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HCCS1 inhibits the stemness of human pancreatic cancer stem-like cells

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Abstract. – OBJECTIVE: SW1990-spheroid enrichment (SW1990-SE) cells were isolated using a new type of consecutive spheroid enrichment in this study. Cell surface markers were determined by flow cytometry for identification. *In vivo* tumorigenicity was applied by subcutaneous transplantation in nude mice for verifying the stemness characteristics of SW1990-SE cells.

MATERIALS AND METHODS: SW1990-SE cells were subjected to lentivirus infection for establishing the SW1990-SE cell line stably low-expressing HCCS1 (SW1990-SE-shHCCS1) and negative control cell line (SW1990-SE-LV3NC). The stemness regulatory effects of HCCS1 on SW1990-SE cells were evaluated by cell counting kit-8 (CCK-8) assay and 96-wells plate single cell cloning assay *in vitro*. Subcutaneous transplantation in nude mice was conducted for evaluating the *in vivo* stemness regulation of HCCS1 on SW1990-SE cells..

RESULTS: HCCS1 knockdown in SW1990-SE cells did not markedly change the cell proliferation and doubling time, whereas the *in vitro* spheroid diameter and single cell cloning efficacy remarkably increased. *In vivo* experiments showed that HCCS1 knockdown greatly enhanced the tumorigenicity of SW1990-SE cells in nude mice.

CONCLUSIONS: This study first obtains the human pancreatic cancer stem-like cells SW1990-SE through consecutive spheroid enrichment. Both *in vivo* and *in vitro* experiments verified that HCCS1 knockdown largely enhanced the stemness of SW1990-SE cells. Our study provides an important reference for the research of tumor stem cells.

Key Words
Human pancreatic cancer stem-like cells, Floating spheroid, HCCS1.

Introduction

Pancreatic cancer is one of the most severe malignancies in the world and has become a global public health problem due to its complex etiology and poor response to treatment[1-3]. Some authors[4,5] have shown that pancreatic cancer stem-like cells are associated with tumor metastasis, recurrence and drug resistance. Tumor stem cells exert infinite proliferative capacity, self-renewal ability, and multi-directional differentiation potential, accounting for a small part in tumor tissues. They are the root of tumor cells with various degrees of differentiation and tumor tissues with continuous growth[6-9]. Hence, explorations on the biological functions of tumor stem cells are important.

The self-renewal mechanism of tumor stem cells has been well explored in recent years. In addition to some important transcription factors, the regulatory effect of the tumor microenvironment on the stemness of tumor stem cells has attracted much more attention. HCCS1 (hepatocellular carcinoma suppressor gene 1) is a newly discovered candidate tumor-suppressor gene located in the 17p13.3 region of the chromosome. The complementary deoxyribose nucleic acid (cDNA) of HCCS1 has a total length of about 2.1×10^3 bp and contains 18 exons[10]. Hypermutation of HCCS1 is found in liver cancer tissues, and HCCS1 expression is lowly expressed in tumor tissues than that of paracancerous tissues. It is reported that high expression of HCCS1 can promote calcium influx and induce apoptosis, whereas HCCS1 deficiency promotes excessive cell proliferation[11]. Low expression of HCCS1 is negatively correlated to malignant level, recurrent and metastatic rate of non-small cell lung cancer, and colon cancer[12,13]. So far, HCCS1 has become one of the potential targets for gene therapy of liver cancer and other malignant tumors[14,15]. This work investigated the effect of HCCS1 on the stemness, proliferation, and tumorigenicity of SW1990-SE cells, so as to provide a theoretical basis for developing novel therapeutic targets for pancreatic cancer.

In this study, SW1990-SE cells were isolated using consecutive spheroid enrichment and verified for their stem cell characteristics. The stemness regulatory effects of HCCS1 on protein se-

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cretion and microenvironment change of tumor cells were explored by determining *in vivo* and *in vitro* stemness markers. Our study provides an important reference for elucidating the stemness mechanism of tumor-like stem cells.

Materials and Methods

Cells

Human pancreatic cancer stem-like cells SW1990 were provided by ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1% penicillin-streptomycin and 1% amphotericin B. SW1990 cells were maintained at 37°C, 5% CO_2, and 95% humidity. Most of the SW1990 cells were floating, spindle differentiated, and clonal aggregated. Few cells were suspended as a single cell. Pairing floating cells were occasionally seen. Cell passage was conducted every 4 days. For establishing SW1990-SE cells, the culture medium of SW1990 cells with 80% of confluence was harvested for centrifugation at 1 000 r/min for 5 min. The precipitate was cultured and centrifuged again as the above demonstrated for 8 times.

Experimental Animals

The BALB/cA-nu nude mice were purchased from Beijing Huakangkang Bioscience Co., Ltd. (Beijing, China) authorized by the Animal Experimental Center of Peking University. All the experimental animals passed through the quality test of the Institute of Laboratory Animal Sciences, CAMS&PUMC. The experimental animal procedures were approved by the Animal Ethics Committee and conformed to Guideline for Ethical Review of Animal Welfare.

Lentiviral Infection and Fluorescence Activated Cell Sorter (FACS) of SW1990-SE Cells

SW1990-SE cells in the logarithmic growth phase were infected with lentivirus containing HCCS1 inhibition plasmid or negative control. The multiplicity of infection (MOI) was set to 50 and the polybrene concentration was 10 μg/mL. After 72 hours of cell infection, the infection efficiency was confirmed by a fluorescence microscopy. FACS (Nikon Eclipse E 800, Tokyo, Japan) was performed until 80% of infection efficiency. Cells were sorted by FACS, purified and cultured for subsequent usage.

Real Time-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and preserved in a -80°C refrigerator. The extracted RNA was subjected to reverse transcription using a cDNA first strand synthesis kit (TaKaRa, PrimeScript RT Master Mix, RR036A, Dalian, China). The reverse transcription synthesis was performed at 37°C for 15 min and 85°C for 5 s, and finally preserved at 4°C. PCR amplification of the target gene HCCS1 and the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out using SYBR Premix Ex Taq II, RR820A (TaKaRa, Dalian, China). Primer sequences of HCCS1 were: Forward, 5'-GCAGATCTATGATGGAGGAGGAGGAACT-3', reverse: 5'-GCCTCGAGCTACGTCCATCTCACCTGTT-3'. Primer sequences of GAPDH were: Forward, 5'-AGAAGGCTGGGGCTCATTTG-3', reverse: 5'-AGGGGCCATCCACAGTCTTC-3'. RT-PCR was conducted at 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s, for a total of 40 cycles. The expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Cells were lysed in protein lysate, shaken on ice for 10 min (shaken for 30 s and maintained on ice for 15 s) and centrifuged at 4°C, 12 000 r/min for 5 min. The supernatant was mixed with loading buffer and boiling for 5 min. Subsequently, 10% concentrated and 5% separation gel were prepared for electrophoresis. Total protein loading was 30 μg per well. After electrophoresis at 120 V for 60 min and 350 mA for 60 min, proteins were transferred on the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were then incubated with primary and secondary antibodies. Relative gray scale of the exposed bands was analyzed using ImageJ software. Tubulin was utilized as the internal reference.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 1 000 cells/well. Five duplicate wells and one blank well were set at each time point. For absorbance measurement, 10 μL of cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added and incubated for 2 h in a 37°C incubator. The absorbance (OD) at 450 nm was measured using a microplate reader for 5 days. OD value was cal-

culated as the average of 5-day records and plotted the growth curve. Doubling time (DT) = t×lg2÷(lgNt–lgNo), in which t was the time interval between the selected two time points, No was the OD value at the first time point and Nt was the OD value at the second time point.

96-Wells Plate Single Cell Cloning Assay

The cell suspension was prepared with 10 cells/mL. In a 96-well plate, 100 μL of the prepared single cell suspension was added to each well and incubated for 10 days. The amount of wells with spheroids containing 50 cells was calculated. Percentage of 96-wells plate single cell cloning = the amount of wells where spheroids formed / the amount of wells with successful seeding × 100%.

Subcutaneous Transplantation in Nude Mice

Cell suspension with different doses was diluted with Matrigel at a ratio of 1:1 on ice. 100 μL of diluted suspension was subcutaneously injected in the posterior fossa of the left and right limbs in avoidance of puncturing into mouse abdominal cavity. The needle was carefully pulled out to avoid liquid leakage. Tumorigenicity was daily observed one week later. Mice were sacrificed until the average tumor volume was up to 1 cm^3.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were expressed as mean±SD and analyzed using the *t*-test analysis. $p<0.05$ was considered statistically significant.

Results

Establishment of SW1990-SE Cell Line

During the repeated collection and culture of floating SW1990 cells, previously adherent, spindle-differentiated SW1990 cells gradually became aggravated spheroids. At the 8th time of cell collection, adherent cells were barely seen. SW1990-SE cells were routinely cultured and passaged (Figure 1A).

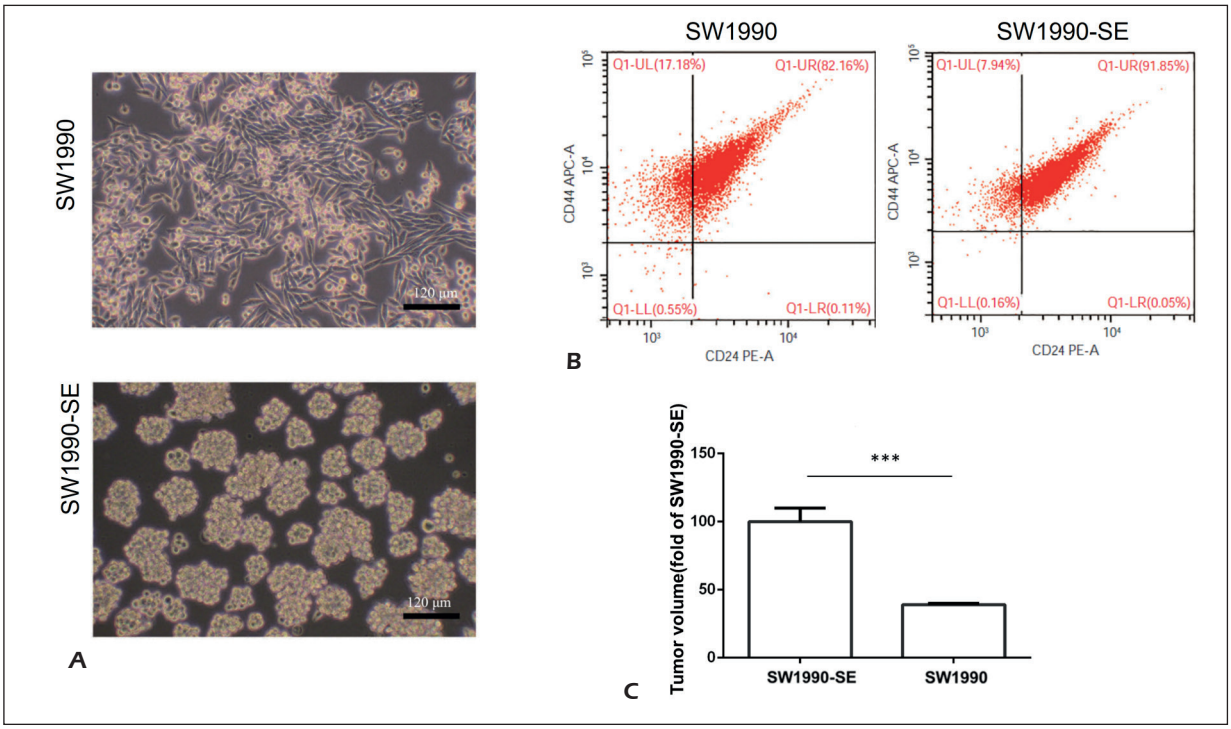

Figure 1. Establishment and identification of SW1990-SE cell line. **A**, Morphology of SW1990 and SW1990-SE cells. Above: spindle-differentiated, adherent SW1990 cells; below: aggravated floating SW1990-SE cells *(Magnification: 40×)*. **B**, Expression level of CD44+CD24+ in SW1990 and SW1990-SE cells determined by FCM. Left: expression level of CD44+CD24+ in SW1990 cells; right: expression level of CD44+CD24+ in SW1990-SE cells. **C**, Tumorigenicity in nude mice subcutaneously transplanted with SW1990 or SW1990-SE cells.

Expression Level of CD44+CD24+ in SW1990 and SW1990-SE Cells

High expression of CD44+CD24+ is a hallmark of stem-like cells in pancreatic cancer. FACS analyzed that cell proportion with highly expressed CD44+CD24+ in SW1990 and SW1990-SE cells was 82.16% and 91.85%, respectively (Figure 1B). It is suggested that consecutive spheroid enrichment could markedly elevate the ratio of pancreatic cancer stem-like cells. Moreover, SW1990-SE cells were found with stem cell characteristics relative to SW1990 cells.

Tumorigenicity in Nude Mice Subcutaneously Transplanted with SW1990 or SW1990-SE Cells

To further elucidate the stem cell characteristics of SW1990 and SW1990-SE cells, tumorigenicity was evaluated in nude mice. As the results showed, SW1990 cells exerted weaker tumorigenicity in nude mice than SW1990-SE cells (Figure 1C). Hence, we confirmed the tumor-like stem cell characteristics of SW1990-SE cells both *in vivo* and *in vitro*.

HCCS1 Expression in SW1990 and SW1990-SE Cells

RT-PCR results showed that HCCS1 expression differed in SW1990 cells and SW1990-SE cells. The mRNA level of HCCS1 in SW1990-SE cells was (35.74±0.024)% of SW1990 cells, indicating a significant difference in HCCS1 expression between adherent spindle-differentiated subtype and aggravated floating subtype (Figure 2A, $p<0.001$).

Transfection Efficacy of shHCCS1

Transfection efficacy of shHCCS1 was verified after lentivirus infection by determining protein and mRNA levels of HCCS1. RT-PCR results showed that the ratio of mRNA level of HCCS1 in SW1990-SE-shHCCS1 cells was only (31.61±0.95)% of SW1990-SE-LV3NC cells (Figure 2B, $p<0.001$). Western blot results identically showed lower protein level of HCCS1 in SW1990-SE-shHCCS1 cells than SW1990-SE-LV3NC cells, indicating that lentiviral infection effectively downregulated HCCS1 expression (Figure 2C, 2D, $p<0.001$).

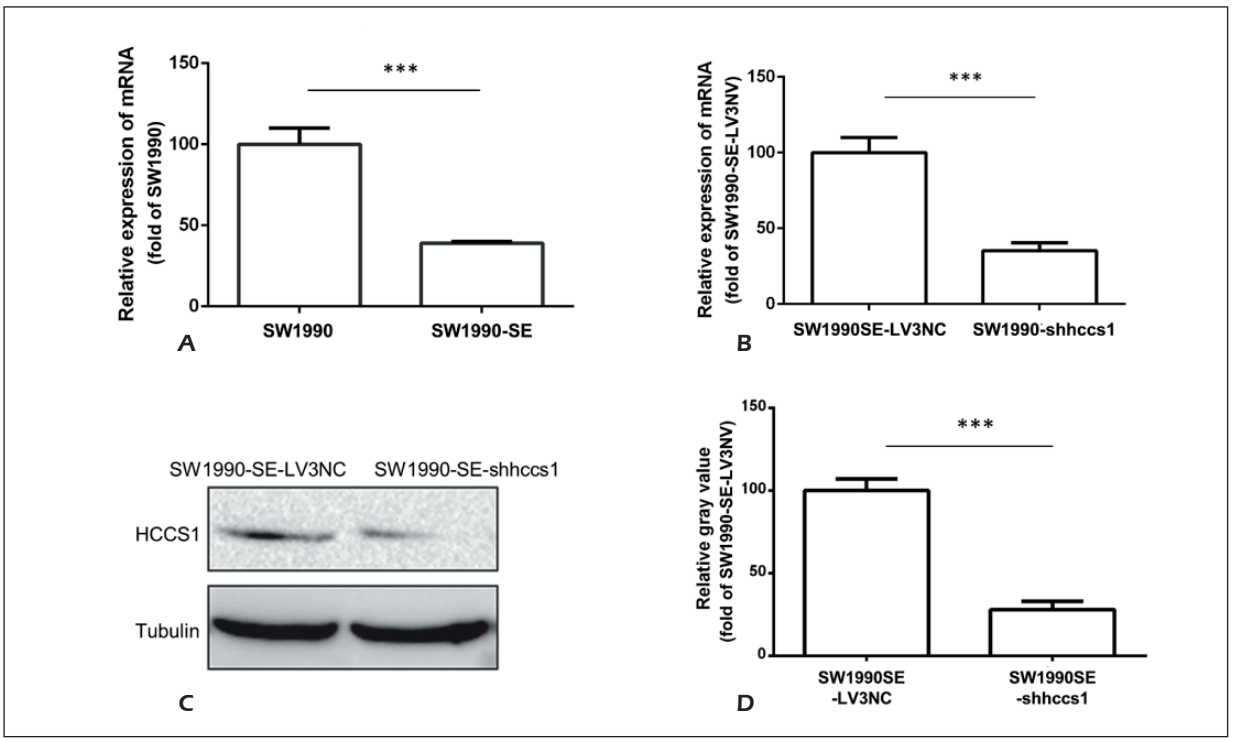

Figure 2. The mRNA level of HCCS1 determined by RT-PCR and protein level of HCCS1 determined by Western blot. **A**, The mRNA level of HCCS1 in SW1990 and SW1990-SE cells (***$p<0.001$); **B**, The mRNA level of HCCS1 in SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells (***$p<0.001$). **C**, Western blot analysis of HCCS1 in SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells; **D**, The protein level of HCCS1 in SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells (***$p<0.001$).

HCCS1 Knockdown Did Not Affect Proliferation of SW1990-SE Cells

Proliferative characteristics of SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells were detected by the CCK-8 assay. Growth curves were plotted for calculating DT. No significant difference in proliferation was observed between SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells (Figure 3A, $p>0.05$). It is indicated that HCCS1 knockdown did not affect the proliferative characteristics of SW1990-SE cells.

HCCS1 Knockdown Increased Spheroid Diameter of SW1990-SE Cells

After SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells were grown for 72 h, the spheroid diameter was observed under a microscope. Thirty randomly selected spheroids in each group were photographed. Spheroid diameter in SW1990-SE-shHCCS1 cells was (1.84±0.03) times larger compared with SW1990-SE-LV3NC cells (Figure 3B, $p<0.001$). It is indicated that HCCS1 knockdown markedly increased the spheroid diameter of SW1990-SE cells.

HCCS1 Knockdown Increased Single Cell Cloning Ability of SW1990-SE Cells

96-wells plate single cell cloning assay was conducted to further elucidate the influence of HCCS1 on stemness of SW1990-SE cells. The single cell cloning ability in SW1990-SE-shHCCS1 cells was (2.75±0.05) fold of SW1990-SE-LV3NC cells (Figure 3C, $p<0.01$). We may conclude that HCCS1 knockdown could enhance the single cell cloning ability, thus improving the stemness of SW1990-SE cells.

HCCS1 Knockdown Enhanced the Tumorigenicity of SW1990-SE Cells

Subcutaneous transplantation in nude mice is a classic animal model for verifying stemness. Here, we injected different numbers of SW1990-

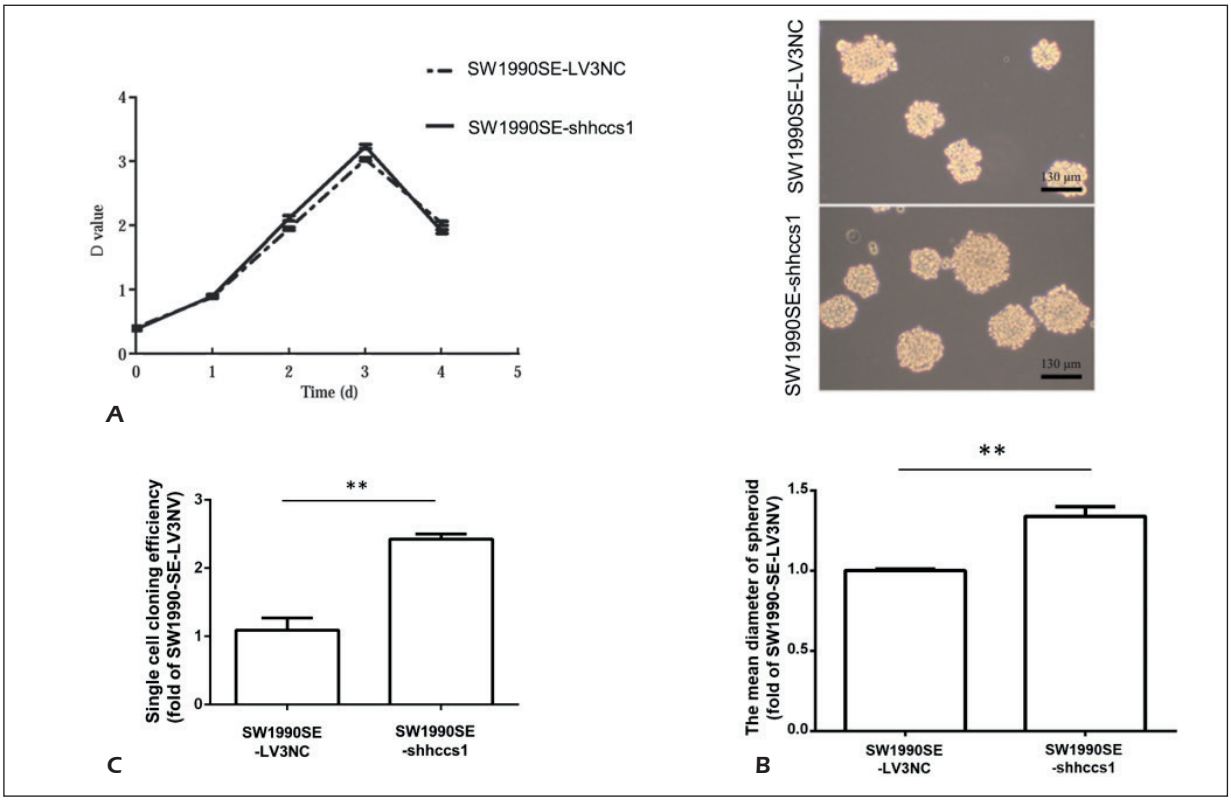

Figure 3. HCCS1 knockdown increases SW1990-SE cells stemness. **A**, Proliferation of SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells with HCCS1 knockdown. **B**, Spheroid diameter of SW1990-SE cells with HCCS1 knockdown. Above: images of single cell cloning of SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells at the same time point; below: the mean spheroid diameter of SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells at the same time point (**$p<0.01$) *(Magnification: 40×).* **C**, Single-cell cloning ability of SW1990-SE-LV3NC and SW1990-SE-shHCCS1 cells with HCCS1 knockdown (**$p<0.01$).

Table I. The subdermal tumorigenesis of different cell amounts.

Cell amount	Cell line	Tumor formation rate	Tumor volume (cm^3)
10^6	SW1990-SE-LV3NC	2/2	2.567/1.283
	SW1990-SE-shHCCS1	2/2	1.574/0.389
500	SW1990-SE-LV3NC	2/2	2.980/1.291
	SW1990-SE-shHCCS1	2/2	1.535/0.814
100	SW1990-SE-LV3NC	2/2	1.900/1.464
	SW1990-SE-shHCCS1	2/2	1.420/1.086
10	SW1990-SE-LV3NC	2/2	3.050/0.503
	SW1990-SE-shHCCS1	2/2	1.185/0.499
1	SW1990-SE-LV3NC	2/10	2.917/1.450
	SW1990-SE-shHCCS1	0/10	0

SE cells with HCCS1 knockdown in mice to evaluate the stemness changes. The data revealed that HCCS1 knockdown markedly enhanced the tumorigenicity. More importantly, stemness difference between SW1990-SE-shHCCS1 cells and SW1990-SE-LV3NC cells was gradually pronounced with the transplanted cell number decreased (Table I). It is worth noting that only SW1990-SE-shHCCS1 cells exerted subcutaneous tumorigenicity when accurately transplanting one single cell prepared by a pipette, fully confirming the tumorigenic ability of SW1990-SE-shHCCS1 even at the single-cell level. HCCS1 knockdown was proved to be able to promote the *in vivo* stemness of SW1990-SE cells.

Discussion

Tumor stem cells are widely involved in pathological processes of tumorigenesis, tumor survival, proliferation, metastasis, and recurrence[6]. Basically, the vitality of tumor cells and tumor tissues are maintained by tumor stem cells through self-renewal and infinite proliferation[8]. Therefore, targeting tumor stem cells are expected to be the possible therapeutic way to cure cancers[9]. Here, we used a new type of consecutive spheroid enrichment as previously described to isolate the SW1990-SE cells. SW1990-SE cells were aggravated, floating spheroids isolated from adherent SW1990 cells. HCCS1 knockdown in SW1990-SE cells markedly enhanced spheroid diameter, single cell cloning ability, and tumorigenicity. Strikingly, even a single cell of SW1990-SE with HCCS1 knockdown showed tumorigenicity in nude mice. It is the first time to report the single-cell tumorigenicity of pancreatic cancer stem-like cells. HCCS1 is involved in the regulation of metastasis of different types of tumors, whereas its role in stemness regulation is rarely reported. This study investigated the possibility of the autocrine effect of pancreatic cancer stem-like cells in the stemness regulation, which is an interesting direction for stemness treatment in tumors deserving to be further explored. The potential regulatory role of HCCS1 in the microenvironment of tumor stem cells is undergoing. The nuclei of normal stem cells are relatively large that require for a simpler external environment than normal cells. Tumor stem cells may have a similar mechanism and complex environment to induce cell differentiation. Therefore, the maintenance of tumor stem cells may be achieved by the reduction of secreted proteins.

This study reported for the first time that HCCS1 regulated stemness of pancreatic cancer stem-like cells. However, the downstream genes of HCCS1 have not been explored. Some scholars[16-18] pointed out that some inflammatory factors have a regulatory effect on the stemness of tumor stem cells. Inflammatory factors are normally secreted through the classical secretory protein pathway, which may be a potential target site. Some autophagy-regulated proteins also have an autocrine regulatory effect. Autophagy has been reported[19-21] to have a positive regulatory effect on the stemness of tumor stem cells. In addition, some extracellular matrix proteins enhance the association among tumor stem cells[22-24]. Therefore, protein profiles of pancreatic cancer stem-like cells are needed to be further analyzed through the database.

In summary, this study explored the role of HCCS1 in the stemness regulation of pancreatic cancer stem-like cells. We provided a possible theoretical support for the involvement of tumor microenvironment in stem cell maintenance.

Conclusions

We found that HCCS1 knockdown largely enhances the *in vivo* and *in vitro* stemness of SW1990-SE cells. Our study provides an important reference in the research of tumor stem cells.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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