Synergistic Inhibition of GSK3 and MEK Induced Cancer Stem Cell Generation

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Abstract

Cancer stem cells (CSCs) are supposed to be tumor initiation cells, responsible for tumor invasive growth and dissemination to distant organ sites. Typically, the radiation treatmentand the chemotherapy of cancer should target at them. However, the current research and therapy of cancer is hampered by the difficulty of isolating pure CSCs and maintaining them in vitro. Here, we report that synergistic inhibition of glycogen synthase kinase3 (GSK3), mitogen-activated protein kinase kinase (MEK) by small molecules can efficiently generate CSCs from immortalized human mammary epithelial cells (HMLEs) and result in the acquisition of mesenchymal traits and the expression of stem cell markers. These converted cells have an increased ability to form mammospheres and regenerate tumors when we injected them into SCID mouse. In addition to these properties, they also exhibit remarkable chemotherapy resistance. This finding shows us a practically strategy to generate CSCs by small molecular in vitro which provides a cell resource for drug screening. It also reveals synergistic roles of Wnt and MEK pathways in tumourgenesis.

Keywords

Cancer stem cell (CSCs), Glycogen synthase kinase3 (GSK3), Mitogen-activated protein kinase kinase (MEK), Epithelial to mesenchymal transition (EMT), Small molecules.

1. Introduction

There is accumulating evidence suggests that cancers are hierarchically organized and only a small fraction of tumor cells is essential to tumor initiation, invasive growth and possibly dissemination to distant organ sites, through blood or lymphatic vessels[1]. These few cells accepted as cancer stem cells(CSCs) posses an enhanced self-renewal capacity and the ability to differentiate into multiple lineages of the bulk tumors[2-3]. Although some of the properties of it have been explored, the deeper research is hampered by the difficulty of isolating pure CSCs and maintaining them in vitro[4].

Many attempts have been tried, but there is a lack of robust ways to meet the expectations. Recently, some cases have proven the difficulty to confirm markers that originally appeared to robustly distinguish tumorigenic from non-tumorigenic cells. The methods which highlight the importance of surface markers in isolating CSCs turn to be unreliable[5-7]. In addition, the safety of the gene transduction method in vitro hinders the clinical application of the technology, due to a genomic integration that may cause tumor formation[8].

In our attempts to generate CSCs from norm cancer cells by small molecules, we fortuitously created a homogenously converted cell population by combined treatment of two small molecules CHIR99021 and PD184352 (referred to hereafter as CHIR and PD, respectively). Apparently, the cells share the properties similar to CSCs, appeared to self-renew and the stability in maintaining the characteristics. As an inhibitor of glycogen synthase kinase3 (GSK3), CHIR is implicated in the self-renewal of embryonic stem cells, activating canonical Wnt signaling[9-10]. PD is a small inhibitor of MEK, has been implicated in suppressing the proliferation of cells[11]. The subsequent research showed that the levels of proteins[12-13] which mediated migration and invasion, changed tremendously with the process of transition. Our work reprogrammed cancer cells, conveniently and concisely, to generate stable CSCs by two small molecules, comparing to ways mentioned above. In

addition, our method provided a practical strategy to generate CSCs for drug screening, by small molecules in vitro.

2. Material and method

2.1 Cell Culture

The immortalized human mammary epithelial cells (HMLE) were maintained in DMEM: F12 media (1:1) supplemented with insulin, EGF, hydrocortisone, and 5% calf serum. The HMLE cells were treated with 3μM PD and 2μM CHIR. Cultured cells were photographed on day 3. Mammosphere culture was performed as described in Dontu et al. (2003). Essentially, single cells were transferred to 50% Matrigel and cultured in MEGM medium (Clonetics)supplemented with 10ng/ml bFGF for additional 7 days. The structures were then photographed.

2.2 Immunocytochemical analysis

Samples were washed once with PBS (Invitrogen; without Ca2+ and Mg2+) and were fixed with a 4% formaldehyde solution containing 0.15% picric acid (Sigma-Aldrich) in PBS for 20 min,followed by three washes with PBS. Blocking and permeabilization were done with 10% donkeyserum (Jackson ImmunoResearch) and 0.3% Triton X-100 (Sigma-Aldrich) solution in PBS for 1hour at room temperature. All primary antibodies were diluted in 1% BSA and incubated overnight at 4°C. After 1 hour of washing with 0.1% BSA in PBS, samples were incubated with Alexa-555– or Alexa-488–conjugated secondary antibodies (Invitrogen) for 1 hour at roomtemperature and nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich).All images were taken using a Nikon Eclipse Ti microscope equipped with a PhotometricsCoolSnap HQ2 camera and processed with NIS Elements Basic Research Software (Nikon).The following primary antibodies were used: E-cadhere and Vimentin.

2.3 Reverse Transcriptase PCR Analysis

Total RNA was extracted from samples at the designated time points using the RNeasy Plus mini kit with QiaShredder columns (Qiagen). One microgram of total RNA per sample was reverse-transcribed using the iScriptcDNA synthesis kit (Bio- Rad) and the cDNA was diluted with 100 μL of water. A total of 1/50 of the diluted cDNA was used for quantitative PCR with iQ SYBR Green Supermix on the CFX96 system (Bio-Rad). All qPCR reactions were done in triplicate, expression levels were analyzed using CFX manager software (Bio-Rad), with levels normalized to GAPDH. Each set of reactions was repeated using cDNA from at least three independent experiments.Primers sequence used to amplify genes are listed in the Table1.

Table 1 Primers sequence

Genes	Forward	Reverse
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
Vimentin	CTTCAGAGAGAGGAAGCCGA	ATTCCACTTTGCGTTCAAGG
E-cadherin	TTGACGCCGAGAGCTACAC	GACCGGTGCAATCTTCAAA
Twist	CCTTCTCGGTCTGGAGGAT	TCCATTTTCTCCTTCTCTGGAA

2.4 FACS Analysis

Cells were washed with PBS and dissociated with Accutase (Innovative Cell Technologies). After harvesting, the cells were washed twice with ice-cold FACS buffer (HBSS supplemented with 10 mMHepes, 2% FBS, and 0.1% sodium azide; Sigma-Aldrich). Non-dissociated cells were removed by passing the cell suspension through a cell strainer (BD) twice. Cells were incubated with PE-conjugated anti-human CD24 antibody and APC-conjugated anti-human CD44 antibody(eBioscience) for 30 min at 4°C. After incubation, the cells were washed twice with five volumes of FACS buffer, fixed, and suspended in 4% paraformaldehyde solution (Electron Microscopy Sciences) in PBS. More

than 20,000 cells were analyzed using FACSCalibur and CellQuest software (BD). Further analysis was performed using FlowJo software (Tree Star).

2.5 Statistical analysis

All data are presented as mean ± SEM except stated. When two groups were compared, the Student's t test was used ($p < 0.05$ was considered significant).

3. Results

3.1 PD and CHIR treatments induced morphologic transformation of HMLE from epithelial to mesenchymal

To determine the effects of PD and CHIR in inducing CSCs in vitro, we first observed the changes in morphology of the HMLE cells following the treatments with PD and CHIR. The untreated cells were of an oval or cobblestone shape with tight intercellular junctions (the left panel of Fig.1A). After the treatment for 3 days, the cells loss of contacts with an added acquisition of elongated fibroblastic morphology (the right panel of Fig.1A). We also measured the ability of proliferation of the two cells. At the first two days, the treated cells proliferated faster than the control cells, but at the third day, the treated cells turned slower in growth than the untreated ones (Fig.1B) which was consistent with the property of E-cadherin knock down generating CSCs[14].

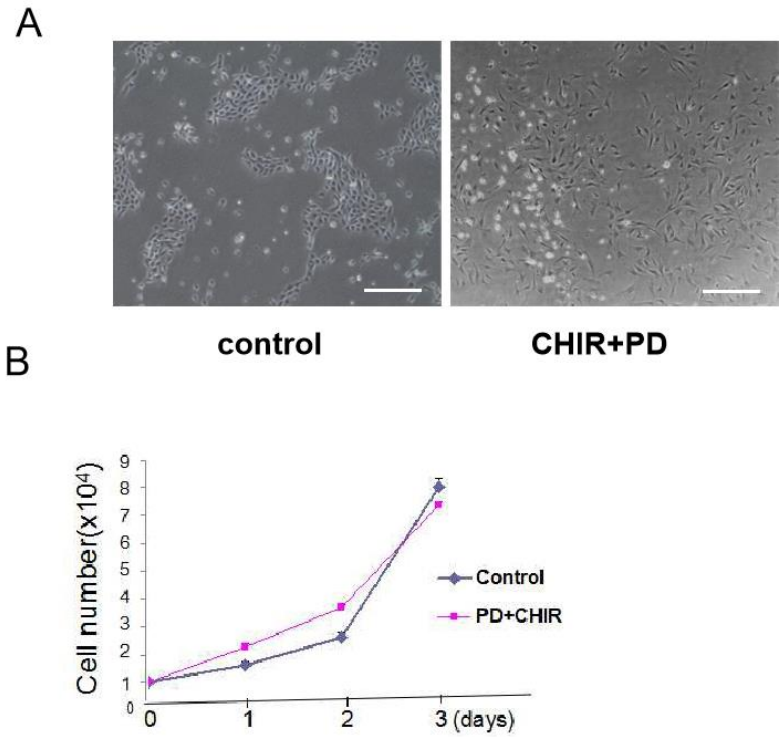


Fig. 1 Morphology of cancer cells after treatment with compounds.(A)The left panel of representative photomicrographs shows a oval or cobblestone-like appearance of normal HMLER cells ;the right panel shows a slight increase in cell size, accompanied with cell flattening, a partial loss of cell-cell adhesion and the formation of the spindle-like cells, after the stimulation with compounds for 3 days. (Scale bar 100μm)(B) Seeding 10000 cells in 6-well plates, Viable cells were counted by Trypan Blue dye exclusion at day1,2, and 3. Bars denote the standard error (n = 3)

3.2 Cells treated with CHIR and PD exhibited cancer stem cells markers

To determine whether the morphologic transformation was accompanied with the phenotypic switch, we detectedthe expression of specific epithelial marker E-cadherinand the stem cell specific marker Vimentin, before and after the treatmentwith compounds in HMLE cells .The controlHMLE cellsshowed a high level of E-cadherin protein, which was significantly decreased following the

treatment,and the expression of it was almost undetectable indeed after 48 hours(the left panel of Fig.2A and Fig.2B). On the contrary, the level of Vimentin protein was very low in these controlcells, but markedly increased after the treatmentfor 48hours, and the amount of it almost double after 48hours(the right panel of Fig.2A).We also examined another specific marker Twist, and the level of Twistalso experienced a rapid growth that the amount in 48 hours was twice as much as that in 24 hours.(Fig.2C).Moreover, we observed that most if not all of the wild type HMLEare CD44+/ CD24+cells,and after the treated with the compounds,these cells shift into CD44high/ CD24 lowones (Fig.2D).

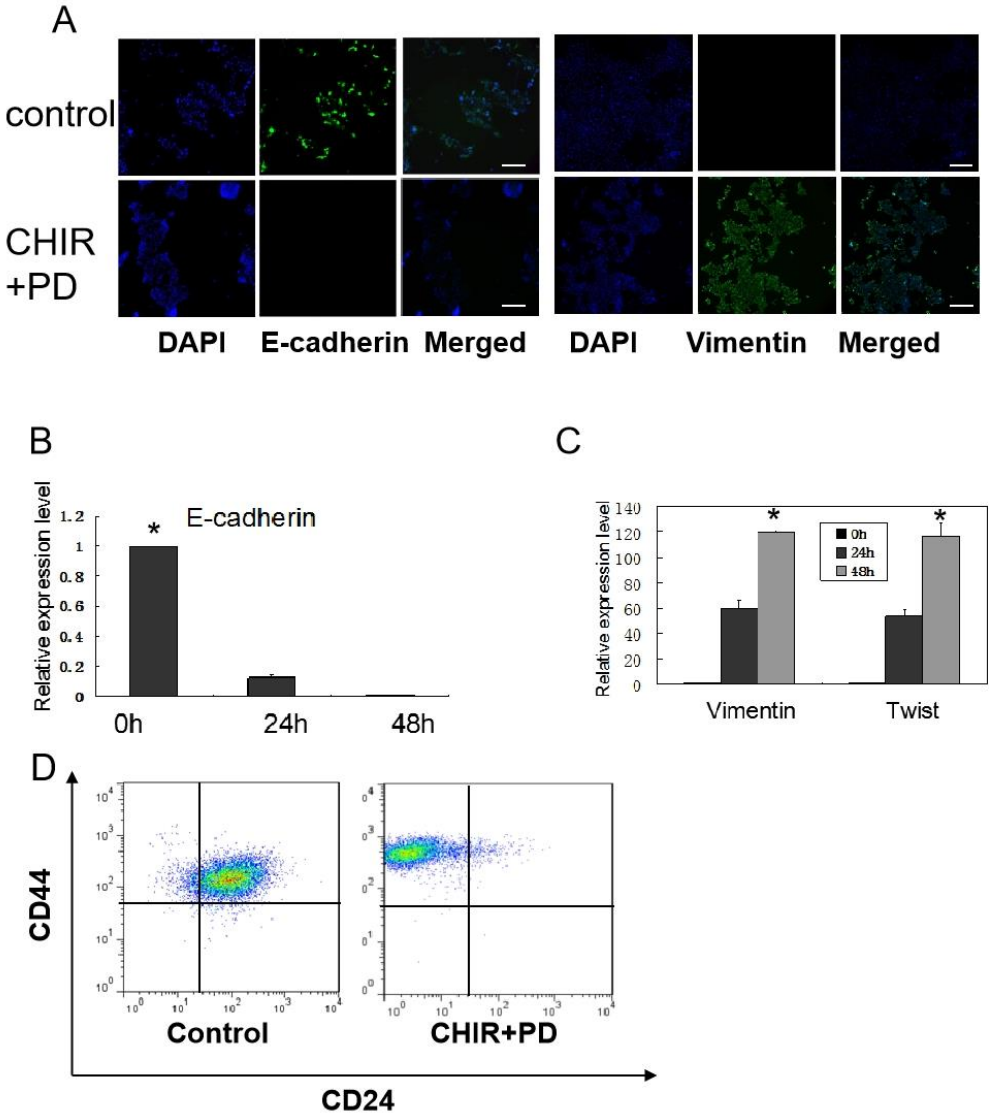
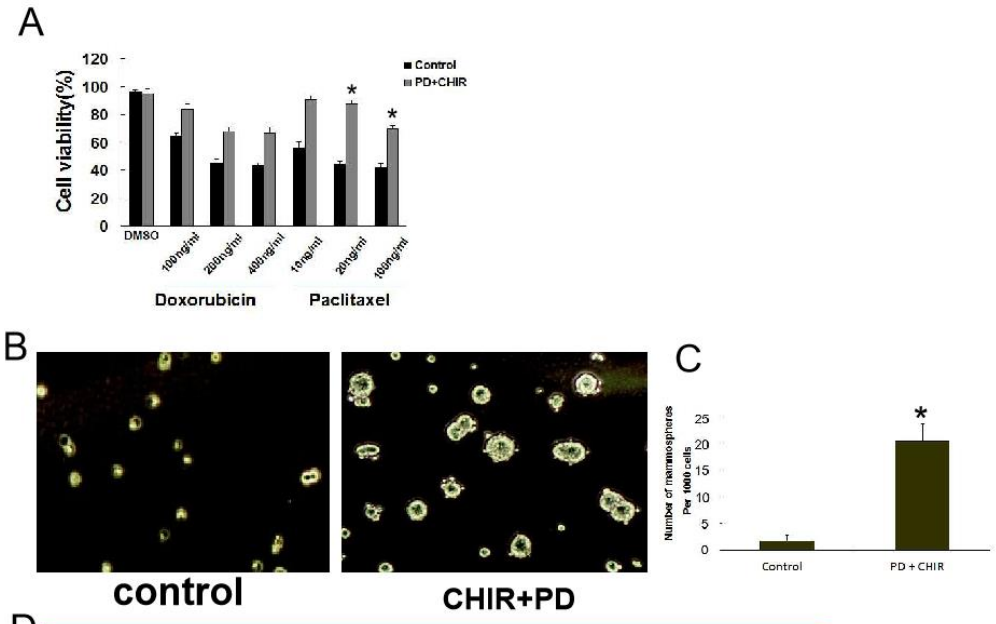


Fig2. Cells treated with CHIR and PD exhibited cancer stem cells markers. (A) immunofluorescence images of cells stained using antibodies against E-cadherin and Vimentin. the nuclei stained by DAPI. (B) The mRNA level of E-cadherin was measured by Real-time PCR analysis during the time course at 0, 24 and 48 hours ($p<0.05$).(C) The mRNA levels of vimentin and twist were measured by Real-time PCR analysis during the time course at 0, 24 and 48 hours ($p<0.05$). (D) FACS analysis of CD44 and CD24 for control cells and cells treated with compounds.

3.3 Cells treated with CHIR and PD displayed the properties of cancer stem cells

To investigate whether the cells treated with CHIR and PD displayed the properties of cancer stem cells.We, firstly, treated both of the cells with Doxorubicin or Pirfenidone of serial dilutions for 24 hours, and find that the percentages of the treated cells which showed the ability of drug resistance

were much higher than the untreated ones. Then, we plated HMLE cell at very low densities in plates containing serum-free with or without the compounds, and measured the number ofnewly formed mammospheres. Comparing withthe control cells,the treated cells generated much more and biggermammospheres (Fig3B).As shown in Fig3C, the treated cells could form 20 spheres per 1000 cells while the control cells just formed 2 spheres per 1000 cells (Fig.3C).To test the ability of tumor initiation of the converted cells, we respectively injected 103,104,105,106 cells into SCID mouse. We found tumors in all of the mouse injected treated cells ,while about104 of the control cells were required to initiate tumor formation and only three-quarter mouse formed tumors when they were injected 105control cells.(Fig.3D).



D

Cells injected	Control	CHIR+PD
1×10^6	4/4	4/4
1×10^5	3/4	4/4
1×10^4	1/4	4/4
1×10^3	0/4	4/4

Fig3. The cells treated with CHIR and PD display the properties of cancer stem cells ($p<0.05$). The cells treated with compounds were resistant to drug Doxorubicin and Paclitaxel. (B) Phase-contrast images of mammospheres seeded by the control and the treated cells. (C) Quantification of mammospheres formed by cells from the control and treated cells populations. The data are reported as mean ± SEM ($p<0.05$). (D)Tumor Incidence of treated cells and then injected into host mice in limiting dilutions

4. Discussion and Conclusion

Realizing the effects of CSCs on tumor-initiation and therapeutic resistance [15-16], the methods of isolation and expansion them in vitro need to be developed. Although CSCs have been distinguished by a certain cell surface marker (known as a CSC marker) in a variety of tumors, the cancer cells which were surface markers negative also exhibited the property of proliferation in the native states. These findings demonstrated that the cautions should be taken when using surface markers independently to identify cancer stem cells due to the phenotypic plasticity of tumor cells.[17-18]. However, in our study, we treated the non-stem cancer cells with GSK3 inhibitor (CHIR99021) and MEK inhibitor (PD184352) synergistically. These treated cells expressed highly raised CSC markers,

exhibited remarkable tumorigenicity and therapeutic resistance. Our observation indicates, at least in part, that it is possible to capture CSCs by stimulating with specific molecules rapidly and effectively [19-21].

Recent studies suggest that Gsk3 plays key roles in many fundamental processes, including mediating signaling downstream of Wnt, FGF, Hh during the process of cancer [22-23]. Up-regulated Wnt ligands led by blocking Gsk3 bind to the frizzled/lRP co-receptor complex, which leads to the stabilization and nuclear translocation of β-catenin. β-catenin acts as a powerful trans activator of LEF/TCF transcription factors, which regulate important downstream target genes that promote cell proliferation, differentiation, and tissue development. The Mitogen-activated protein kinase kinase (MEK) pathway comprises several key signaling components and phosphorylation events that play important role in tumorigenesis. These activated kinases regulate cell growth, differentiation, proliferation, and migration functions. [24] During our study, we find an interesting phenomenon that the cancer cells treated with compounds to inhibit MEK signal pathway grow faster than the control cells. One possible explanation for this is that CHIR not only inhibits signaling pathway, but also engages in cross talk with FGF signaling to enhance the expansion [25]. In addition, a very recent studies showed that there exists a bidirectional crosstalk between Erk and Wnt [25-26]. After blocking the upstream molecular MEK, the accumulation of β-catenin led by the inhibition of CHIR resulted in the activation of downstream molecular Erk and stimulated the up regulated of oncogenes, such as c-myc, ras and so on[27].

EMT has been postulated to play a critical role in the acquisition of malignant traits by carcinoma cells, especially in the process of metastasis [28-29]. During EMT, epithelial cells lose cell–cell junctions and polarity, leading to a more migratory, mesenchymal cell phenotype. In this study, we examined that the treatment with the compounds induced the specific molecular changes consistent with EMT, such as downregulation of E-cadherin expression, upregulation of Twist, and Vimentin expression. In addition, mammosphere culture formed and the tumors found in SCID mouse in the study prevented the success of transition and capturing CSCs. [30-31]. These findings demonstrated that the hypothesis of EMT could be one of the sources of this phenomenon. However, Jenniet al. found that MAPK/Erk activation by ionizing radiation co-operated with TGF-β1 to induce EMT in normal mammary epithelial cells, and inhibition of MAPK/Erk activity clearly prevented downregulation of E-cadherin protein levels and restored cell–cell contacts. Galina S et al. also reported that GSK3 inhibition affected the proliferation rate of stem cells by causing slight accumulation of G1-phase cells. Noteworthy, the treated cancer cells, in our study, not only showed the conversion of surface markers, the property of cancer stem cells, but also experienced a rapid growth. The phenomenon prevented that the compounds retained the advantages of the two small molecular, and get rid of the disadvantages of them. One of the possible causes implied is that the compounds resulted in the reducing of the β-catenin/E-cadherin-mediated adhesion rather than the β-catenin-dependent transcription of EMT- or cell cycle-related genes. However, the underlying molecular mechanisms of these conditions are poorly understood.

Collectively, our finding demonstrated that co-stimulate of PD and CHIR could generate stem-like cancer cells efficiently. More importantly, these results also indicated that the process of inducing differentiated breast epithelial tumor cells is sufficient to promote the initiation and establishment of tumor. Moreover, the mechanisms implied in our process of inducing remain largely unknown and merit further explorations

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