Original Article

Construction of human colon cancer stem cells cDNA library and identification of novel proteins interacted with stratifin

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Abstract: Cancer stem cells play an important role in tumorigenesis and therapeutics. However, research has been hampered by lack of cancer stem cells cDNA libraries. Stratifin acts as a tumour suppressor protein and its inactivation is crucial in tumorigenesis. Colon cancer spheres were cultured from primary human colon cancer cells in a serum-free suspension culture system. CD133/CD166/CD44 triple positive cells were isolated from these spheres using flow cytometry. SMART cDNA Library Construction Kit was used to constructed cDNA library of human colon cancer stem cells and the quality of it was evaluated. After stratifin gene was cloned and transformed into yeast strain AH109, yeast two-hybrid screening assay was performed by mating AH109 with Y187 that contained colon cancer stem cells cDNA library plasmid. After the positive colonies were sequenced, the sequences were blasted against sequences in GenBank to analyse the function of the genes. Using the coimmunoprecipitation assay, we confirmed the proteins that interacted with stratifin. At last, the expression of two genes was knocked down using siRNA lentiviral vector and proliferation of colon cancer stem cells were observed. A mean of 1.2% of cells were CD133/CD166/CD44 triple positive in primary colon cancer spheres. Human colon cancer stem cells cDNA library was constructed successfully. The titer of the primary library was 0.23 × 10⁷ CFU/ml, the content reached 0.92 × 10⁷ CFU. After the yeast two-hybrid screen, forty eight true-positive colonies were selected and four coding genes with known functions were identified, including BAD, MPRIP, chrm 17 and TMEM45B. We confirmed the relations between stratifin with BAD, MPRIP, chrm 17 and TMEM45B proteins. Knock-down expression of the stratifin and BAD proteins significantly inhibited cell proliferation and colony formation of colon cancer stem cells. We constructed a plasmid cDNA library with human colon cancer stem cells successfully and screened the proteins that interacted with stratifin from the library. BAD and stratifin proteins may affect the proliferation and colony formation of colon cancer stem cells.

Keywords: Stratifin, cDNA library, yeast two-hybrid system, colon cancer stem cells

Introduction

Colon cancer is the leading cause of cancer-related death in developed countries [1]. Although its incidence has significantly reduced with the development of novel diagnostic and treatment methods, the mechanism of tumorigenesis, progression, and metastasis of colon cancer is not completely understood. Cancer stem cells (CSCs) are a sub-population of cancer cells that possess characteristics associated with normal stem cells, such as self renewal and the ability to differentiate into multiple cell types. CSCs are tumorigenic, in contrast to most cancer cells, which are thought to be non-tumorigenic. Recently, an increasing number of studies have described CSCs in solid tumors such as ovarian [2], colon [3], lung [4], breast [5], liver [6], melanoma [7] and pancreatic [8] tumors, raising the possibility that the CSC hypothesis applies to most neoplastic systems. CSCs are the most critical tumor cell type because they are capable of self renewing, differentiating, and maintaining tumor growth and heterogeneity, and thus play an important role in tumorigenesis and therapeutics. In our previous study, we found that CD133+/CD166+/CD44+ spheroid cells cultured from primary
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colon cancer cells displayed several features typically seen in stem cells and this marker combination was very useful in the identification of colon CSCs (unpublished data).

14-3-3 protein family are highly conserved proteins consisting of seven isotypes in human cells (β, γ, ε, η, α, τ, ξ). They play crucial roles in regulating multiple cellular processes, including the maintenance of cell cycle checkpoints and DNA repair, the prevention of apoptosis, the onset of cell differentiation and senescence, and the coordination of cell adhesion and motility. Among the 14-3-3 proteins, stratifin (also named 14-3-3σ) is the isoform most directly linked to cancer [9]. There are several lines of evidence indicating that stratifin acts as a tumour suppressor gene and that its inactivation is crucial in tumorigenesis. The down-regulation of stratifin by CpG methylation is detected in many tumours [10-16]. In our former study, we found the expression of stratifin was obviously increased in colon CSCs compared with differentiated cancer cells [17]. Stratifin may be involved in the course of self-renewal, proliferation and differentiation of colon CSCs.

To better understand the role of stratifin in the tumorigenesis, self-renewal, and differentiation of stem cells, we constructed a cDNA library from CD133+/CD166+/CD44+ spheroid cells and used the yeast two-hybrid system to identify novel binding proteins that interact with stratifin. Using this system, we found novel binding proteins from the cDNA library that closely interact with stratifin. This investigation provides functional clues for further exploration into novel cancer-related proteins for the treatment of colon cancer.

Materials and methods

Yeast strains, plasmids and chemical agents

All yeast strains and plasmids for the yeast two-hybrid experiments were obtained from Clontech (Palo Alto, CA, USA) as components of the MATCHMAKER two-hybrid system 3. The pGBK7 T DNA-BD cloning plasmid, pGADT7 AD cloning plasmid, pGBK7-53 control plasmid, pGADT7 and pGBK7-Lam control plasmid were obtained from Clontech Ltd. Taq DNA polymerase, T4 DNA ligase, and restriction endonu-

clase were purchased from Takara Company, Japan. The yeast culture media were purchased from Clontech, USA.

Primary cultures

Colonic adenocarcinoma tissues were collected from patients who underwent curative surgery for colon cancer at Huadong Hospital, Shanghai, China. Written informed consent was obtained from each patient recruited, and the study protocol was approved by the Clinical Research Ethics Committee of Huadong Hospital. After washing with phosphate-buffered saline (PBS), colon samples were minced into 1.0 mm³ fragments and dissociated enzymatically with 0.25% (w/v) trypsin-0.53 mM EDTA. Tumor fragments were incubated with pre-warmed trypsin-EDTA for 100 min at 37°C. The cell suspension was then filtered and washed with RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum. After dissociation, the cells were purified using Ficoll-Hypaque density centrifugation. Finally, the recovered cell population was washed and resuspended in RPMI 1640 medium with 10% fetal bovine serum and antibiotics (penicillin G 100 IU/mL, streptomycin 100 μg/mL, metronidazole 1 μg/mL, amphotericin B 2.5 μg/mL, gentamicin 20 μg/mL) (Yihe Biological). Cells were seeded into 96-well plates at a concentration of 10,000 cells/well and cultured at 37°C and 5% CO₂ for 10 days.

Culture of colon cancer spheres

The serum-supplemented medium (SSM) consisted of RPMI 1640 medium supplemented with 10% fetal bovine serum. The serum-free medium (SFM) was 1:1 (v/v) Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12; HyClone) containing B27 supplement (1:50; Gibco), 20 ng/mL epidermal growth factor (PeproTech), 10 ng/mL basic fibroblast growth factor (PeproTech), 10 ng/mL leukemia inhibitory factor (Chemicon), 2 mM L-glutamine, 4 U/L insulin, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Primary cultured colon cancer cells from surgery samples were washed with PBS and digested with trypsin (Amresco), followed by resuspension in SFM. After colon cancer spheres were generated, they were collected by 800 rpm centrifugation, dissociated to single cells, and cultured to generate second-generation spheroids.
Flow cytometry

Cells were collected separately from colon cancer spheres by trypsin digestion, followed by washing and resuspension in PBS at a concentration of 5 × 10⁷/mL. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD44 and phycoerythrin (PE) conjugated anti-CD133/CD166 mono-clonal antibodies for 30 min at 4°C. The percentage of positive tumor cells was assessed by measurement of the fluorescence intensity of the abovementioned cell-surface markers. All samples were analyzed on a FC500 flow cytometer (Beckman Coulter), which recorded 100,000-200,000 events per sample.

Total RNA and mRNA isolation from CD133+/CD166+/CD44+ spheroid cells

Total RNA was isolated with TRizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The total RNA was measured at 260 and 280 nm with a spectrophotometer (Ultraspec 2000, Pharmacia Biotech). The 260/280-nm ratios and a 1% agarose-formaldehyde gel stained with ethidium bromide (EB) were used to confirm the RNA quality of the samples. Fast track Kit (Invitrogen) was used for isolation of mRNA directly from total RNA according to the manufacturer’s instructions. The eluted mRNA in DEPC H₂O was dried in a cold vacuum and directly used for cDNA synthesis without ethanol precipitation.

cDNA synthesis and size fractionation

SMART™ PCR cDNA Synthesis Kit (Clontech) was used for cDNA synthesis. Briefly, 2 μg of total mRNA was added to a reaction mixture containing a final concentration of 0.1 M CDS and 3 μM SMART Oligo IV primer. Then the mixture was incubated at 80°C for 2 min and kept on ice. After centrifuged for 1 min, the supernatant was combined with dNTP and DTT at final concentrations of 0.25 and 2 mM, respectively, and with 20 units MMLV reverse transcriptase (Promega, USA); first-strand buffer (5 ×) was added. Subsequently, PCR reaction was prepared for a final reaction volume of 200 μl, including 6 μl of product, 0.2 μM 5’ PCR primer, 0.2 μM CDS III/3’ PCR primer, 0.2 mM dNTP, 4 μl advantage 2 polymerase mix (50 ×), 20 μl of advantage 2 PCR buffer (10 ×). The PCR conditions were 95°C for 30 s, followed by 24 cycles of 95°C for 10 s, and 72°C for 3 min. Double stranded cDNA was subjected to sfiI digestion under 50°C for 2.5 h. Then digested cDNA was size-fractionated on 1% agarose gel with 6 V/cm electrophoresis and the size fraction of 500 bp to 10 kb was excised. Then cDNA in each gel slice was extracted using the MinElute Gel Extraction kit (Qiagen, USA).

Construction of cDNA library

The SMART cDNA Library Construction Kit (Clontech) was used to construct human colon cancer stem cells cDNA library. Briefly, 2 μg cDNA, added 4 μl hybridization buffer (4 ×) and 5 μl sterile H₂O, was incubated at 98°C for 2 min and 68°C for 5 h. Then 1 ul of the cDNA mixture was amplified with primers M1 and M2. The PCR conditions were 96°C for 30 s, followed by 30 cycles of 96°C for 10 s, 66°C for 15 s, and 72°C for 3 min. After digested with proteinase K and SfiI, the cDNA was separated on a Sepharose CL-B column. Purified cDNAs were directionally cloned into the pDNR-LIB cloning vector (Clontech, USA). For ligation reaction, 50 ng cDNA, 0.5 μl empty vector, 0.5 μl T4 DNA ligase and 0.5 μl buffer were used at 16°C overnight. Transformation of 1 μl product into competent cells was accomplished by electroporation. Then the cells were plated on selective blue-white screening LB-agar medium with X-gal, IPTG, and carbenicillin over night at 37°C. Individual colonies were handpicked with sterile wooden toothpicks into wells containing 50 μl of selective LB with ampicillin and incubated overnight at 37°C.

cDNA library evaluation and cDNA clone analysis

The titer of the cDNA library was calculated by determining the number of growth colonies on the agar plate. According to the titer formula of cDNA library construction, the titer of primary library = total number of clone spots × dilution ratio × package volume. Blue-white plaque selection method was used to calculate the recombinant rate. Recombinant rate = negative plaques/(negative plaques + positive plaques). PCR method was used to amplify cDNA from 24 clones randomly picked using vectorspecific primers to check the insert size and the recombination rate. Overnight bacterial cultures were used to seed 25 μl PCR reactions in a 96-well PCR plate thermal cycler. The PCR
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RT-PCR was performed to amplify stratifin gene from CD133+/CD166+/CD44+ spheroid cells. Then the 747 bp PCR products were cloned into the pMD18-T vector. The primary structure of the insert was confirmed by direct sequencing. pMD18-T-stratifin vector was used as a mode to amplify stratifin gene by PCR method. The sequences of the primers containing the SfiI restriction enzyme sites were: SNF-F 5'-TTGGCCATTACGGCCATGGAGAGAGCCAGTCTG-3'; SNF-R 5'-AAGGCCGAGGCGGCCTCAGCTGGTGCTGAGCTCTG-3'. The PCR conditions were as follows: 94°C for 30 s, followed by 30 cycles of 94°C for 10 s, 58°C for 5 s, and 68°C for 2 min, and then 4°C at the end of the program. The PCR products were analyzed by 1% agarose gel electrophoresis.

**Construction of bait plasmid and expression of stratifin protein**

RT-PCR was performed to amplify stratifin gene from CD133+/CD166+/CD44+ spheroid cells. Then the 747 bp PCR products were cloned into the pMD18-T vector. The primary structure of the insert was confirmed by direct sequencing. pMD18-T-stratifin vector was used as a mode to amplify stratifin gene by PCR method. The sequences of the primers containing the SfiI restriction enzyme sites were: SNF-F 5'-TTGGCCATTACGGCCATGGAGAGAGCCAGTCTG-3'; SNF-R 5'-AAGGCCGAGGCGGCCTCAGCTGGTGCTGAGCTCTG-3'. The PCR conditions were as follows: 94°C for 30 s, followed by 30 cycles of 94°C for 10 s, 58°C for 5 s, and 68°C for 2 min, and then 4°C at the end of the program. The PCR products were analyzed by 1% agarose gel electrophoresis.

**Isolation and characterization of bait plasmid**

The bait plasmids pGBK7-stratifin were transformed into super-competence E. coli Top10 by electroporation. Transformants were plated on ampicillin SOB selection media and grown under selection conditions. Subsequently, the plasmids were re-isolated following the standard protocol from randomly chosen three transformants, analyzed by PCR amplification, agarose gel electrophoresis and direct sequencing.

**The toxicity and autonomous activation assays**

The purified bait plasmid was transformed into the AH109 strain and the strain was then cultured on SD/-Trp/agar plates for detection. Approximately 2 mm of AH109 colonies, transformed by pGBK7-stratifin and pGADT7, were incubated in 3 ml YPDA liquid medium at 30°C for 16 h with shaking. The absorbance values at 600 nm in different groups were compared. Additionally, 12 randomly chosen transformants containing the pGBK7-stratifin and pGADT7 plasmids were transferred to SD-Trp-Leu-His+5 mM 3AT and SD-Trp-Leu-His-Ade plates at 30°C for 5 days. LacZ assay was used to detect the toxicity and autonomous activation in yeast. In parallel, AH109 cells transformed by pGBK7-P53+pGADT7-LargeT and pGBK7-LaminC+pGADT7-LargeT served as the positive and negative controls, respectively.

**Screening of the CD133+/CD166+/CD44+ spheroid cells cDNA library by the yeast two-hybrid system**

One large colony of AH109 (bait) was inoculated into 50 ml SD/-Trp and incubated and shaken at 250 r/min at 30°C overnight. Then, cells were pelleted by centrifuging at 1000 r/min for 10 min. After the supernatant was decanted, the cell pellet was resuspended in the residual liquid. The cDNA library was cloned into pGADT7 and the yeast reporter strain Y187. The entire AH109 (bait) culture and 1 ml of the CD133+/CD166+/CD44+ spheroid cells cDNA library were combined and cultured in a 2-L sterile flask; 45 ml of 2 × YPDA/Kan was added and swirled gently. After a 20-hour mating period, the cells were pelleted, re-suspended and spread on 4 and 40 large (150 mm) plates containing 100 ml SD-Trp-Leu and SD-Trp-Leu-His+5 mM 3AT, respectively. After 14 days, the yeast colonies were transferred onto plates containing SD-Trp-Leu-His+5 mM 3AT and SD-Trp-Leu-His-Ade plates and detected with LacZ assay.

**Bioinformatics analysis**

The positive colonies were sequenced and blasted against sequences in GenBank to analyze the function of the genes (http://www.ncbi.nlm.nih.gov/blast). Other bioinformatics analyses, including their molecular weight, theoretical PI, estimated half-life, secondary structure prediction, and so on, were respectively performed with different software tools (www.expasy.ch/tools/protparamhtml; www.cmpharma.ucsf.edu/~n-omi/nn-predict.html; http://www.ch.embnet.org/software/COILS_form.html; www.expasy.org/tools/protscale.html; http://www.ch.embnet.org/software/TMPRED_form.html; http://www.cbs.dtu.dk/services/SignalP/).

**Plasmid constructs and transfection**

The plasmids in positive yeast clones were isolated. The stratifin, BAD, MPRIP, chrn 17 and TMEM45B genes were PCR amplified with spe-
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ACUUCUA-3’ and 5’-AAGAAGGGACTTCTCGCC-G-3’ were designed and synthesized by Shanghai Haike Corporation. The nonsilencing sequence 5’-TTCTCCGAACGTGTCACGT-3’ was used as a scrambled control. Pairs of complementary oligonucleotides with these sequences were synthesized, annealed, and ligated into a linearized pGCSIL-GFP plasmid vector. These plasmids were amplified in E. coli DH5. Lentivirus was generated in 293T cells by cotransfection of the recombinant pGCSIL-GFP vector with pHelper 1.0 and pHelper 2.0 plasmids using Lipofectamine 2000 (Invitrogen). The lentiviral particles were harvested 48 h after transfection and purified by ultracentrifugation (2 h at 50,000 × g), and they are referred to as Lv-si-stratifin, Lv-si-BAD and Lv-si-NC (negative control). For cell infection, CD133+/CD166+/CD44+spheroid cells were incubated with lentiviruses at a multiplicity of infection of 50 for 48 h, and the medium, which contained puromycin (10 mg/mL; Sigma-Aldrich, USA), was replaced to select stable clones.

Cell proliferation assay and colony formation in soft agar

CD133+/CD166+/CD44+spheroid cells and siRNA lentiviruses-infected cells were seeded at a density of 1 × 10⁵ in 35-mm Petri dishes. The cultured cells stained with trypan blue were observed and counted in triplicate over 6 weeks. The cells were disassociated, suspended in medium containing 0.3% agar, and plated onto a bottom layer containing 0.6% agar. The cells were plated at a density of 3 × 10⁴ cells/6-cm dish, and the number of colonies that were > 0.5 mm in diameter was counted 14 days later.

Statistical analysis

All data were statistically analyzed using Student’s t test or repeated one-way ANOVA with Dunnett post hoc test (GraphPad Prism 6, CA, USA). In all statistical analysis, P value of < 0.05 was considered significant.

Results

Primary human colon cancer cultures from fresh tumor tissue and colon cancer spheres formation

Fresh tumor tissue were digested and cultured in SSM. On the third day, some cells began to
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Figure 1. Primary cultured human colon cancer cells and colon cancer spheres. A. Primary cultured human colon cancer cells under light phase-contrast microscopy (× 200). B. Spheres of human colon cancer cells in the SFM suspension culture system.

RNA quality characterization

Total RNA was extracted from CD133⁺/CD166⁺/CD44⁺ spheroid cells and yielded approximately 725 μg of high purity total RNA. The absorbance ratios of the RNA at 260/280 and 230/260 nm were 2.03 and 2.01, respectively, indicating that the RNA was of the highest quality and was therefore useful for the following experiments. After the isolation using Fast track Kit, we got 5.9 μg of total mRNA for the construction of CD133⁺/CD166⁺/CD44⁺ cells cDNA library (Figure 2A).

cDNA library generation and evaluation

Fractionated cDNA was cloned into the sfiI sites of the vector and transformed into competent cells. The average cDNA insert size and their distribution were analyzed by random sampling of cDNA clones from randomly selected plates. A plasmid cDNA library with CD133⁺/CD166⁺/CD44⁺ spheroid cells was constructed successfully. The result showed that the titer of the primary library was 0.23 × 10⁷ CFU/ml, content of the library reached 0.92 × 10⁷ CFU in which the percentage of recombinant clones was 96%. The harbored foreign cDNA inserts with average size was 1.6 Kbp (Figure 2B). The titer of the amplified library was 3.5 × 10⁶ CFU/ml, content of the library reached 1.05 × 10⁷ CFU, and the harbored foreign cDNA inserts with average size was 1.5 Kbp (Figure 2C).

The toxicity and autonomous activation assays

The A600 nm values in the AH109-pGBKT7-stratifin and AH109-pGBKT7 groups suggested that the pGBKT7-stratifin plasmid was not toxic to yeast and had no effect on the growth of the yeast. Furthermore, the AH109-pGBKT7-stratifin clones were white and were detected on the SD/-Ade/-His/-Leu/-Trp/X-α-gal plates (Figure 3). Therefore, stratifin protein was believed to have no autonomous activation effect.

Screening for positive clones

Diploids were detected under an inverted microscope 20 h after co-incubation, which indicated that yeast mating was successful. Forty eight positive colonies grew on the SD/-Ade/-His/-Leu/-Trp/X-α-gal agar medium and restre-
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Figure 2. mRNA extraction and cDNA library construction of CD133+/CD166+/CD44+ spheroid cells. A. Quality identification of mRNA extracted from CD133+/CD166+/CD44+ spheroid cells. M: DNA Marker. B. Different lengths of cDNA fragments from white clones amplified by PCR. Lanes 1-24: randomly selected white clones in the primary cDNA library, Lane M: DNA Marker. C. Different lengths of cDNA fragments from white clones amplified by secondary PCR. Lanes 1-24: randomly selected white clones in the amplified cDNA library, Lane M: DNA Marker.

aked three times (Figure 4). Finally, forty eight putative positive yeast colonies were obtained (Table 1).

Gene sequencing and analysis
Forty eight positive yeast colonies were selected, and sequencing analysis was performed. Four genes that interacted with stratifin, including BAD, MPRIP, chrm 17 and TMEM45B, were identified (Table 2).

Plasmid constructs and transfection
The PCDEF-Flag-stratifin, PCDEF-Myc-BAD, PCDEF-Myc-MPRIP, PCDEF-Myc-chrm 17, and PCDEF-Myc-TMEM45B plasmids were successfully constructed. The sequences of the plasmids were further confirmed by DNA sequencing. After 48 h the plasmids transfected, CD133+/CD166+/CD44+ spheroid cells were observed under a fluorescence microscope.
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Figure 3. Autonomous activation assays of plasmid transfected yeasts. Number 1-12: randomly selected 12 transformants, AD-T+BD-53: positive control, AD-T+BD-Lam: negative control. A. Randomly selected 12 transformants and control yeasts grown on SD-Trp-Leu plates. B. Randomly selected 12 transformants did not grow on SD-Trp-Leu-His+5mM3AT plates. Positive control yeasts grown and negative control did not. C. Randomly selected 12 transformants did not grow on SD-Trp-Leu-His-Ade plates. Positive control yeasts grown and negative control did not. D. In LacZ assay, randomly selected 12 transformants did not turn blue. Positive control turned blue and negative control did not.

Figure 4. Screening of the CD133+/CD166+/CD44+ spheroid cells cDNA library for positive clones. After the bait yeast and CD133+/CD166+/CD44+ spheroid cells cDNA library mated, yeasts grown for 14 days. Then the positive colonies were transferred onto plates containing SD-Trp-Leu-His+5mM3AT and SD-Trp-Leu-His-Ade plates and detected with LacZ assay. Number 1-48: All the positive transformants of screening CD133+/CD166+/CD44+ spheroid cells cDNA library, AD-T+BD-53: positive control, AD-T+BD-Lam: negative control. A. SD-Trp-Leu-His+5mM3AT plate; B. SD-Trp-Leu-His-Ade plate; C. LacZ assay.

Confirmation of interaction by coimmunoprecipitation

Four interactions detected in this two-hybrid screen were further confirmed in CD133+/CD166+/CD44+ spheroid cells by specific coimmunoprecipitation of Flag-tagged bait proteins with the four Myc-tagged prey proteins. CD133+/CD166+/CD44+ spheroid cells stably expressed the PCDEF-Myc-BAD, PCDEF-Myc-MPRIP, PCDEF-Myc-chrm 17 and PCDEF-Myc-TMEM45B plasmids. An anti-Myc antibody was used to immunoprecipitate Myc-BAD, Myc-MPRIP, Myc-chrm 17 and Myc-TMEM45B from cell lysates. The presence of Flag-stratifin protein in the immunoprecipitated complex was determined by western blot analysis (Figure 5). These coimmunoprecipitations confirm that several of the novel interactions identified in the present two-hybrid screen are reproducible in the context of mammalian cells and therefore validate the results obtained by the two-hybrid assay.

Inhibition of cell proliferation and colony formation of CD133+/CD166+/CD44+ spheroid cells by siRNA lentivirus-infection

After Lv-si-stratifin or Lv-si-BAD expression vectors infection, knock-down of the stratifin and BAD proteins in CD133+/CD166+/CD44+ spheroid cells was observed (Figure 6A). We tested for differences in the proliferation rate between CD133+/CD166+/CD44+ spheroid cells and Lv-siRNA-infected cells. The cells were examined from week 1 to week 7 after seeding. As shown in Figure 6B, there was a difference in the growth rate between the infected and control cells. The infected cells grew slowly and showed growth inhibition after week 3. The self-renewing capacity of the infected cells was also examined with the colony formation assay. When plated at a density of 100 cells/well, Lv-si-stratifin, Lv-si-BAD and Lv-si-stratifin+Lv-si-BAD infected cells generated a lower mean number of tumour spheres (60.3 ± 4.5, 38.7 ± 3.4 and 17.1 ± 1.9, respectively) compared to the CD133+/CD166+/CD44+ spheroid cells (73.5 ± 4.9) (Figure 6C).
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Table 1. Activation of reporter genes in 48 positive transformants of screening colon cancer stem cells cDNA library

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<tr>
<td>PC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NC</td>
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</table>

No.: clone number; PC: positive control; NC: negative control.

Discussion

Identification and localization of CR-CSCs and the pathways disrupted in CRC remain difficult and highly controversial owing to the lack of widely accepted specific molecular markers. Isolation of CR-CSCs can be achieved by targeting and selecting subpopulation of tumor cells based on expression of one or multiple cell-surface markers associated with cancer self-renewal, such as CD133, CD166, CD44, CD24, beta1 integrin/CD29, Lgr5, EpCAM, ALDH-1, MSI1, DCAMLK1 or EphB receptors [18, 19]. CD133, CD166 and CD44 are the three main markers that have recently been associated with CR-CSCs.

In our study, we found that there was no obvious difference in CD133 expression between the adherent and spheroid cells isolated from human colon cancer tissue. However, the proportions of CD166+ and CD44+ cells within the adherent cell population were both much smaller than in the spheroid cell population. The expression of CD133, CD166 and CD44 in colon cancer spheres was significantly higher than in primary colon cancer cells cultured in SSM, suggesting that CD133, CD166 and CD44 are primarily expressed in undifferentiated cancer cells. Our results revealed, for the first time, that this marker combination (CD133+/CD166+/CD44+) could be very useful in the identification of colon CSCs.

cDNA libraries play an indispensable role in the isolation and characterization of mammalian genes. However, there are no human cancer stem cells cDNA libraries until now. CSC research has been hampered by the lack of genetic resource for identifying and isolating full-length genes and functional units from CSC genome. In this study, we constructed a human colon cancer stem cells cDNA library and identified the quality of it successfully. The CSC cDNA library may provide molecular resources for analysis of genes involved in the tumorigenesis, progression, and metastasis of colon cancer. The yeast two-hybrid system 3 was used to screen proteins interacting with stratifin. At last, we obtained the sequences of four types of genes with known functions.

In addition to their well-known pro-proliferative and anti-apoptotic effects, stratifin proteins have also been found to suppress cell growth and cell-cycle progression, especially after DNA damage, indicating functions in tumour suppression [20, 21]. Of the 14-3-3 protein family, the tumour-suppressor activity has most clearly been defined for stratifin [22, 23]. Stratifin is unique among the 14-3-3 proteins in that it is expressed primarily in epithelial cells and forms homodimers almost exclusively. Further insight as to why the loss of stratifin might facilitate tumor formation comes from the discoveries by Wilker and colleagues that stratifin is a crucial regulator of translation during mitosis and that stratifin function is required for proper mitotic exit and cytokinesis. They show that stratifin is needed for the mitotic switch from cap-dependent to cap-independent translation and that stratifin appears to mediate this switch by binding to eIF4B and perhaps other factors involved in cap-dependent translation [24].
Colon cancer stem cells and stratifin

Table 2. Positive genes of screening colon cancer stem cells cDNA library

<table>
<thead>
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<th>Name</th>
<th>Accession Number</th>
<th>Description</th>
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<tr>
<td>1</td>
<td>BAD</td>
<td>NM_032989.2</td>
<td>Homo sapiens BCL2-associated agonist of cell death (BAD), transcript variant 2, mRNA</td>
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<td>2/8/9/10</td>
<td>MPRIP</td>
<td>NM_015134.3</td>
<td>Homo sapiens myosin phosphatase Rho interacting protein (MPRIP), transcript variant 1, mRNA</td>
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<td>3</td>
<td>Chrm 17</td>
<td>NW_004078092.1</td>
<td>Homo sapiens chromosome 17 genomic scaffold, alternate assembly CHM1_1.0</td>
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<tr>
<td>19</td>
<td>TMEM45B</td>
<td>NM_138788.3</td>
<td>Homo sapiens transmembrane protein 45B (TMEM45B), mRNA</td>
</tr>
</tbody>
</table>

14-3-3 acts as an adaptor or “chaperone molecule” and interacts with various cellular proteins. More than a hundred small molecules interact with 14-3-3 in a phosphorylation-dependent manner [25, 26]. However, a few proteins interact with 14-3-3 in a phosphorylation-independent manner such as Bax. Recently, using direct proteomic analysis, researchers have identified a large number of polypeptides (> 200) that can associate with 14-3-3 proteins. These polypeptides are involved in numerous cell functions, including fatty acid synthesis, reductive metabolism, iron and other metabolisms, DNA/chromatin interactions including transcription factors, RNA binding, protein synthesis, protein folding and processing, proteolysis, protease inhibitors, ubiquitin metabolism, cellular signaling and apoptosis, actin dynamics, cellular trafficking and transporters, signaling kinases, cell division, nuclear proteins, oncogenic signaling, and cytoskeletal proteins [27, 28]. A study demonstrated that some of the 14-3-3 binding proteins were involved in the regulation of the cytoskeleton, GTPase functions, membrane signaling, and cell fate determination [29]. In this study, we found that stratifin could interact with the proteins BAD, MPRIP, chrm 17 and TMEM45B.

Inhibition of apoptosis can confer a growth advantage to cells and contribute to cancer development. Anti-apoptotic effects of stratifin are ascribed to its interaction with and inhibition of the pro-apoptotic proteins BAD and BAX. Phosphorylated BAD induces apoptosis by binding to and inhibiting the anti-apoptotic effects of Bcl-xL and Bcl-2. Stratifin sequesters phosphorylated BAD in the cytosol, and then the stratifin-BAD complex is sequestered from mitochondrial localized Bcl-xL and thus inhibits BAD-induced apoptosis [30].

BAD is a BH3 domain-containing protein that activates apoptosis and negatively regulated by pathways under the control of extracellular growth factors. BAD may be phosphorylated at multiple sites, prominently serine 112, a MAP kinase-dependent site [31], and serine 136, a site phosphorylated by Akt kinase [32]. Recently, some other BAD residues (Ser155 and Ser170) were identified as apoptosis related phosphorylation sites [33, 34]. Phosphorylation of these sites causes BAD to bind to stratifin, sequestering it in the cytoplasm and preventing its proapoptotic effects [35]. More recently it was reported that Cdc2 was capable...

**Figure 5.** Interactions of stratifin and BAD, MPRIP, chrm 17 or TMEM45B protein in CD133<sup>+</sup>/CD166<sup>+</sup>/CD44<sup>+</sup> spheroid cells. Western blot of coimmunoprecipitations using Flag-tagged bait and Myc-tagged prey proteins. Four of the interactions identified in the yeast two-hybrid screening were confirmed by coimmunoprecipitations of Flag-tagged bait and Myc-tagged prey proteins. Different plasmids were cotransfected into CD133<sup>+</sup>/CD166<sup>+</sup>/CD44<sup>+</sup>spheroid cells and cell lysates were prepared 48 h later. A part of the total lysate was kept aside as input control and the rest was divided to two. Anti-Myc antibody was added to one part and vehicle (PBS) to the other. Protein G agarose beads were added to the lysate and the complexes formed were collected and washed. The proteins were subsequently separated by PAGE, transferred to NC membrane and detected by ECL method.
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Active Cdc2 catalyzes the phosphorylation of the BH3-only protein BAD at a distinct site, serine 128, and thereby induces BAD mediated apoptosis in primary neurons by opposing growth factor inhibition of the apoptotic effect of BAD. The phosphorylation of BAD serine 128 inhibits the interaction of growth factor-induced serine 136-phosphorylated BAD with stratifin [36].

Kim et al [37] found overexpression of p-BAD in the colon cancers, which could block the apoptotic activity of BAD. A recent study revealed that phosphorylation status of each serine residue in BAD protein was different in a prostate cell line according to the apoptosis stimuli and p-BAD (Ser136) was expressed in 57.4% of the colorectal cancers but not in normal colon epithelium [18]. These data suggest that phosphorylation of BAD both at Ser112 and Ser136 may be involved in the colorectal cancer pathogenesis.

TMEM45B encodes a putative membrane protein with unknown function and shows properties of thermal aggregation when it was expressed in COS7 cells. Some researchers dissected the region of TMEM45B responsible for this aggregation, and found that of the seven putative transmembrane domains, a region comprising the 4th to 7th ones was essential for the thermal aggregation properties [38]. Its role in colon carcinogenesis might be worth exploring.

In summary, we constructed a plasmid cDNA library with human colon cancer stem cells suc-
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cessfully. Using the yeast two-hybrid system, we found novel binding proteins (BAD, MPRIP, chrm 17 and TMEM45B) from the colon cancer stem cells cDNA library that closely interact with stratifin. Knock-down the expression of stratifin and BAD protein significantly inhibited cell proliferation and colony formation of human colon cancer stem cells.

Acknowledgements

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Disclosure of conflict of interest

None.

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