SNAILs promote G1 phase in selected cancer cells

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Abstract. Cells can acquire a stem-like cell phenotype through epithelial-mesenchymal transition (EMT). However, it is not known which of the stem-like cancer cells are generated by these phenotype transitions. We studied the EMT-inducing roles of SNAILs (the key inducers for the onset of EMT) in selected cancer cells (lung cancer cell line with relatively stable genome), in order to provide more implications for the investigation of EMT-related phenotype transitions in cancer. However, SNAILs fail to induce completed EMT. In addition, we proved that Snail accelerates the early G1 phase whereas Slug accelerates the late G1 phase. Blocking G1 phase is one of the basic conditions for the onset of EMT-related phenotype transitions (e.g., metastasis, acquring stemness). The discovery of this unexpected phenomenon (promoting G1 phase) typically reveals the heterogeneity of cancer cells. The onset of EMT-related phenotype transitions in cancer needs not only the induction and activation of SNAILs, but also some particular heredity alterations (genetic or epigenetic alterations, which cause heterogeneity). The new connection between heredity alteration (heterogeneity) and phenotype transition suggests a novel treatment strategy, the heredity alteration-directed specific target therapy. Further investigations need to be conducted to study the relevant heredity alterations.

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Introduction

Cancer stem cell (CSC) is a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor (1). Although it has been proven that epithelial-mesenchymal transition (EMT) can generate cells with stem cell properties (2), it is still unknown which of the stem-like cancer cells are generated by these phenotype transitions.

The function of Snail genes is best known for induction of EMT. Both in development and during carcinoma progression, Snail1 (Snail) is expressed at the onset of the transition, whereas Snail2 (Slug), Zeb genes, E47 and Twist are subsequently induced to maintain the migratory mesenchymal state (3). As transcriptional repressors, Snail and Slug can regulate the expression of genes mediating therapeutic resistance and acquiring of stem-like phenotype in ovarian cancer cells (4).

Snail and Slug have complicated interactions with many key molecules. Snail can cause functional deficiency of p53 in tumor cells with mutant KRAS (5). We know GSK3β, the downstream molecule of Akt (6), can phosphorylate Snail to cause the degradation or the nuclear export of Snail (7). However, KRAS can interact with the PI3K/Akt pathway (8), and then affect GSK3β. Slug can escape degradation when p53 is mutant in lung cancer (9). In addition, the experssion of Slug can be regulated by mutant KRAS in colon cancer cells (10). Snail and Slug are also connected to other key molecules (e.g., EGFR, ERCC1) (11-13).

Certain key molecules, including KRAS, p53, EGFR, ERCC1, are all connected to EMT (11,14-16) and stem cell biology (17-20). This makes the SNAIL-related phenotype transitions much more complicated (Fig. 1A). Notably, these key molecules are also found to be of great value in non-small cell lung cancer patients (21-24). In addition, the great value of personalized therapy suggests the important role of genetic background in lung cancer (25). Snail and Slug are important regulators in the stemness of lung cancer (26,27). Plenty of studies have already revealed SNAILs can induce EMT-related phenotype transition of lung cancer. Accordingly, we were interested in investigation of whether the in lung cancer cells with wild-type of these key molecules can provide some significant clarification.

We conducted the currently study to investigate the EMT-inducing roles of SNAILs in selected cancer cells (lung cancer cell line with relatively stable genome, selection criteria are described in Materials and methods), in order to provide further knowledge for the investigation of EMT-related phenotype transitions in cancer.

Materials and methods

Antibodies and reagents. Primary antibodies were: anti-Snail, anti-Slug, anti-γ-H2AX (Abcam), anti-Snail, anti-Slug, anti-Bax, anti-Bcl-2, anti-Bcl-xl, anti-cytochrome *c*, anti-caspase 3 (Cell Signaling Technology), anti-total (Ser473)-Akt, anti-phospho (Ser473)-Akt, anti-total (Ser9)-GSK3β, anti-phospho (Ser9)-GSK3β, anti-Myc (Signalway Antibody), anti-β-actin (Beijing Biosynthesis Biotechnology Co., Ltd.), anti-p53, anti-p21, anti-ERCC1, anti-E-cadherin, anti-Vimentin, anti-CK8/18 (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Secondary antibodies were: HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.), Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probe). PI, Hoechst 33342, cisplatin (DDP) and G418 were purchased from Sigma.

Cell lines. Human cell lines A549, H460, H292, HUVEC and HBE were purchased from and tested by the American Type Culture Collection (ATCC; Manassas, VA, USA). All the cell lines were used within 6 months after receipt or resuscitation. All the cell lines were maintained in appropriate medium that contained 10% FBS (both from Life Technologies), penicillin (100 U/ml) and streptomycin (100 U/ml) (both from Thermo).

Selection of the cell line to establish stable cell lines. In order to maximally avoid the interference caused by genomic mutation (genomic instability), H292 cell line was used to establish stable cell lines. In the known non-small cell lung cancer cell lines, we found only H292 cells possess the relatively stable genome (with wild-type *KRAS*/*TP53*/*EGFR*/*ERCC1*/*Keap1*/*Nrf2*) (28-36).

Plasmids construction. RNAi sequences of Snail and Slug were previously described (11). The shRNA constructs were synthesized by Shanghai GeneChem Co., Ltd. The plasmids carrying the full-length human cDNA of Snail or Slug were purchased from OriGene. The target fragments were cut out and inserted into the overexpression vector, pcDNA3.1 pre-inserted fragments encoding EGFP. The two fragments shared the same promoter and each of them had their own initiation and termination codons. All plasmids were confirmed by PCR sequencing.

Cell transfection and construction of stable cell lines. All clones presented in this report were generated by stable transfection. We performed transfection by Lipofectamine 2000 reagent (Invitrogen) following the instructions. Selected by G418 (400 μg/ml), mono-clones were picked up and confirmed by western blotting. Stable clones were cultured with 15% FBS and 200 μg/ml G418. The medium described in cell cultures were used when testing. Cells carrying pGCsil-vector were used as control after stable cell lines were established.

DDP cytotoxicity and cell growth. For cytotoxicity, cells were seeded on 96-well plates and viability was detected by Cell Counting Kit-8 (DojinDo). There were 5-6-wells for each concentration of DDP. For the cell growth curve, cells were seeded in 24-well plates and the cell number was counted three times a day after subculture (for 6 days). We counted 4-wells for each cell line every day. The difference between day 5 and 6 after subculture is equal to day 6 minus day 5. For EdU test, cells were seeded on 6-well plates and performed by Cell-Light™ EdU Apollo®567 In Vitro Flow Cytometry kit (Guangzhou RiboBio Co., Ltd.).

Irradiation, serum deprivation and stimulation. Cells cultured in culture vessels at suitable times were irradiated with 8 Gy by a biological irradiation instrument (Rad Source, USA). For p53 and p21 detection, 8 h after irradiation, cells were fixed in 4% paraformaldehyde for immunofluorescence. For G1/S phase arrest after irradiation, experimental conditions were the same as in p53 and p21 detection. For γ-H2AX detection, cells were fixed 30 min after irradiation. For serum deprivation, cells were seeded on 6 cm dishes and cultured overnight. The day after seeding, culture medium was switched into serum-free medium. For late apoptosis, cells were cultured in serum-free medium for 54 h. For western blotting, cells were cultured and harvested at 0, 2, 4 and 6 days after medium switching. For serum stimulation, cells were seeded on 6 cm dishes and cultured overnight. On the day after seeded, culture medium was switched into serum-free medium. Following culture in serum-free medium for 24 h, culture medium was switched into complete medium with 10% serum. Cells were then harvested at 0, 4 and 8 h after medium switching. Cells were washed with pre-warmed PBS three times before medium switching.

Immunofluorescence. Cells were fixed in 4% paraformaldehyde for 1 h at RT, and then blocked in 3% BSA/PBS. Primary antibodies were incubated in a routine manner, and the secondary antibodies were incubated for 30 min at RT. Images were collected with a microscope (Axio Scope.A1; Carl Zeiss) using Plan-Apochromat objective lens (10X, 0.45; 20X, 0.8; and 63X, 1.4, Oil) and a camera (AxioCam MRm; Carl Zeiss) at 25˚C, illumination with single wavelength LED fluorescent light source (365, 470 and 590 nm). AxioVision Rel. 4.7 software (Carl Zeiss) was used to acquire images. Images were auto-adjusted using the Office Picture Manager. All cell lines were tested.

Scratch-test. For migration ability, cells were seeded on 6-well plates and cultured until 100% confluent. Then, culture medium was switched to serum-free medium and the cell culture continued for 24 h. Then, a straight scratch was made in the middle of the dish. After washing by pre-warmed PBS three times, cells were cultured with complete medium for another 24 h. Then images were collected by inverted microscope (Olympus IX71-22FL/PH). DP controller 1.1.1.65 software (Olympus IX) was used to acquire images. All cell lines were tested. The results with significant difference are reported.

Flow cytometric analysis. For the cell cycle, cells were fixed in 70% ice-cold alcohol and stained with PI (20 μg/ml) solution

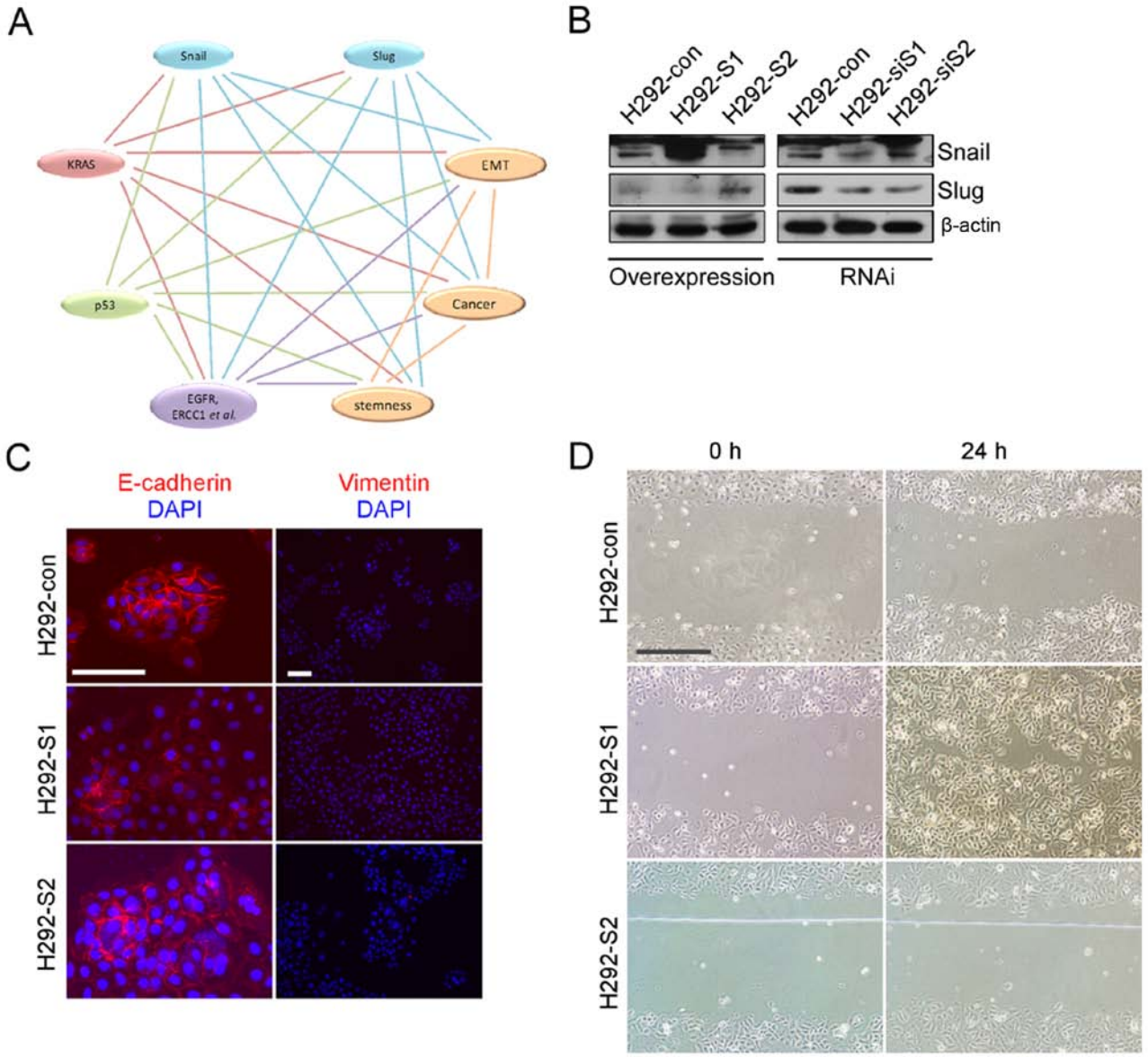

Figure 1. (A) Schematic illustration of the complicated connections between many key molecules and relevant biological behavior. (B) The efficiency of overexpressing and silencing Snail or Slug. (C) Expression of EMT markers in H292-con, H292-S1 and H292-S2. Bar, 100 μm. (D) Scratch-test results in H292-con, H292-S1 and H292-S2. Bar, 500 μm.

containing DNase-free RNase (200 μg/ml). For evaluation of apoptosis, cells were harvested and stained with Hoechst 33342 (25 μg/ml) and PI (1 μg/ml) solution. All samples were analyzed on a BD FACSAria flow cytometry. For qualitative analysis of the cell cycle, the results are shown in a pseudocolor form. All cell lines were tested. Only the results with significant difference are shown.

Protein extraction and western blot analysis. Whole cell proteins were obtained by the Total Protein Extraction kit (KeyGen Biotech). Western blotting was performed with the Mini-PROTEAN Tetra Cell and Mini Trans-Blot Cell systems (BioRad). Immunoblots were detected by chemiluminescence using the ECL kit (Pierce).

Statistical analysis. The data are expressed as means ± SD from the number of independent experiments as indicated. Statistical analysis was performed using Student's t-test. P-values of <0.05 were considered statistically significant.

Results

Establishment of stable cell lines. We established stable cell lines following the description of Materials and methods. Because all the clones of the same transfection possess the same phenotype, we chose one clone of the transfection for further examinations (Fig. 1B).

SNAILs fail to induce completed EMT. Although expression of E-cadherin was weakened and cell migration was enhanced in H292 overexpressing Snail (H292-S1), Vimentin was still negative (Fig. 1C and D). Overexpressing Snail or Slug did not induce completed EMT. Obvious changes were shown in cell growth and DDP treatment. We analyzed in detail the

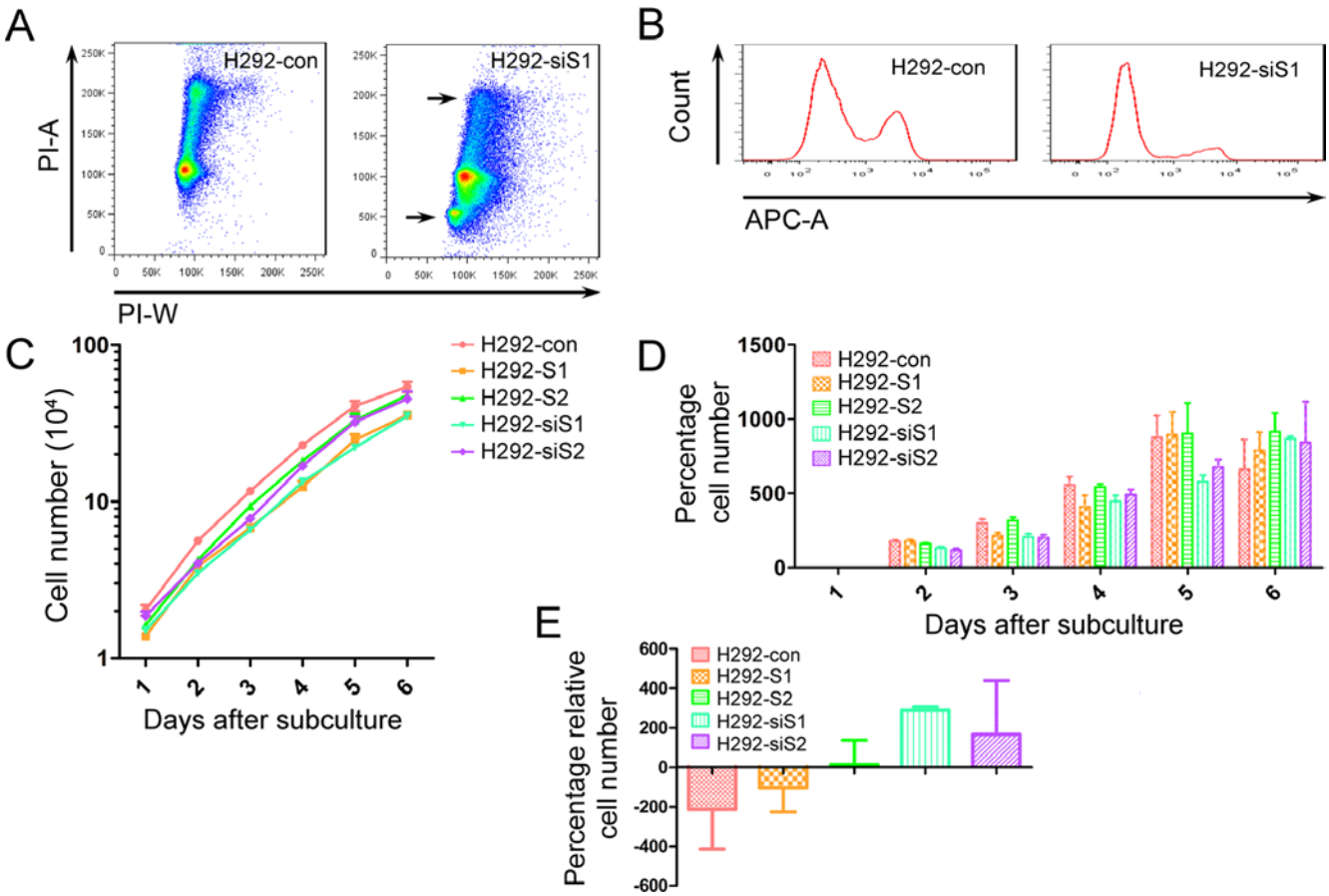

Figure 2. (A) Cell cycle in H292-con and H292-siS1. The higher arrow indicates G2/M subset and the lower arrow indicates apoptotic subset. (B) Number of EdU-positive cells in H292-con and H292-siS1. (C) Cell growth curve of stable cell lines. (D) Increased cell number in each day. (E) Differences of increased cell number between day 6 and 5. Experiments were repeated three times independently. Error bars represent the SD of measurements.

mechanism of changes of cell growth and DDP treatment to investigate why SNAILs failed to induce completed EMT.

Better cell growth needs SNAILs. To confirm the effects of SNAILs on cell growth, we utilized three methods: cell cycle, EdU test and growth curve. The percentage of G2/M phase in H292 silencing Snail (H292-siS1) obviously decreased compared with H292-con and an apoptotic subset was detected in H292-siS1 (Fig. 2A). EdU-positive cells also obviously decreased in H292-siS1 (Fig. 2B). Although there were no significant differences on the cell cycle and EdU test compared to the others, downregulation of Snail or Slug was not conducive to cell culture. For better understanding of growth differences, we drew cell growth curves for all stable cell lines (Fig. 2C). To avoid the potential different effects of chemo-biological reaction between each stable cell line, CCK-8 test was not used for the cell growth curve. Through comparing the percentage of increased cell number, we found cell growth was limited more in H292-siS1 and H292-siS2 (Fig. 2D and E). During the late stage of subculture, cell growth was limited less in H292-S1 and H292-S2. This can be the result of weakened contact inhibition. The results indicate better cell culture needs for SNAILs.

SNAILs respond differently to DDP treatment. To confirm the effects of SNAILs on DDP treatment, we investigated three aspects: cell viability, late apoptosis and cleaving of caspase 3. The cell viability of H292-siS2 reduced to a maximum of 60% when the concentration of DDP was up to 10 μg/ml (Fig. 3A). We utilized double stain of Hoechst 33342/PI to detect late apoptosis, in order to avoid false-positive Annexin V causing membrane damage during cell digestion (digestion is time-consuming in H292 compared with other lung cancer cell lines). The rate of late apoptosis also significantly reduced in H292-siS2 compared with the others (Fig. 3B). There were no statistical differences in the rate of late apoptosis of H292-siS1 compared with H292-con, H292-S1 and H292-S2, but we observed increasing tendency of late apoptosis in H292-siS1. Through analyzing the cleaved caspase 3, we confirmed DDP-related apoptosis significantly increased in H292-S2 and H292-siS1, and decreased in H292-S1 and H292-siS2 (Fig. 3C).

SNAILs affect the apoptotic stage after DDP treatment. Surprised by the responses of SNAILs to DDP treatment, we first checked the response process after DDP interaction with DNA. We did not check the intracellular concentration of DDP because it may not be appropriate in our situation (the drug has passive entry and ATP-dependent efflux). The process of cellular response mainly includes three parts: damage perception, repairing, and outcome (survival or apoptosis) (37). The expression of p53 and γ-H2AX, regulated by ATM/ATR (38), the main molecules conducting

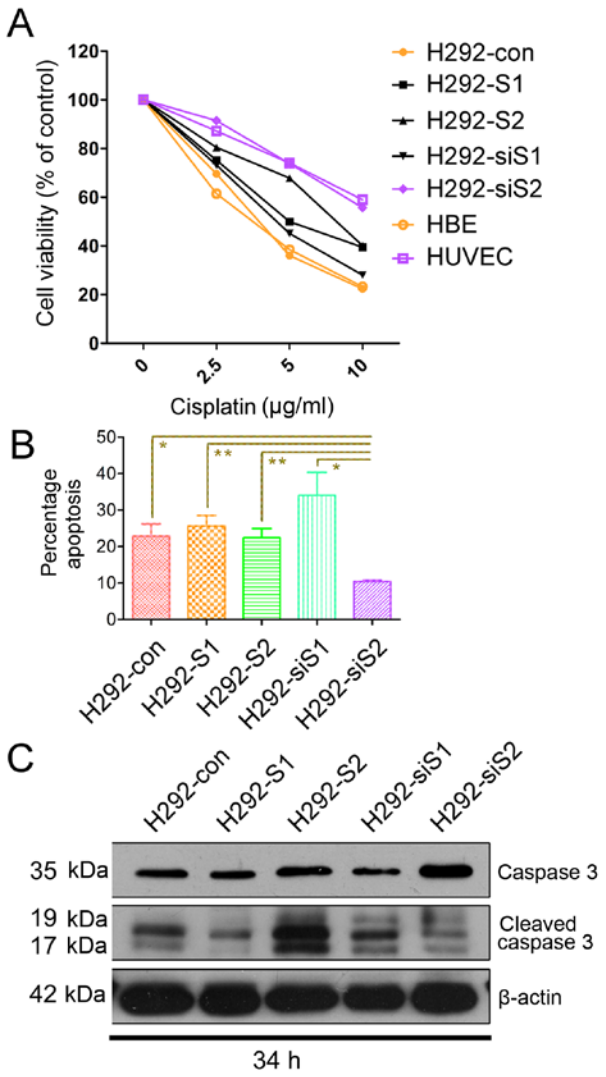

Figure 3. (A) Cell viability alteration after DDP treatment for 24 h. (B) The rate of late apoptosis after DDP (5 μg/ml) for 30 h. (C) Cleaving caspase 3 after treatment with DDP (5 μg/ml) for 34 h. Experiments were repeated three times independently. Error bars represent the SD of measurements. *P<0.05. **P<0.01.

DNA damage signal during DDP treatment (39), were upregulated after irradiation, but had no significant differences among the stable cell lines (Fig. 4A and B). Thus, the ability of DNA damage exists, but there was no significant differences among the stable cell lines. The expression of ERCC1, the main repair factor in DDP-related DNA damage, was also similar at mRNA and protein levels among each stable cell line (Fig. 4C and D). This indicates the capacity of DDP-related DNA damage repair, but again no significant difference was observed in stable cell line.

At the early stage of DPP-induced apoptosis, the expression of apoptotic signal molecules were increased in each stable cell line (Fig. 4E). Except cytochrome *c* (cyto *c*), manipulating either Snail or Slug did not change the expression of Bax, Bcl-2, Bcl-xl, pro-caspase 3 and cleaved-caspase 3. The expression of cyto *c*, reflecting the coordinating effect of pro-apoptotic (e.g., Bax) and pro-survival (e.g., Bcl-2, Bcl-xl) signals, was significantly increased in H292-siS1 compared with H292-con, but significantly decreased in H292-S1, H292-S2 and H292-siS2. The higher level of cyto *c* in H292-siS1 indicates the absence of Snail enhancing the activation of apoptotic signal. The lower level of cyto *c* in both H292-S2 and H292-siS2 suggests the effect of Slug on the DDP-related apoptosis is not dominated by DDP-induced apoptotic signal.

SNAILs affect DDP-related apoptosis via different approaches. Survival pathways can affect DDP sensitivity (37). In addition, Snail can activate survival pathways in serum deprivation (SD) (40). Our results show the absence of Snail enhanced activation of the apoptotic signal. Hence, we speculated Snail enhances the pro-survival signal (e.g., Bcl-xl, of which the function can be enhanced by survival pathways) to affect the apoptotic signal. We treated cells with SD, and the rate of late apoptosis after SD was higher in H292-siS1 (Fig. 5A). Then, we analyzed the expression alteration of Bcl-xl after SD (Fig. 5B). At each check point, the level of Bcl-xl did not increase in H292-siS1, but was always higher in H292-S1. These results support that Snail utilizes pro-survival signal to decrease DDP-related apoptosis. Moreover, the rate of late apoptosis was not significantly different between H292-S2 and H292-siS2 (Fig. 5A). This also suggests that Slug utilizes a different approach to affect DDP-related apoptosis.

DDP sensitivity can also be affected by the function of G1 phase monitoring point. Knockout of *TP53* in MCF-7 cells greatly improved DDP sensitivity through functional deficiency of G1 phase arrest or nucleotide excision repair (NER) (41). We wondered whether the effect of Slug to DDP sensitivity is related to G1 phase monitoring point. Cells, with normal function of G1 phase monitoring point, will show a G1/S phase arrest after irradiation (42). Thus, we investigated G1/S phase arrest after irradiation. H292-S2 did not show the arrest after irradiation (Fig. 5C). Since we studied changes at only one time point, it only supports that overexpressing Slug does delay the generation of G1/S phase arrest.

p21 is a key effector molecule guiding G1/S phase arrest. Hence, we suspected Slug affects the function of p21, and weakens the function of G1 phase monitoring point. We used immunofluorescence to detect changes of p21 expression before and after irradiation. The fluorescence expression of p21 had no significant differences between each irradiated stable cell lines (Fig. 5D). Thus Slug has no direct effects on the monitoring point, considering no significant alteration of expression of p53 was seen after irradiation. The generation of G1/S phase arrest is the result of interaction between the forward momentum of cell cycle and the resistance of monitoring point in G1 phase. It was clear that monitoring point has no significant functional deficiency. Slug enhances the forward momentum of the cell cycle, and delays the emergence of G1/S phase arrest. i.e., Slug promotes G1/S phase transition. This causes more DNA damage to enter G2/M phase when DNA damage stimuli (e.g., DDP, irradiation) exist. As a consequence, cell death will be more easily triggered in mitotic phase.

SNAILs promote different stages of G1 phase via different pathway. The aforementioned results have shown that SNAILs differently affects DDP-related apoptosis via different molecular mechanisms. Considering the impact on cell growth, we speculated that the mechanism of integrated effects of SNAILs will be a change of cell cycle kinetics in

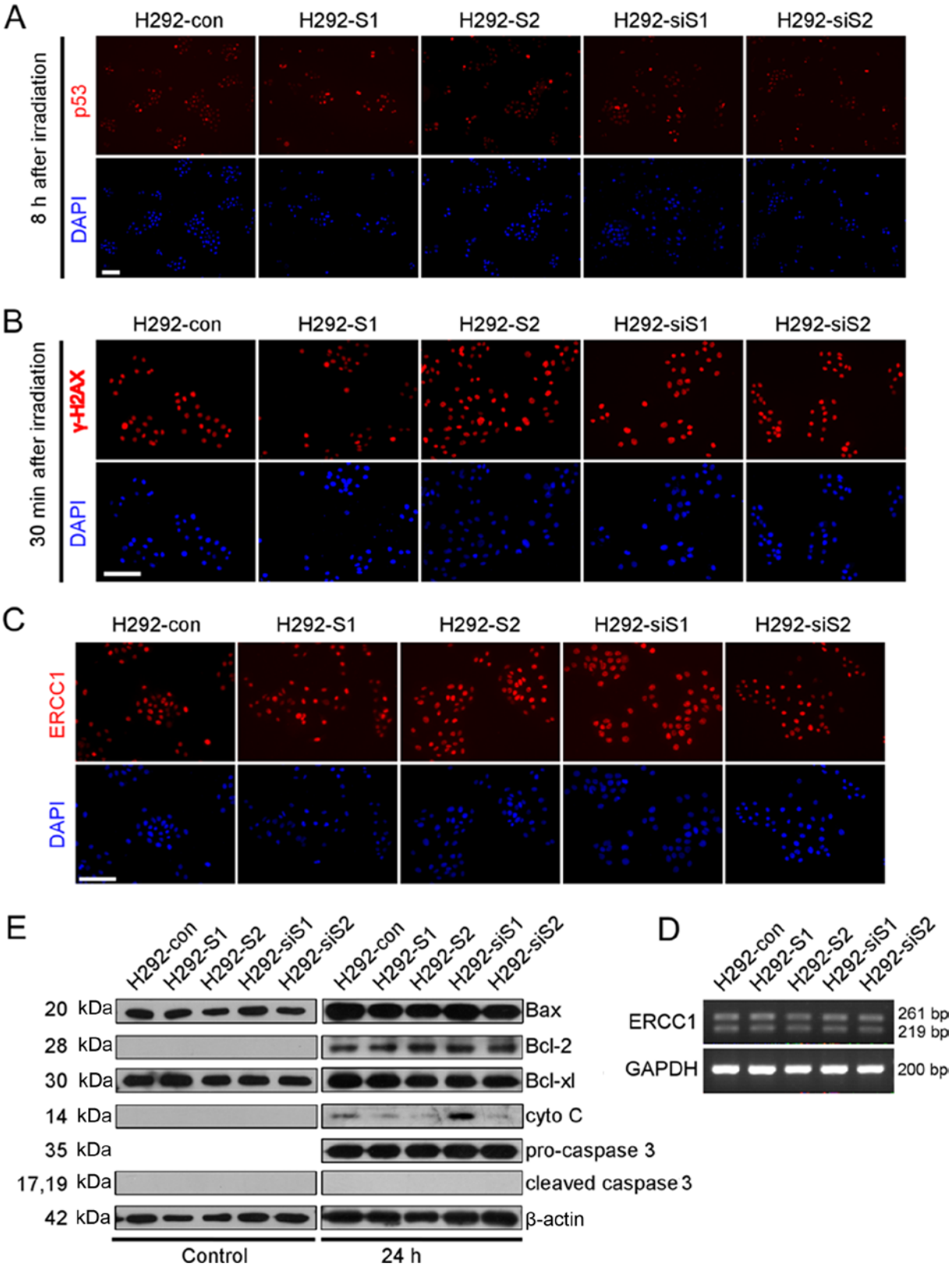

Figure 4. (A) Expression of p53 after irradiation. (B) Expression of γ-H2AX after irradiation. (C) Protein expression of ERCC1. (D) The mRNA expression of ERCC1. (E) Expression of apoptotic molecules after treatment with DDP (5 μg/ml) for 24 h. Experiments were repeated three times independently. Bar, 100 μm.

G1 phase. To prove this hypothesis, we treated cells with serum stimulation and detected the dynamic alteration of expression of cyclin D1 and E1. The expression alterations of cyclin D1 and E1 were combined to reveal the progression kinetics of G1 phase (both early and late stages), and also to partly reveal the functional status of each other.

After serum stimulation (Fig. 6A), the expression of cyclin D1 was significantly lower in H292-S1, but higher in

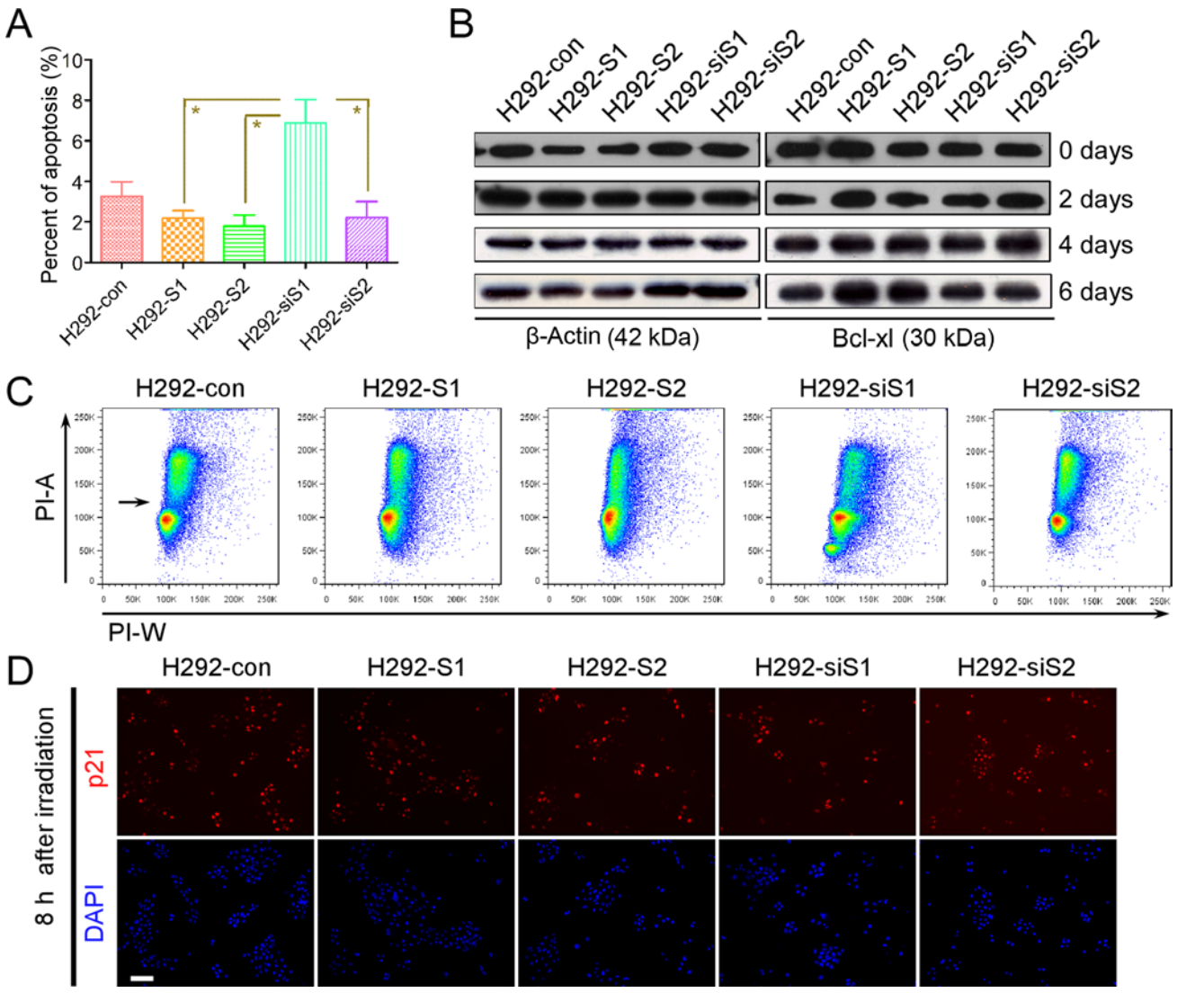

Figure 5. (A) The rate of later apoptosis after serum deprivation (SD). (B) The expression alteration of Bcl-xl after SD. (C) G1/S phase arrest after irradiation. Arrow indicates G1/S phase arrest. (D) p21 expression after irradiation. Bar, 100 μm. Experiments were repeated three times independently. Error bars represent the SD of measurements. *P<0.05.

H292-siS1. The expression of cyclin E1 did not significantly differ between the two cell lines. The results indicate H292-S1 more easily enter into the late stage of G1 phase compared with H292-siS1. This supports that the progression of early G1 phase is faster in H292-S1, while slower in H292-siS1. It does not indicate whether the function of cyclin D1 is enhanced or weakened in H292-S1 and H292-siS1. The expression of cyclin D1 had no significant differences between H292-S2 and H292-siS2. However, the expression of cyclin E1 had significant differences in the two sets compared with the control group. Cyclin E1 was increasing faster in H292-S2 than in H292-con and H292-siS2. Although the baseline expression of cyclin E1 in H292-siS2 was significantly increased, the speed was slower than H292-con and H292-S2. These results illustrate that the late G1 phase progression is faster in H292-S2 than in H292-siS2. The expression alteration of cyclins indicates the function of cyclin E1 is weakened in H292-siS2.

In conclusion, both Snail and Slug promote G1 phase. We speculated the process of promoting G1 phase may involve Akt or c-Myc pathway (Fig. 6B), and detected relevant molecules. The expression of Akt, phospho-Akt, and phospho-GSK3β significantly reduced in H292-siS1 (Fig. 6C). This indicates Snail is essential for the Akt/GSK3β pathway. The expression of c-Myc was significantly upregulated in H292-S2 (Fig. 6D), supporting that Slug can upregulate c-Myc. These results indicate Snail and Slug can utilize Akt or c-Myc to promote G1 phase.

Although upregulation of Akt/GSK3β pathway was not detected in H292-S1 (Fig. 6C), overexpressing Snail can still facilitate G1 phase progression after cell cycle synchronization by SD, via the ability of activating Akt in the absence of serum (40). Besides, downregulation of c-Myc was not detected in H292-siS2 either (Fig. 6D). Knockdown of Slug can downregulate c-Myc in *Xenopus laevis* embryos (43). Because KRAS can activate c-Myc through MAPK (44), no-decline of c-Myc expression in H292-siS2 can result by the neutralizing effect of KRAS activated by culture medium containing serum. Moreover, combined with the weakened function of cyclin E1 in H292-siS2 (Fig. 6A), the expression

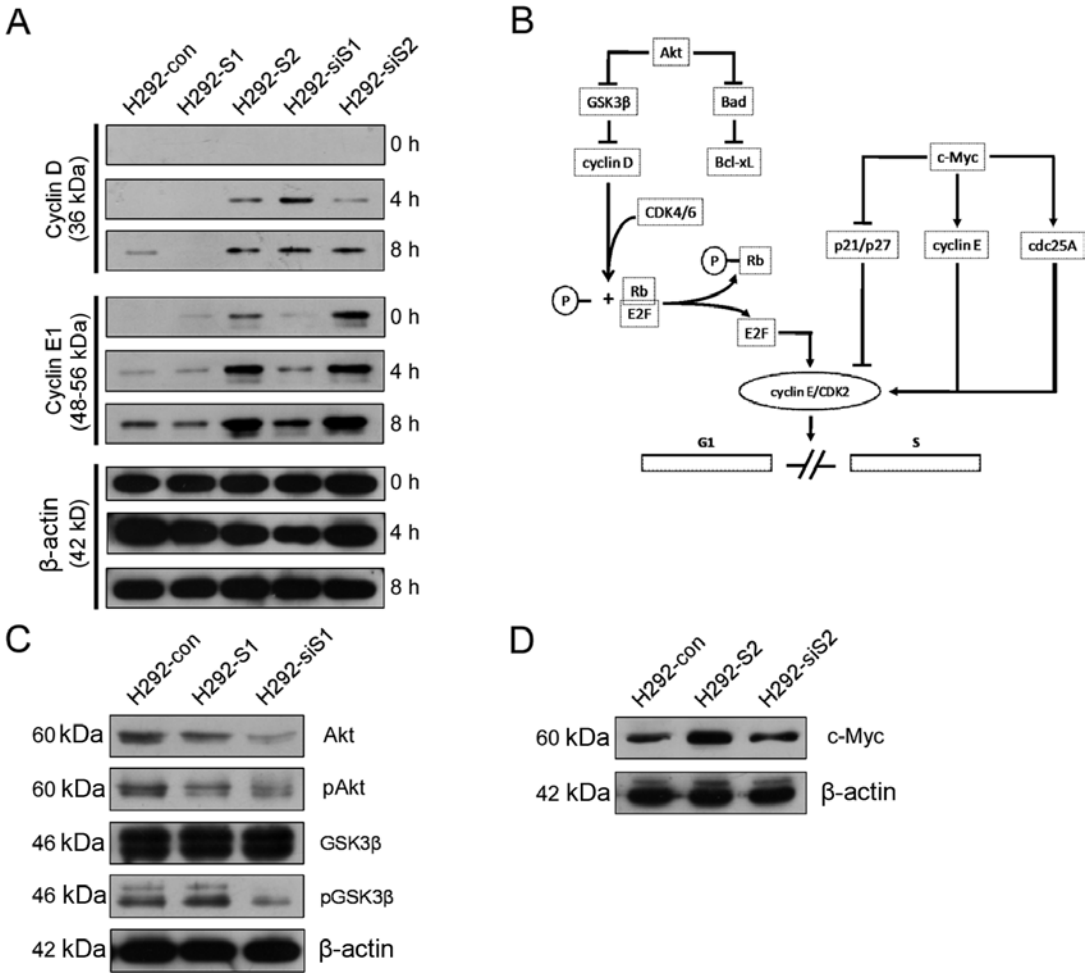

Figure 6. (A) Alterations of expression of cyclin D1 and E1 after serum stimulation. (B) Mode of Akt and c-Myc regulated G1 phase progression. (C) Expression of Akt/GSK3β pathway. (D) Expression of c-Myc. Experiments were repeated three times independently.

of c-Myc in H292-S2 and H292-siS2 indicates Slug enhances not only the expression but also the function of c-Myc. Hence, downregulation of Slug can still block G1 phase progression after cell cycle synchronization by SD.

Discussion

The most important finding of this investigation is the promoting action of SNAILs to G1 phase. It is contrary to the acknowledged blocking effect (40,45). Drug-induced downregulation of Snail was connected with upregulation of p21 and G1 phase arrest (46). However, those who first found the repression effect of Snail on p21 (47), already indicate it may be dependend on the type of the cell line. In an epithelial cell line (MDCK cells), Snail induced G0/G1 arrest through increased expression of p21. In a mesenchymal cell line (MG63 cells), Snail suppressed E2A-dependent activation of the p21 promoter. In contrast, the G1 phase-promoting effect reported by us is inherent in an epithelial cell line and not related to the drug. Our results revealed a novel value of the heredity alteration during phenotype transition.

The G1 phase-blocking effect is one of the basic roles that make Snail genes the regulators of epithelial phenotype and of cell adhesion and movement (3,48). Snail genes as regulators of phenotype transition in development, are also very important to many pathological processes (e.g., tumor metastasis, tissue repair/regeneration). Considering the connections of EMT, tumor metastasis and tissue repair/regeneration (49), Mani *et al* further proved that EMT can generate cells with stem cell properties (2). Hence, blocking G1 phase is one of the basic conditions for the onset of EMT-related phenotype transitions. However, SNAILs fail to block G1 phase in deliberately selected cancer cells. The discovery of this unexpected phenomenon typically reveals the heterogeneity of cancer cells. In fact, heterogeneity is caused by heredity alteration (genetic or epigenetic). Hence, our findings reveal the onset of EMT-related phenotype transitions in cancer needs not only the induction and activation of SNAILs, but also some particular heredity alterations (Fig. 8).

The unexpected phenomenon is very rare. We cannot deny its existence, even though the results mainly came from single cell clonal populations derived from a single cell line. After all, the results of each stable cell line can be supported by each other. In addition, all clones of the same transfection possess the same phenotype (data not show).

According to our results, we cannot say that the genotype of interest (wild-type *KRAS*/*TP53*/*EGFR*/*ERCC1*/*Keap1*/*Nrf2*) is the right heredity alteration. In addition, the results of studies involving phenotype and these molecules also indicate the situation is much more complicated than first thought.

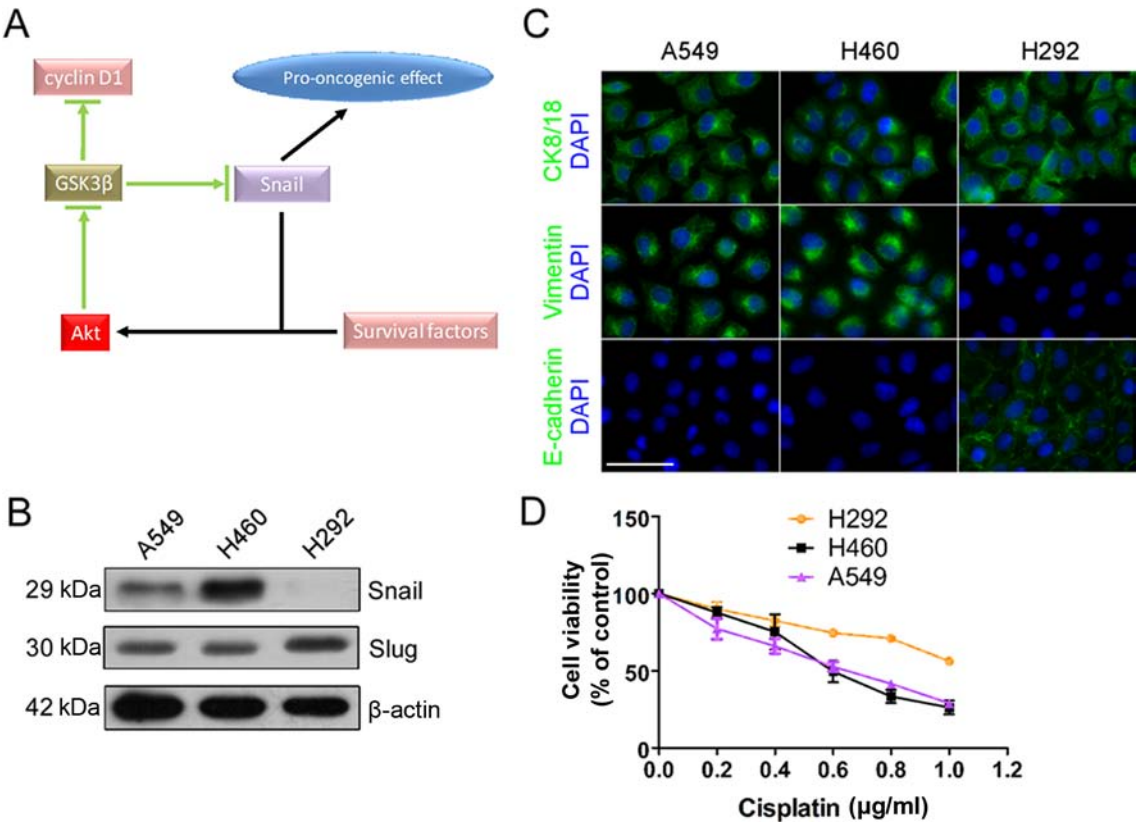

Figure 7. (A) Snail/Akt/GSK3β constitutes a signal loop. (B) Expression of Snail and Slug in A549, H460 and H292. (C) Expression of EMT markers in A549, H460 and H292. Bar, 50 μm. (D) DDP sensitivity in A549, H460 and H292. Experiments were repeated three times independently.

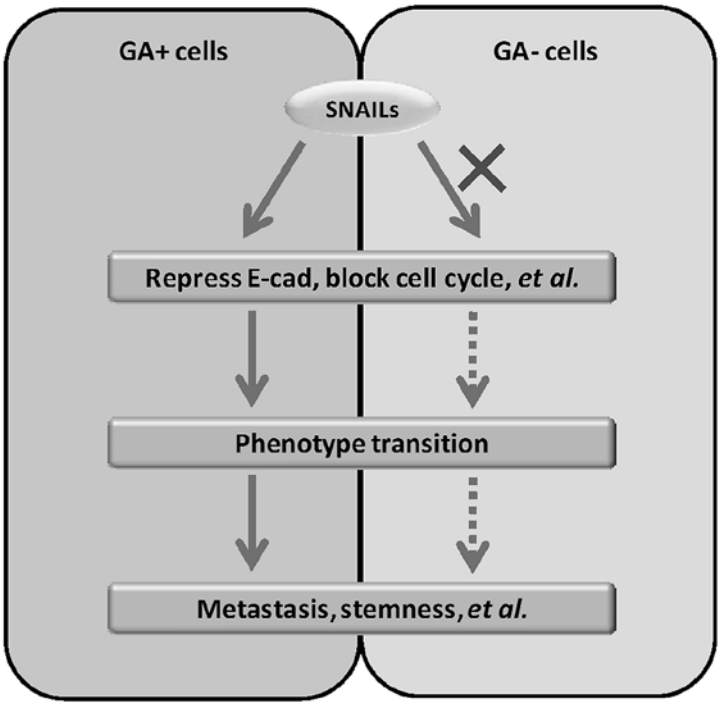

Figure 8. Schematic illustration of the relationship between genetic alteration and SNAIL-induced phenotype transition in cancer cells. Dotted arrow indicates the process cannot happen. GA, genetic alteration.

We discovered the unexpected phenomenon, but we do not know which hereditary factors caused the phenomenon. The unknown heredity alterations can be genetic or epigenetic. To identify the actually relevant heredity alterations in human body, comparison of alteration between primary and matched metastatic tumor tissue (50,51) may be an efficient approach.

However, the results of published studies are inconsistent. Hence, further studies need to be conducted to establish the most efficient and feasible approach.

Our study uncovered a new connection between heredity alteration and phenotype transition. This connection will offer new clues and rationales for distinguishing particular cancer cell populations (e.g., metastatic and non-metastatic cancer cells, 'innate' and 'acquired' CSCs). Heredity alteration-based distinguishing will identify the right target cells, no matter when or whether they begin phenotype transitions. If the correct heredity alterations can be identified, heredity alteration-directed specific target therapy would be an efficient approach to prevent tumor recurrence and metastasis.

Via EMT, cancer cells can acquire a stem cell phenotype and capacity of tumorigenesis, metastasis and therapeutic resistance (3). Hence, the origin of CSCs is being questioned (52). According to our results, the generation of 'acquired' CSCs (induced by EMT) at least needs some particular heredity alterations. In fact, genetic heterogeneity was already found to exist in lung CSC populations, and related to intrinsical cellular diversity (53,54). Notably, Kreso *et al* proposed their own hypothesis regarding genetic alteration and CSC (two mutually exclusive models for tumor hererogeneity) (55). They speculated tumor-initiating cells (T-ICs) can evolve and acquire additional genetic mutations. However, studies cited to support their speculation are mainly the description of correlation, and importantly, there is no direct evidence indicating that non-T-ICs do not generate T-ICs after acquiring aggressive mutations. Taken together, more attention to heredity alteration is required, whether investigating the origin of CSCs or the causality of mutually exclusive models.

Overexpressing Snail activated Akt signaling in the absence of serum (Fig. 5A and B) (31). When serum existed, overexpressing Snail did not enhance the Akt/GSK3β pathway, but Snail was essential for pathway activation (Fig. 6C). GSK3β can inhibit Snail and be inhibited by Akt. So, we proposed Snail/Akt/GSK3β constitutes a signal loop (Fig. 7A). In cells with minimal amount of Snail (e.g., H292 cells, compared with A549 and H460, Fig. 7B), survival factors (e.g., some kind of growth factors in serum) activate Akt and inhibit GSK3β, and then induce upregulation of Snail and stabilization of cyclin D1. However, the high level of Snail no longer enhances the activity of Akt. KRAS can interact with PI3K/Akt pathway and mutant KRAS is needed for the pathological roles of Snail in pancreatic fibrotic disease (56). In A549 and H460 cells with mutant *KRAS* and wild-type *TP53/EGFR/ERCC1* (H460 also has *PI3K* mutation), we observed high level of Snail (Fig. 7B), and a mesenchymal phenotype (Fig. 7C). DDP sensitivity was increased in A549 and H460 (Fig. 7D), accompanying with significantly increased proliferation capacity. We speculate that KRAS participates in the Snail/Akt/GSK3β signal loop and is one of the guarantors of the canonical roles of Snail, but more study to confirm this is required.

In conclusion, we uncovered an unexpected role of SNAILs in selected cancer cells, and provided significant knowledge to the investigation of EMT-related phenotype transitions. However, more questions are raised than answered. More study is necessary in order to achieve better effect of personalized therapy in cancer patients.

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