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MicroRNA expression profile of colon cancer stem-like cells in HT29 adenocarcinoma cell line

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ABSTRACT

Increasing evidence has suggested cancer stem cells (CSCs) are considered to be responsible for cancer formation, recurrence, and metastasis. Recently, many studies have also revealed that microRNAs (miRNAs) strongly implicate in regulating self renewal and tumorigenicity of CSCs in human cancers. However, with respect to colon cancer, the role of miRNAs in stemness maintenance and tumorigenicity of CSCs still remains to be unknown. In the present study, we isolated a population of colon CSCs expressing a CD133 surface phenotype from human HT29 colonic adenocarcinoma cell line by Flow Cytometry Cell Sorting. The $CD133^+$ cells possess a greater tumor sphere-forming efficiency in vitro and higher tumorigenic potential in vivo. Furthermore, the $CD133^+$ cells are endowed with stem/progenitor cells-like property including expression of "stemness" genes involved in Wnt2, BMI1, Oct3/4, Notch1, C-myc and other genes as well as self-renewal and differentiation capacity. Moreover, we investigated the miRNA expression profile of colon CSCs using miRNA array. Consequently, we identified a colon CSCs miRNA signature comprising 11 overexpressed and 8 underexpressed miRNAs, such as miR-429, miR-155, and miR-320d, some of which may be involved in regulation of stem cell differentiation. Our results suggest that miRNAs might play important roles in stemness maintenance of colon CSCs, and analysis of specific miRNA expression signatures may contribute to potential cancer therapy.

1. Introduction

Colon carcinoma is the third leading cause of deaths from cancer [1], which represents a major therapeutic challenge. Recently, the cancer stem cell hypothesis has been proposed to explain the functional heterogeneity and the carcinogenesis process of cancer. According to this model, a subpopulation of cancer cells, which exhibit stem-like features, sustains tumor formation, metastasis, and resistance to therapy [2–4]. Several studies have been carried out to investigate the protein-coding genes and its products that participate in the stemness maintenance and tumorigenicity of CSCs [5–7]. However, the underlying mechanisms remain largely unknown.

miRNAs are a class of endogenous, small non-protein coding RNA molecules, which are crucial for post-transcriptional regulators of gene expression. Previous studies have shown that miRNAs participate in several biological functions, including cellular proliferation, differentiation and apoptosis [8]. miRNAs are also known to contribute to preserving stemness of embryonic stem cells and human CSCs [9–13]. Especially in breast cancer, Let-7 was considered to be responsible for breast CSCs properties including self renewal and multipotent differentiation potential [12]. However, the role of miRNAs in human colon CSCs is still unknown, and a miRNA signature has never been evaluated. Therefore, we isolated and identified a population of colon CSCs expressing a CD133 surface phenotype from human HT29 colonic adenocarcinoma cell line. Further, we performed miRNA array analyses to investigate miRNA expression signature characteristic of colon CSCs between $CD133^+$ and $CD133^-$ cells. Our results suggest that miRNAs might play important roles in maintaining stemness of colon CSCs. Investigation of miRNA expression profiles in colon CSCs will be crucial for elucidating characters of colon CSCs, which will benefit to develop drugs or novel therapeutic methods to target CSCs.

2. Materials and methods

2.1. Cell culture and colon sphere formation

Human colonic adenocarcinoma cell line HT-29 was obtain from ATCC and was maintained in McCoy's 5A medium (GIBCO) containing 10% Fetal Bovine Serum (FBS, GIBCO) at 37 °C and 5% CO_2, and the medium was changed every three days. Cells were passaged at 80% confluence and seeded at 20% confluence to keep at optimal proliferating conditions.

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For colon sphere formation, single-cell suspensions were cultured in a DMEM/F-12 basal serum-free medium (GIBCO), containing 2 mM L-glutamine, 1 mg/ml $NaHCO_3$, 4 μg/ml heparin, 100 μg/ml transferrin, 25 μg/ml insulin, 30 nM sodium selenite anhydrous, 20 nM progesterone (Sigma) and supplemented with 20 ng/ml EGF (R&D) and 10 ng/ml FGF-2 (R&D). Cultures of differentiated cells were obtained from colon spheres after growth factors removal and addition of 10% FBS.

2.2. Flow cytometry cell sorting and flow cytometry analysis

For isolation of $CD133^+$ and $CD133^-$ populations, single-cell suspensions were incubated with phycoerythrin (PE)-conjugated anti-human CD133/1 (AC133 clone; Miltenyi) and FcR blocking reagent (Miltenyi) in staining solution containing 1% BSA and 2 mM EDTA for 10 min at 4 °C. Isotype-matched mouse immunoglobulin served as control. Samples were analyzed and sorted with a FACS Aria (Becton Dickinson). For the positive and negative population, only the top 10% most brightly stained cells or the bottom 10% most dimly stained cells were selected, respectively. After cytofluorimetric sorting, viability was assessed using trypan blue exclusion, cell purity was controlled by flow cytometry with an antibody against CD133/2 (293C3-PE, Miltenyi). Colon spheres and differentiated cells were analyzed for CD133 expression using CD133/2 (293C3-PE, Miltenyi) or isotype-matched control.

2.3. Real-time PCR

Total RNA was extracted from the isolated $CD133^+$ and $CD133^-$ cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized by using ImProm-II reverse transcriptase (takara) using random hexamers. To determine fold changes in each gene, real-time qPCR was performed using the first strand cDNA, 0.5 μM forward and reverse primers, and the SYBR Green PCR master mix (takara). The primer sequences and PCR conditions are summarized in Supplementary Table S1. Reaction and signal detection were measured by the Mini Opticon real-time PCR system (BioRad). Expression levels were calculated as the relative expression ratio compared to β-actin. The qPCRs were performed three times in triplicate independently.

2.4. In vivo tumorigenicity

Cells were resuspended in 50 μL of phosphate-buffered saline after sorting, and cell aliquots were diluted 1:1 with growth factor reduced matrigel matrix (BD Biosciences) before injection. Six week-old BALB/c nude female mice were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Science (SLAC-CAS) and maintained under standard conditions according to the guidelines of the Medical Institute of Shanghai Jiao Tong University. Mice were subcutaneously injected with isolated cells and killed by cervical dislocation 8 weeks after transplantation. The grafts were removed, fixed with 10% buffered formalin, and stained with hematoxylin and eosin.

2.5. miRNA microarray and statistical analysis

Human miRNA microarrays (Agilent Technologies, Santa Clara, CA) were used to compare the expression profiles of $CD133^+$ and $CD133^-$ cells. The microarray contains probes for 866 human miRNAs from the Sanger database v.13.0 (http://microrna.sanger.ac.uk/sequences). $CD133^+$ and $CD133^-$ cells (5×10^6) were obtained from HT29 cell line by FACS, and total RNA was isolated using mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quality control, labeling, and hybridization were performed at Shanghai Biochip Company according to the protocols in the Agilent miRNA microarray system. Arrays were scanned using an Agilent Microarray Scanner (Agilent, Santa Clara, CA, USA); the microarray image information was converted into spot intensity values using Scanner Control Software Rev. 7.0 (Agilent Technologies, Santa Clara, CA). Average values of the replicate spots of each miRNA were background subtracted, normalized, and further analyzed. Normalization was done using quantiles (log2 scale) before statistical analysis. A paired *t*-test was used to identify differentially expressed miRNAs between $CD133^+$ and $CD133^-$ comparisons. miRNAs were considered statistically significant if their p value was less than 0.05.

3. Result

3.1. Isolated $CD133^+$ cells exhibit stem-like features in vitro

Previous studies had proved that human prominin-1 (CD133) was a powerful marker to detect colon CSCs in both primary tumor and cancer cell lines [14–17]. In this study, Human HT29 colonic adenocarcinoma cell line was employed as a model, which expressed CD133 in 42% of the cells (Fig. 1A), we then purified $CD133^+$ and $CD133^-$ cells from HT29 cell line by FACS (Fig. 1B). The purity of the $CD133^+$ populations was generally greater than 95% as revealed by postsorting analysis (Fig. 1C). To study the molecular features of colon CSCs, the expression of genes involved in stem cell related pathways was investigated by real-time PCR in $CD133^+$ and $CD133^-$ populations. As shown in Fig. 1D, significant increased expression level of "stemness" genes, like CD133, Sox2, Oct4, Nanog, Notch1, C-myc, Bmi1, Wnt2, and transporter and motility genes, like ABCG2, and CXCR4, was consistently observed in $CD133^+$ cells compared with the $CD133^-$ counterpart ($p < 0.05$).

It is reported that $CD133^+$ cells isolated from primary colon tumors were able to expand as sphere-like cellular aggregates in serum-free stem cell medium [16,17]. Similarly, we found that $CD133^+$ cells sorted from the HT29 cell line were also able to generate bigger and greater numbers of tumor spheres than $CD133^-$ cells ($p < 0.01$) (Fig. 1E and F). As expected, sorted $CD133^+$ HT29 cells were cultivated in serum-free stem cell medium for sustaining undifferentiated state, after 10–14 days of culture, floating sphere-like cellular aggregates were formed as observed by the inverted phase contrast microscope (Fig. 1G). Moreover, colon cancer spheres displays a bigger volume and more compact sphere structure following the third cell-culture passage (data not show), suggesting they retained self-renewal capacity and progression potential. In order to evaluate the differentiation potential, colon spheres were cultivated with growth factors removal and addition of 10% FBS, and then monitored for the expression of CD133 as well as their morphology. After one day of culture, floating spheroid attached to the plastic, gradually migrating from colon spheres and changed into adherent cells (data not show). Following differentiation, adherent cells acquired a cobblestone-like morphology closely resembling their parental HT29 cells (Fig. 1H). Comparative flow cytometry analysis of colon cancer spheres and differentiated adherent cells demonstrated that CD133 expression was significantly decreased from 80.2% to 62.4% during differentiation by day 5 (Fig. 1I). Our results demonstrated that the $CD133^+$ HT29 cells are endowed with stem/progenitor cells-like property including expression of "stemness" genes as well as self-renewal and differentiation capacity.

3.2. $CD133^+$ cells possess higher tumorigenic potential in vivo

To determine whether $CD133^+$ colon cells are more tumorigenic than their $CD133^-$ counterparts in vivo, we carried out tumor

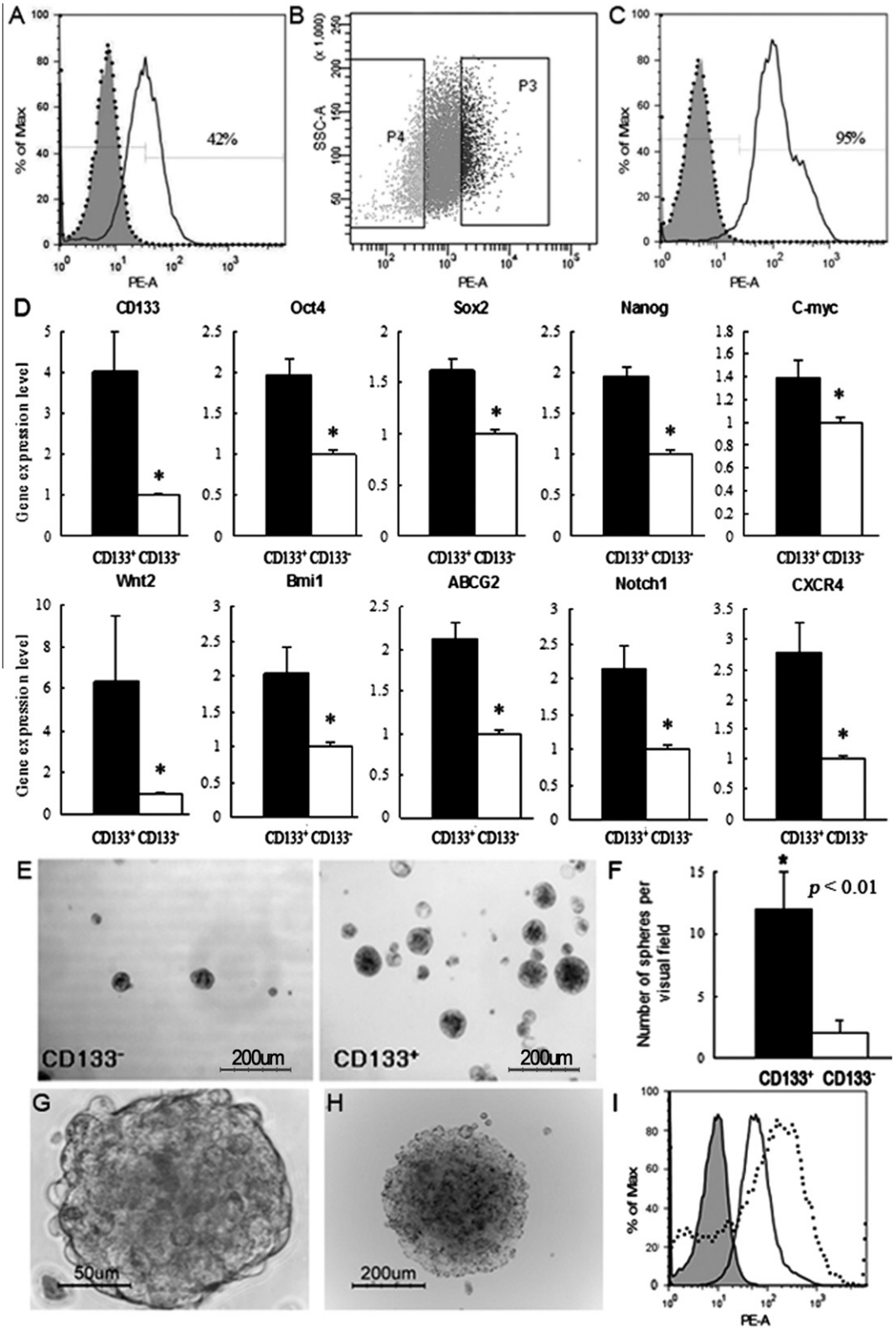

Fig. 1. Isolation and characterization of colon CSCs in HT29 cell line. (A) Flow cytometry histogram showing expression of $CD133^+$ cells (42%) in the HT29 cell line. (*Dotted line*, isotype control; *solid line*, CD133 expression of HT29 cells). (B) Methodology of flow cytometry cell sorting in HT29 cell line. *P4*, $CD133^-$ sorting area; *P3*, $CD133^+$ sorting area. (C) Example of analysis for purity of $CD133^+$ cells following cell sorting. (*Dotted line*, isotype control; *solid line*, sorted $CD133^+$ cells). (D) "Stemness" genes expression of sorted $CD133^+$ and $CD133^-$ cells assessed by real-time PCR.$^*p < 0.05$. (E, F) Colon sphere-forming capacity of different phenotypic subpopulations, the $CD133^+$ cells produced bigger and greater numbers of tumor spheres ($p < 0.01$). (G) $CD133^+$ colon cancer cells expanded in vitro as undifferentiated spheres in serum-free medium containing EGF and FGF-2. Picture show a typical colon sphere observed by the inverted phase contrast microscope. (H) Microscopically analysis of colon cancer spheres cultivated in differentiation conditions (growth factors removal and addition of 10% FBS) for 5 days. (I) CD133 expression was significantly decreased from 80.2% to 62.4% during differentiation by flow cytometry analysis. (*Solid curve*, negative control; *Continuous line*, CD133 expression of differentiated cells; *dotted line*, CD133 expression of colon cancer spheres).

development experiments. Nude mice were subcutaneously injected with either 5×10^3 or 5×10^4 sorted CD133$^+$ cells or CD133$^-$ cells from HT29 cell line. The CD133$^+$ cells showed significantly higher tumorigencity than CD133$^-$ cells (Fig. 2A, Table 1). When 5×10^3 and 5×10^4 CD133$^+$ cells were inoculated into nude mice, all mice (6/6) developed tumors. In contrast, only one mouse inoculations of 5×10^4 CD133$^-$ cells grew tumors (5×10^3 group could not developed tumors). Transplanted tumors were confirmed as colon cancers with hematoxylin-eosin staining (Fig. 2B).

3.3. miRNAs expression profile in colon CSCs

To investigate the miRNA expression profile of human colon CSCs, we performed miRNA microarray analyses between the isolated CD133$^+$ colon CSCs and CD133$^-$ control cells. To pursue the precise results, we performed 3 times independent microarray analyses using fresh isolated cells, respectively. All data were aggregate analyzed using a paired *t*-test statistical method ($p < 0.05$). Consequently, the microarray identified 19 differentially expressed miRNAs comprising 11 miRNAs that were up-regulated in CD133$^+$ colon CSCs and 8 miRNAs that were down-regulated compared with CD133$^-$ control cells (Fig. 3, Supplementary Table S2). Among these miRNAs, notably, miR-429, and miR-155, the significant changed miRNAs observed in our study, have already been reported to be involved in regulating cancer stem cell differentiation and the epithelial–mesenchymal transition (EMT) processes [18,19]; while some others, for example, miR-185, miR-320, miR-494, miR-221, and miR-31, which are known to contribute to carcinogenesis [20–24].

4. Discussion

There has been accumulating evidence supporting the fact that CSCs may be the root source of human cancers, including colon cancer [2–4]. Recently, CD133 is regarded as a specific marker for the isolation and identification of the CSCs in both primary colon cancer and colon cancer cell lines [14–17]. Considering limitations of high heterogeneity and variability of primary cancer cells isolated from patients, the human cancer cell lines can provide a renewable and stable genetic tumor material to study the fundamental CSCs features. Furthermore, almost all colon cancers are primary adenocarcinomas, which accounts for 95% of cases [1], therefore, the human colonic adenocarcinoma cell line HT29 was chosen as a model in this study.

We purified a population of colon CSCs expressing a CD133 surface phenotype by FACS. The CD133$^+$ cells displayed a greater tumor sphere-forming efficiency in vitro and higher tumorigenic potential in vivo. These results are consistent with other reports of characteristics of CD133$^+$ cells [14]. Furthermore, CD133$^+$ cells were able to expand as colon cancer spheres in serum-free medium and differentiate in presence of 10% FBS, suggesting they retained self-renewal capacity and differentiation potential [17]. Importantly, CD133$^+$ cells preferentially express the markers which important for stemness-keeping and fate-determining properties of progenitor cells including CD133, Sox2, Oct4, Nanog, Notch1, C-myc, Bmi1, Wnt2, and transporter and motility genes, like ABCG2 and CXCR4. Many of these molecular markers have been previously reported to be involved in stemness maintenance of human CSCs [25–28]. Our results demonstrated that the CD133$^+$ HT29 cells are endowed with stem/progenitor cells-like property.

miRNAs are a class of endogenous, non-protein coding RNA molecules, which are able to regulate expression of hundreds of target mRNAs simultaneously. Several studies have revealed the significance of miRNAs in stem cell self-renewal and differentiation [29–31]. More recently, miRNAs are also known to contribute to preserving stemness of human CSCs. One of the most studied miRNAs, Let-7, was markedly reduced in breast CSCs and increased

Table 1
In vivo tumor development experiments of CD133$^+$ and CD133$^-$ cells sorted from HT-29 cell lines in nude mice.

Cell type	Cell numbers injected	Tumor incidence
CD133$^+$	50000	3/3
	5000	3/3
CD133$^-$	50000	1/3
	5000	0/3

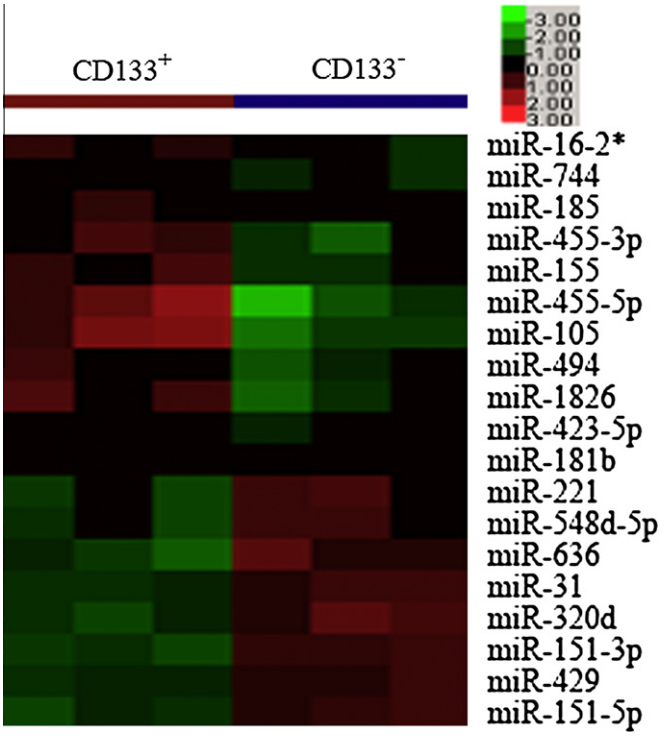

Fig. 3. miRNAs differentially expressed in CD133$^+$ colon CSCs versus CD133$^-$ comparison. Hierarchical clustering of the 19 miRNA genes with a significantly different expression ($p < 0.05$) in colon CSCs. Rows represent individual genes; columns represent three separate samples. Pseudo-colours indicate transcript levels below, equal to, or above the mean (green, black, and red, respectively). The scale represents the intensity of gene expression (log2 scale ranges between −3 and 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

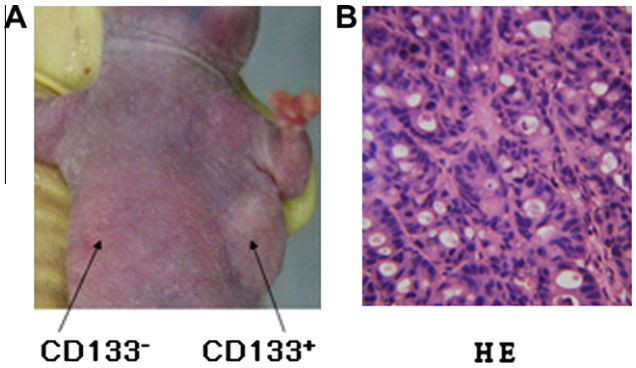

Fig. 2. Tumorigenecity of CD133$^+$ cells in vivo. (A) The photograph of a representative mouse was taken at 8 weeks after cell transplantation. (B) The grafts of the CD133$^+$ cells were excised at 8 weeks after transplantation and then were stained with H.E. (original magnification 100×).

with cell differentiation [12]. Furthermore, Yohei Shimono found that miR-200c provide a molecular link that connects normal stem cells with breast CSCs [13]. Similarly, Hilah Gal et al. examined the microRNA profiles of Glioblastoma stem cell ($CD133^+$) and non-stem cell ($CD133^-$) populations and found miR-451 may be involved in regulation of brain differentiation [9]. These findings reveal that miRNAs have crucial regulatory functions in various CSCs. However, the role of miRNAs in human colon CSCs is still unknown, and a miRNA signature has never been evaluated. Therefore, in this study, we performed microRNA array to examine the miRNA expression profile of colon CSCs and hopefully find potential players in stemness maintenance and tumorigenic ability. Consequently, the microarray identified 19 differentially expressed miRNAs in $CD133^+$ colon CSCs compared with $CD133^-$ control cells. Some of these significant changed miRNAs, such as miR-429 and miR-155, have been found to be involved in EMT processes [18,19]. Intriguingly, Morel et al. reported that $CD44^+CD24^-$ breast CSCs can originate from $CD44^-CD24^+$ human mammary epithelial cells after aberrant activation of the Ras/MAPK pathway by EMT induction [32]. Thus, our results suggested that such specific miRNAs in colon CSCs may serve as a bridge between cancer stem cell and EMT processes, and we were also embarking on further study of this (date not show). While some others, for example, miR-494, miR-320 and miR-185, have been known to contribute to carcinogenesis. miR-494 acts as a microoncogene by regulating the expression of PTEN in malignant transformed 16HBE cells [20]; moreover, miR-320 has been proved to be correlated with the probability of recurrence-free survival in stage II colon cancer patients [21]; recently, Zhang et al. found miR-185 was significant increased when normal intestinal cell line 6 (IEC-6) cells were transformed by treatment with cancerogenic agent [22]. These findings demonstrate that miRNAs play an essential role in colon cancer pathogenesis. Interestingly, two inflammation related miRNAs, miR-155 and miR-105, were up-regulated in colon CSCs. Recently, Shuai Jiang reported that miR-155 exerts its oncogenic role by negatively regulating a tumor suppressor gene suppressor of cytokine signaling 1 (socs1) in human breast cancer [33]. Moreover, miR-105 can induced reduction of cytokine production (interleukin-6 and TNF-α) by modulation of TLR2 protein expression in human oral keratinocytes, thus play a crucial role in modulating immune function [34]. The incidence suggested that up regulation of theses miRNAs in colon CSCs may serve as a bridge between inflammation and cancer.

In summary, we revealed a global miRNA expression signature in colon CSCs, suggesting that miRNAs might play important roles in maintaining stemness of colon CSCs. The identification of a miRNA profiles will exciting new insight into the molecular mechanism for elucidating characters of colon CSCs, it may represent a novel tool which contributes to potential cancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.106.

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