Isolation of colorectal cancer stem-like cells

Eunice Dotse · Yuhong Bian

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Abstract This study is aimed at isolating colorectal cancer stem-like cells in vitro using a neurosphere assay method employed in isolating glioblastoma multiforme tumor cells. This was followed with confirmation of the isolated cells by flow cytometry, pluripotent genes expression and in vivo tumorigenicity assay. Using this culture assay, stem-like and non-stem-like CRC cells were isolated and expanded in vitro from purchased Balb/c mice induced with CT26 colorectal cancer (CRC) cell line. The procedure includes an initial mechanical dissociation and chemical digestion of tumor tissue and subsequently plating the resulting single cell suspension in serum-free medium (SFM) or serum-containing medium (SCM). This selectively permits growth of cancer stem-like cells in SFM and eliminates non-stem-like cancer cells through the process of anoikis or apoptosis. CRC stem cells derived cultures proliferated as non-adherent spheres in vitro in different shapes and sizes. These cells expressed cell surface markers previously reported for tumor stem cells, including CD44, CD133, CD166 and CD26 and formed tumors when implanted in severe combined

immunodeficient mice in a concentration dependent manner. Importantly, the stem-like cells had self-renewal properties with significantly higher expression of the pluripotent stem cell genes NANOG, OCT4, and SOX2 compared to the adherent non-stem cells. Collectively, the results of this study indicate that SFM is a defined culture medium that enriches for CRC stem-like cells and represents a suitable in vitro model for the study of CRC stem-like cells. This finding may be useful in developing therapeutic strategies aimed at eradicating the tumorigenic subpopulation within colorectal cancer.

Keywords Colorectal cancer · Cancer stem cells · Non-cancer stem cells

Introduction

Colorectal cancer (CRC), comprising both colon and rectal cancer, is the third most common cancer in men and the second in women worldwide (Globocan Statistics 2012). The majority of colon cancer arises sporadically, the incidence of which increases markedly after the age of 50 years; only about 25 % of the hereditary cases occur earlier than the sporadic colon cancer (Lynch and Smyrk 1996). Over the past years, evidence has emerged to suggest that cancers, including CRC, can be considered as stem cell disease, implication of which suggests that, cancers grow as

E. Dotse · Y. Bian (✉)
Tianjin University of Traditional Chinese Medicine, 312
Anshan West Road, Nankai District, Tianjin 300193,
People's Republic of China
e-mail: bianyuhong_2012@163.com

E. Dotse
Noguchi Memorial Institute for Medical Research,
University of Ghana, LG 581, Legon, Accra, Ghana

normal tissues of the body in a strictly organized system with cancer stem cells at the top of the tree, giving rise to all other cancer cells (Clarke et al. 2006; Blainpain et al. 2007). The cancer stem cell (CSC) theory posits that both primary and metastatic tumors develop from a small population of cancer cells possessing the characteristics of self-renewal and pluripotency and are responsible for initiation and maintenance of tumors (Anderson et al. 2011). CSCs are of clinical significance as it has been shown that they are more resistant to both chemotherapy and radiotherapy than other malignant cells (Elrick et al. 2005; Vlasi et al. 2009). Isolation and identification of CSCs are essential for a better understanding of their role in the tumorigenic process and for the development of CSC-specific therapies. CSCs are relatively scarce and lack a unique morphology that is easily distinguished from its progeny in vivo (Boman and Huang 2008). Of late, these cell populations from different solid tumors are isolated based on expression of specific cell surface markers. Colorectal cancer stem cells have been shown to express CD44, CD166, CD133 and CD 26 (Dalerba et al. 2007b; Snippert et al. 2009; Zhu et al. 2009; Pang et al. 2010; Chen et al. 2011; Sanders and Majumdar 2011) cell surface markers. CD44 is the major hyaluronan receptor and is important for the homing and settling of adult stem cells, metastasizing tumor cells and cancer initiating cells. Upregulated expression of CD44 increases tumor growth and has an anti-apoptotic effect (Keysar and Jimeno 2010). CD133, a five-transmembrane domain antigen is found on stem-like cells of various tissues and cancers like pancreatic, prostate, kidney and colorectal cancer (Mizrak et al. 2008). CD26 is a 110 kDa cell surface glycoprotein belonging to the serine protease family with intrinsic dipeptidyl peptidase IV (DPP-IV) activity expressed on a variety of cell types and plays a significant role in tumor pathogenesis and progression (Pang et al. 2010). CD166, a cell adhesion protein is pathologically correlated with aggressive forms of the disease. CD166-positive cells appear at multiple stages of intestinal carcinoma progression, including benign and metastatic tumors (Levin et al. 2010). Cells expressing these surface markers have the ability to form tumors at a much diluted concentration in Severe Combined Immuno-deficient (SCID) mice that resemble the primary tumor from which they were derived (O'Brien et al. 2007). This study is aimed at isolating colorectal cancer stem-

like cells in vitro using a neurosphere assay method initially used in isolating glioblastoma multiforme tumor cells (Azari et al. 2011) and subsequent confirmation of isolated cells by flow cytometry and pluripotent genes expression as well as in vivo tumorigenicity assay.

Method

Primary isolation stem-like and non-stem cells

Four to six weeks old female Balb/c mice (18–20 g) were obtained from Center of Laboratory Animal, Academy of Medical Sciences, Chinese People's Liberation Army and housed on a 12–12 h light–dark cycle at room temperature ($23 \pm 1^\circ\text{C}$). Tumor was induced in the mice with 1×10^5 CT26 colorectal cancer cells obtained from the Institute of Animal Research in Beijing (China). The cell line was cultured in RPMI (Invitrogen, Beijing, China), 10 % Foetal Bovine Serum (Biological Industries, Kibbutz Beit-Haemek, Israel), 1 % L-glutamine (Lonza, Shanghai, China) and 1 % Penicillin/Streptomycin (Hyclone, Beijing, China). The mice were allowed food and water ad libitum and kept in a controlled animal housing unit for one week. The mice were sacrificed by cervical dislocation according to the ethics regulation of Tianjin University of Traditional Chinese Medicine. The mice were then submerged in 70 % ethanol for 5 min to eliminate external microbes. They were then removed and placed on a sterile plate in a sterile hood. The mice were dissected using scissors and forceps and the tumor taken out and placed in a sterile 50 ml centrifuge tube. Washing was carried out three times with 10 % antibiotics (Penicillin/streptomycin (Hyclone)) in Phosphate Buffer Saline (0.1 M PBS, PH 7.4) and finally once with PBS without antibiotics. The tissues were then minced and digested in 10–15 ml of pre-warmed 0.05 % Trypsin–EDTA (T:E) (Gibco by Life Technologies, Grand Island, NY, USA) for 15 min with gentle shaking every 5 min in a 37°C incubator. An equal volume of soybean trypsin inhibitor solution was added to stop the enzymatic trypsin reaction after the incubation period. Soybean trypsin inhibitor solution was prepared by adding 0.14 g of soybean trypsin inhibitor (Gibco by Life Technologies) to 10 ml of 1 mg/ml DNase (Solarbio, Beijing, China) solution. The combined volume was made up to 1 l using HEM

[a mixture of 1×10^1 packet of MEM (Gibco by Life Technologies) and 160 ml of 1 M HEPES (Solarbio) in a total volume of 8.75 l using distilled water (final pH of 7.4)]. The suspension was pelleted down by centrifuging at 800 rpm (110 g) for 5 min. The supernatant was discarded and the tissue pieces were re-suspended in 15 ml of sterile cell medium (DMEM/Ham's F12 (Hyclone) and sieved through a 200 μ m mesh to discard clumps. The suspension was centrifuged at 800 rpm (110 g) for 5 min. The supernatant was discarded and washed once with PBS to obtain cell pellets. The pellets were re-suspended in 5 ml sterile cell medium for counting. A 15 μ l of the cell suspension was added to 15 μ l of 0.04 % Trypan blue in a 1 ml Eppendorf tube and a 20 μ l volume was counted using Nexcalom Bioscience (Lawrence, MA, USA) mini cellometer.

The obtained cells were split in two culture media conditions:

1. Serum Containing Medium (SCM). This is made up of DMEM/Ham's F12 (1:1) (Hyclone), 10 % Foetal Bovine Serum (FBS) (Biological Industries), 1 % penicillin/streptomycin (P/S) (100 U/ml) (Hyclone), and 1 % L-glutamine (LG) (Lonza)
2. Serum-Free Medium (SFM). This contains DMEM/Ham's F12 (1:1), epidermal growth factor (EGF, 20 ng/ml), basic fibroblast growth factor (bFGF; 10 ng/ml) both from PeproTech (Rocky Hill, NJ, USA), 2 μ g/ml of 0.2 % heparin (Solarbio) and 1 % P/S

The cells were seeded at a density of 2×10^5 /ml and 1×10^5 /ml for SFM and SCM cells, respectively, in a T75 or T25 cell culture flask (Corning Inc., Corning, NY, USA). The cells were incubated at 37 °C and 5 % CO₂ for 5–7 and 6–8 days for SFM and SCM, respectively. Individual culture flasks were photographed and scored for the presence of cell monolayers and colonospheres (floating spheres) morphologies.

Subculture of colorectal cancer non stem and stem-like cells

Non-stem cells

The adherent cells obtained from the SCM were harvested by trypsinization to obtain single cells and then expanded in SCM medium to obtain more cells.

These cells were scored as non-cancer stem cells (nCSC).

Colon cancer stem-like cells

Obtained colonospheres from SFM were scored as cancer stem-like cells (CSC). The colonospheres were collected, centrifuged, washed and trypsinized with 1 ml T: E for 60 s and inhibited with soybean trypsin inhibitor in a 1:3 ratio.

Characterization of obtained stem-like and non-stem-like cells

In vitro colonospheres regeneration assay

Primary cultures of colonospheres were harvested after 7 days in culture and collected after centrifugation, dissociated with T:E and re-plated in 24-well culture dishes in fresh medium at 5×10^4 /ml. The formation and morphology of spheres were evaluated after at least 1 week of culture by microscopic observation. Other expansions in SFM were carried out to obtain more cells for storage and further work.

In vitro differentiation assay

To examine the differentiation ability of the obtained cells from SFM, colonospheres were dissociated into single cells and differentiation was induced by transferring SFM cells into SCM.

Flow cytometry

Flow cytometry, was employed to characterize isolated colorectal cancer cells cultured in the two different media conditions and the expression of a panel of differentiating markers were visualized. The cells were trypsinized into single cell suspension. They were then washed twice with PBS and resuspended in PBS at a concentration of 1×10^7 cells/ml. Staining was performed by adding 20 μ l (6 μ g/ml) of titrated antibodies PE anti-mouse CD166 (e-Bioscience, San Diego, CA, USA), APC anti-mouse CD26 (Biolegend, San Diego, CA, USA), FITC anti-mouse/human CD44 (Biolegend) and PE anti-mouse CD133 (Biolegend) to 100 μ l of the cell suspension. After incubation for 30 min at room temperature in the dark, the unbound antibodies were washed off by adding

Table 1 RT-qPCR Primers and expected product sizes

Gene	Forward primer 5'	Reverse primer 3'
Gapdh	AACTTTGGCATTGTGGAAGG	CACATTGGGGGTAGGAACAC
Nanog	AAGCAGAAGATGCGGACTGT	GTGCTGAGCCCTTCTGAATC
Sox2	AAAGGGTTCTTGCTGGGTTT	AGACCACGAAAACGGTCTTG
Oct4	CAAGGCAAGGGAGGTAGACA	ATGAGTGACAGACAGGCCAG

400 µl of PBS and centrifuging at 400×g for 5 min. The cells were resuspended in 0.2 ml of PBS and analyzed using FACSCalibur Flow Cytometer and cell quest Pro software (BD Bioscience, Franklin Lakes, NJ, USA).

In vivo tumorigenicity

To test the ability of isolated cells to initiate tumor growth in vivo, eighteen 4–6 weeks old SCID mice (18–20 g) were obtained from Beijing Huafukang Biology Science Company. The mice were divided into 6 groups and kept under controlled conditions in Gongchengsuo animal facility (Tianjin, China) and allowed to acclimatize for 1 week. The CSCs from SFM as well as nCSCs from SCM, were collected by enzymatic dissociation into single cells, washed in PBS and kept on ice before being used for injection. Different concentrations (1×10^5 , 1×10^4 and 5×10^3 viable cells/ml) of nCSCs and CSCs per mice were injected subcutaneously into the right flanks of the mice and monitored for tumorigenicity. Tumor width and length were measured every day, starting the day after first appearance for 20 days. Tumor volumes in mm³ were calculated according to the formula $V = W^2 \times L$ where W is the width and L is the length of the tumor.

Stem cell gene expression of isolated cells

RNA extraction Total RNA of cells were extracted using Bio-tek Omega E.Z.N.A. total RNA kit (Norcross, GA, USA). Obtained cells were analyzed for expression of stem cell pluripotent genes SOX2, OCT4 and NANOG. Cells grown in both culture media were washed twice with PBS. The cells were then lysed with TRK lysis buffer in an RNase free microfuge tube. For each 1 ml of TRK Lysis buffer, 20 µl of 2-mercaptoethanol was added. The suspension was then centrifuged at 14,000×g for 5 min. The supernatant (350 µl) was then transferred

into a new 1.5 ml tube and an equal volume of 70 % ethanol was added and mixed thoroughly. The mixture was then added to Hi-Bind RNA mini column attached to a 2 ml collection tube and centrifuged at 10,000×g for 1 min at room temperature (RT). The flow-through was discarded and the column washed twice with 300 and 500 µl RNA wash buffer 1 followed with a successive wash with 500 µl RNA wash buffer 2. The column was then dried by spinning the cartridge attached to an empty collection tube at 10,000×g for 2 min. The RNA was eluted into a clean microfuge tube by adding 30–50 µl of DEPC-treated water. The purity and total RNA recovered was measured at 260 and 280 nm with a K5500 spectrophotometer, with the 260 nm reading used in estimating the concentration of total RNA.

Real time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized from 1 µg of total RNA using All-in-one first strand cDNA synthesis kit (Genecopoeia, Rockville, MD, USA) according to the manufacturer's protocol. Real time qPCR was carried out with IQ5 (BIO-RAD Laboratories, Beijing, China) using Syber green based All-in-one qPCR mix (Genecopoeia, USA) according to manufacturer's instruction. The synthetic oligonucleotides used as primers are listed in Table 1. GAPDH was used as the housekeeping gene. The RT-qPCR conditions and amplification efficiency for the genes were optimized at 95–98 % for a total of 40 cycles. Two step protocols were used, 95 °C for 10 min; 95 °C for 10 s, 58 °C for 20 s and 72 °C for 20 min. This was followed with a melting curve at 72 °C for 10 s. All DNA templates were analyzed in triplicates.

Statistical analysis

Data were expressed as mean ± standard deviation where applicable. Statistical analyses were performed

Fig. 1 **a, b** Morphology of isolated colon cancer cells cultured in SFM growing as non adherent spheres (colonosphere), **c, d** culture of isolated colon cancer cells in SCM growing as adherent cells

using SPSS Version 16.0 and differences in measurements were compared using Student's *t* test. A *p* value < 0.05 was considered statistically significant.

Results

Primary isolated colorectal cancer stem-like and non-stem cells

Results from the present study indicated that, SFM promoted formation of cancer stem-like cells in vitro comparable to cultured stem-like cells derived from human CRC. Cells cultured for 48–72 h in this condition exhibited a round morphology with only few cells showing an epithelial-like morphology after 96–120 h. All cells rounded up to form anchorage-independent spheroids (Fig. 1a, b). Few cultures

displayed small adherent colonies, readily detachable from tissue culture plastic after gentle tapping. These cells had self renewal abilities when sub cultured. Conversely, corresponding primary cultures immediately plated in SCM after the dissociation, proliferated as adherent monolayers showing a predominance of epithelial-like cells, spindle in shape, having the capacity to grow in culture after several passages (Fig. 1c, d).

Characterization of stem-like and non-stem cells

In vitro differentiation of obtained cancer stem-like cells

Transfer of cells from SFM to SCM resulted in differentiation characterized by epithelial-like morphology and adherence in about six days. However,

Fig. 2 **a** Primary colonosphere, **b** secondary colonosphere, **c** in vitro differentiation of floating colon spheres into adherent differentiated colon cancer cells

Fig. 3 Pictorial representation of singly labeled surface marker expression of isolated CSC and nCSC. *FITC* Fluorescein Isothiocyanate, *PE* phycoerythrin, *APC* allophycocyanin

there were still some cell aggregates in culture (Fig. 2).

Flow cytometric analysis of isolated cells

Comparatively, majority of cells in the SFM culture (93.63 %) were positive for CD44 while 39.99 % of cells cultured in SCM stained positive for the same marker. Furthermore, the isolated cells were also positive for other CRC stem cell markers but in low

percentages. Percentage expressions for CSCs were CD166 (8.39 %), CD133 (3.08 %), CD26 (3.09 %) while those of nCSCs were CD 166 (1.66 %), CD133 (0.49 %) and CD26 (1.63 %) (Figs. 3, 4a).

Pluripotent gene expression

The qPCR results of the present study indicated a higher expression of pluripotent genes of isolated CSCs compared to those of nCSCs. The mean gene

Fig. 4 **a** Graphical representation of single labelled cell surface marker expression of isolated CSC and nCSC, **b** graphical representation of SOX2, OCT-4 and NANOG gene expression of CSC and nCSC

Table 2 Days and number of mice with tumor appearance post injection

Concentration (Cells)	Number of mice and days of appearance	
	CSCs	nCSCs
1×10^5	9 (3/3)	10 (3/3)
1×10^4	11 (3/3)	16 (3/3)
5×10^3	14 (1/3)	10 (3/3)

expression was lowest for SOX2 and highest for NANOG with OCT4 in-between. Statistically, there was a significant difference in all genes under consideration with *p* values of 0.021, 0.024 and 0.016 for SOX2, OCT4 and NANOG, respectively (Fig. 4b).

In vivo tumorigenicity

All mice injected with 1×10^5 CSCs had visible tumors on day 9 but those injected with 1×10^5 nCSCs had visible tumors on day 11. Tumors were

Fig. 5 Different tumor sizes excised from SCID mice injected with different concentrations of isolated CSCs and nCSCs

Fig. 6 **a** Mean tumor volume of SCID mice injected with 1×10^5 concentration of isolated CSCs and nCSC, **b** mean tumor volume of SCID mice injected with 1×10^4 concentration of CSCs and nCSCs, **c** mean tumor volume of SCID mice injected with 5×10^3 concentration of CSCs and nCSCs, **d** tumor index obtained from SCID mice injected with different concentrations of CSCs and nCSCs

measured on day 11 and 12 for CSCs and nCSCs injected mice, respectively. As the cell concentration decreased, the time of tumor appearance also decreased in both cell types. As observed, 1×10^4 and 5×10^3 CSCs injected mice had a tumor appearance by day 11 and 14 respectively. A similar trend was seen in the nCSCs, however, with a more prolonged delay as compared to CSC cells. For the respective days that tumor appeared, all mice injected with 1×10^5 and 1×10^4 of CSCs and nCSC had visible tumors. But only 1 out of 3 mice injected with 5×10^3 had visible tumors for both cell types. However, at the end of the experiment, all mice injected with 5×10^3 CSCs showed tumor appearance while 2 out of 3 nCSCs injected mice developed tumor (Table 2). Tumor sizes as well as indices were photographed, measured (Fig. 5) and volumes calculated. Tumor volumes were higher for CSCs injected mice compared to those of nCSCs injected mice in a concentration dependent manner (Fig. 6).

Discussion

It has long been recognized that tumors are composed of a heterogeneous population of cells with various levels

of cellular differentiation and morphologic features. At the same time, most tumors are believed to be monoclonal in origin (Fialkow 1976; Vogelstein et al. 1985), supporting the notion that the originating tumor must be capable of giving rise to various cell types that make up the tumor. Portions of this model have recently been challenged by increasing evidence that tumor growth and progression are supported by a small population of tumor cells with stem-like properties, and the reinvigoration of the CSC theory. While most normal tissues are supported by a small population of slowly cycling and self-renewing stem cells, the CSC theory proposes the existence of a similar tumor cell hierarchy with a CSC residing at the apex (Dalerba et al. 2007a). Here we isolated and expanded murine colorectal cancer stem cells in vitro using a neurosphere assay previously employed in isolating glioblastoma multiforme cells. This assay proved to be a valuable method that could be employed to isolate and study the concept of colorectal CSCs. The present study established colorectal cancer stem and non-stem cell lines and investigated the stem cell properties in vitro and in vivo. This method which employed the use of serum free media supplemented with basic fibroblast growth factor, epidermal growth factor and heparin, selectively promoted growth of cancer stem cells and inhibited growth of non-stem cells

in vitro. These cells grew as non-adherent spherical floating cells and could maintain an undifferentiated state (self renewal properties), a major stem cell characteristic. However, upon withdrawal of growth factors from the media and introduction of 10 % FBS, there was loss of stem cell characteristics visualized as cells differentiated and had adherent properties as those of the primarily isolated non-cancer stem cells. Colon cancer stem or stem-like cells have been shown to express CD44, CD166, CD133 and CD 26 (Dalerba et al. 2007b; Snippert et al. 2009; Zhu et al. 2009; Pang et al. 2010; Chen et al. 2011; Sanders and Majumdar 2011) cell surface markers among others. The question of whether these cell surface markers have functional relevance to the CSC population or whether they act simply as surrogate markers for CSCs remains unclear. Many of these proteins, such as CD133, have unknown function. Others, such as CD44 (hyaluronic acid receptor) and CD26 (dipeptidyl peptidase IV), have known functions; however, their functional relevance to tumorigenesis is uncertain and it is quite likely that these proteins have additional, currently unknown roles which may be relevant to cancer initiation or progression (Anderson et al. 2011). A phenotype study was performed to examine and compare the expression of multiple cancer stem cell markers on the isolated cells. In our study, expression of CD44, CD133, CD 166, and CD 26 were significantly higher in cells cultured in SFM compared to cells cultured in SCM. However, a higher percentage of the cells from SFM expressed CD44 as compared with the other stem cell makers considered. Other markers were in low percentages; however, their expressions were higher in SFM cells than SCM cells. Our results are in line with the previous work by Du et al. (2008) which suggested that, CD44 is a more selective colon CSC marker than CD133, a work which showed that, as few as 1 of 100 CD44⁺ cells from human tumors were capable of initiating tumor formation in SCID mice. Our results support the view that CD44 is a more robust marker for colorectal CSC than the reported marker CD133 (O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Du et al. 2008). However, it is clearly not sufficient to define a stem cell based only on surface markers. Moreover, none of the markers used to isolate stem cells in various normal and cancerous tissues is expressed exclusively by the stem cell fraction. Indeed most markers used for colon CSC isolation are chosen either because they are expressed in normal stem cells or as they were found to identify CSCs in other

malignancies, either hematological or solid (Puglisi et al. 2013). Thus several of these markers, have problems with specificity, and while overlaying stem cell populations, they also mark other non-stem cells (Buczacki et al. 2011). Tumorigenicity is considered to be an indispensable prerequisite for fulfilling the definition of CSCs, which should also possess self-renewal and differentiation capacities. The xenograft transplantation assay showed that SFM cultured cells (CSCs) are highly tumorigenic. In all cell concentrations used, the CSCs induced tumor in the immunodeficient mice quicker as compared to the adherent nCSCs. For groups injected with the highest concentration of cell types, occurrence of tumor as well as volume was similar however mice injected with lower concentration showed a more distinctive result between the two cell types. There were as much as 15 and 22 days delay between injection day and tumor appearance in SCM cells as compared to the SFM cells which occurred by days 12 and 15, respectively. The possibility that some of the cells from the SCM medium formed tumors could be because the cells primarily isolated, were still young and though cultured in SCM, some may still retain their stem cell properties. This correlates with the flow cytometry results where the adherent cells expressed as much as 39.99 % of CD44⁺ cell surface marker. Many properties of cancer cells are reminiscent of those in normal stem cells. Uncontrolled self-renewal plays a direct function in the progression of different types of carcinomas. The same molecular pathway that manages self-renewal in normal stem cells also seems to manage cancer stem cells (Amini et al. 2014). Genes important to stem cell development have been significantly implicated in the etiology and clinical outcome of colorectal cancer (Yang et al. 2012). The isolated cells were further examined at the molecular level. SOX2, OCT4 and NANOG genes were considered. Compared with SCM obtained cells, our results indicated that the SFM derived cells expressed higher stem cell genes with NANOG having the highest expression followed by OCT4 and then SOX2. The differences in the expression of these pluripotent genes were highly significant. Expression of OCT4 and SOX2 are associated with clinical outcome in various human malignancies including lung (Sholl et al. 2010), esophageal (Bass et al. 2009), ovarian (Zhang et al. 2010a), cervical (Ji and Zheng 2010) and gastric (Zhang et al. 2010b) carcinomas. Expression of NANOG has been identified as a component of an embryonic stem (ES) cell signature in

various human carcinomas (Santagata et al. 2007; Zbinden et al. 2010). NANOG interacts with the Hedgehog pathway (Zbinden et al. 2010) and epithelial–mesenchymal transition (Meng et al. 2010) where NANOG may play a role in maintaining pluripotency that is necessary for generating tumor heterogeneity. Thus, NANOG expression may be a critical co-factor for the neoplastic progression, the highest gene expression observed in the isolated CSCs in our study.

Conclusion

This work reports the isolation and phenotypic characterization of colorectal cancer murine-derived stem/progenitor cells using the neurosphere assay, showing in vitro properties such as self-renewal, proliferation and in vivo tumorigenicity. By culturing obtained cells in a serum free stem cell permissive medium, highly enriched CD44-positive murine colorectal cancer stem-like cells were isolated. These cells had the ability to survive and proliferate as floating colonospheres in vitro with different shapes and sizes. These cells expressed genes associated with colorectal cancer stem cells and somatic stem cells and had the ability to induce tumor in vivo when injected into SCID mice. The stem-like cells exhibited differentiation potential when cultured in a serum containing medium and showed changes in morphologies and reduced tumorigenic potential. This is a simple and suitable method that could be employed in isolating colorectal cancer stem cells for studies into its biology and testing of therapeutic strategies that is aimed at eradicating the stem cell populations.

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Conflict of interest The authors declare no conflict of interest.

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