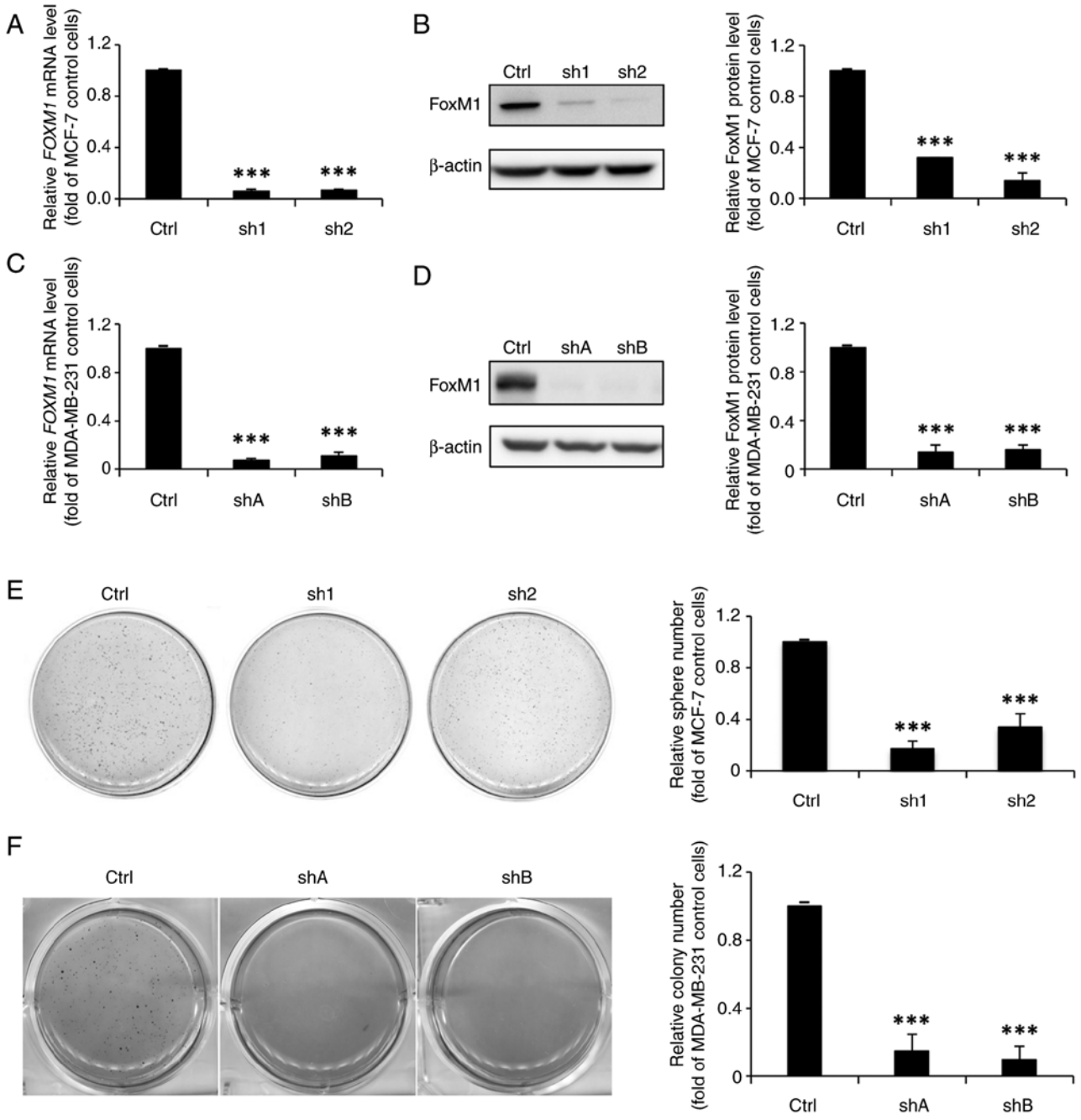

Figure 5. FoxM1 knockdown in MCF-7 and MDA-MB-231 cells diminishes their stemness properties. (A and C) Total RNAs (5 μg) extracted from the *FOXM1*-knockdown stable clones derived from MCF-7 (sh1 and sh2 in panel A) and MDA-MB-231 (shA and shB in panel C) cells, and their Ctrl cells were subjected to reverse transcription-quantitative PCR analysis to determine the mRNA expression levels of *FOXM1*. (B and D) Total lysates (20 μg) prepared from the FoxM1-knockdown stable clones were subjected to western blot analysis, using the primary antibody against FoxM1. β-actin was used as a loading control. The semi-quantitative results (right panels in B and D) were obtained via densitometry. (E) Cells ($1x10^4$) from the FoxM1-knockdown MCF-7 clones were cultured on low attachment dishes with defined media for 14 days to form spheres. (F) A soft agar colony formation assay was conducted by seeding cells ($5x10^3$) from the FoxM1-knockdown MDA-MB-231 clones in 0.3% agar for 21 days. The numbers of spheres or colonies were counted using MetaMorph software after they were stained with MTT. Data are presented as the mean ± SD of three independent experiments. ***P<0.001 vs. Ctrl cells (using one-way ANOVA). Ctrl, wild-type cells; sh, short hairpin RNA; FoxM1, forkhead box M1.

the self-renewal of different types of breast cancer (BC) remains controversial (24,28,29).

Dinaciclib is a novel CDK1/2/5/9 inhibitor, and its therapeutic effects on several types of cancer, including BC, leukemia and lymphoma, are being assessed in various clinical trials (31,40-42). Accumulating evidence suggests the involvements of CDK1/2/5/9 in regulating the stemness of normal and cancer cells, as well as the cancer stem cell (CSC)-suppressive effects of various selective inhibitors against these CDKs. For example, overexpression of CDK1 enhances the spheroid-forming ability, tumorigenic potential and tumor-initiating capacity of human melanoma cells by increasing the phosphorylation, nuclear localization and transcriptional activity of Sox2 (43). Moreover, the aberrant activation of cyclin E/CDK2 oncogenic signaling is essential for the maintenance and expansion of the breast

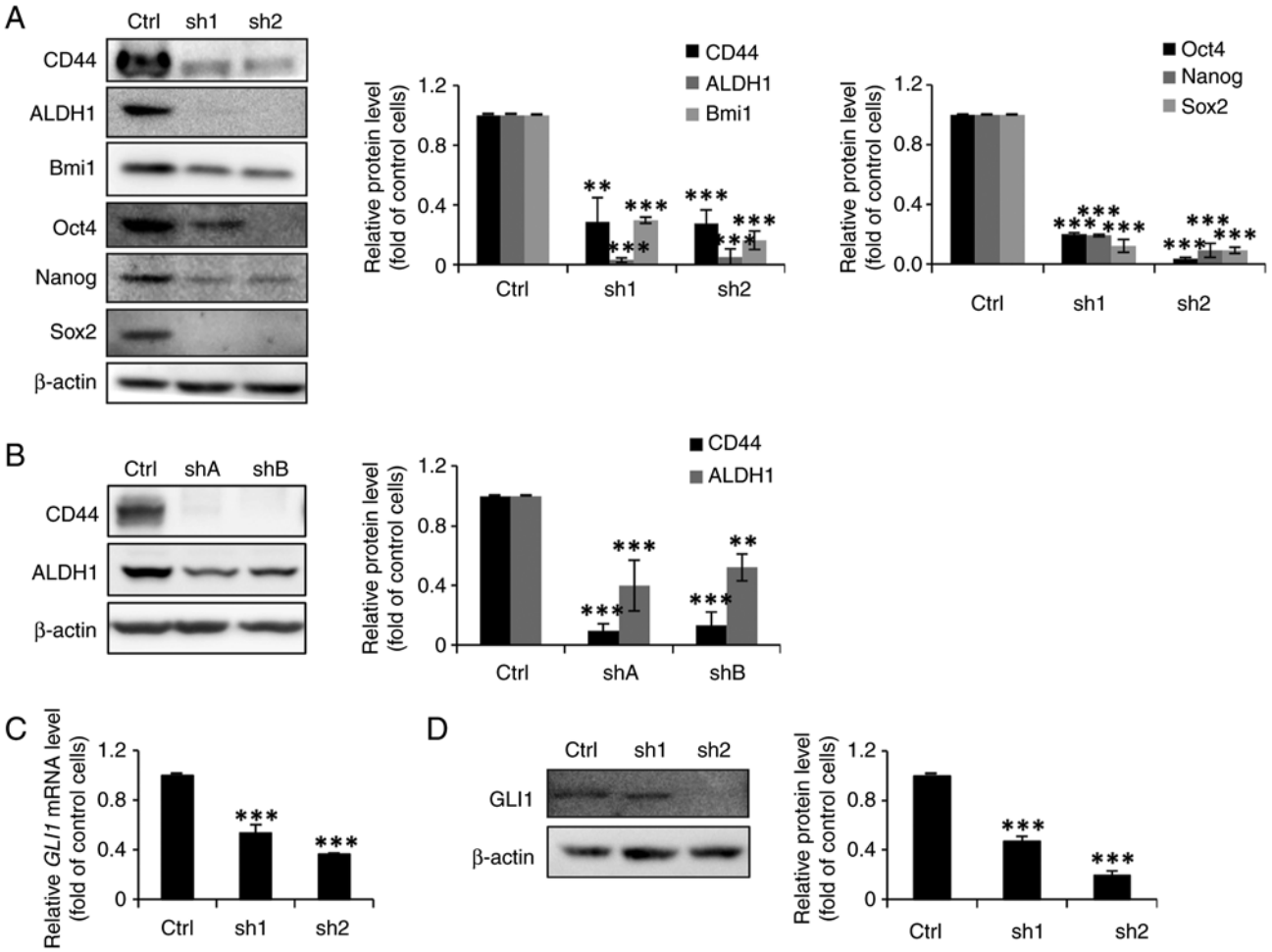

Figure 6. FoxM1 knockdown in MCF-7 and MDA-MB-231 cells decreases the protein expression levels of several stem cell markers and GLI1. (A, B and D) Total lysates (20 μg) prepared from the FoxM1-knockdown stable clones and their Ctrl MCF-7 (sh1 and sh2 in panels A and D) and MDA-MB-231 (shA and shB in panel B) cells were subjected to western blot analysis, using primary antibodies against CD44, ALDH1, Bmi1, Oct4, Nanog, Sox2 and GLI1. β-actin was used as a loading control. The semi-quantitative results were obtained via densitometry. (C) Total RNAs (5 μg) extracted from the MCF-7 FoxM1-knockdown stable clones were subjected to reverse transcription-quantitative PCR analysis to determine the mRNA expression levels of *GLI1*. Data are presented as the mean ± SD of three independent experiments. **P<0.01 and ***P<0.001 vs. Ctrl cells (using one-way ANOVA). Ctrl, wild-type cells; sh, short hairpin RNA; FoxM1, forkhead box M1; GLI1, GLI family zinc finger 1; ALDH1, aldehyde dehydrogenase 1 family member A1; Bmi1, BMI1 proto-oncogene, polycomb ring finger.

cancer stem cell (BCSC) subpopulation, which is more sensitive to SU9516, a specific CDK2 inhibitor (18). The Nestin/CDK5/dynamin-related protein 1 axis inhibits mitochondrial oxidative phosphorylation, which is essential for the maintenance of the stemness of neural stem/progenitor cells (43). In addition, CDK9 signaling plays an important role in brain tumor-initiating cell maintenance, and dinaciclib treatment can attenuate tumor growth and prolong the survival of tumor-bearing mice (44).

The present study aimed to identify whether dinaciclib could inhibit the stemness of human BC cells. Estrogen receptor-positive (ER$^+$) MCF-7 and triple-negative breast cancer (TNBC) HCC-1806 cell lines were selected as models, and it was found that the former was more susceptible to dinaciclib. Interestingly, although the protein expression levels of total and p-FoxM1 were significantly reduced by dinaciclib in both MCF-7 and HCC-1806 cells, the effects on the former could be accounted mainly by the drug-induced transcriptional suppression, whereas those in the latter may be attributed to the decreased stability of FoxM1 due to the inhibition of CDK1/2-mediated phosphorylation of this transcription factor (TF) (15). In terms of the regulation of FoxM1 protein, multiple CDK phosphorylation sites on FoxM1 variants (FoxM1b and c) have been identified, and the phosphorylation on some of them (e.g., Ser35) can enhance both protein stability and transcriptional activity of FoxM1 (15). As the protein level of total FoxM1 should be composed of both the unphosphorylated and phosphorylated forms of FoxM1, it was expected to see a more dramatic decrease in total FoxM1 protein level. Thereby, it was not too surprising to find that both total and phospho-(Ser35)-FoxM1 levels were increased by MG-132 co-treatment (Fig. 3).

In agreement with earlier observations made by other investigators, the present study demonstrated that dinaciclib could suppress the sphere formation and induce sphere shrinkage in MCF-7 and HCC-1806 cells, as well as inhibit the anchorage-independent growth of the former, thereby confirming the stemness-suppressive effects of this drug in human BC cells. Additionally, it was discovered that dinaciclib significantly decreased the protein expression levels of not only the three BCSC markers CD44, ALDH1 and Bmi1, but also of the three ESC markers Oct4, Nanog and Sox2.

Regarding the inhibitory effects of this drug on the protein expression levels of three ESC markers, previous studies have shown that respective CDK-mediated phosphorylation at the Ser/Thr-Pro motifs of Nanog and at the Ser12-Pro motif of Oct4 could promote their interactions with Pin1, leading to the increases of their stability and transcriptional activity (45,46). Moreover, the cyclin E/CDK2 and cyclin D3/CDK6 complexes have been reported to phosphorylate Nanog, Oct4 and Sox2, thereby promoting their interactions with Pin1 and protecting them from polyubiquitylation and proteasomal degradation (34,43). It has also been shown that the protein expression levels of Nanog, Oct4 and Sox2 in murine ESCs, patient-derived glioblastoma tumor-initiating cells or TNBC cells are notably decreased by CVT-313 treatment, which is a highly selective inhibitor of CDK2 (34). CDK1 has also been reported to bind to Sox2 and phosphorylate its Ser249, 250 and 251, resulting in an increased nuclear translocation and transcriptional activity of this factor, ultimately promoting the tumor-initiating potential of human melanoma cells (34). In agreement with the aforementioned observations, the present study identified that the decreased protein expression levels of two ESC markers, as well as those of total and p-FoxM1, triggered by dinaciclib were significantly restored by the co-treatment with MG-132, a proteasome inhibitor. These findings suggested that dinaciclib may inhibit the stemness of human BC cells by suppressing the expression of FoxM1 and three core pluripotency TFs in ESCs.

FoxM1 has been implicated in stimulating not only BCSC (47), but also TNBC/basal-like BC phenotypes (48). Although FoxM1 has been reported to be a downstream component of the Wnt signaling pathway critical for β-catenin transcriptional function in human glioma cells (38), the current study failed to detect any effect of dinaciclib on the nuclear β-catenin expression in MCF-7 cells (data not shown). Neither was Notch signaling, which has not yet been associated with FoxM1, to the best of our knowledge, affected by this drug (data not shown). Therefore, the current study assessed the effects of dinaciclib on the Hedgehog/Gli signaling pathway in MCF-7 cells, since Gli1 has been shown to activate FoxM1 expression in murine cerebral neural SCs (49), as well as in human basal cell carcinomas (50) and colorectal cancer cells (51). As expected, dinaciclib reduced the nuclear protein expression level of Gli1. Furthermore, this drug also suppressed the expression of Gli1 and PTCH1, two well-known downstream target genes of Gli1 in MCF-7 cells. The present study also detected a significant decrease in IPO7 mRNA expression in these cells after they were treated with 10 nM dinaciclib, which may be due to the drug-induced reduction of FoxM1, a known transcriptional activator of IPO7 (37). Additionally, as IPO7 has been shown to facilitate the nuclear translocation of Gli1 in glioblastoma multiforme cells (37), its downregulation may also account in part for the decreased nuclear Gli1 expression in drug-treated cells. Based on these findings, it was suggested that FoxM1 may also act as an upstream regulator of the Hedgehog signaling pathway. In line with this suggestion, it was further demonstrated that the self-renewal ability and protein expression levels of three embryonic SC (ESC) and three BCSC markers, as well as those of Gli1, in MCF-7 cells were notably suppressed by FoxM1 knockdown. Data of the time course experiments of FoxM1 downregulation were not provided as previous results (data not shown) suggested limited cytotoxic effects of FoxM1 knockdown on cell growth which was in contrast to the findings in a number of previous studies which showed a positive role of FoxM1 in regulating the growth of various types of cancer cells (reviewed in ref. 52) and the reasons to explain the discrepancy between our and others' observations need further dissection. On the other hand, transient upregulation of FoxM1 in HCC-1806 cells markedly increased their resistance to both the cytotoxic and sphere-forming inhibitory effects of dinaciclib, which indicated FoxM1 as the major target of this novel CDK inhibitor.

In conclusion, the present study demonstrated that dinaciclib, a CDK1/2/5/9 inhibitor, not only kills MCF-7 (ER-positive) and HCC-1806 (triple-negative) human BC cells, but also effectively suppresses their stemness. In addition, the stemness-inhibitory effects of this drug in the former cells are likely achieved by diminishing the expression and phosphorylation of FoxM1, resulting in decreases in the expression of three core pluripotency TFs (Nanog, Oct4, and Sox2) as well as in the Hedgehog signaling pathway. Thus, the present findings support the clinical development of dinaciclib for BC treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ANT was a major contributor who performed the dinaciclib (and MG-132) treatment on both wild-type MCF-7 and HCC-1806 cell lines to generate, analyze, and interpret the data regarding sphere formation and shrinkage assays, soft agar colony formation assays, western blot and RT-qPCR, and the MTT assay. ANT established the FoxM1-knockdown stable clones from MCF-7 cell line, performed the experiments regarding these clones, and generated, analyzed, and interpreted the data. YSC established the FoxM1-knockdown stable clones from MDA-MB-231 cell line and the doxycycline-inducible FoxM1-expressing HCC-1806 cells, and used the aforementioned cells to perform western blot analysis, RT-qPCR, and soft agar colony formation assays. YCL used the doxycycline-inducible FoxM1-expressing HCC-1806 cells established by YC to perform the MTT assay. ANT analyzed and interpreted the raw data generated by YCL and YSC. YS and TCC were the principal investigators who supervised this study and provided significant guidance and instructions for each result. All authors read and approved the manuscript

and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work, in particular the data, are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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