Identification of CD200⁺ colorectal cancer stem cells and their gene expression profile

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Abstract. CD200 is a cell surface glycoprotein that has been implicated in a variety of human cancer cells. It has been proposed as a cancer stem cell (CSC) marker in colon cancer and is closely related to tumor immunosuppression. However, there is little functional data supporting its role as a true CSC marker, and the mechanism by which CD200 contributes to colorectal cancer has not been elucidated. In the present study, CD200⁺ and CD200⁻ COLO 205 colorectal cancer cells were sorted out by flow cytometry, and colonosphere formation and Transwell migration assays were performed. Affymetrix Human U133 Plus2.0 arrays were used to screen the gene expression profiles of CD200⁺ and CD200⁻ colorectal cancer cells. The results suggest that there are differentially expressed genes between the two subpopulations, including several important genes that function in cell proliferation, metastasis, apoptosis and the immune response. Pathway analysis revealed that the Wnt, MAPK and calcium signaling pathways were differentially expressed between CD200⁺ and CD200⁻ cells. Moreover, several key genes upregulated in CD200⁺ cells were also highly overexpressed in CD44⁺CD133⁺ colorectal stem cells compared to the CD44⁻CD133⁻ fraction of the same cell line. In the present study, we showed for the first time a correlation between CD200 expression and the Wnt signaling pathway in colon cancer cells.

Introduction

Cancer stem cells (CSCs) are a type of tumor cell that can self-renew and are pluripotent. They have been closely linked to tumor occurrence, development, metastasis, recurrence and drug resistance. Over the past several decades, the use of

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specific stem cell surface markers to identify stem cells has been widely used in CSC research, and several molecular markers have been thought to be relatively precise stem cell markers in colorectal cancer, including CD44, CD133, CD24, Lgr5 and EpCAM (1-6). However, the use of a single stem cell marker to purify stem cells has been controversial; thus, using two markers in combination, such as CD44 and CD133, is considered a more accurate approach to purify and define stem cells (7,8).

CD200 is a type I membrane glycoprotein with two extracellular domains, a single transmembrane region, and a cytoplasmic tail with no known signaling motifs. It is a member of the highly conserved immunoglobulin family, and it is expressed in myeloid cells, such as macrophages, dendritic and mast cells, and eosinophils. Interactions between CD200 and its receptor CD200R act as an immune tolerance signal, which reduces myeloid cell activity and change their migration ability (9-12). In recent years, high CD200 expression was found in colon cancer, myeloma, breast and brain cancer, melanoma and normal mesenchymal stem cells. Moreaux *et al* (13) previously observed significant CD200 overexpression in colon, head and neck and renal carcinoma, malignant mesothelioma and testicular cancer, MGUS myeloma and chronic lymphocytic leukemia compared to normal cell or tissue counterparts. Moreover, CD200 expression plays important roles in tumor progression of various types of cancers (14,15). Based on these findings, CD200 has been proposed as a new CSC marker in colon cancer. However, further experiments have not been performed to confirm its accuracy as a colorectal CSC marker. In our previous study, xenograft transplantation assays confirmed that CD200⁺ cells were more tumorigenic than CD200⁻ cells. Continuous monitoring of xenograft tumor growth revealed that at the same cellular dosage, CD200⁺ cells initiated tumor growth much faster than CD200⁻ tumors, resulting in a larger tumor mass. These data provided preliminarily evidence that CD200 is a reliable colon cancer stem cell (CCSC) marker. In the present study, we performed *in vitro* experiments to further confirm CD200 as a stem cell surface marker.

Since CSCs are thought to initiate tumor formation, development, metastasis and recurrence, they must be able to escape tumor immune killers. It was previously hypothesized that during the process from immune clearance to immune balance or during immune escape, CSCs express specific

surface molecules to secrete immune inhibitory factors, protect against tumor-specific immune responses and increase immune inhibition responses to escape from immune killers. Since CSCs and tumor immune protection are causes of tumor recurrence and metastasis, we hypothesized that CSCs reduce tumor immune responses by expression of specific markers. There are clear links between CSC marker expression and immune tolerance. Numerous previous studies have shown that tumor cells overexpressing CD200 can better escape from immune damage and evade the immune system (14,16). Therefore, CD200 is thought to be a crucial regulator of CSC immune escape, and it likely links colorectal CSCs (CCSCs) to immune evasion. Therefore, an in-depth understanding of CD200 and CD200-related genes is important to explore tumor immunosuppression mechanisms and tumor targeting therapy.

To address these issues, we compared the FACS-sorted CD200+ population with the CD200- population for their self-renewal abilities *in vitro*, which is a key feature of CSCs. Moreover, long-term cultured CD200+ cells were assayed for invasion ability, another defining characteristic of CCSCs. We also investigated the expression pattern of genes commonly modulated by CD200 in human COLO 205 colorectal cancer cells. After clustering and molecular function analysis, biological process and related pathway analysis, we chose several important functional genes and pathways with specific connections to CD200 as targets for further research.

Materials and methods

Cell culture. The human colon cancer cell lines, COLO 205, LoVo, SW620, SW480, SW1116 and HT29 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin in a humidified incubator under 5% CO_2 and 95% air at 37˚C. Cells were grown to logarithmic phase in serum medium, the upper medium containing cells in suspension were removed, and the adherent cells were digested using 0.25% trypsin + 0.02% EDTA into a single cell suspension. Cells were replated and cultured in serum-free medium/F12 (DMEM/F12; HyClone) supplemented with epidermal growth factor (EGF; 20 ng/ml), basic fibroblast growth factor (bFGF; 20 ng/ml), leukemia inhibitory factor (LIF; 10 ng/ml) (all from PeproTech, Rocky Hill, NJ, USA), and insulin (4 U/l; Sigma, St. Louis, MO, USA) onto ultralow attachment T25 slide dishes (Corning, Tewksbury, MA, USA). At least 1,000 cells/ml were used for colonosphere formation. Cells were maintained at 37˚C in a humidified incubator under 5% CO_2 and 95% air.

Flow cytometry and FACS. CD200, CD133 and CD44 expression was analyzed by flow cytometry. The cells were diluted into a single cell suspension, washed twice and suspended in 100 μl assay buffer [phosphate-buffered saline (PBS) 0.5% BSA, 2 mM EDTA, pH 7.2] with 10 μl APC-conjugated anti-CD200 or 10 μl PE-conjugated anti-CD133 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) or 20 μl FITC-conjugated anti-CD44 antibody (BD Biosciences,

San Jose, CA, USA). Mouse PE-IgG2b, APC-IgG2b (both from Miltenyi Biotec) and FITC-IgG2b antibodies (BD) were used as isotype controls. Cells were incubated in the dark at 4˚C for 20 min, washed twice with 1 ml assay buffer, and centrifuged at 300 g for 10 min. The cells were resuspended in assay buffer for analysis and sorting on a FACSCalibur flow cytometer or subjected to fluorescence-activated cell sorting (FACS) using a BD FACsAria II sorter (both from BD Biosciences). Side scatter and forward scatter profiles were used to eliminate cell doublets. Cell purity was analyzed after sorting and was typically >95%.

Colonosphere formation assay. To evaluate the self-renewal potential of CD200+ and CD200- subpopulations in COLO 205 cells *in vitro*, FACS sorted cells were collected and enzymatically digested with Accutase (Millipore, Billerica, MA, USA) to obtain a single cell suspension (100 cells/ml). A total of 100 μl was added to each well of a 96-well plate (Corning). The cells were cultured in serum-free medium/F12 (DMEM/F12) supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml LIF (all from PeproTech) and 4 U/l insulin (Sigma). All growth factors were added to the culture medium every 3 days. Cells were cultured for 14 days, and the number of floating colonospheres containing >50 cells was counted in each well. Colonosphere forming efficiency (CFE) was calculated in each well as the number of colonospheres/10. In addition, each generation of colonospheres was diluted into a single-cell suspension and re-seeded to test CFE for each indicated passage. The colonosphere formation assay was confirmed with at least 3 replicates for each cell population.

Matrigel-coated Transwell assay. A Transwell assay was conducted to evaluate the invasiveness of CD200+ and CD200- cells. Each cell population was added to an 8.0-μm pore Matrigel-coated Transwell (BD) insert at a cell density of $5x10^4$/insert in DMEM/F12 basic medium. The lower chambers were filled with DMEM/F12 medium supplemented with 10% FBS as a chemoattractant, and the cells were cultured for 24 h. Cells that passed through the membrane to the lower chamber were stained with crystal violet. A light microscope (Olympus IX71) was used to calculate the number of invaded cells per high power field. At least 10 microscopic fields were randomly selected for each experiment.

Microarray analysis. Gene expression profiling of CD200+ and CD200- COLO 205 cells was performed using the Affymetrix Human U133 Plus2.0 GeneChip according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Chips were scanned with a GeneChip® Scanner 3000 (Cat#00-00212; Affymetrix). The raw data were read with the Command Console Software 3.1 (Affymetrix). Quality qualified data were normalized with the Gene Spring Software 11.0 (Agilent Technologies, Santa Clara, CA, USA). The MAS5.0 algorithm was used. NetAffx (http://www.affymetrix.com) was used to obtain information of each probe on the Human U133 Plus2.0 chip. Differentially expressed genes were identified through data analysis of the SAS system. Fold-change was used to calculate the expression differences of the signal value between the two sets after homogenization (fold-change = $2^{mean2-mean1}$) A gene expression value of 2 was set as the ratio cut-off of 2, as it is commonly adopted for microarray data analysis.

The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://niaid.abcc.ncifcrf.gov/) and the SAS Analysis Systemb(http://sas.ebioservice.com/) were used for further analysis of the selected differentially expressed genes.

RT-PCR. Total cellular mRNA was extracted from FACs-sorted cells with TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. mRNA expression was determined by RT-PCR using SYBR® Premix Ex *Taq*™ (Takara, Otsu, Shiga, Japan) on a LightCycler 480 real-time genetic analyzer (Roche), with the indicated amplicon length. The PCR conditions were as follows: initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 20 sec and extension at 65°C for 15 sec, and finally followed by melting curve analysis. GAPDH expression served as an internal control.

Sodium butyrate treatment. COLO 205 cells were diluted into a single-cell suspension, and then, $1x10^6$ cells were seeded in 10-cm plastic dishes at day 0. Sodium butyrate (NaBT; Wako, Osaka, Japan) was added on day 1 at concentrations of 0, 3, 5, 8 or 12 mM, and incubated for 24 h. Enzyme-linked immunosorbent assay (ELISA) with SensoLyte™ pNPP alkaline phosphatase assay kit (AnaSpec, Inc., Fremont, CA, USA) was used to detect changes in alkaline phosphatase levels according to the manufacturer's protocol.

Western blotting. Whole cell lysates were prepared as previously described (17). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% resolving polyacrylamide gel and transferred onto a Hybond-P polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked in 5% bovine serum albumin (BSA)/0.1% Tween-20 in PBS solution (TPBS) for 60 min and then incubated overnight at room temperature with β-catenin, Wnt3a, pLRP6, DVL2, Wnt5a, LRP6 or Naked1 rabbit mAbs (Cell Signaling Technology, Danvers, MA, USA) at a concentration of 1:1,000. The membrane was washed 3 times in 0.1% TPBS for 5 min each, then, incubated for 1 h at room temperature with a horseradish peroxidase-linked secondary antibody (Sigma) (1:10,000; anti-rabbit IgG) diluted in 5% BSA/PBS containing 0.1% Tween-20. After 3 washes, the membrane was visualized using enhanced chemiluminescence reagents (Thermo Scientific, Waltham, MA, USA). The monoclonal anti-β-actin antibody (Sigma) was used at 1:2,000 as a loading control.

Statistical analysis. Data are reported as means ± SEM of triplicate experiments. Statistical analysis (analysis of variance) was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered to indicate a statistically significant result.

Results

Isolation of CD200⁺ colorectal cells from human colon cancer cell lines. We preliminarily screened 6 human colon cancer cell lines (COLO 205, LoVo, SW620, SW480, SW1116

and HT29) for CD200 expression by flow cytometry. The proportion of CD200⁺ cells in all 6 colorectal cancer cell lines was <3% (Fig. 1A). However, when we verified the cell purity, it was <90%, with yields ranging from 40-60%. Since our previous study showed that COLO 205 colonospheres contain cancer stem-like cells under serum-free culture conditions (18), we used COLO 205 cells in the present study in serum-free culture experiments (Fig. 1B). We examined the ratio of CD200⁺ cells by flow cytometry at different time points after transfer to serum-free medium. We found that the ratio of CD200⁺ COLO 205 cells was highest in colonospheres cultured for 2 weeks in serum-free medium, and its expression was high enough to accurately distinguish and sort CD200⁺ cells from C200⁻ cells. We sorted and collected 10% of the cells with strongest positive signal and 10% of the cells with the strongest negative signal by FACS. We verified the purity of the sorted CD200⁺ and CD200⁻ cells by flow cytometry, and it was always >95% for both cell fractions (Fig. 1C).

CD200⁺ COLO 205 cells have strong self-renewal and invasive abilities. We performed colonosphere formation assays using sorted CD200⁺ and CD200⁻ cells to determine the self-renewing capability of each fraction. We found that CD200⁺ cells more efficiently generated colonospheres upon serial passage compared to CD200⁻ cells (Fig. 2A). For primary sphere formation, 46.6±6.4% of CD200⁺ cells formed spheres compared to 9.3±2.1% of CD200⁻ cells. Moreover, the CFE of CD200⁺ cells increased upon passaging, while the CFE of CD200⁻ cells decreased (Fig. 2A). These results suggest that CD200⁺ COLO 205 cells have a stronger self-renewing capacity compared to CD200⁻ cells. In parallel, we performed a Transwell migration assay, which is an *in vitro* indicator of metastatic potential, to evaluate the effects of CD200 expression on migration ability of COLO 205 cells. As shown in Fig. 2B, after 48 h of incubation, the number of CD200⁺ cells that passed through the membrane was significantly higher than the number of CD200⁻ cells.

Microarray analysis of CD200⁺ COLO 205 cell gene expression compared to CD200⁻ cells. To identify potential genes associated with the distinct cellular behavior of the 2 populations, we generated gene expression profiles of CD200⁺ COLO 205 and CD200⁻ COLO 205 cells. The gene chip results showed that a total of 958 genes were upregulated or downregulated at least 2-fold in CD200⁺ COLO 205 cells; 433 genes were upregulated and 525 genes were downregulated (Fig. 3A). Gene Pathway analysis revealed that the differentially expressed genes were distributed in multiple pathways, including metabolic, cancer, immune response, apoptosis and several classical signaling pathways. Integrating data from Gene Ontology with the published data, we identified several genes that may be related to the tumor-initiating ability of CD200⁺ COLO 205 cells. Genes associated with tumor proliferation and invasion, such as GLI2, RAC3 and PRKCB were upregulated. These genes have been reported to be involved in proliferation and metastasis regulation in various cancers (19-21). CD200⁺ cells also showed increased PPARD expression, a PPARS family member that regulates lipid metabolism (22), proliferation (23) and the inflammatory response (24).
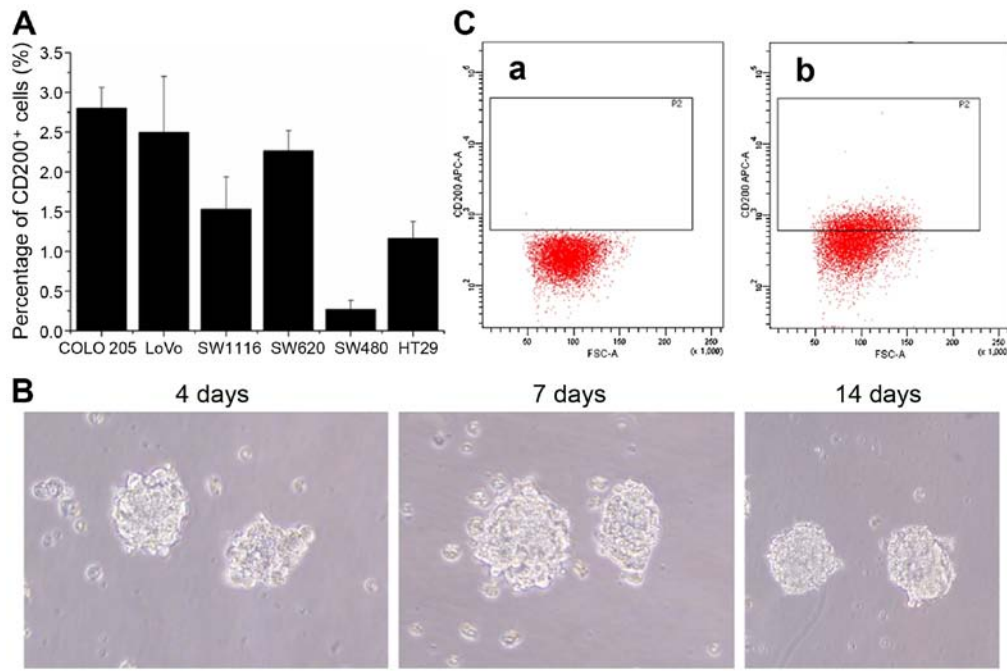
Figure 1. (A) Analysis of the proportion of CD200+ cells in 6 types of colorectal cancer cell lines by flow cytometry. CD200+ cells were all <3% in all of the 6 cell lines. (B) COLO 205 colorectal cells induced colonospheres in serum-free medium, and the colonospheres gradually tended to be regular round with serial passages. (C-a) The proportion of CD200+ fraction in COLO 205 colorectal cells in the culture medium containing 10% FBS. (C-b) The proportion of CD200+ cells in serum-free medium for 2 weeks.
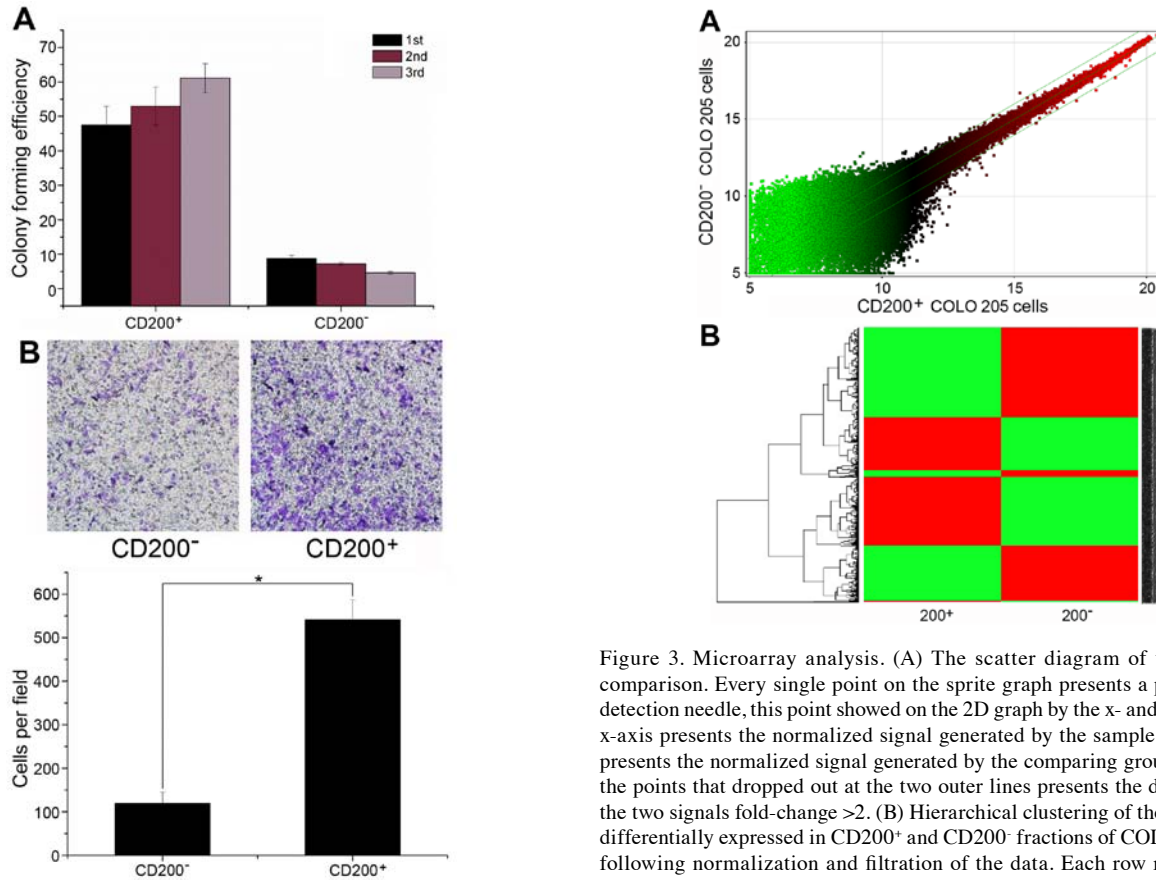

Figure 2. (A) Colonosphere forming efficiency (CFE). Colonosphere formation of single sorted CD200+ and CD200- cells in serum-free medium upon serial passage. (B) Transwell assay showing metastatic capacity of CD200- and CD200+ cells, which are representative images of migratory cells and quantification of at least 10 randomly chosen microscopic fields (*P<0.05).

Figure 3. Microarray analysis. (A) The scatter diagram of two-sample comparison. Every single point on the sprite graph presents a point on the detection needle, this point showed on the 2D graph by the x- and y-axes. The x-axis presents the normalized signal generated by the sample. The y-axis presents the normalized signal generated by the comparing group. Whereas the points that dropped out at the two outer lines presents the difference of the two signals fold-change >2. (B) Hierarchical clustering of the transcripts differentially expressed in CD200+ and CD200- fractions of COLO 205 cells following normalization and filtration of the data. Each row represents a single gene, while samples are in columns. Colors used in this image are commonly used by microarray users (green color) downregulated genes, red color (upregulated genes). For cluster analysis of the differently expressed genes, we used the following selection criteria: i) at least 2-fold difference between CD200+ and CD200-COLO 205 cells; ii) appearance of the signal in at least 3 slide microarray replicates from each treatment.
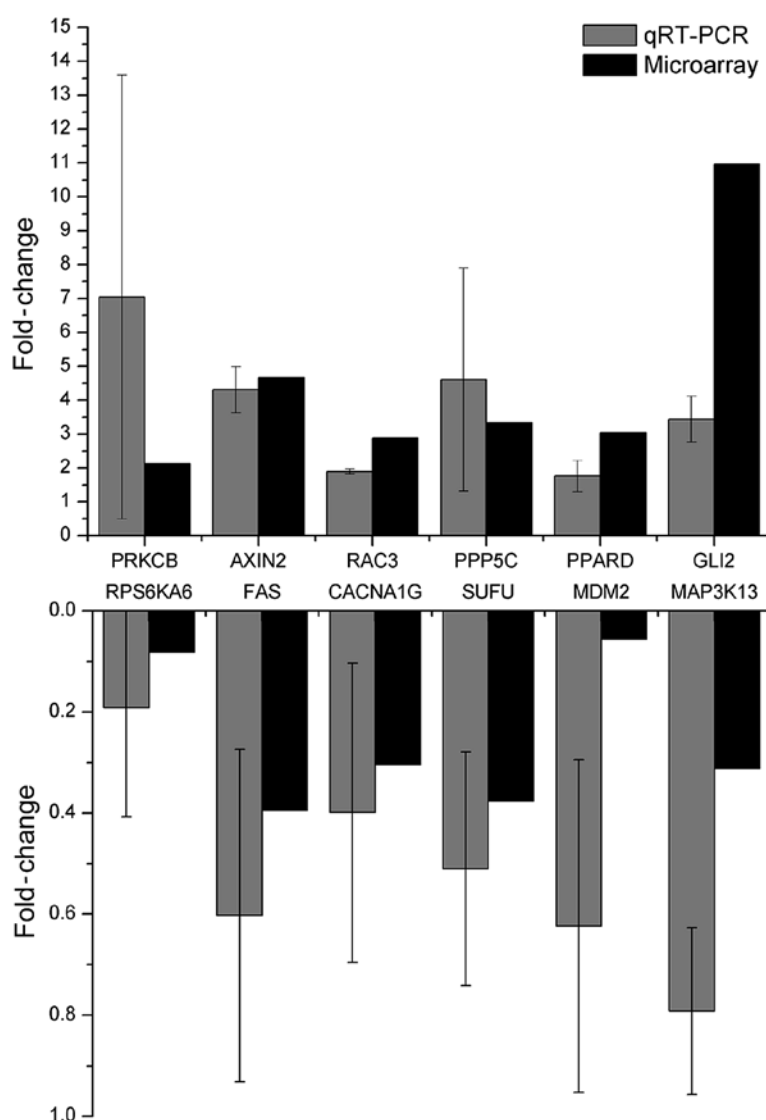
Figure 4. Real-time PCR validation of genes are differentially expressed between CD200⁺ and CD200⁻ fractions of colorectal cancer cells. A subset of candidate genes found to be overexpressed or underexpressed in COLO 205 CD200⁺ cells by microarray analysis were assessed by real-time PCR on CD200⁺ fractions isolated from COLO 205 cells and compared to CD200⁻ counterparts. Levels of the transcripts were normalized against GAPDH RNA. The qRT-PCR data are presented as mean ± SEM of 3 independent determinations.

In contrast, genes with tumor-suppressor activities, such as CACNA1G, RPS6KA6 and the pro-apoptotic gene FAS were downregulated in CD200⁺ cells compared to CD200⁻ cells. Among these, CACNA1G (Cav3.1) encodes a T-type, low-voltage activated calcium channel, which along with FAS, may contribute to the promotion of apoptosis and the repression of tumor proliferation (25,26). CACNA1G is considered a target of abnormal methylation and silencing in colorectal and lung cancer, and several other tumors (27). RPS6KA6 may be an important tumor-suppressor gene by modulating senescence induction and regulating cell proliferation (28).

We validated the expression of a subset of the genes upregulated (GLI2, RAC3, PRKCB, PPARD, PPP5C and FGF14) or downregulated (FAS, RPS6KA6, CACNA1G, SUFU, MDM2 and MAP3K13) in CD200⁺ COLO 205 cells by qRT-PCR. We selected candidate genes that represented diverse biological functions that could be involved in proliferation, invasion and stemness, and that reflects the extent of differential gene expression. Notably, qRT-PCR assays confirmed the same trends observed in the microarray analyses (Fig. 4).

CD44⁺CD133⁺ population is enriched in CD200⁺ cells and shares a similar gene expression profile. To investigate whether there was a correlation between the CD44⁺CD133⁺ population and CD200 expression, we sorted CD44⁺CD133⁺ and CD44⁻CD133⁻ COLO 205 cells by FACS. Flow cytometric analysis showed that CD200 expression was always higher in the CD44⁺CD133⁺ population compared to the CD44⁻CD133⁻ population (Fig. 5). To further explore the relationship between CD200 expression and CD44⁺CD133⁺ cells, we examined the expression of genes that were upregulated in the CD200⁺ cells in the CD44⁺CD133⁺ and CD44⁻CD133⁻ cells by qRT-PCR. The results showed that some of the genes upregulated in the CD200⁺ cells were also upregulated in the CD44⁺CD133⁺ fraction compared to the CD44⁻CD133⁻ fraction (Fig. 6).
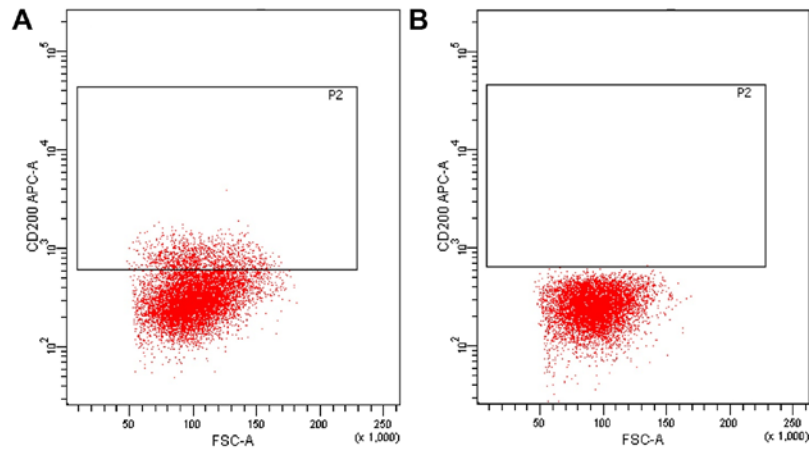
Figure 5. (A) Proportion of CD200⁺ fraction in the CD44⁺CD133⁺ COLO 205 cells. (B) The proportion of CD200⁺ cells in the CD44⁻CD133⁻ cells.

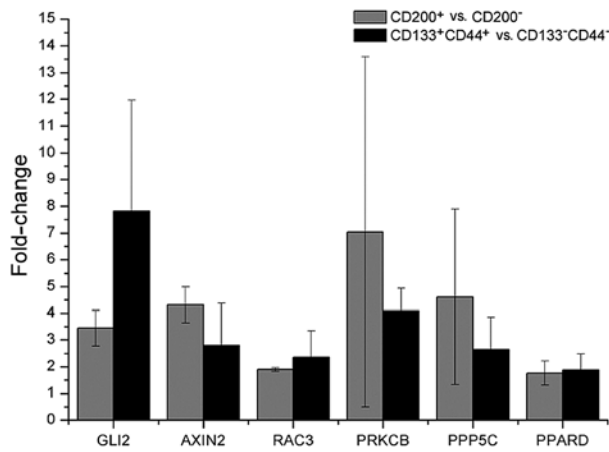

Figure 6. Comparative expression profile between CD200⁺ and CD133⁺CD44⁺ fractions in COLO 205 cells. A representative example of comparison, by quantitative RT-PCR analysis, for 6 genes that showed increased expression in CD200⁺ cells compared to CD200⁻ cells. Light and dark grey bars represent the mean fold-differential expression between CD200⁺ and CD133⁺CD44⁺ fractions compared to their respective counterparts. Data are presented as mean ± SEM of 3 independent determinations.
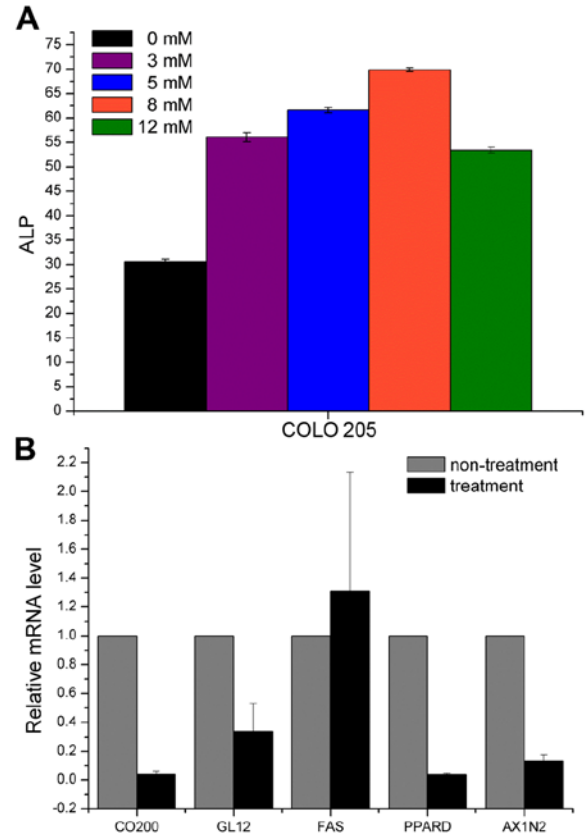

Figure 7. (A) NaBT-induced differentiation in colon cancer cell lines. COLO 205 was treated with various concentrations of sodium butyrate (NaBT). The optimum density of the NaBT was evaluated by the change of alkaline phosphatase (ALP) level with ELISA. The expression level of ALP was the highest in COLO 205 at the 8 mM NaBT concentration. (B) Comparative expression profile between untreated and NaBT-treated COLO 205 cells. A representative example of comparison, by quantitative RT-PCR analysis, for 5 genes that showed decreased expression in NaBT-treated COLO 205 cells relative to untreated cells. Dark and light grey bars represent the mean fold-differential expression between untreated and NaBT-treated fractions. Data presented are mean ± SEM of 3 independent determinations.

NaBT-induced differentiation alters CD200, GLI2, FAS, PPARD and AXIN2 expression. NaBT is known to induce colonic epithelial cell differentiation in colon cancer (29,30). Thus, we examined the expression of several stem cell markers (GLI2, FAS, PPARD and AXIN2) and CD200 before and after NaBT treatment of COLO 205 cells. We estimated the efficiency of NaBT treatment by ELISA for the well-known differentiated colonocyte marker alkaline phosphatase (ALP). ALP expression was highest after treatment with 8 mM NaBT (Fig. 7A). NaBT treatment significantly decreased CD200, GLI2, PPARD and AXIN2 expression. In contrast, FAS expression increased upon NaBT treatment (Fig. 7B). These findings confirm that CD200, GLI2, PPARD and AXIN2 are useful markers of undifferentiated or primitive colon cancer cells.

CD200 activates the Wnt/β-catenin signaling pathway. We identified 12 differentially expressed genes associated with the Wnt signaling pathway, by pathway analysis. Thus, we hypothesized that CD200 regulation of colorectal cancer occurrence and development may be closely associated with the Wnt signaling pathway. Western blot analyses confirmed that β-catenin, Wnt3a, Plrp6 and DVL2 were upregulated in
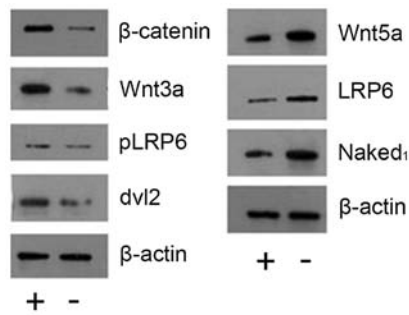
Figure 8. Total protein extracts were prepared from CD200+ and CD200- COLO 205 cells and analysed for the expression of β-catenin, Wnt3a, Plrp6, dvl2, Wnt5a, LRP6 and Naked₁ protein by western blot analysis. Actin expression in the same samples is shown as control for loading.

the CD200+ population, while Wnt5a, LRP6 and Naked1 were downregulated (Fig. 8). These results suggest that CD200 likely activates the Wnt/β-catenin signaling pathway.

Discussion

Colorectal cancer is one of the most common digestive carcinomas in China, and its morbidity rate has increased in recent years. The CCSC model has the potential to revolutionize the colorectal cancer field from both a pathogenetic and therapeutic point of view. However, the identity of CCSCs has proved to be a difficult task. CD200 upregulation in several human cancers and its tumor immune suppression properties suggest that it is a potential CSC marker. In our previous study, we found that CD200+ cells have stronger tumor formation abilities than CD200- cells *in vivo*, which preliminarily confirmed the reliability of CD200 as a CCSC marker. In the present study, we compared the CD200+ population to the CD200- population in *in vitro* colonosphere formation ability. We demonstrated that CD200+ COLO 205 cells showed stronger self-renewal ability. In addition, high-passage CD200+ colonosphere cells were highly invasive *in vitro*. We also screened CD200+ cell and CD200- cell gene expression profiles by gene chip, and identified a set of upregulated genes in the CD200+ fraction. We selected several genes associated with proliferation, invasion, stemness and immune processes for further study. We also examined the correlation between CD200 expression and CD44+CD133+ CSCs. By comparing the gene expression profiles of CD200+ and CD44+CD133+ COLO 205 cells, we found that many genes were upregulated in both populations, thus further supporting a role for CD200 molecule in the CSC signature.

Overall, the present study further confirmed the reliability of CD200 as a CCSC marker by *in vitro* experiments. We also showed a relationship between CD200 and FAS expressions, which may help in understanding the CD200 role in the colorectal cancers immune tolerance process. Indeed, Fas (CD95) belongs to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor superfamilies, and it is expressed on the cell surface. Upon binding to its receptor FasR, Fas triggers the death signal cascade and induces apoptosis of sensitized cells (31). Fas-mediated apoptosis is involved in functions such as tolerance acquisition and thymocyte clonal deletion, immune response termination, T cell-mediated

cytotoxicity and T cell activation-induced cell death. Loss of Fas function and expression of functional FasL by tumor cells may contribute to the evasion of host immune surveillance by triggering apoptosis of tumor-specific T lymphocytes (32,33). Decreased FAS and/or increased FASL expression promotes malignant transformation and progression of pancreatic, breast, colorectal and gastric cancer, and many other tumors (34-37). In summary, FAS is downregulated in CD200+ COLO 205 cells. Decreased Fas expression leads to tumor escape from immune surveillance. Thus, we hypothesize that FAS is associated with the immune inhibitory effect of CD200.

Another gene of interest is PPARD. This gene encodes a peroxisome proliferator-activated receptor (PPAR) family member. PPARs mediate several biological processes and are closely related to several chronic diseases, including obesity, diabetes, atherosclerosis and cancer (38-40). PPAR expression is increased in a wide variety of cancers, such as colorectal, lung, prostate and bladder cancer (41-44). Additionally, PPARD is involved in the inhibition of inflammation. PPARγ inhibits inflammatory gene expression activation and can negatively interfere with pro-inflammatory transcription factor signaling in inflammatory cells (39). PPARD agonists suppress monocyte elaboration of inflammatory cytokines at agonist concentrations (24). Moreover, PPARD plays a central role in stem cell maintenance in multiple systems (45). Therefore, PPARD may play an important role in carcinogenesis and immune process regulation. In the present study, we found that PPARD expression was much higher in CD200+ cells compared to CD200- cells, suggesting that PPARD functions in the CD200-regulated tumor immunosuppression process.

Notably, pathway analysis revealed that CD200 is associated with Wnt/β-catenin signaling pathway activation. Wnt signaling plays important roles in embryonic development and tumorigenesis. Aberrant signaling leads to a variety of developmental defects and some common human malignancies (46). Despite the well-known function of Wnt signaling in cell growth, apoptosis, differentiation and cell cycle, it also plays a role in immune regulation. It participates in immune cell differentiation and development and controls peripheral immune cell function (47,48). As previously suggested (49), constitutively activate Wnt/β-catenin signaling may contribute to anti-melanoma T cell response suppression in both the induction and effector phases by impairing DC and T cell function, partially through IL-10 and the generation of the immunosuppressive microenvironment. In the present study, we found that β-catenin, Wnt3a, pLRP6 and DVL2 were upregulated in CD200+ COLO 205 cells compared to CD200- cells, while Wnt5a, LRP6 and Naked1 were downregulated.

Wnt3a is a canonical Wnt signaling pathway activator, and treatment with Wnt3a results in an increase in β-catenin targets (50,51). Upon Wnt stimulation, LRP6 is phosphorylated by kinases at multiple sites. Phosphorylated LRP6 recruits axin to the membrane and presumably activates β-catenin signaling (52). DVL2 associates with actin filaments and cytoplasmic vesicular membranes (53) to mediate Fzd receptor endocytosis after Wnt stimulation (54). Dvl overexpression has been observed in certain cancers (55). High expression of β-catenin, Wnt3a, pLRP6 and DVL2 suggest that the Wnt/β-catenin signaling pathway is activated. Moreover, Mikels and Nusse (56) suggested that Wnt5a signals through both the

canonical and non-canonical Wnt pathways. Purified Wnt5a inhibits Wnt3a-induced canonical Wnt signaling by downregulating β-catenin-induced reporter gene expression. However, Wnt5a can also activate β-catenin signaling in the presence of Frizzled-4. As shown by Zhang *et al* (57), Wnt5a inhibits melanoma proliferation and melanogenesis via non-canonical Wnt/Ror2 pathway activation and inhibition of the canonical Wnt pathway. Naked1 is a negative Wnt signaling regulator, and it inhibits the canonical Wnt/β-catenin pathway by binding to dishevelled proteins and directs dishevelled activity towards the planar cell polarity pathway (58,59). Taken together, these data suggest that the Wnt/β-catenin signaling pathway is activated in CD200⁺ COLO 205 colorectal cancer cells. Wnt/β-catenin signaling is a target of CD200-mediated immunosuppression. However, our studies only examined expression of several common proteins related to Wnt signaling pathway. Future studies may focus on dual-luciferase assays to determine whether CD200 activates the Wnt/β-catenin signaling pathway.

In summary, *in vitro* experiments revealed that CD200⁺ COLO 205 cells have greater colony formation ability, higher invasion and CD200 expression is enriched in CD133⁺CD44⁺ cells. These findings further confirm CD200 as a CSC surface marker. By analyzing gene expression profiles, we examined genes of particular interest that are likely involved in CSC properties. Numerous genes identified in the present study, may also be good CCSC marker candidates, and their evaluation could increase specificity in CCSC identification. Although further studies are required to identify the functional roles of these genes in the CSC phenotype and their relationship with the CD200 immunosuppression mechanism, these findings hold promise for potential future approaches in elucidating the mechanism of colorectal cancer and developing new tumor targeting therapies.

Acknowledgements

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