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Hes1 is involved in the self-renewal and tumourigenicity of stem-like cancer cells in colon cancer

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A small subpopulation of cancer cells with stem cell-like features might be responsible for tumour generation, progression, and chemoresistance. Hes1 influences the maintenance of certain stem cells and progenitor cells and the digestive systems. We found upregulated Hes1 in poorly differentiated cancer samples compared with well-differentiated tumour samples, and most of the adenocarcinomas exhibited significantly higher levels of Hes1 mRNA compared with that observed in matched normal colon samples. Moreover, Hes1 expression was found to be correlated with the expression of stem cell markers in colon cancer samples, and Hes1 upregulates the expression of stemness-related genes in colon cancer cells. In addition, Hes1 enhances the self-renewal properties of the stem-like cells by increasing the sizes of CD133+ cells and SP cells and the ability of tumour sphere formation. Additionally, the Hes1-overexpressing cells formed significantly larger and higher number of colonies, as determined through the colony and the soft agar assays. More importantly, Hes1 enhances the tumourigenicity of colon cancer cell lines in nude mice and exhibits a strong tumour-formation ability at a cell density of 1×10^3. Taken together, our data indicate that Hes1 induces stem-like cell self-renewal and increases the number of tumour-initiating cells in colon cancer.

The cancer stem cell theory was first proposed in 1983 by Mackillop[1], who proposed that a few of all cancerous cells act as stem cells that reproduce themselves and thus sustain the cancer; as a result, these cells were called cancer stem cells (CSCs). Based on the cancer stem cell theory, many new anti-cancer therapies have been evaluated.

To date, CD133 has been recognised as the most important marker of colon tumours, and CD133-positive (CD133+) cells possess the potential to initiate and sustain tumour growth[2,3]. Side population (SP) cells, which exhibit stem cell characteristics and are robustly able to expel the Hoechst 33342 dye, have also been shown to exist in various types of tumours, including colon cancer[4,5].

It has been reported that Hes1 plays an important role in the tumourigenesis of biliary neuroendocrine tumours[6] and that the blocking of Hes1 expression initiates the differentiation of human neural stem cells and telencephalic progenitor cells[7,8]. Another study showed that Hes1 might modulate the therapeutic resistance in breast cancer[9]. Previous studies have indicated that Hes1 influences the maintenance of certain stem cells and progenitor cells and partially influences the digestive systems through the Notch signalling pathway, i.e., Hes-Notch interactions play a role in digestive organ development[10]. In addition, Hes1 acts as a marker of normal colon stem cells[11]; however, an increase in Notch signalling, including the Notch target Hes1, may contribute to the initiation of colon cancer[12]. Because the roles of Hes1 in the pathogenesis of colon cancer are not well elucidated, these aforementioned findings prompted us to investigate whether Hes1 is related to the progression and stemness of human colon cancer.

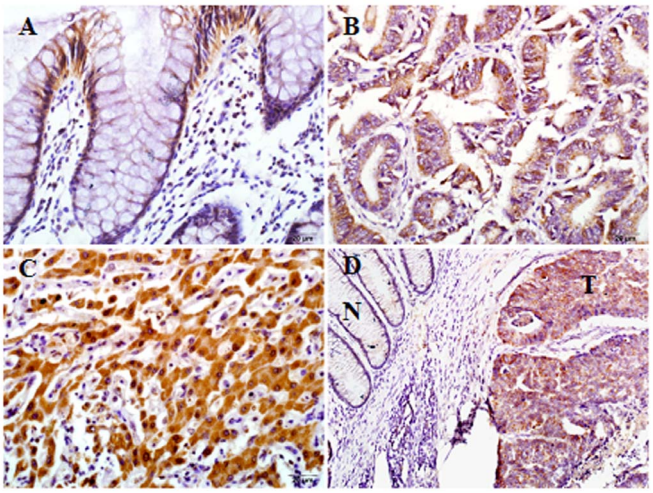
The data obtained in the present study demonstrate that Hes1 is expressed in almost all normal tissues, particularly at the bottom of the crypts, which are often considered to be rich in stem cells[11], and is expressed in all tumour tissues. However, the expression of Hes1 in poorly differentiated cancer samples was upregulated compared to its expression in well-differentiated tumour samples, and most of the adenocarcinomas exhibited

significantly higher levels of Hes1 mRNA than their matched normal colon samples. In addition, Hes1 expression was found to be correlated with the expression of stem cell markers in colon cancer samples. Moreover, the results of this study provide the first demonstration that Hes1 expression increases the number of CD133+ cells and the number of SP stem-like cells in colon cancer. The results from this study indicate that Hes1 plays a quantitative role in the development and progression of colon cancer and the maintenance of the stemness of cancer stem cells, which remains to be fully characterised.

Results

Hes1 expression during human colon tumourigenesis. Figure 1 shows the results of the immunohistochemical analysis of sections from human normal colon tissue (Figure 1A), well-differentiated colon cancer tissue (Figure 1B), and poorly differentiated colon cancer tissue (Figure 1C, D). We found that Hes1 is expressed in almost all normal tissues, particularly at the bottom of the crypts, and in all cancer tissues, including moderate and poorly differentiated cancer samples and well-differentiated cancer samples. In addition, the expression of Hes1 in poorly differentiated cancer samples was higher than that observed in well-differentiated tumour (Figure 1E).

Hes1 expression correlates with the expression of stem cell markers in colon cancer samples. As mentioned previously, Hes1 influences the maintenance of certain stem cells and might act as a marker of normal colon stem cells. To determine whether any correlation exists between Hes1 expression and the expression of representative markers of stem cells in colon cancer biopsy samples, we obtained RNA from 28 normal samples and 28 colon cancer samples and analysed the expression levels of ABCG2, CD133, ALDH1, Nanog, Bmi-1, and CD44 through RT-PCR. The results show that Hes1 and the analysed stem cell markers are upregulated in the colon cancer tissue compared with the normal samples (Figure 2A). We also found that the Hes1 expression is positively correlated with the expression of ABCG2, CD133, ALDH1, Nanog, Bmi-1, and CD44 (Figure 2B).



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Differentiation	Hes1 expression			
	n	Low	High	*p* Value
Well	13	8	5	0.000#
Poor	36	7	29	

Chi-square test.

Figure 1 | Induction of Hes1 expression in human colon carcinogenesis. Immunohistochemical analysis of Hes1 in human normal colon tissue (A), well differentiated colorectal cancer tissue (B) and poorly differentiated colorectal cancer (C, D), 40×. Normal colonic mucosa (N) can be seen to the left of the carcinoma (T) in (D), 20×. (E) Statistical analyse of the relation between Hes1 staining and clinic differentiation.

Hes1 upregulates stemness-related gene expression in colon cancer cells. Our findings and those of other groups suggest that Hes1 might play an important role in the maintenance of the stemness of colon cancer. We thus overexpressed Hes1 in colon cancer SW620 and HCT116 cells to further explore the roles of Hes1 in colon cancer. Representative stem cell markers were analysed by RT-PCR and western blot. As shown in Figure 3A, Hes1 overexpression upregulated the stem cell markers CD133, ABCG2, Nanog, ALDH1, CD44, and Bmi-1 at the transcriptional level compared with the control (LV-con). The increase in Hes1, CD133, ABCG2, Nanog, and ALDH1 was further confirmed at the protein level (Figure 3B, S1), which indicates that overexpression of Hes1 in colon cancer cell lines induces alterations in the levels of stem-like cellular markers.

Hes1 induces stem-like properties in colon cancer cells. Because CD133+ cells and SP cells have been reported to exhibit cancer stem cell characteristics, we explored whether the increased expression of stem cell markers that was observed in the Hes1-overexpressing cells was caused by an increase in the size of the CD133+ cells and the stem cell-like SP cells. As shown in Figure 4A, Hes1 increased the percentages of CD133+ cells in cultures of SW620 and HCT116 cells. As shown in Figure 4B, the stable expression of Hes1 dramatically increased the size of the SP cells in the SW620 (from 0.6% to 1.2%) and HCT116 (from 0.5% to 1.9%) cell lines.

We then analysed whether the increase in the sizes of CD133+ cells and SP cells in colon cancer was due to the enhanced self-renewal properties of the stem-like cells in the cell culture. Hes1-overexpressing and control cells were cultured in suspension to generate spheres. As shown in Figure 4C, the Hes1-overexpressing cells formed a higher number and larger spheres compared with the vector controls cells. As a result, we conclude that Hes1 can enhance the self-renewal properties of the stem cells and thereby increase the size of these populations.

We then isolated CD133+ and CD133-negative (CD133−) cells for further study. As shown in Figure 4D, the western blot analysis revealed an increased expression of Hes1 and CD133 in the CD133+ cells compared with the CD133− cells and an increased expression of Hes1 and CD133 in tumour spheres compared with adherent cells from the SW620 and HCT116 colon cancer cell lines. This finding illustrates that Hes1 expression is correlated with the expression of CD133 and might play an important role in the maintenance of the stemness of colon cancer stem cells.

Hes1 enhances the transforming ability of colon cancer cells. To investigate whether Hes1 can enhance the transforming ability of colon cancer cells, we used both a colony formation and an anchorage-independent growth assay in soft agar. We plated 200 colon cancer cells in triplicate wells of 6-well plates for the colony formation assay. After 14 days of culture, the Hes1-overexpressing cells formed colonies that were significantly larger than those formed by the vector control cells (Figure 5A, left panel). The statistical analysis showed significant differences in the number of colonies between the Hes1-overexpressing and the vector control cell lines ($p < 0.05$; Figure 5A, right panel). In addition, the transforming ability of the Hes1-overexpressing cells was also determined using a soft agar assay. As shown in Figure 5B, the Hes1-overexpressing cells formed significantly larger and a significantly higher number of colonies compared with the vector cells.

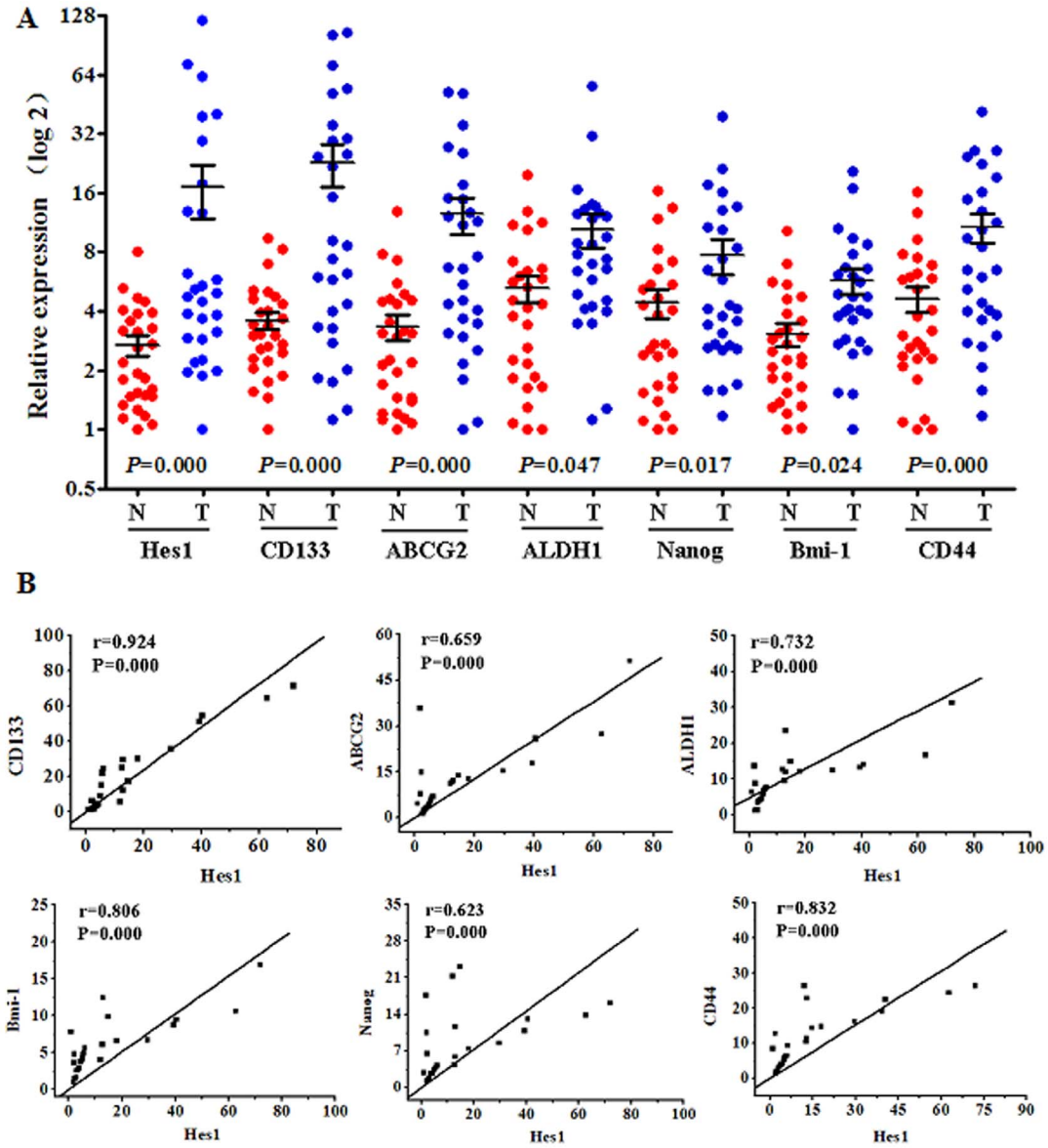


Figure 2 | The expression of Hes1 correlates with the expression of stem cell-related markers in colon cancer tissues. (A) The mRNA level of Hes1, ABCG2, CD133, ALDH1, Nanog, Bmi-1 and CD44 expression in colon cancer biopsy samples and control normal samples measured by real time RT-PCR. N = normal, T = tumour. (B) Hes1 correlates positively with ABCG2, CD133, ALDH1, Nanog, Bmi-1 and CD44 expression in colon cancer tissue samples.

Hes1 increases the number of tumour initiating cells in vivo. Because SP cells and CD133+ cells are enriched in tumour-initiating cells, we then evaluated the effects of Hes1 on the tumourigenicity of colon cancer cell lines in nude mice. The data presented in Figure 6A and Figure 6E show that the injection of 1×10^6 and 1×10^5 Hes1-overexpressing colon cancer cells into nude mice resulted in 100% (4/4 and 6/6, respectively) of the mice developing tumours. In addition, the injection of 1×10^4 Hes1-overexpressing colon cancer cells into nude mice resulted in the development of tumours in 100% (10/10) of the mice, whereas only 80% of the mice (8/10) developed tumours after injection of the control cells. The injection of 1×10^3 cells control cells resulted in the development of small tumours in 50% (6/12) of the mice, whereas the injection of the same number of Hes1-overexpressing cells led to the formation of tumours. As shown in Figures 6B and 6E, the injection of 1×10^6 Hes1-overexpressing and control cells resulted in the formation of palpable tumours that appeared at a similar time. However, the growth rates of the Hes1-overexpressing tumours were found to be slightly higher than those of the controls. As the number of injected cells was reduced (1×10^5, 1×10^4, and 1×10^3), the growth rates of the Hes1-overexpressing tumours were markedly higher than those of the controls that were injected with the same number of cells.

In addition, the first palpable tumour in the Hes1-overexpressing group injected with 1×10^3 cells appeared five days earlier compared with the control. The mice were sacrificed 24–27 days after injection, and the tumours were then weighed (Figure 6C). In all cases, the sizes of the tumours formed by the Hes1-overexpressing colon cancer cells were larger than those observed in the groups injected with the vector control cells. Furthermore, we surveyed the expression levels of Hes1, ALDH1, and Bmi-1 through immunohistochemistry and found higher expression of Hes1, ALDH1, and Bmi-1 in the Hes1-overexpressing tumours compared with the control tumours (Figure 6D). Hence, Hes1 increases the number of tumour-initiating cells in colon cancer.

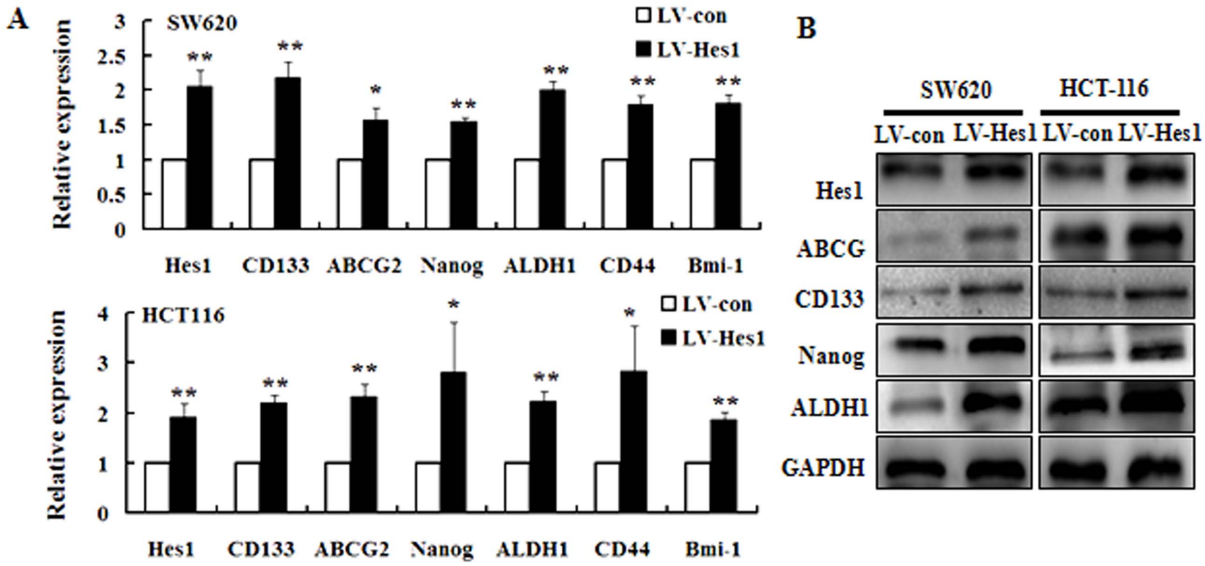


Figure 3 | Overexpression of Hes1 in colon cancer cell lines induces stem-like cellular marker alterations. (A) qRT-PCR demonstrates an increased expression of Hes1, CD133, ABCG2, Nanog, ALDH1, CD44 and Bmi-1 in Hes1-expressing (LV-Hes1) cells in comparison with the vector control (LV-con). (B) Western blot reveals an increased expression of Hes1, CD133, ABCG2, Nanog, ALDH1 in Hes1-expressing cells.

Discussion

An increasing body of evidence suggests that human cancers are stem cell diseases and that only a small subpopulation of cancer cells with stem cell-like features might be responsible for tumour generation, progression, and chemoresistance[13]. Tumour recurrence after curative surgery might also be partly due to the existence of cancer stem cells (CSC)[14]. According to the CSC hypothesis, colon cancer can be considered a disease in which mutations convert normal stem cells into aberrant counterparts that are responsible for tumour generation and propagation[15].

The results of our current study indicate a pivotal role for Hes1 in the progression of colon cancer through the induction of stem-like cancer cells. The novel roles of Hes1 in increasing the size of the SP and CD133+ cells, enhancing the self-renewal properties of stem-like cancer cells, and increasing the number of cancer-initiating cells confirm the involvement of this protein in oncogenic processes through the modulation of the CSC population in colon cells. Many studies have recently focused on the role of Hes1 in influencing the differentiation decision of cells in the gastrointestinal tract. However, we found that Hes1 is expressed in almost all normal tissues, particularly at the bottom of the crypts, which are enriched in stem cells, and in all cancer tissues, including moderate and poorly differentiated cancer tissues and well-differentiated cancer tissues. In addition, the expression of Hes1 in poorly differentiated cancer samples was found to be higher than that obtained in well-differentiated tumour samples. We obtained RNA from 28 normal samples and 28 colon cancer samples, and the results demonstrated that Hes1 was upregulated in the colon cancer tissue samples compared with the normal samples. Thus, we conclude that Hes1 might play an important role in the tumourigenesis and development of colon cancer.

It has been reported that CD133 is negatively correlated with the differentiation of human colon cancer cells[16]. Previous studies have suggested that CD133 expression is an independent prognostic marker for low survival in colon cancer[17]. We observed a positive correlation between Hes1 and CD133 expression in colon cancer specimens. In addition, we found upregulated CD133 expression and an increased number of CD133+ cells in a culture of Hes1-overexpressing colon cancer cells and that Hes1 is upregulated in CD133+ colon cancer cells compared with CD133− cells.

SP cells have been identified in many tissues with high regenerative capacities, and ATP-binding cassette (ABC) transporter family members, such as ABCG2, are hypothesised to contribute to the high Hoechst dye-efflux capabilities of SP cells[18]. Our current study demonstrated that Hes1 induces a stem cell-like state, as evidenced by an enhanced self-renewal capacity, and increases the number of tumour-initiating cells *in vivo*. This finding was confirmed by the increased SP size and the higher expression of some stem cell markers, such as ABCG2, CD133, ALDH1, Nanog, Bmi-1, and CD44. This study also demonstrates that ABCG2, CD133, ALDH1, Nanog, Bmi-1, and CD44 are upregulated in colon cancer tissue compared with normal tissue. Previous studies have shown that ABCG2, CD133, ALDH1, Nanog, Bmi-1, and CD44 play essential roles in the tumourigenesis and progression of colon cancer[19–21]. More importantly, the positive correlation between Hes1 and these genes, including ABCG2, ALDH1, Nanog, Bmi-1, and CD44, in colon cancer specimens provides a valuable clue to the further elucidation of the processes underlying clinical metastasis and recurrence in colon cancer.

The above results illustrate that Hes1 expression is positively correlated to the levels of stem cell markers (e.g., ABCG2, CD133, ALDH1, Nanog, Bmi-1, and CD44) and might increase the number of stem-like cancer cells in colon cancer. However, the complex regulatory machineries associated with Hes1 during cancer stem cell induction have not been fully elucidated. Hence, the elucidation of the underlying signalling network that regulates these Hes1-associated pathways through bioinformatics predictions and the use of various Hes1 mutants and different pathway inhibitors will provide important insights into the exact role of Hes1 in the emergence of cancer stem cells.

Methods

Ethics statement. All animal work was conducted under the institutional guidelines of Guangdong Province and approved by the Use Committee for Animal Care. Approval from the Southern Medical University Institute Research Ethics Committee was obtained, and written informed consent was provided by each human subject.

Cell lines and cell culture. Human colon cancer cell lines (SW620 and HCT116) were cultured in RPMI1640 medium supplemented with 10% foetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37°C.

Clinical samples. The biopsies of 77 colon cancer patients and 38 non-tumoural tissues were collected from the Department of Pathology at Nanfang Hospital of Guangzhou City in China between 2010 and 2012. None of the patients received preoperative radiotherapy or chemotherapy.

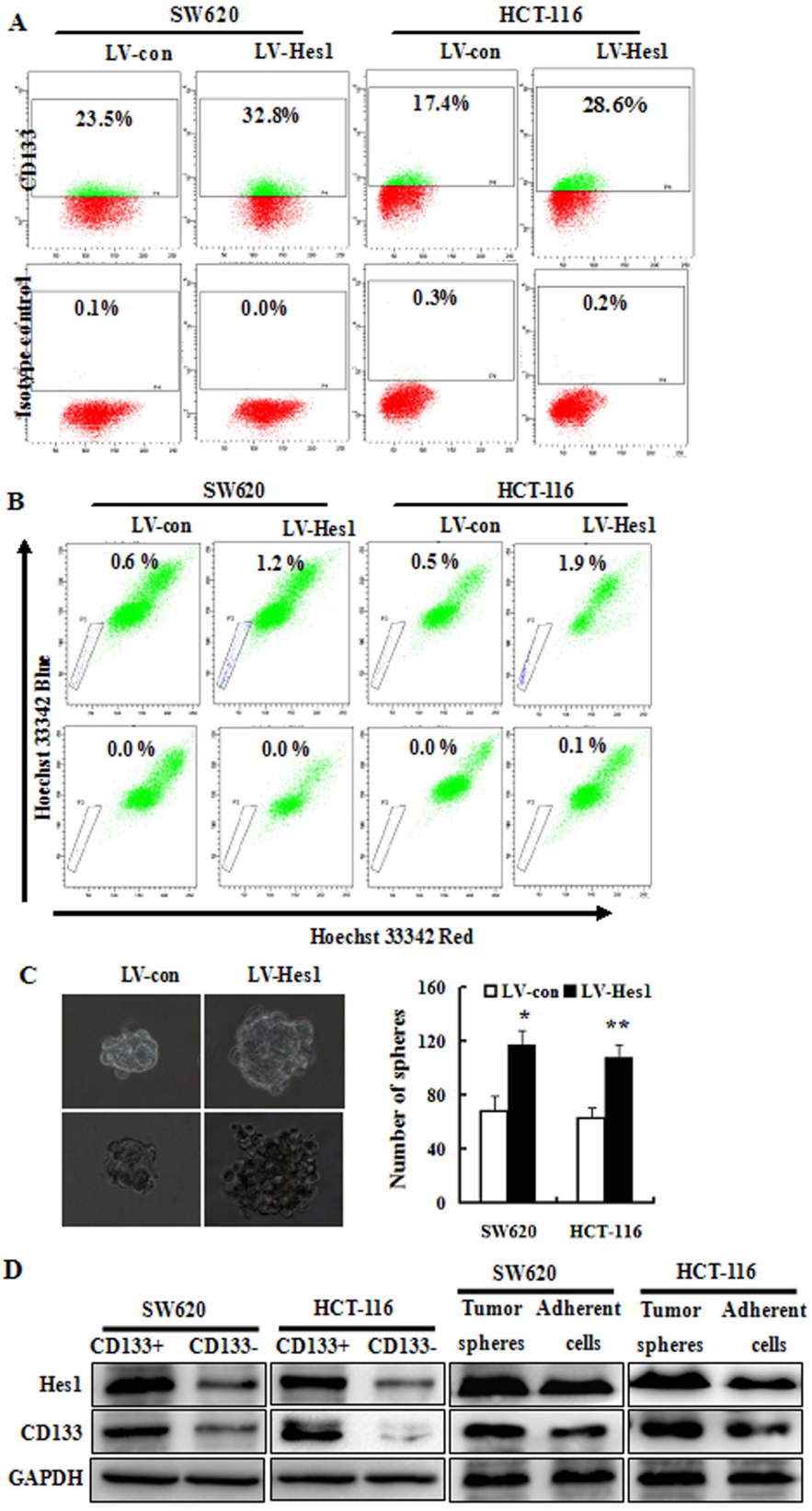


Figure 4 | Hes1 induces stem-like properties in colon cancer cells. (A) Hes1 increases the percentages of CD133+ cells in Hes1-overexpressing SW620 and HCT116 cells. Flow cytometric profiles of cells in the presence of isotype control are shown in the bottom panels. (B) Hes1 increases the size of the side population (SP) cells. Flow cytometric profiles of SP cells among the SW620 and HCT116 cell lines after stable expression of Hes1. SP cell profiles in the presence of verapamil are shown in the bottom panels. The percentages of SP cells are indicated. (C) Hes1 induces stem cell-like self-renewal properties. Sphere sizes are shown in the left panels, and the numbers of spheres is shown in the right panels. (D) Westen blot reveals an increased expression of Hes1 and CD133 in CD133-positive cells in comparison with CD133-negative cells, and an increased expression of Hes1 and CD133 in tumour spheres in comparison with adherent cells in SW620 and HCT116 colon cancer cell lines.

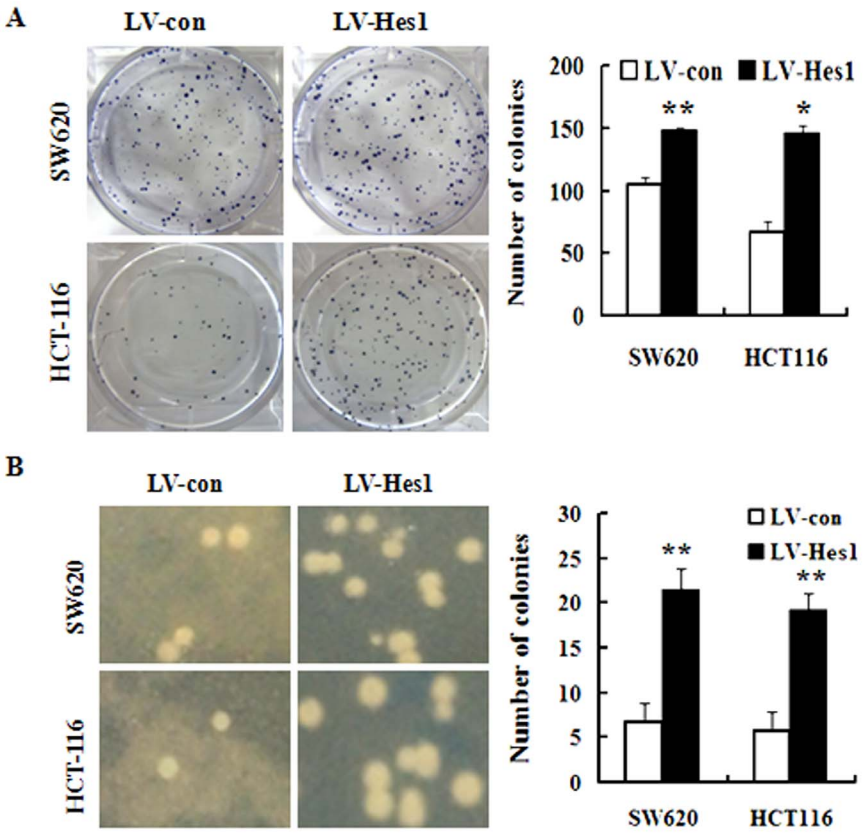


Figure 5 | Hes1 enhances the transforming ability of colon cancer cells. (A) Colony formation assay of the SW620 and HCT116 cell lines. (B) The anchorage-independent growth in soft agar of SW620 and HCT116 cells with or without exogenous Hes1 expression. Upon the stable expression of Hes1, these cells form bigger (left panel) and more colonies (right panel) compared with the control cells.

Immunohistochemistry. The immunohistochemistry analysis was performed as described below. After deparaffinisation and rehydration, the TMA sections were subjected to high pressure for 2 min to achieve antigenic retrieval. The slides were incubated overnight at 4°C with the following primary antibodies: Hes1 (1 : 500 dilution; Bioss), ALDH1 (1 : 500 dilution; Abcam), and Bmi-1 (1 : 500 dilution; Abcam). The sections were then incubated with DAB for 2 min. In every run, the primary antibodies were substituted with PBS for the negative controls.

For the evaluation of the IHC results, the proportion of stained tumour cells was evaluated using four grades: 0, no positive tumour cells; 1, <10% positive tumour cells; 2, 10–50% positive tumour cells; and 3, >50% positive tumour cells. Similarly, the scoring criteria for the staining intensity were the following: 0, no staining; 1, weak staining; 2, modest staining; and 3, strong staining. The final score was calculated by multiplying the tumour staining area by the intensity score (0, 1, 2, 3, 4, 6, and 9). According to this method of assessment, staining scores ≤4 and ≥6 were regarded as tumours with low and high expression, respectively.

RNA isolation, reverse transcription, and qRT-PCR. For the mRNA analyses, the total RNA was extracted using the Trizol Reagent (TaKaRa) according to the protocol provided by the manufacturer. The total RNA was reversely transcribed using the PrimeScript RT reagent Kit (TaKaRa). The qRT-PCR for the analysis of mRNA expression was performed on a Stratagene Mx3005P qRT-PCR system using the SYBR Green qRT-PCR master mix (TaKaRa) and GAPDH for normalisation. The primers used for the amplification of the indicated genes are listed in Table S1. All of the samples were normalised to the internal controls, and the fold changes were calculated through relative quantification ($2^{-\triangle\triangle Ct}$).

Western blot analysis. The protein lysates were separated by 10%–15% SDS-PAGE and electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore). The membrane was incubated with antibodies, Hes1 (Bioss), CD133 (Santa Cruz), ABCG2 (Santa Cruz), Nanog (Abcam), ALDH1 (Santa Cruz). Then the membrane was incubated with HRP-labelled goat-anti-mouse or rabbit IgG, and the proteins were detected by high sensitivity chemiluminescence imaging system (BIORAD). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the protein-loading control.

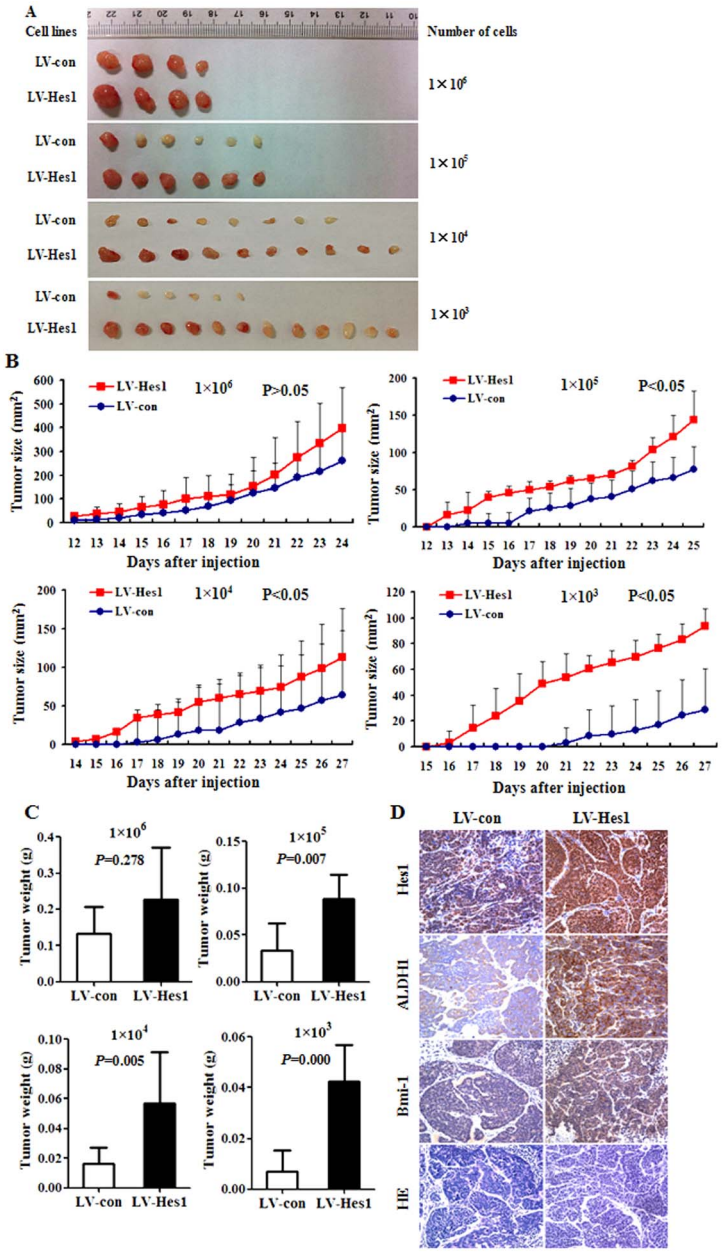
Immunofluorescence analysis. The cell lines were plated on culture slides (Costar, MA, USA). After 24 hours, the cells were rinsed with PBS, fixed in 4% paraformaldehyde for 10 min, and then permeabilised with 0.05% Triton X-100. The cells were blocked for 30 min in 10% BSA (Sigma, MO, USA) in PBS and then incubated with primary monoclonal antibodies in PBS for 1 h at 37°C. After three washes in PBS, the slides were incubated for 1 h in the dark with secondary goat anti-mouse or goat anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA). After three washes, the slides were stained with 4-,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) for 5 min to visualise the nuclei and examined using a confocal laser-scanning microscope (Olympus FV1000).

Tumour sphere formation assay. The cells were digested with 0.25% trypsin (Sigma, St. Louis, MO, USA), washed twice with calcium/magnesium-free PBS, suspended in sphere formation medium (DMEM-F12 50 ml + 100 μg/ml EGF + 100 μg/ml bFGF + B27 supplement 1 ml), and seeded in 10-cm or 6-well plate (1000 cells/ml). The cells were cultivated for 5 ~ 14 days (depending on the cell type), and the spheres were then counted under a microscope.

Sorting of SP cells by flow cytometry. After reaching the logarithmic growth phase (24 hours after replating), the cells were analysed by FACS. The cells were digested with 0.25% trypsin (Sigma, St. Louis, MO, USA), washed twice with calcium/magnesium-free PBS, and resuspended in ice-cold RPMI 1640 culture (supplemented with 2% FBS) at a concentration of 1×10^6 cells/mL. The DNA binding dye Hoechst 33342 (Sigma, St. Louis, MO, USA) was then added to obtain a final concentration of 5 mg/mL, and the samples were incubated for 70–90 min in the dark with periodic mixing. The cells were then washed twice with PBS. Then, 1 mg/mL propidium iodide (Sigma, St. Louis, MO, USA) was added to the cells, and the cells were maintained at 4°C in the dark prior to sorting by flow cytometry (BD FACSAria).

Because Hoechst 33342 extrudes from cells treated with verapamil (a calcium ion tunnel antagonist) through sensitive ABC transporters, a subset of the cells were incubated with 50 mmol/L verapamil for 30 min at 37°C before the addition of Hoechst 33342 to determine whether this treatment blocks the fluorescent efflux of SP cells.

Sorting of CD133+ cells by flow cytometry. The cells were analysed by FACS according to the protocol provided by the manufacturer (MACS Miltenyi Biotec). The cell suspension was washed and resuspended in D-Hank's solution. Then, 10 μl of the CD133/1 (AC133)-APC antibodies and the human or mouse IgG1 isotype control antibodies were added per 100 μl of suspension. The suspension was then mixed well and incubated for 25 minutes in the dark at 2–8°C. After two washes, the cell pellet was resuspended in a suitable amount of buffer for analysis by flow cytometry.



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Cell number	Cell name	Number of tumors/ Number of injections: Days after injection 12	13	14	15	16	17	18	19	20	21	22	23	24	25
1×10⁶	LV-con	1/4	2/4	2/4	3/4	3/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	-
	LV-Hes1	3/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	-
1×10⁵	LV-con			1/6	1/6	1/6	4/6	4/6	4/6	5/6	5/6	6/6	6/6	6/6	6/6
	LV-Hes1		3/6	3/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
1×10⁴	LV-con						1/10	2/10	4/10	4/10	6/10	6/10	6/10	8/10	8/10
	LV-Hes1			1/10	2/10	4/10	8/10	8/10	8/10	10/10	10/10	10/10	10/10	10/10	10/10
1×10³	LV-con									1/12	2/12	2/12	3/12	4/12	6/12
	LV-Hes1				1/12	5/12	7/12	9/12	11/12	11/12	12/12	12/12	12/12	12/12	12/12

Figure 6 | Hes1 enhances the initial tumour cell population in SW620 cells. (A) Tumours formed after injection of nude mice with Hes1 (LV-Hes1) or vector control (LV-con) expressing SW620 cells. (B) Tumour growth curves after injection of nude mice with Hes1 (LV-Hes1) or vector control (LV-con) expressing SW620 cells. Once they become palpable, the Hes1 tumour cells grow at a higher rate than the vector control cells in all cases. Differences were not evident when the injected cell number was above 10^6. However, at 10^5, 10^4 and 10^3 cells, the differences were significant. (C) Tumour weights after injection of nude mice with Hes1 (LV-Hes1) or vector control (LV-con) expressing SW620 cells. Once they become palpable, the Hes1 tumour cells grow at a higher rate than the vector control cells in all cases. This difference becomes more pronounced when the injected cell number is 10^5, 104 and 103. (D) Expression of Hes1, ALDH1, Bmi-1 in Hes1 (LV-Hes1) or vector control (LV-con) expressing tumours detected by using immunohistochemistry. (F) Tumours become palpable at an earlier timepoint and grow at a faster rate in Hes1-expressing cells compared with vector control cells. Tumour formation was monitored for 24–27 days after injection of nude mice. In the case of injections with 10^4 cells, all tumours arose in 10 mice for Hes1-expressing cells, whereas only 8 of 10 mice infected with vector control cells formed tumours. When 10^3 cells were injected, all mice formed tumours in the Hes1-expressing cell group, but only 6 of 12 mice did so in the vector control group.

Anchorage-independent growth assay. First, 6-well plates were coated with a layer of 0.6% agar in medium supplemented with 20% foetal bovine serum. The cells were prepared in 0.3% agar and seeded in triplicate. The plates were then incubated at 37°C in a humid atmosphere with 5% CO_2 for two weeks until colonies had formed. Each experiment was repeated at least three times. The colonies were photographed between 18 and 24 days (at a final magnification of 20×) under a phase contrast microscope, and the colonies that were larger than 50 mm in diameter were counted under a light microscope.

Statistical analysis. The data are presented as the mean ± SEM of at least 3 independent experiments unless otherwise indicated. The statistical analyses were performed using the SPSS 13.0 software package. Two-tailed Student's t-test was used for the comparisons of two independent groups ($^{*}p < 0.5$; $^{\#}p < 0.01$). The Origin 7.5 software was used to generate the correlation analysis graphs.

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Author contributions

F.G., Y.Q.Z., S.C.W. and Y.Q.l. carried out experiments; L.Z., J.Q.Y., W.H. and Y.Q.L. gave assistance in collecting tissue samples; Y.F.Y. took on the statistical analysis; F.G., D.X. and W.R.L. conceived experiments and analyzed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Additional information

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