Snail-induced epithelial-mesenchymal transition promotes cancer stem cell-like phenotype in head and neck cancer cells

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is known to have a poor prognosis. The resistance to treatment and distant metastasis are important clinical problems in HNSCC. The epithelial-mesenchymal transition (EMT) is a key process in successful execution of many steps such as the invasion and metastasis for cancer cells. Snail is one of the master regulators that promote EMT in many types of malignancies including HNSCC. Recently, it has been shown that Snail-induced EMT could induce a cancer stem cell (CSC)-like phenotype in a number of tumor types. In this study, we investigated the role of Snail in inducing EMT properties and CSC-like phenotype in HNSCC. We established HNSCC cell lines transfected with Snail. E-cadherin was analyzed using western blot analysis and immunofluorescence staining. Cell migration and invasion were assessed using wound-healing assay and modified Boyden chamber assay, respectively. CSC markers of HNSCC, CD44 and aldehyde dehydrogenase 1 (ALDH1), were also evaluated with western blot analysis, and chemosensitivity was assessed with WST-8 assay. Introduction of Snail induced EMT properties in HNSCC cells and enhanced cell migration and invasion. Moreover, Snail-induced EMT gained CSC-like phenotype and was associated with increased chemoresistance. These results suggest that Snail could be one of the attractive targets for the development of therapeutic strategies in HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer in the world and also known for its rapid clinical progression and poor prognosis (1,2). The mortality is mainly caused by locoregional recurrence and cervical lymph node metastasis and occasionally by distant metastasis (3). Notably, regional and distant metastases in HNSCC correspond to an extremely poor prognosis with limited treatment options. The treatment resistance and tumor recurrence are important clinical problems in the management of HNSCC. To improve the therapeutic outcome of HNSCC, more effective treatment strategy is urgently needed.

Epithelial-mesenchymal transition (EMT) is a critical process in tumor progression that causes epithelial cells to acquire a migratory mesenchymal phenotype (4,5). EMT is thought to be a crucial step in the induction of cell invasion and tumor metastasis (4). Furthermore, it has also been shown that cells with an EMT phenotype are more resistant to chemoradiotherapy in HNSCC (6).

Snail, a zinc-finger transcription factor, plays an important role in EMT by directly repressing epithelial marker such as E-cadherin and by upregulating mesenchymal markers (7-12). Several studies have shown that Snail-related transcription factors play a transcriptional and regulatory role in invasion, metastasis, and poor outcome for different type of malignancies, including HNSCC (13,14).

It has been suggested in recent reports that Snail-induced EMT causes the cancer stem cell (CSC)-like properties in different type of malignant tumors and that both EMT and CSC-like phenotype are associated with treatment resistance (11,15,16). Prince *et al* showed that the purified CD44^{+} population of HNSCC cells possesses the self renewing properties of CSCs (17). Aldehyde dehydrogenase 1 (ALDH1) has also been shown to be a putative marker of CSC in HNSCC (18-20). Furthermore, Chen *et al* showed that CD44^{+}/ALDH1^{+} cells

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resist radiotherapy and may serve as a reservoir for developing tumors and metastasis (21). These findings suggested that Snail expression may regulate the CSC-like properties in HNSCC via EMT. On the contrary, there is also a report that Snail expression did not correlate with prognosis (22).

The key role of Snail in HNSCC has not been fully elucidated. In this study, we demonstrate that introduction of Snail in HNSCC cells confers EMT properties such as increased cell motility and invasiveness *in vitro*. In addition, we report that Snail-induced EMT gains HNSCC cell CSC-like phenotype and are associated with chemoresistance.

Materials and methods

Cell lines and culture. Human HNSCC cells, SAS and HSC-4, were employed in this study. SAS cells and HSC-4 cells, obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan), were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA) at 37˚C in 5% CO_2.

Transfection with Snail in SAS and HSC-4 cells. cDNA fragment encoding human Snail (NM_005985.2) was inserted into pCR 3.1 mammalian expression vector (Invitrogen). SAS and HSC-4 cells ($1.5x10^5$ cells) were plated into 6-well culture plates and allowed to adhere for 12 h. Then, SAS and HSC-4 cells were transfected with 2 μg of either pCR 3.1-Snail or pCR 3.1-vector (without insert DNA) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. We established SAS-Snail and HSC-4-Snail as transiently Snail-expressing cell lines, and their respective control cell lines. All assays were performed 24 h after transfection.

Immunoblot analysis and antibodies. Snail, E-cadherin, CD44 and ALDH1 signaling on SAS and HSC-4 cells after the transfection with or without Snail were evaluated with western blot analysis. Cells were collected and frozen in 100 μl RIPA buffer, and stored at -30˚C. Briefly, total protein extracts were prepared according to the freeze-thawing lysis method and protein concentrations were measured with Bovine Serum Albumin (BSA) Protein Assay. Sample of extract containing 20 μg of protein were then separated by sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes, after washing with phosphate-buffered saline with Tween-20 (PBST), the membranes were incubated first with rabbit anti-Snail, rabbit anti-E-cadherin (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1,000), rabbit anti-CD44 and goat anti-ALDH1 (Abcom, Cambridge, MA, USA; diluted 1:2,000 and 1:500, respectively) at 4˚C overnight and then with peroxidase-conjugated secondary anti-rabbit or goat immunoglobulin G (IgG) (Cell Signaling Technology; diluted 1:1,000) for 1 h. After rinsing in PBST (4 times, 5 min each), immunodetection was accomplished using an ECL western blot analysis detection reagent and analysis system. The membranes were subsequently exposed to X-ray film as described previously (23).

Immunofluorescence staining. Cells were cultured in Labtech chamber slide system (Thermo Scientific, Waltham, MA, USA), and then fixed with 4% paraformaldehyde for 20 min at room temperature. After rinsing with phosphate-buffered saline (PBS), the cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min. Then, they were blocked with 1% BSA and 0.1% Tween-20 in PBS for 1 h at room temperature and incubated overnight at 4˚C with rabbit anti-E-cadherin antibody (diluted 1:200). After rinsing with 0.1% Tween-20 in PBS, chamber slides were incubated with fluorescent-labeled secondary antibody (goat anti-rabbit-IgG-Alexa Fluor 594; Invitrogen; diluted 1:1,000) for 1 h at room temperature in the dark. The slides were then mounted with Prolong gold antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). The fluorescent images were visualized by confocal image microscopy (Keyence, Osaka, Japan).

Wound healing migration assay. Equal number of cells was plated onto 24-well culture plates in DMEM medium and cultured for 24 h. The cell monolayer was scraped with a 200-μl pipette tip, washed with PBS and changed to fresh medium. The wound closure was photographed at 24 h after wounding with control at 0 h under phase contrast microscope. The wound width was measured in three points per image. This experiment was repeated at least three times on each cell line. The cell migration potency was determined by calculating a difference between wound width at 0 and 24 h.

Invasion assay. Invasion assays were performed using 24-well Matrigel-coated Transwells (BD Bioscience, Bedford, MA, USA) (24). Cells ($4x10^4$) were suspended in 200 μl of serum-free DMEM medium and placed in the top chambers, and 700 μl DMEM medium containing 10% FBS was added to the bottom chambers. After 24 h of incubation at 37˚C, non-invading cells were removed from the top of the Matrigel with a cotton swab, while invading cells on the bottom surface of the filter were fixed in 4% paraformaldehyde and stained with Giemsa (Sigma-Aldrich, Dorset, UK) for 30 min. The invading cells were then visualized at x200 magnification and counted in five fields for each filter.

Chemotherapy for cultured cells. In chemotherapy, cells were treated with cisplatin (Nihonkayaku Co., Tokyo, Japan) at concentration of 1.0 or 10 μM. Chemosensitivity was assessed by Cell Counting Kit-8 (WST-8 cleavage; Dojindo, Mashikimachi, Japan) as described previously (23). The cell viability after the chemotherapy for the cells was evaluated with the WST-8 cleavage. The cells were seeded in 96-well plates at an initial density of $4x10^3$ cells/well and incubated for 24 h. For chemotherapy, cisplatin (0-10 μM) was added to each well. Following incubation for an additional 48 h, 10 μl of WST-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to each well, and the plate was incubated for further 2 h. The absorbance of each well at 450 nm (reference wave length at 620 nm) was measured by a Multiscan FC Microplate Photometer (Thermo Scientific). The measurement was repeated at least three times for each cell line.
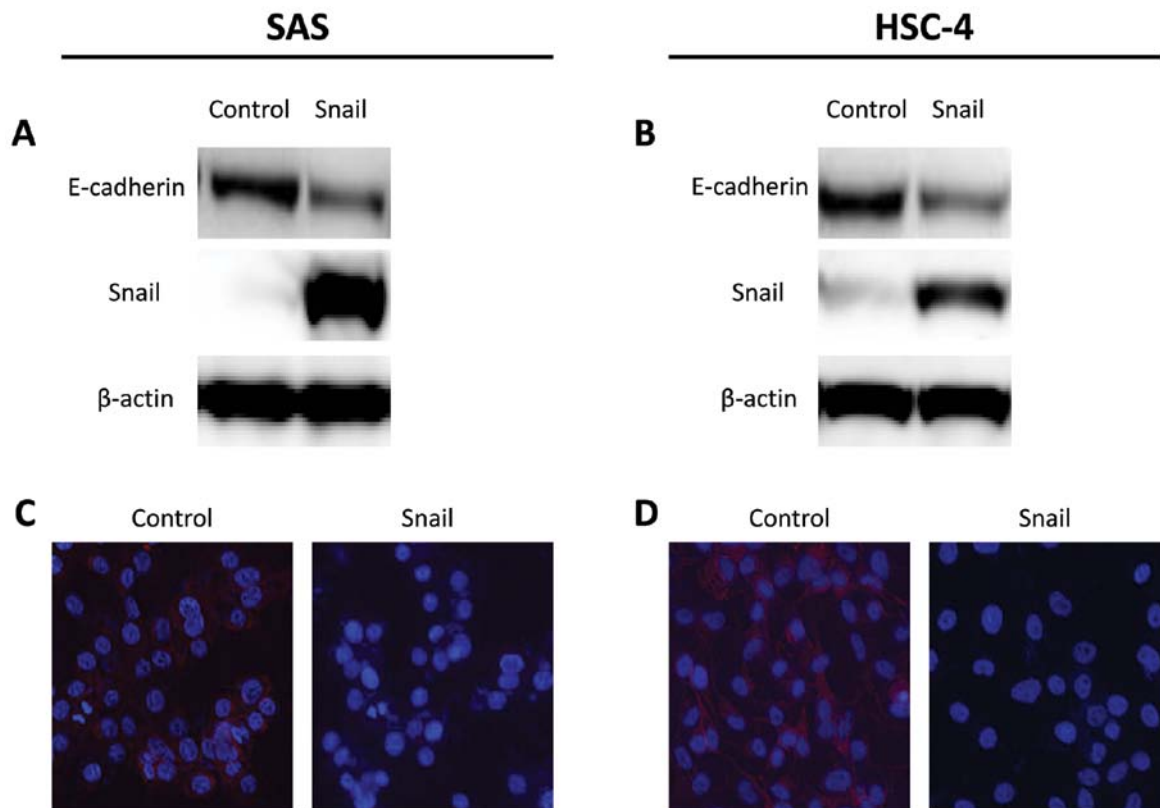
Figure 1. Snail regulates E-cadherin expression. SAS and HSC-4 cells were transfected with control or Snail expression vector. (A and B) E-cadherin and Snail protein levels were determined by immunoblot analysis using β-actin as the loading control. (C and D) Cells were stained with E-cadherin antibody [red, E-cadherin; blue, 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei].

Statistical analysis. Data were presented as mean ± standard error (SE). Experimental differences between groups were assessed with the t-test. The differences were considered to be significant at P<0.05.

Results

Snail regulates EMT properties in HNSCC cells. SAS and HSC-4 cells were tranfected with Snail or control vector. In western blot analysis, introduction of Snail enhanced the suppression of E-cadherin protein levels in SAS and HSC-4 cells (Fig. 1A and B). In addition, cellular staining pattern of E-cadherin was examined by immunofluorescence analysis. E-cadherin staining also decreased on the cell membrane in SAS-Snail and HSC-4-Snail cells, whereas each control cell line stained positively for E-cadherin (Fig. 1C and D).

A wound healing migration assays showed that migrating cells significantly increase in SAS-Snail and HSC-4-Snail cells more than those in the control cells (Fig. 2). Furthermore, in invasion assays, both these cell lines tranfected with Snail displayed more invasive ability compared to the control cells with significant difference (Fig. 3). These results suggested that Snail was able to induce EMT in HNSCC cells.

Snail expression induces CSC-like phenotype in HNSCC cell lines. We demonstrated that EMT by Snail expression induced a stem cell-like phenotype in HNSCC cells. The expression of CSC surface markers in HNSCC cells was evaluated with

western blot analysis. Both CD44 and ALDH1 protein levels increased in SAS-Snail and HSC-4-Snail cells compared with their control cells (Fig. 4). These results implied that Snail-induced EMT could elicit a CSC-like phenotypic change as CD44^{+}/ALDH^{+} in HNSCC cells.

Snail expression enhances chemoresistance. The cells transfected with Snail showed significantly low chemosensitivity at 1.0 and 10 μM, as compared with the control cells (Fig. 5). Thus, these results implied that the acquisition of CSC-like phenotype caused by EMT results in enhancement of chemoresistance in HNSCC cells.

Discussion

In a variety of solid human tumors, the capacity to initiate and maintain cancer growth and recurrence has been found to reside in the small populations of cells within tumors, termed cancer stem cells (CSCs). CSCs have the ability to undergo self-renewal and produce differentiated progeny. These characteristics allow CSCs to maintain a pluripotent phenotype, while also producing a tumor composed of a heterogeneous cell population (25,26). Several studies have shown that CSC is implicated in tumor invasion and metastasis, and that tumor recurrence after therapy is correlated with therapeutic resistance of CSCs (27-30). CSC populations in HNSCC were first identified using CD44, which has been used as a marker of CSC in different type of malignant tumors including HNSCC
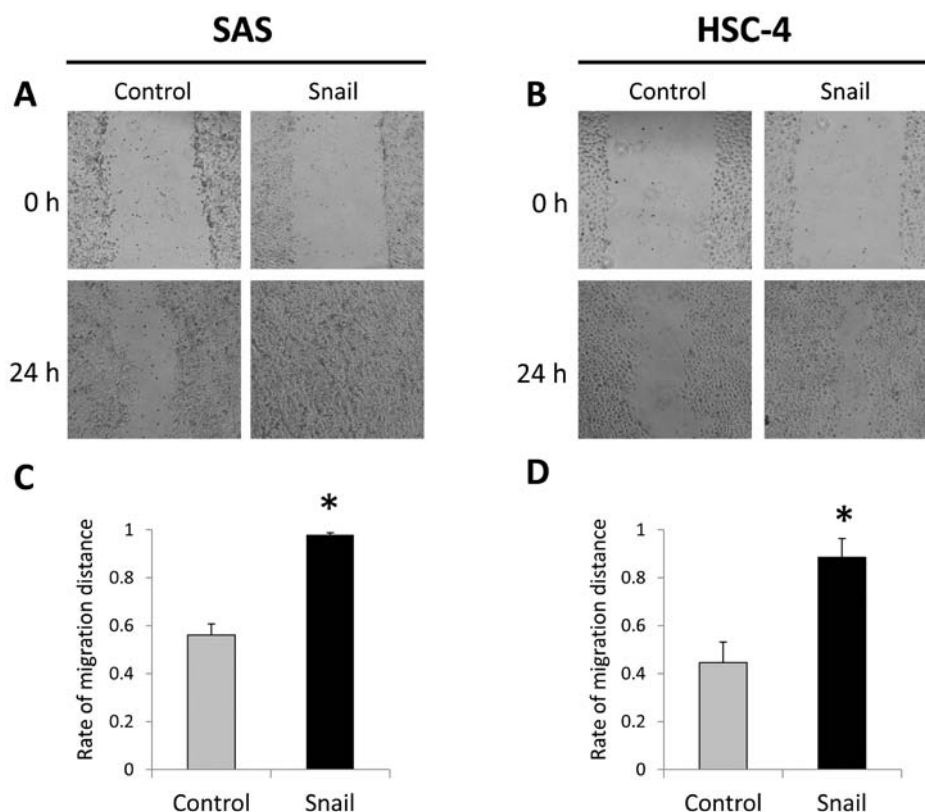
Figure 2. Snail expression increases cell migration in SAS and HSC-4 cells. (A and B) Phase contrast images were taken at 0 and 24 h after wounding. (C and D) The migration potency was determined by calculating a difference of wound width. Data are shown as mean ± SE. *P<0.05.
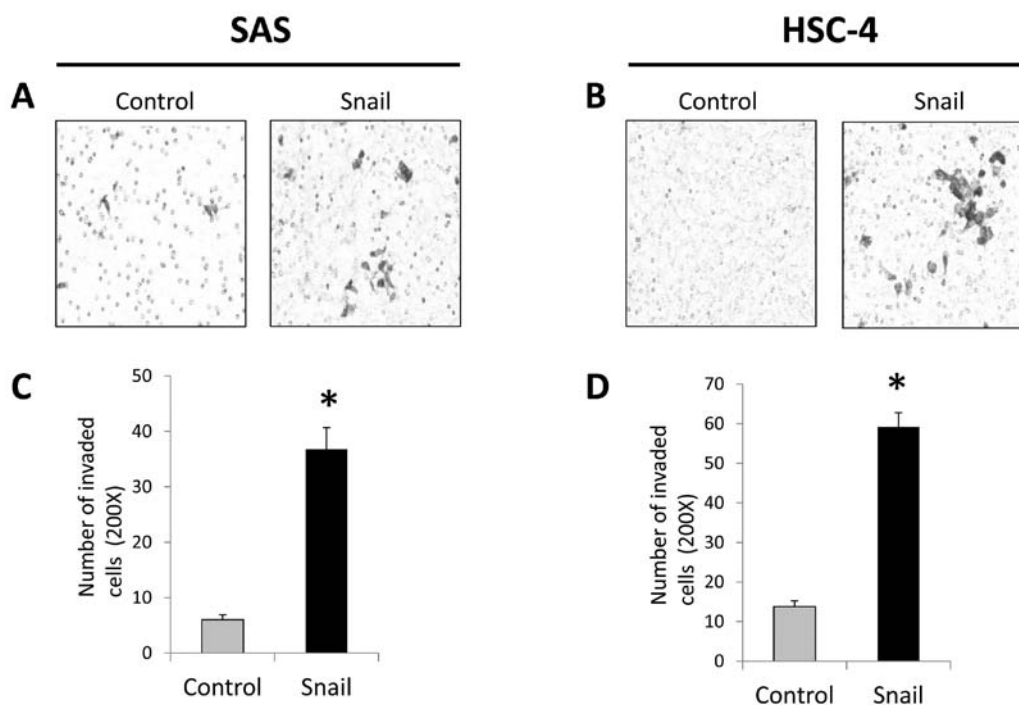

Figure 3. Snail expression increases cell invasion in SAS and HSC-4 cells. (A and B) Invading cells were visualized at x200 magnification. (C and D) Number of invading cells was counted in five fields for each filter. Results are expressed as mean ± SE. *P<0.05.

(17,31,32). However, HNSCC in CSCs are not precisely defined by CD44 expression alone (33). Recently, ALDH1 has been shown to be a marker of CSC. ALDH1 has also been used to identify the CSCs (34). In addition, Kirshnamurthy *et al* found that the combination of CD44 and ALDH1 is more selective for CSC populations than either marker used alone (35).
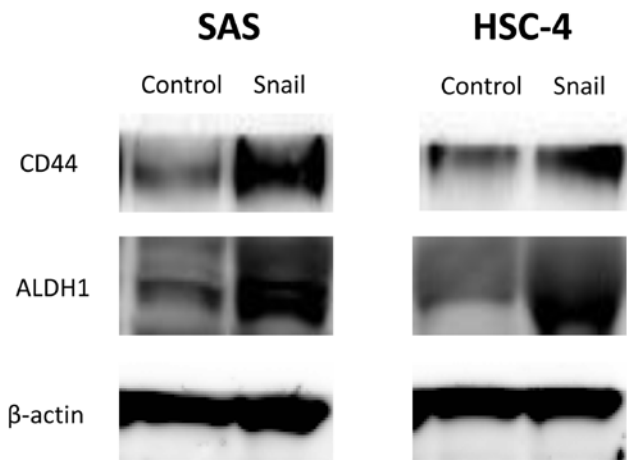
Figure 4. Snail induces expression of cancer stem cell (CSC) markers in SAS and HSC-4 cells. CD44 and aldehyde dehydrogenase 1 (ALDH1) protein levels were determined by western blot analysis with β-actin used as the internal control.

Epithelial tumor cells that undergo EMT lose cell-cell adhesion properties and acquire more mesenchymal properties, including invasiveness, motility and increase resistance to apoptosis. EMT is an important biological process that plays a critical role in tumor cell invasion, metastasis and recurrence, and is commonly observed in tumor samples from HNSCC patients (36,37). Moreover, the connection between CSC and EMT has become more evident. It has been described that induction of EMT results in cells gaining CSC-like properties and treatment resistance (16,38-40). Therefore, the importance of EMT in treatment resistance has recently been targeted for investigation of CSCs in different type of cancers, including HNSCC.

Snail is one of the master regulators that promotes EMT by repressing epithelial markers and upregulating mesenchymal markers and that mediates invasiveness as well as metastasis in many different types of malignant tumors including HNSCC

(41). Furthermore, it has been reported that Snail expression may regulate the treatment resistance and CSC-like properties of HNSCC (34,42). Medelsohn et al have recently reported that Snail is an independent marker of tumor metastasis in patients with HNSCC (43).

In this study, we showed that induction of Snail could suppress E-cadherin expression and increase motility and invasiveness of HNSCC cells. These results suggested that Snail could promote EMT and mediate tumor invasion. In addition, we demonstrated that induction of EMT via Snail could lead HNSCC cells to adopt CSC-like phenotype and chemoresistance for cisplatin. Previous data imply that CSCs also rely on a microenvironment, called the CSC niche, which controls their differentiation and proliferation (44-47). The CSC niche has a complex anatomical unit and is composed of diverse stromal cells, such as a vascular network, mesenchymal and immune cells, extracellular matrix (ECM), and soluble factors derived from niche cells (47-50). It has been suggested that interactions of CSC with CSC niche could induce tumor invasion and treatment resistance. The detail of interactions between CSCs and their niche are still unknown, and so the precise mechanisms should be verified in further investigation. However, understanding the interactions between the CSCs by Snail-induced EMT and their niche microenvironments, which contribute to treatment resistance, may pave the way for the development of novel strategies for treating cancer including HNSCC.

In summary, we obtained EMT properties by the overexpression of Snail in HNSCC cells. Moreover, these data suggest that Snail also acquires CSC-like phenotype via EMT and enhances treatment resistance. This Snail-induced EMT is considered to play an essential role in tumor progression and treatment resistance of HNSCC. Although the precise involvement of EMT and CSC by Snail remains to be elucidated, they could be involved in the latent effect. The critical mechanisms still need to be further investigated. However, the strategy targeting EMT-regulating Snail could be useful for cancer treatments, as the inhibition of EMT may serve
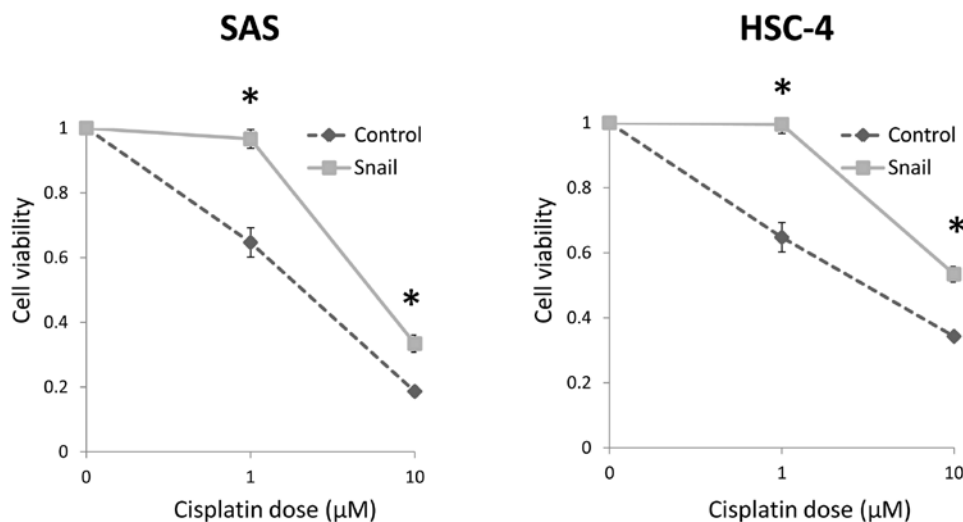

Figure 5. Snail expression enhances chemoresistance for cisplatin in SAS and HSC-4 cells. Cell viability was assessed using WST-8 assay for chemosensitivity. Cells were incubated for 24 h at first. For chemotherapy, cells were treated with various concentration of cisplatin. Following an additional 48 h, cell viability was determined by WST-8 assay. Data are shown as mean ± SE. *P<0.05.

to block not only cancer invasion and metastasis but also the formation of CSC.

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