# Suspension culture strategies to enrich colon cancer stem cells

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**Abstract.** How to efficiently obtain high-purity cancer stem cells (CSCs) has been the basis of CSC research, but the optimal conditions for serum-free suspension culture of CSCs are still unclear. The present study aimed to define the optimal culture medium composition and culture time for the enrichment of colon CSCs via suspension culture. Suspension cell cultures of colon cancer DLD-1 cells were prepared using serum-free medium (SFM) containing variable concentrations of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to produce spheroids. Culture times were set at 10, 20 and 30 days. A total of nine different concentrations of EGF and bFGF were added to SFM to generate nine experimental groups. The proportions of $CD44^+$, $CD133^+$, and $CD44^+CD133^+$ double-positive spheroid cells were detected via flow cytometry. mRNA expression of stemness-, epithelial-mesenchymal transition- and Wnt/β-catenin pathway-associated genes was determined via reverse transcription-quantitative PCR. Self-renewal ability was evaluated by a sphere-forming assay. Tumorigenesis was studied *in vitro* using a colony formation assay and *in vivo* via subcutaneous cell injection in nude mice. It was found that the highest expression proportions of $CD133^+$ and $CD44^+$ spheroid cells were observed in group (G)9 (20 ng/ml EGF + 20 ng/ml bFGF) at 30 days (F=123.554 and 99.528, respectively, P<0.001), $CD133^+CD44^+$ cells were also observed in G9 at 30 days (and at 10 days in G3 and 20 days in G6; F=57.897, P<0.001). G9 at 30 days also displayed the highest expression of Krüppel-like factor 4, leucine-rich repeat-containing G protein-coupled receptor 5, CD44, CD133, Vimentin and Wnt-3a (F=22.682, 25.401, 3.272, 7.852, 13.331 and 17.445, respectively, P<0.001) and the lowest expression of E-cadherin (F=10.851, P<0.001). G9 at 30 days produced the highest yield of cell spheroids, as determined by a sphere forming assay (F=19.147, P<0.001); colony formation assays also exhibited the greatest number of colonies derived from G9 spheroids at 30 days (F=60.767, P<0.01), which also generated the largest mean tumor volume in the subcutaneous tumorigenesis xenograft model (F=12.539, P<0.01). In conclusion, 20 ng/ml EGF + 20 ng/ml bFGF effectively enriched colon CSCs when added to suspension culture for 30 days, and conferred the highest efficiency compared with other combinations.

## Introduction

Colon cancer is a highly aggressive malignancy of the digestive system that carries a poor prognosis; according to the results of the National Center for Health Statistics, the number of new cases of colon cancer is 104,610, and the number of deaths is 53,200 in the United States (1). It constitutes the third leading cause of cancer diagnosis and mortality in both males and females, despite the reduced mortality rates in females in recent decades, and reduced overall incidence and mortality rates due to colon cancer screening (2). Traditional surgery, radiotherapy and chemotherapy primarily target tumor cells in the proliferative phase, but have limited killing effects on cells in the non-proliferative phase, which leads to metastasis, recurrence, and drug resistance (3). Cancer stem cells (CSCs) may be responsible for these poor clinical outcomes (4,5).

CSCs are a self-renewing subpopulation of neoplastic cells that promote tumorigenesis, proliferation and growth (6,7). Originally discovered in leukemia, CSCs have been identified and isolated from solid tumors, such as those of the prostate, brain, colorectum, pancreas and breast (8). CSC isolation and

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identification methods include fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS) and serum-free medium (SFM) cultures (9). FACS separates CSCs via flow cytometry using cell surface markers; however, it is inefficient, expensive, and can injure cells. MACS relies on the labeling of cells with monoclonal antibodies tagged with magnetic beads, which are then sorted using a strong magnetic field; however, this technique produces small-scale cell yields of low purity that are inadequate to meet experimental requirements. CSCs survive and proliferate in serum-free suspension culture environment, but cancer cells cannot (10,11). Therefore, the SFM suspension culture method can effectively expand and enrich CSCs. Advantages include high yields, improved cell viability, ease of operation and low cost (9,12).

In the study of CSCs derived from breast cancer (13), prostate cancer (14), and endometrial cancer (15), researchers have applied the method of SFM suspension culture to enrich CSCs. The obtained CSCs were further sorted and purified for subsequent research, or directly used in the follow-up experiments, allowing studies on CSCs to be carried out successfully.

In the present study of colon CSCs, SFM suspension culture protocols were used to effectively obtain CSCs. In order to maintain the integrity of signaling pathways in CSCs, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were added to the culture medium. However, the concentration to be used for the supplementation of these two growth factors remains a controversial issue; some researchers have used 10 ng/ml EGF + 10 ng/ml bFGF in SFM suspension culture (16,17), whereas others have used 20 ng/ml EGF + 10 ng/ml bFGF (18,19), and others still have used only a single growth factor (20). The selection of the concentrations of the two growth factors is not very rigorous. Another important aspect is that the culture time required for enriching CSCs by SFM suspension culture is controversial. Certain groups have opted for a culture time of 7-10 days (21-24), whereas others have reported using cultures of up to 14 days (25,26), or even >28 days (27). Insufficient time for suspension culture may lead to the incomplete formation of cell spheroids. Conversely, a prolonged culture time will weaken cell stemness and induce abnormal differentiation of spheroid cells.

Although the abovementioned culture strategies can enrich CSCs, the efficiency of their enrichment remains to be studied, particularly as variable serum-free suspension culture strategies produce dissimilar enrichment efficiencies. Insulin can participate in the metabolism and stemness of CSCs through EGF/EGF receptor pathways, making CSCs more malignant (28). Previous studies reported adding insulin to SFM to promote the proliferation of CSCs and maintain stemness (27,29). The currently preferred effective SFM suspension culture strategy is to add insulin, cell culture additive B27 and other basic nutrients to DMEM/F12, followed by the addition of EGF and bFGF (30). However, to our knowledge, there have been no systematic studies into the effects of culture medium composition, EGF and bFGF concentrations, or culture time on CSC enrichment. Consequently, the aim of the present study was to determine the optimal EGF and bFGF composition strategy and culture time for the serum-free suspension method. An optimized, efficient and reliable enrichment strategy that generates high yields of CSCs may facilitate preclinical drug development and other areas of basic and clinical research.

## Materials and methods

*Materials*. The human colon cancer DLD-1 cell line was obtained from the American Type Culture Collection. DMEM/F12 and RPMI 1640 media were purchased from HyClone (Cytiva). EGF and bFGF were purchased from PeproTech, Inc. Fetal bovine serum (FBS) and bovine serum albumin (BSA) were purchased from Amresco, LLC. Insulin was purchased from MilliporeSigma. SFM supplement factor B27 was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). CD133-phycoerythrin (PE; cat. no. 130-098-826), CD44-FITC (130-095-195) were purchased from Miltenyi Biotec GmbH. Corresponding isotype control antibodies Mouse IgG1κ Isotype Control PE (12-4714-41) and FITC (cat. no. 11-4714-41) were purchased from eBioscience. PrimeScript™ RT Master Mix kits were purchased from Takara Biotechnology Co., Ltd. Phosphate-buffered saline (PBS), crystal violet dye and trypsin were purchased from Wuhan Boster Biological Technology, Ltd. PCR primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The BD FACSCanto II Flow Cytometry Instrument was purchased from ACEA Bioscience, Inc. (Agilent Technologies, Inc.), and the PCR thermocycler was purchased from Roche Diagnostics. Sevoflurane was purchased from MilliporeSigma. BALB/C-Nu/Nu mice were purchased from Beijing HFK Bioscience Co., Ltd.

*Experimental group design*. SFM cultures were prepared by combining DMEM/F12 with 10% BSA, 5 mg/ml insulin, B27 cell culture additive (1:50), 100 U/ml streptomycin, 0.1 mg/ml penicillin and variable concentrations of growth factors. A total of nine experimental groups were prepared using different combinations of EGF and bFGF: Group 1 (G1), 5 ng/ml EGF; G2, 5 ng/ml bFGF; G3, 5 ng/ml EGF + 5 ng/ml bFGF; G4, 10 ng/ml EGF; G5, 10 ng/ml bFGF; G6, 10 ng/ml EGF + 10 ng/ml bFGF; G7, 20 ng/ml EGF; G8, 20 ng/ml bFGF; and G9, 20 ng/ml EGF + 20 ng/ml bFGF.

*DLD-1 monolayer cell culture*. After thawing, DLD-1 cells adhered in a monolayer in RPMI 1640 medium containing 10% FBS. The medium was changed every 2 days. When cells reached the logarithmic growth phase, they were digested with EDTA-containing trypsin for 4 min at 37˚C, followed by the addition of 4 ml RPMI-1640 and transfer to a centrifuge tube. After centrifugation at 111.8 x g for 5 min with 4˚C, the supernatant was discarded, and cells were resuspended in new medium. Finally, cells were cultivated in a new culture dish according to the number of cells. All cell cultures were maintained in a humidified incubator at 37˚C and 5% $CO_2$.

*Suspension culture of DLD-1 spherical cells*. Adherent DLD-1 cells were cultured to the logarithmic growth phase, and then cultured in DMEM/F12 for 24 h at 37˚C. Cultures underwent trypsin digestion for 4 min to create single-cell suspensions in DMEM/F12, followed by cell counting. A volume of 20 ml of the G1-G9 culture media preparations were added to super slip plastic culture flasks, followed by the addition of $3x10^4$ cells to each experimental group flask. Cultures were then incubated

with the addition of 2 ml of the corresponding group medium daily at 37°C and 5% $CO_2$. Follow-up tests were performed at days 10, 20 and 30.

*Flow cytometric analysis.* Suspended cell spheroids were cultured to days 10, 20 and 30, and then were subjected trypsin digestion and quantification. Single-cell suspensions were obtained in PBS. Aliquots of $5x10^5$ cells were rinsed twice with PBS, followed by the addition of staining buffer blocking solution (pH 7.2, containing 2% FBS and 0.4% BSA) and storage at 4°C for 1 h. The suspension was centrifuged (251.55 x g for 5 min with 4°C) and the supernatant was discarded. Subsequently, the cells were treated with the addition of 25 $\mu$l staining buffer and 1.25 $\mu$l of either anti-CD133 antibodies, anti-CD44 antibodies, or a mixture of both anti-CD133 and anti-CD44 antibodies (all 1:50), and protected from light at 4°C for 30 min. Finally, the supernatant was discarded after centrifugation (251.55 x g for 5 min with 4°C). CSCs were then suspended in 500 $\mu$l PBS and detected via flow cytometry. The data were analyzed using FlowJo software version 10.0 (BD Bioscience, ACEA Bioscience, Inc.).

*RT-quantitative (q)PCR analysis.* RNA was extracted from spheroids with chloroform and precipitated with absolute ethanol at days 10, 20 and 30. mRNA was then reverse-transcribed into cDNA (37°C for 15 min, 85°C for 5 min, 4°C for 5 min). GAPDH was used as an internal reference. PrimeScript™ RT Master Mix kit and TB Green® Premix Ex Taq™ (Tli RNaseH Plus; cat. no. RR420A; both from Takara Biotechnology Co., Ltd.) were used according to the manufacturer's instructions to detect the mRNA expression level in each sample using the $2^{-\Delta\Delta Cq}$ method (31). PCR was executed according to the follows: 1 cycle of pre-incubation at 95°C for 10 min, followed by 40 cycles of amplification for 10 sec at 95°C, 10 sec at 60°C, 10 sec at 72°C, then 1 cycle of melting at 95°C for 10 sec, at 65°C for 60 sec, at 97°C for 1 sec, and cooling at 50°C for 30 sec. The primer sequences used are presented in Table I.

*Sphere-forming assay.* A total of 100 spheroid cells were collected from each group and cultured in 24-well plates with 1 ml of the corresponding group medium for 14 days at 37°C. The number of spheroids in each group was then counted in three fields of view under a light microscope at x100 magnification for statistical analysis. Count three times for each sample.

*Colony formation assay.* Spheroid cells were trypsinized, suspended and counted. A total of 500 spheroid cells were collected from each group and seeded in 6-well plates with 5 ml RPMI-1640 medium containing 10% FBS for 7 days at 37°C. Cells were fixed with 75% ethanol solution at 37°C for 15 min and stained crystal violet alcohol solution (Shangbao Biological Company) at 37°C for 30 min. Colonies (>3 cells) counted manually under a light microscope at 40x magnification for statistical analysis. The experiments was performed three times.

*Subcutaneous tumorigenesis in nude mice.* All animal experiments were approved by the Ethical Committee of Huazhong University of Science and Technology (approval no. S255). A total of 81 female nude mice (age, 4-6 weeks; weight, 15-21 g)

Table I. Primer sequences of spheroid cell genes analyzed via reverse transcription-quantitative PCR.

| Gene | Primer sequence (5'→3') |
|---|---|
| GAPDH | F: GGGGAGCCAAAAGGGTCATCATCT |
| | R: GACGCCTGCTTCACCACCTTCTTG |
| KLF4 | F: CGAACCCACACAGGTGAGAA |
| | R: TACGGTAGTGCCTGGTCAGTTC |
| Lgr5 | F: CTCTTCCTCAAACCGTCTGC |
| | R: GATCGGAGGCTAAGCAACTG |
| CD44 | F: CTGCCGCTTTGCAGGTGTA |
| | R: CATTGTGGGCAAGGTGCTATT |
| CD133 | F: ACCGACTGAGACCCAACATC |
| | R: GACCGCAGGCTAGTTTTCAC |
| E-cadherin | F: GCCCTGCCAATCCCGATGAAA |
| | R: GGGGTCAGTATCAGCCGCT |
| Vimentin | F: GCTTCAGAGAGAGGAAGCCGAAAA |
| | R: CCGTGAGGTCAGGCTTGGAAA |
| Wnt-3a | F: AGCTACCCGATCTGGTGGTC |
| | R: CAAACTCGATGTCCTCGCTAC |

KLF4, Krüppel-like factor 4; Lgr5, leucine-rich repeat-containing G protein-coupled receptor 5; F, forward; R, reverse.

were randomly divided into 27 groups (n=3 mice/group), and housed in a specific pathogen-free environment (18-26°C; humidity, 40-50%, 12/12-h light/dark cycle and free access to food and water) for 1 week to facilitate adaption to the environment. Spheroid cells were trypsinized, collected and counted. Suspensions of $1x10^5$ cells in 200 $\mu$l physiological saline were prepared for later use. According to the grouping of nude mice, the corresponding cells were injected subcutaneously using a 1-ml syringe, and the injection tracks were clamped for 20 sec to prevent leakage. Nude mice were observed daily to evaluate their general condition and changes in body weight. After 30 days, sevoflurane (5%) was used to anesthetize the nude mice via breathing inhalation, then nude mice were sacrificed via cervical dislocation; after confirming the onset of rigor mortis, the tumors were harvested and measured, and tumor volumes were calculated using the following formula: Volume=length x width$^2$ x 0.5.

*Statistical analysis.* SPSS 25.0 (IBM Corp) was used for statistical analysis, and GraphPad Prism 7 (GraphPad Software, Inc.) was used for graphing of results. The normal distributions of data were presented as the mean ± SD, and one-way analysis of variance and Tukey's post hoc tests were used to compare statistical differences between each group. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed ≥3 times.

## Results

*Proliferation of adherent cells and suspension culture spheroids.* Adherent cells were arranged individually and grew
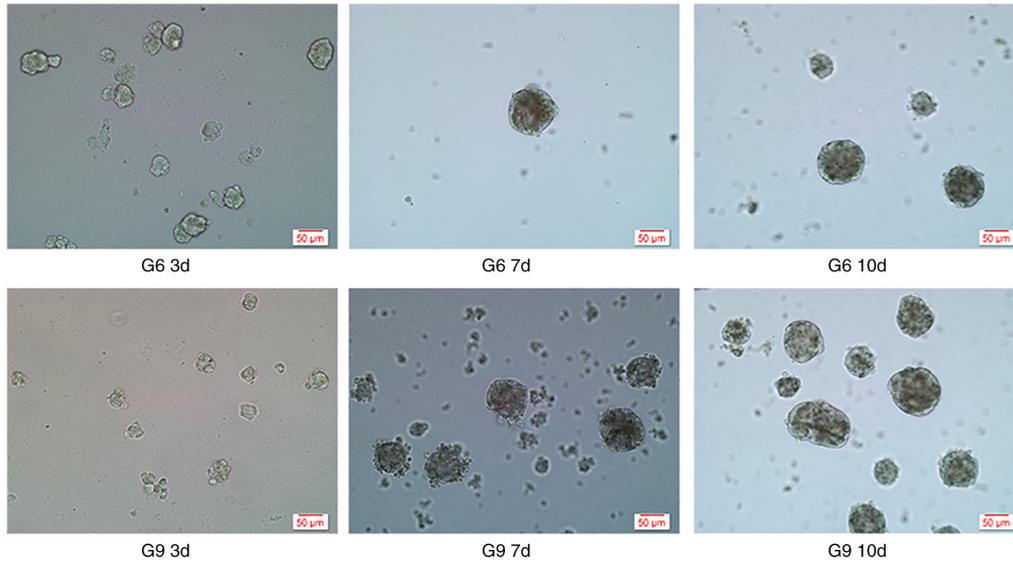
Figure 1. Morphology of spheroids. The morphology of spheroids cultured in G9 and G6 medium suspension. Spheroids began to form on day 3, and stabilized from days 7 to 10. Serum-free medium applied to the G9 and G6 groups formed stable spheroids; G6 and G9 are the groups with the most optimal spheroid cell growth trends. G, group; d, day.

close to the bottom of the culture flasks. Cells in each group began to assume a spheroid morphology on day 3, and tended to be suspended in the culture medium as a single oval unit at day 7, after which the volume of cell spheroids gradually increased. There were no intergroup differences in spheroid volume (Fig. 1). The suspension cultures started to produce stable spheroids after the seventh day of culture.

*Flow cytometry.* Flow cytometry revealed the percentages of CD133+, CD44+ and CD133+CD44+ double-positive spheroid cells of the nine groups at days 10, 20 and 30 of culture (Table II). The percentages of CD133+ and CD44+ cells were highest in G9 at 30 days, and were significantly elevated compared with the other combinations (F=123.554 and 99.528, respectively, P<0.001). The percentage of CD133+ cells in the G9 group at day 30 was 2.558±0.085%, which was similar to the results for G7 at day 10, 20 and 30, and G8 at day 10 and 30 (P>0.999, P=0.686, P>0.999, P=0.282 and 0.818, respectively); however, the percentage of CD44+ cells was 72.990±2.188%, which was higher compared with in any other group. The percentage of CD133+CD44+ double-positive spheroid cells in G9 at day 30 was second only to G3 at day 10 and G6 at day 20 and G9 at day 30, and G9 at day 30 was significantly higher compared with other combinations (except G3 at day 10 and G6 at day 20) (F=57.897, P<0.001). The difference between G3 at day 10 and G6 at day 20 was not statistically significant (P=0.574). These results suggested that the G9 suspension-cultured spheroids at 30 days of culture exhibited more prominent CSC characteristics than those grown under the other experimental conditions.

*RT-qPCR.* RT-qPCR detected the mRNA expression of stemness genes [Krüppel-like factor 4 (KLF4), leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), CD44, CD133], epithelial-mesenchymal transition (EMT) genes (E-cadherin, Vimentin) and Wnt/β-catenin pathway genes (Wnt-3a; Fig. 2). The mRNA expression levels of KLF4

(Fig. 2A), Lgr5 (Fig. 2B), CD44 (Fig. 2C), CD133 (Fig. 2D), Vimentin (Fig. 2E) and Wnt-3a (Fig. 2F) were significantly altered between the culture groups (F=22.682, 25.401, 3.272, 7.852, 13.331 and 17.445, respectively, P<0.001). with the highest expression values most commonly observed in G9 at day 30. Comparing the spheroids of each group with G9 at day 30, CD44 expression in G2 at day 20, G3 at days 20 and 30, G5 at day 20, G7 at day 30, and G9 at days 10 and 20 was similar (P=0.218, 0.830, 0.069, 0.162, 0.123, 0.060 and 0.133, respectively; Fig. 2C). There were no significant differences in CD133 expression between G9 at day 30 and G3 at day 20, G8 at days 20, G8 at days 30 (P=0.876, 0.304 and 0.664, respectively; Fig. 2D). There were no significant differences in Vimentin expression between G3 at day 30 and G9 at day 30 (P=0.350; Fig. 2E). Wnt-3a mRNA expression was similar between G9 at day 30 and G2, 3, 4 and 6 at day 20 (P=0.339, 0.235, 0.09 and 0.198; Fig. 2F). E-cadherin expression was lowest in the G9 group at day 30 (F=10.851; P<0.001), but was not significantly different compared with 15 other culture conditions (Fig. 2G). Increased expression of Wnt-3a indicated activation of the Wnt/β-catenin signaling pathway, whereas high expression of Vimentin and low expression of E-cadherin indicated that EMT had occurred, and was accompanied with substantially increased expression of stemness genes. In summary, these results indicated that cell spheroids exhibited the characteristics of CSCs. Comprehensive analysis of the data showed that G9 at day 30 exhibited the most upregulated expression of stemness genes and the lowest expression of E-cadherin, indicative of the most characteristic gene expression of CSCs (32,33).

*Sphere-forming assay.* Self-renewal ability is a landmark feature of CSCs, which is closely associated with cancer occurrence, development, recurrence and treatment resistance (34). The purpose of the sphere-forming assay is to test the self-renewal ability of spheroid cells. The sphere count was significantly different between the different conditions (F=19.147, P<0.001; Fig. 2H), with the highest yield

Table II. Surface marker expression in spheroid cells.

A, Proportion of CD133$^+$ cells

| Group | 10 days (%) | 20 days (%) | 30 days (%) |
|---|---|---|---|
| G1 | 0.227±0.124 | 0.127±0.049 | 0.317±0.050 |
| G2 | 0.140±0.040 | 0.693±0.163 | 0.507±0.051 |
| G3 | 0.593±0.091 | 0.590±0.053 | 1.363±0.060 |
| G4 | 0.035±0.005 | 0.720±0.061 | 0.597±0.040 |
| G5 | 0.840±0.110 | 0.090±0.027 | 0.195±0.005 |
| G6 | 0.177±0.015 | 1.223±0.105 | 0.217±0.023 |
| G7 | 2.470±0.149 | 2.277±0.047 | 0.336±0.507 |
| G8 | 2.210±0.297 | 1.087±0.006 | 2.300±0.131 |
| G9 | 1.820±0.046 | 1.030±0.035 | 2.558±0.085 |

B, Proportion of CD44$^+$ cells

| Group | 10 days (%) | 20 days (%) | 30 days (%) |
|---|---|---|---|
| G1 | 20.263±0.738 | 54.620±1.992 | 22.400±2.256 |
| G2 | 37.237±0.240 | 46.813±4.772 | 16.087±2.222 |
| G3 | 36.097±0.415 | 38.027±2.720 | 13.043±4.142 |
| G4 | 38.717±2.546 | 37.453±0.645 | 21.107±0.230 |
| G5 | 4.687±0.075 | 57.383±2.799 | 21.817±1.569 |
| G6 | 31.523±3.441 | 42.490±5.059 | 17.013±5.169 |
| G7 | 20.553±3.572 | 14.013±1.544 | 24.870±1.946 |
| G8 | 36.020±2.017 | 25.470±2.647 | 17.920±1.991 |
| G9 | 41.723±2.430 | 22.147±2.870 | 72.990±2.188 |

C, Proportion of CD133$^+$CD44$^+$ cells

| Group | 10 days (%) | 20 days (%) | 30 days (%) |
|---|---|---|---|
| G1 | 0.267±0.108 | 0.173±0.021 | 0.910±0.114 |
| G2 | 0.027±0.012 | 0.843±0.131 | 0.177±0.040 |
| G3 | 1.643±0.225 | 0.750±0.147 | 0.347±0.160 |
| G4 | 0.193±0.038 | 0.493±0.023 | 0.217±0.032 |
| G5 | 0.127±.038 | 0.273±0.035 | 0.087±0.032 |
| G6 | 0.287±0.047 | 1.597±0.237 | 0.173±0.055 |
| G7 | 0.387±0.091 | 0.353±0.047 | 0.950±0.053 |
| G8 | 0.603±0.055 | 0.557±0.100 | 0.617±0.047 |
| G9 | 1.020±0.046 | 0.407±0.121 | 1.257±0.144 |

of spheroids in the G9 condition from day 30. Of note, the difference between G6 and G9 from day 30 was not statistically significant (P=0.116). These results suggested that G9 spheroid cells cultured for 30 days exhibited the most efficient self-renewal capacity.

*Colony formation assay.* To test *in vitro* tumorigenicity, colony formation assays were performed for each experimental group (Fig. S1). Spheroid cells can redifferentiate into adherent cells. The number of colonies was significantly different between the different conditions (F=60.767, P<0.001), with the largest number of colonies observed

for G9 cells cultured for 30 days. However, there was no statistically significant difference between G9 from day 30 compared with G6 from day 30 or G7 from day 20 (P=0.119 and 0.131; Fig. 3). Therefore, the results suggested that G9 spheroid cells cultured for 30 days exhibited the most prominent *in vitro* tumorigenic capacity.

*Subcutaneous tumorigenesis in nude mice.* In vivo tumorigenesis was analyzed by using the tumor cell xenograft formation assay (Fig. S2). G9 at day 30 promoted the largest tumor volume, which was significantly increased compared with other combinations (F=12.539, P<0.001; Fig. 4). However,
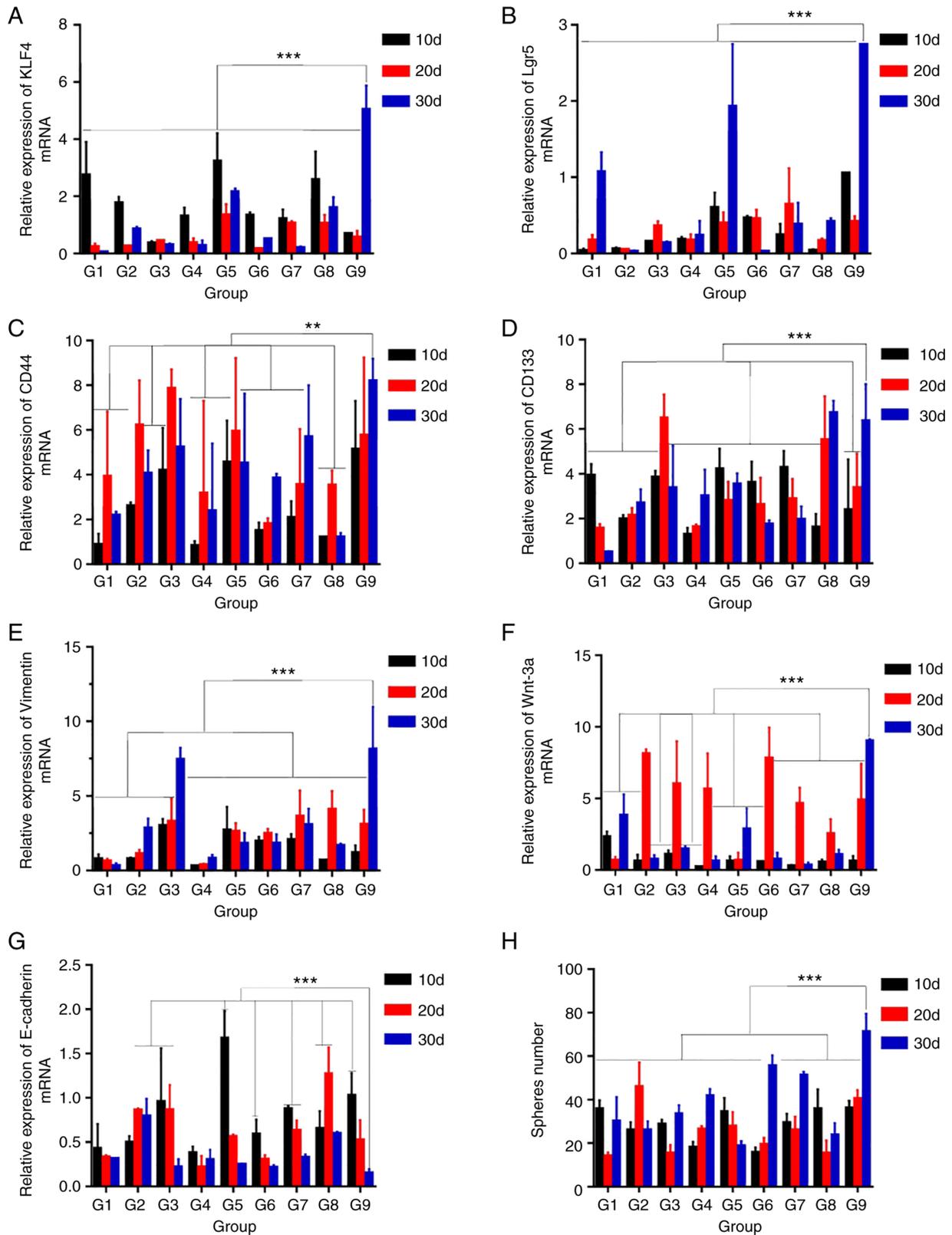
Figure 2. Reverse transcription-quantitative PCR to detect mRNA expression and sphere-forming ability of spheroid cells. All data are presented as the mean ± SD of three independent experiments. mRNA expression of (A) KLF4, (B) Lgr5, (C) CD44, (D) CD133, (E) Vimentin, (F) Wnt-3a and (G) E-cadherin. GAPDH was used as an internal reference. (H) Results of a sphere-forming assay. **P<0.01, ***P<0.001. G, group; d, day; KLF4, Krüppel-like factor 4; Lgr5, leucine-rich repeat-containing G protein-coupled receptor 5.

there were no statistically significant differences between G9 from day 30 compared with G6 from days 20 and 30, G7 from day 30 or G9 from day 20 (P=0.892, 0.940. 0.053 and 0.997,

respectively). It is therefore proposed that G9 spheroid cells cultured for 30 days exhibited the most potent *in vivo* tumorigenicity.
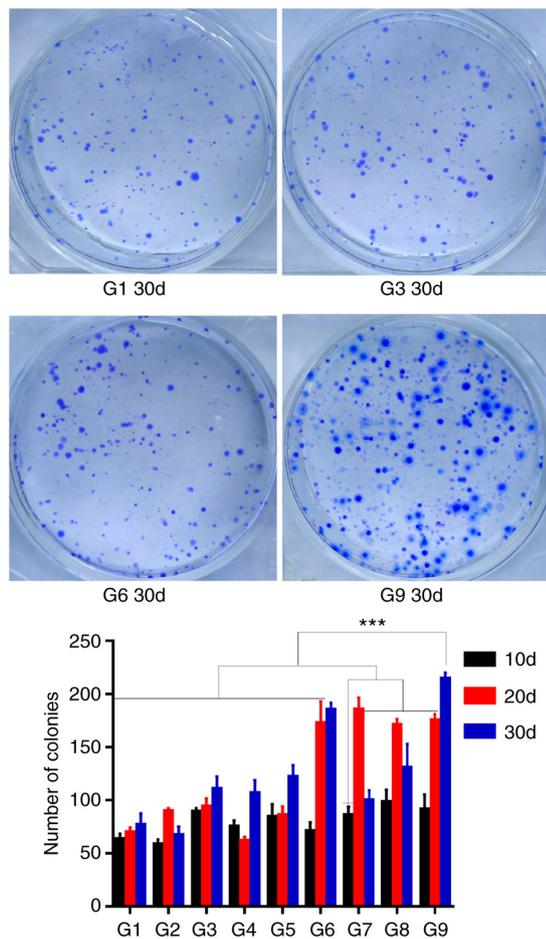
Figure 3. Comparison of *in vitro* tumorigenicity of spheroid cells cultured under different conditions. Data are presented as the mean ± SD of three independent experiments. Results of colony formation assay using cells following culture for 10, 20 or 30 days with different serum-free medium conditions. ***P<0.001. G, group; d, day.

## Discussion

Increasing evidence suggests that targeting and selective killing of CSCs to prevent tumor metastasis, recurrence and drug resistance may improve the prognosis of colon cancer (3,35). Annett and Robson (36) reported that therapeutic candidates that specifically target CSCs are now entering clinical trials. Gupta *et al* (37) reported that monoclonal antibodies that target surface markers on colon CSCs effectively treat colorectal cancer in preclinical *in vivo* models. Jahanafrooz *et al* (3) proposed that the combined targeting of CSCs and the tumor microenvironment (TME) may potentially lead to successful colon cancer eradication. Therefore, it is proposed that targeted therapy directed against colon CSCs will open novel opportunities for colon cancer treatment. However, a prerequisite for all supporting research is the procurement of sufficient and viable colon CSCs.

Acquisition and identification of colon CSCs is essential to targeted therapy research. Obtaining large quantities of high-purity colon CSCs has become a major challenge, due to their scarcity and difficult detection (38). FACS and MACS are traditional methods of obtaining CSCs, but are limited by low cell yields, reduced cellular viability and cost. By

incorporating technological advances, suspension culture has become a complementary method to FACS or MACS, and has been widely recognized as a means to circumvent the aforementioned disadvantages (12). Spheroid cells enriched by SFM suspension culture have prominent characteristics of CSCs, and can either be used directly or purified via FACS or MACS prior to CSC-related research (39). CD133, CD44 (40), high aldehyde dehydrogenase activity (41) and Lgr5 (42,43) are markers of colon CSCs. Combined surface markers can accurately identify and facilitate the isolation of CSCs from spheroid cells (43).

Currently, the most prevalent strategy for enriching CSCs with SFM suspension culture utilizes serum-free suspension medium with various concentrations and combinations of EGF and bFGF, and incubation times ranging from 7 to 15 days (15,44,45). Suspension culture time is crucial for the expression of CSC characteristics. Excessive culture time not only increases costs and the risk of culture contamination, but also increases the risk of *in vitro* culture variation and attenuation of CSC characteristics (46,47). Insufficient culture time produces enriched spheroid cells with diminished CSC characteristics (48). Culture times of either 7 or 14 days are favored in current suspension culture strategies, but there is no empirical validation that these are optimal time intervals (21,22). The present study observed that spheroid cell volume and stability did not change with increasing culture time after the seventh day of culture. However, PCR and flow cytometry results showed that the expression of various genes in spheroid cells were higher at 30 days compared with at 10 or 20 days. Moreover, the results of the sphere-forming and colony formation assays demonstrated that spheroid cells at 30 days exhibited more evident CSC characteristics. It is therefore proposed that CSC characteristics gradually increase through proliferation and accumulation of spheroid cells in suspension culture with increased culture time.

Colon CSCs simulate a CSC niche, where the TME maintains the CSC population and modulates the occurrence and development of cancer (49-51). Ahmed *et al* (52) reported that the chemoresistance, metastasis and recurrence of CSCs are regulated by the TME. Lenos *et al* (49) proposed that CSCs are regulated by cytokines, chemokines and stem cell factors in the TME. Consequently, the TME determines CSC characteristics and capabilities. *In vitro* serum-free suspension culture simulates the TME, as EGF and bFGF are among the most important factors affecting the TME; during CSC enrichment, EGF regulates proliferation, apoptosis and EMT via the EGF signaling pathway (28,53), whereas bFGF promotes self-renewal and maintains stemness (34,54). Varying concentrations of EGF and bFGF have been used previously to enrich CSCs in suspension culture, but to our knowledge, the optimization of growth factor concentrations and culture times has not been addressed. The present study comprehensively analyzed multiple experimental conditions, and found that G9 spheroids after 30 days exhibited the most proficient self-renewal, *in vivo* and *in vitro* tumorigenicity, and characteristic expression of stemness-associated genes compared with the other groups. Although it is possible that higher concentrations of EGF and bFGF may be more effective and require investigation in future studies, it was determined that a 30-day incubation using 20 ng/ml EGF +

Figure 4. Comparison of *in vivo* tumorigenicity of spheroid cells in each group. Subcutaneous tumorigenesis was investigated in nude mouse. Data are presented as the mean ± SD. ***P<0.001. G, group; d, day.
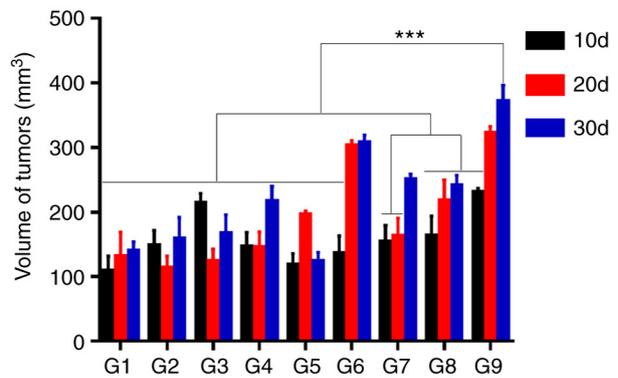
20 ng/ml bFGF medium supplement was the most conducive method for CSC enrichment compared with other strategies.

Next, the mechanisms underlying the effects of EGF and bFGF combination on CSC enrichment were explored. Activation of the Wnt/β-catenin pathway promotes EMT, and Wnt signaling contributes to the maintenance of CSC populations (55). In addition, EMT serves important roles in CSC metastasis, tumorigenesis, self-renewal and drug resistance (56-58). The Wnt/β-catenin pathway and EMT should be considered when identifying or isolating CSCs. The present study showed that Wnt/β-catenin pathway activation and EMT occurred in all groups of spheroid cells. It is worth mentioning that the expression levels of Wnt/β-catenin pathway-(Wnt-3a) and EMT-associated (Vimentin) genes were highest and the expression of E-cadherin was lowest in the G9 condition (20 ng/ml EGF + 20 ng/ml bFGF) after 30 days of culture compared with the other groups. It is proposed that the results observed for G9 after 30 days of culture are at least partly explained by the activation of ETM and the Wnt/β-catenin pathway due to the combination of EGF and bFGF.

Nonetheless, the present study has several shortcomings. The suspension culture method was not applied to a variety of cell lines and primary cells, and the study did not evaluate enrichment after 30 days. In addition, the underlying molecular mechanisms have not been explored completely. Such issues require further investigation.

In summary, the present study reported that SFM suspension cultures can effectively enrich tumor stem cells using the DLD-1 cell line, and that a 30-day culture using medium supplemented with a combination of 20 ng/ml EGF + 20 ng/ml bFGF presented the best enrichment efficiency due to activation of EMT and the Wnt/β-catenin pathway.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

GZ, XL and XZ conducted the cell and animal experiments, the analysis of experimental data, and the writing and revision of the manuscripts. WY and WL contributed to the cell experiments. YF and QX contributed to the subcutaneous tumor formation experiments in nude mice. JL, SJ and ZL made substantial contributions to the experimental design, and analysis and interpretation of data. ZL was involved in drafting the manuscript and revising it critically for important intellectual content. GZ and ZL confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The animal experiments were approved by the Ethical Committee of Huazhong University of Science and Technology (approval no S255; Wuhan, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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