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Maintenance of the stemness in CD44$^+$ HCT-15 and HCT-116 human colon cancer cells requires miR-203 suppression☆

Sy-Yeuan Ju [a], Shih-Hwa Chiou [b,c,*], Yeu Su [a,**]

[a] *Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taiwan*
[b] *Institute of Pharmacology, National Yang-Ming University, Taiwan*
[c] *Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan*

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Abstract The purpose of this study was to isolate cancer stem cells (CSCs, also called tumor-initiating cells, TICs) from established human colorectal carcinoma (CRC) cell lines, characterize them extensively and dissect the mechanism for their stemness. Freshly isolated CD44$^+$ and CD44$^-$ cells from the HCT-15 human colon cancer cell line were subjected to various analyses. Interestingly, CD44$^+$ cells exhibited higher soft agar colony-forming ability and in vivo tumorigenicity than CD44$^-$ cells. In addition, the significant upregulation of the protein Snail and the downregulation of miR-203, a stemness inhibitor, in CD44$^+$ cells suggested that this population possessed higher invasion/metastasis and differentiation potential than CD44$^-$ cells. By manipulating the expression of CD44 in HCT-15 and HCT-116 cells, we found that the levels of several EMT activators and miR-203 were positively and negatively correlated with those of CD44, respectively. Further analyses revealed that miR-203 levels were repressed by Snail, which was shown to bind to specific E-box(es) present in the miR-203 promoter. In agreement, silencing miR-203 expression in wild-type HCT-116 human colon cancer cells also resulted in an increase of their stemness. Finally, we discovered that c-Src kinase activity was required for the downregulation of miR-203 in HCT-15 cells, which was stimulated by the interaction between hyaluronan (HA) and CD44.
Taken together, CD44 is a critical molecule for modulating stemness in CSCs. More importantly, we show for the first time that the downregulation of miR-203 by HA/CD44 signaling is the main reason for stemness-maintenance in colon cancer cells.
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Abbreviations: CRC, colorectal carcinoma; CSCs, cancer stem cells; HA, hyaluronan; EMT, epithelial mesenchymal transition; miRNAs, microRNAs; FCS, fetal calf serum; FACS, fluorescence activated cell sorting; ChIP, chromatin immunoprecipitation; shRNA, short hairpin RNA; PBS, phosphate buffered saline.

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* Correspondence to: S-H. Chiou, Department of Medical Research and Education, Taipei Veterans General Hospital, Institute of Oral Biology, National Yang-Ming University, No. 155, Sec. 2, Linong Street, Taipei 11221, Taiwan, ROC. Fax: +886 2 28745074.
** Correspondence to: Y. Su, Institute of Biopharmaceutical Sciences, National Yang-Ming University, No. 155, Sec. 2, Linong Street, Taipei 11221, Taiwan, ROC. Fax: +886 2 28250883.
E-mail addresses: shchiou@vghtpe.gov.tw (S.-H. Chiou), yeusu@ym.edu.tw (Y. Su).

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Introduction

Stem cells have critical roles not only in the maintenance of organ homeostasis but also in the development of tumors (Jordan et al., 2006). Recent findings confirm the idea that cells with stem cell properties are truly responsible for tumorigenesis (Dean et al., 2005). These stem cell-like cancer cells (also termed cancer stem cells, CSCs) have been defined as a subset of tumor cells with the ability to self-renew and give rise to phenotypically diverse tumor cell populations to drive tumorigenesis. Not surprisingly, CSCs have been identified in a wide variety of cancers thus far (Clevers, 2011).

Although, colorectal CSCs (Co-CSCs) were originally identified as a small group of $CD133^{+}$ cells present in the primary tumors which exhibited high tumorigenicity in immunodeficient mice (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), some studies have shown that $CD44^{+}$ colorectal cancer cells also possess stem-like properties (Dalerba et al., 2007). Interestingly, accumulating evidence demonstrates that CD44 is not only a marker for Co-CSCs but also of functional importance in cancer initiation (Chu et al., 2009; Du et al., 2008). In addition, a recent study has also shown that direct repression of CD44 could inhibit tumor formation in prostate cancers (Liu et al., 2011). These results clearly indicate that CD44 plays a critical role in determining the tumorigenic capacity of certain types of cancers. However, the underlying mechanism of cancer initiation mediated by CD44 in Co-CSCs is poorly understood.

Snail is well-known for its roles in embryonic development and cancer progression (Barrallo-Gimeno and Nieto, 2005). The transcriptional regulation by this factor often involves the recognition and binding of this factor to the consensus core sequence (-CANNTG-) located in the promoter region of its target genes (Cano et al., 2000). It has been reported that normal mammary epithelial cells could be induced to adopt the $CD44^{high}/CD24^{low}$ expression profile that is associated with certain attributes of stem cells when the transcription factors Snail or Twist were conditionally overexpressed in these cells (Mani et al., 2008). Elevated Snail expression not only enhanced drug resistance by antagonizing p53-mediated apoptosis but also enhanced the acquisition of stemness in ovarian cancer cells (Kurrey et al., 2009). In addition, Snail promotes the expression of IL-8 and other genes to induce stem-like activities in colorectal cancer cells (Hwang et al., 2011). Collectively, the above findings strongly suggest that Snail, Twist or ZEB1 may be sufficient to induce a population with the characteristics of cancer stem cells.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression in a post-transcriptional manner (He and Hannon, 2004). In addition to playing roles in regulating cell proliferation, differentiation, apoptosis and immunity, other functions of miRNAs are currently being extensively examined. Among these studies, the association of miRNAs with the formation, angiogenesis, metastasis and chemoresistance of tumors has become one of the core issues in the epigenetic study of cancer. In fact, many reports have shown the important roles of miRNAs in the development of various cancers as well as in the self-renewal and differentiation of embryonic and tissue-specific stem cells (Calin and Croce, 2006). Interestingly, it has recently been reported that the expression of certain miRNAs involved in cancer stemness is modulated by EMT (epithelial–mesenchymal transition) activators such as ZEB1 and Twist via the regulation of their promoter activities (Bourguignon et al., 2010; Wellner et al., 2009). These findings suggest a different mechanism by which these factors influence CSCs. Additionally, ample evidence indicates the involvement of various miRNAs in different signaling pathways leading to colorectal cancer (Liu and Chen, 2010). For example, miR-451 has been shown to negatively regulate the self-renewal capability and tumorigenicity of Co-CSCs by suppressing COX-2-mediated Wnt activation (Bitarte et al., 2011). In addition, miR-21 has been shown to play an important role in regulating the stemness of colon cancer cells by modulating TGFβR2 signaling (Yu et al., 2012). Although miR-203 was originally characterized as a skin-specific microRNA that promotes the differentiation of epidermal cells by repressing stemness (Yi et al., 2008), it was reported recently that it is not only functioning as a tumor-suppressor in hepatocellular carcinoma (Furuta et al., 2010) but also inhibiting the proliferation, migration and invasiveness in prostate cancer cells (Viticchie et al., 2011). Moreover, restoration of miR-203 expression reverses the chemoresistance in p53-mutated colon cancer cells (Li et al., 2011). It appeared that miR-203 downregulates the proliferation of epithelial precursor cells and self-renewal of stem cells by directly targeting p63 and Bmi1, respectively (Lena et al., 2008; Wellner et al., 2009). Nevertheless, the role of miR-203 in tumor initiation in Co-CSC has not yet been delineated.

In this study, we first demonstrated that the $CD44^{+}$ population isolated from the HCT-15 human colon cancer cell line not only exhibited multiple in vitro features of CSCs but also showed higher in vivo tumorigenicity. In accordance, knocking down CD44 expression in HCT-15 cells resulted in reduced soft agar colony-forming ability and the decreased expression of Bmi1, Snail and Twist1, whereas the increased expression of Snail, Twist1 as well as several ESC markers was found in CD44-overexpressing cells. We further showed that CD44 plays crucial roles in cancer initiation and stemness-maintenance in HCT-15 and HCT-116 cells which are modulated by the Snail-mediated suppression of a stemness inhibitor, miR-203. Finally, we further demonstrated that c-Src kinase, which is activated by hyaluronan (HA)/CD44 signaling, is responsible for Snail upregulation in these cells. Collectively, our results show for the first time that CD44 not only can be a single marker used for the isolation of CSCs from human colon cancer lines but also acts as a signal transducer leading to the suppression of miR-203. This suppression is crucial for the maintenance of stemness in these Co-CSCs.

Materials and methods

Cell culture

Human colon carcinoma cell lines (HCT-15, HCT-116 and HT-29) were purchased from the American Type Culture Collection (ATCC). While three parental cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (Biological Industries, USA), 100 units/ml

penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (Biological Industries, USA) at 37 °C in 5% CO_2, several stable transfectants derived from HCT-15, HCT-116 and HT-29 cells were maintained under similar conditions except that 5 μg/ml of puromycin was added to the media. To assess the effects of the hyaluronan (HA, Sigma, USA)—CD44 interaction, cells were cultured on HA (100 μg/ml)-coated or -noncoated dishes for 24 h, followed by treating without or with 10 μM PP2 or 2 μM Dasatinib (Sigma, USA) for another 24 h before being harvested.

Magnetic cell separation and fluorescence-activated cell sorting (FACS)

Dissociated HCT-15 or HCT-116 cells were suspended in regular medium and 1×10^7 cells were then labeled by microbead-conjugated CD44 antibody. Cells were then magnetically separated using a CD44 Cell Isolation Kit (Miltenyi Biotec, Germany). A positive selection column was used to collect the CD44-positive ($CD44^+$) cells. In the meantime, fluorescence-activated cell sorting was used to identify and select $CD44^+$ HCT-15 and HT-29 cells after they were incubated with a FITC-conjugated CD44 antibody. The isolated $CD44^+$ and CD44-negative ($CD44^-$) cells were used for the following experiments.

Xenograft

Cells (1×10^5, 1×10^4 or 1×10^3) were resuspended in 50 μl PBS (phosphate buffered saline) and mixed with an equal volume of Matrigel (BD) before being injected subcutaneously into 8-week-old nude mice (BALB/c strain, obtained from the National Laboratory Animal Center). Tumor size was measured every week with calipers, and the tumor volume was calculated using the formula (length × width^2)/2.

Chromatin immunoprecipitation

ChIP assays were performed following the protocol provided by Upstate Biotechnology to investigate whether Snail binds in situ to the E-boxes present in the miR-203 promoter and whether such binding is affected by the CD44 signaling triggered by HA treatment. Parental or $CD44^+$ HCT-15 cells were cultured on dishes coated with or without HA in the presence or absence of PP2 for 24 h. Cells were then fixed with 1% formaldehyde for 15 min, followed by a 5-min treatment with 1.25 M glycine to quench the reaction. Nuclear extracts were prepared as described previously (Schreiber et al., 1989) and were then sonicated to shear DNA into 500-bp fragments. Precleared lysates were subjected to overnight immunoprecipitation with 2 μg/ml of rabbit anti-Snail antibody or normal rabbit IgG (negative control). DNA samples were recovered after phenol/chloroform extraction and isopropanol precipitation. A volume equal to that of the final precipitate was used for PCR amplification with primers (sense: 5′-TGGCTCCAGACTTGGGGCAA-3′ and antisense: 5′-GGTAGCCCCGTACCTGCCTC-3′) that gave rise to a miR-203 promoter region amplicon containing a putative E-box.

Additional methods

Detailed methods for the soft agar colony forming assay, transwell migration assays, reporter assay, real-time RT-PCR and Western blotting analysis can be found in the Supplementary data section.

Results

$CD44^+$ HCT-15 cells display stem cell properties both in vitro and in vivo

Because CD44 has been shown to be a robust marker for isolating colorectal CSCs (Co-CSCs) from patient tumors (Du et al., 2008), we first examined whether $CD44^+$ cells isolated from the HCT-15 human colon cancer line have more prominent stem cell properties. Both $CD44^+$ and $CD44^-$ subgroups were present in the parental HCT-15 cells, and the former subset was estimated to be 10% of the total population according to our flow cytometric analysis (Supplementary Fig. 1A). Magnetic separation was then applied to purify the $CD44^+$ and $CD44^-$ populations and the RNA and protein levels of CD44 were analyzed by qRT-PCR and Western blotting, respectively. As expected, CD44 RNA and protein levels were significantly higher in $CD44^+$ cells than in $CD44^-$ cells (Supplementary Fig. 1B and 1C). Because anchorage-independent growth and the migration ability of cancer cells have been shown to correlate well with their in vivo capacity for tumorigenesis and me tastasis, respectively (Shin et al., 1975), isolated $CD44^+$ and $CD44^-$ cells were subjected to soft agar colony-forming and transwell migration assays. Our results demonstrated that $CD44^+$ cells exhibited higher clonogenicity in soft agar and higher migration ability than their $CD44^-$ counterparts (Figs. 1A and B). However, a similar proliferative ability of $CD44^+$ and $CD44^-$ cells was observed according to their respective growth curve (Supplementary Fig. 1D).

To investigate whether $CD44^+$ HCT-15 cells were stem-like cells, qRT-PCR was performed to analyze several typical markers for embryonic stem cells (ESCs) including Oct4, Sox2, Nanog, Klf4 and Lin28. The data showed that the expression of the ESC markers was significantly increased in $CD44^+$ cells as was the intestinal stem cell marker, Lgr5 (Fig. 1C). On the other hand, we found that $CD44^-$ HCT-15 cells expressed higher RNA levels of markers for differentiated colonic epithelial cells (e.g., CK20 and CDX1) than $CD44^+$ cells. However, the respective expression levels of the markers for endocrine cells (CHGA) and goblet cells (mucin) did not differ between the two populations (Fig. 1D). To demonstrate that $CD44^+$ HCT-15 cells could differentiate into mature cancer cells, freshly isolated $CD44^+$ cells were maintained in a complete medium for 2 weeks. Treatment with fetal calf serum (FCS) resulted in marked increases of the mRNA levels for both CK20 and CDX1 in a time-dependant manner (Figs. 1E and F). Since a recent study has shown that the introduction of Snail or Twist stimulates cultured mammary cancer cells to adopt CSC characteristics (Mani et al., 2008), therefore besides Oct4 and Sox2, the levels of Snail and Slug in $CD44^+$ and $CD44^-$ HCT-15 cells were also analyzed by Western blotting. As seen

in Fig. 1G, marked increases of two pluripotency factors and two EMT activators were found in $CD44^+$ cells which were accompanied by a dramatic decrease in E-cadherin levels. However, no apparent morphological differences could be detected in the two subpopulations derived from HCT-15 cells (data not shown), suggesting that these alterations did not lead to EMT.

To further assess whether $CD44^+$ HCT-15 cells had the most critical feature of CSCs, high tumorigenicity in the immunodeficient animals, we subcutaneously injected equal numbers of $CD44^+$ and $CD44^-$ cells (mixed with Matrigel) into nude mice. Animals injected with 1×10^4 and 1×10^3 $CD44^+$ cells (Figs. 1H and I) grew visible tumors respectively at 2 and 4–5 weeks after transplantation, but only two mice receiving 1×10^4 $CD44^-$ cells grew visible tumor after 6 weeks. A much higher incidence of tumor growth was also observed in $CD44^+$ cells (Table 1). By contrast, $CD133^+$ HCT-15 cells did not show any advantage over $CD133^-$ cells or the parental cells with regard to tumor formation in nude mice (Supplementary Fig. 2A and B). Based on these data, we concluded that a small group of $CD44^+$ (but not $CD133^+$) HCT-15 cells that form tumors with high efficiency after xenograft represents the cancer stem cell population.

CD44 is required to maintain stem-like properties

Recent studies have indicated that CD44 is not only a marker for Co-CSCs but also a critical factor for cancer initiation (Chu et al., 2009; Du et al., 2008). Hence, several stable CD44-knockdowned clones were established by expressing CD44 shRNA via lentiviral infection of HCT-15 cells. Real-time RT-PCR assay was performed to confirm the downregulation of CD44 in the transfectants. CD44 levels in clones 2 and 4 were decreased to 60% and 50%, respectively, relative to sh-GFP, a negative control clone (Supplementary Fig. 3A). We next performed a soft agar colony-forming assay to examine whether CD44 levels affect the anchorage-independent growth of these cells. As expected, the numbers of colonies generated from clones 2 and 4 were dramatically decreased in comparison to the control group (Fig. 2A). In the meantime, we also noticed that few colonies in the soft agar were grown from both $CD44^-$ cells and CD44-knockdowned clones and we wondered whether CD44 levels in these colonies were altered. After flow cytometric analysis, significant increases of CD44 levels were detected in cells collected respectively from the aforementioned colonies (Supplementary Fig. 4). These results strongly suggested that colon cancer cells, regardless of their origins, exhibited higher anchorage-independent growth abilities if they expressed higher levels of CD44.

Because Nanog, Bmi1, Snail and Twist1 have been shown to be involved in self-renewal, tumor progression, and metastasis, we asked whether their levels were affected by CD44 downregulation. To no surprise, marked decreases in the protein levels of these four factors were found in CD44-knockdowned clones (Fig. 2B).

In the meantime, stable CD44-overexpressing clones (S1 and S2) were also established from HCT-15 cells via retroviral infection. Quantitative RT-PCR and Western blotting confirmed that CD44 levels were much higher in the CD44-overexpressing compared to the cells infected with the viruses carrying the vector alone (Supplementary Fig. 3B and C). Contrary to the clones, the numbers of colonies generated from S1 and S2 were significantly higher than the control group (Fig. 2C). Additionally, immunoblotting demonstrated that the nuclear levels of Snail and Twist1 were significantly increased in CD44-overexpressing transfectants when compared with the vector control cells (Fig. 2D). In accordance with the above results, qRT-PCR results showed that the expression levels of several pluripotency factors of ES cells including Oct4, Sox2, Nanog and Klf4 were significantly increased in S1 and S2 cells (Fig. 2E). Collectively, these data suggested that CD44 plays important roles in maintaining some of the CSC properties of HCT-15 cells possibly by upregulating several ESC markers.

CD44 and miR-203 expression levels are inversely correlated in HCT-15 and HCT-116 cells

MicroRNAs (miRNAs), a class of highly conserved small RNA molecules that regulate gene expression, have been reported as modulators of pathogenesis in various types of cancers (Vazquez-Ortiz et al., 2006). It is well documented that miR-200 family members are crucial modulators of EMT, metastasis and stemness (Wellner et al., 2009). We therefore examined whether miR-200 family members were differentially expressed in $CD44^+$ versus $CD44^-$ HCT-15 cells. Our data showed that the expression of miR-183, miR-200b and miR-203 was significantly decreased in $CD44^+$ cells (Fig. 3A). Subsequently, a time-dependent increase in the expression of miR-203 was observed after FCS treatment in $CD44^+$ HCT-15 cells (Fig. 3B). In addition, only miR-203 expression levels (not miR-183, miR-200a and miR-200b) were consistent in two different CD44-knockdowned clones (Supplementary Fig. 5A–C). We therefore chose miR-203 as a candidate for the following studies. To first elucidate the relationship between CD44 and miR-203 expression, qRT-PCR was performed to analyze miR-203 levels in CD44-knockdowned and -overexpressing clones. Our results showed that the expression of miR-203, a stemness inhibitor, was increased significantly in CD44-knockdowned clones generated from HCT-15 as well as HCT-116 cells (Fig. 3C). Conversely, miR-203 expression was markedly decreased in CD44-overexpressing clones derived from both lines (Fig. 3D). Together, these results suggested that the stemness of HCT-15 and possibly HCT-116 cells might be modulated by miR-203, whose expression in two CRC lines seemed to be negatively regulated by CD44.

Involvement of Snail in the HA/CD44 signaling-mediated downregulation of miR-203

To further assess whether the downregulation of miR-203 in $CD44^+$ HCT-15 cells is directly affected by CD44 signaling, hyaluronan (HA), a natural ligand for CD44 as well as a major component of the extracellular matrix, was used to trigger the signaling of this receptor. Parental HCT-15 cells were cultured on dishes coated without or with HA (100 μg/ml) for 24 hours (h), followed by treatment with or without 10 μM PP2 (an inhibitor of the c-Src family kinases) or 2 μM

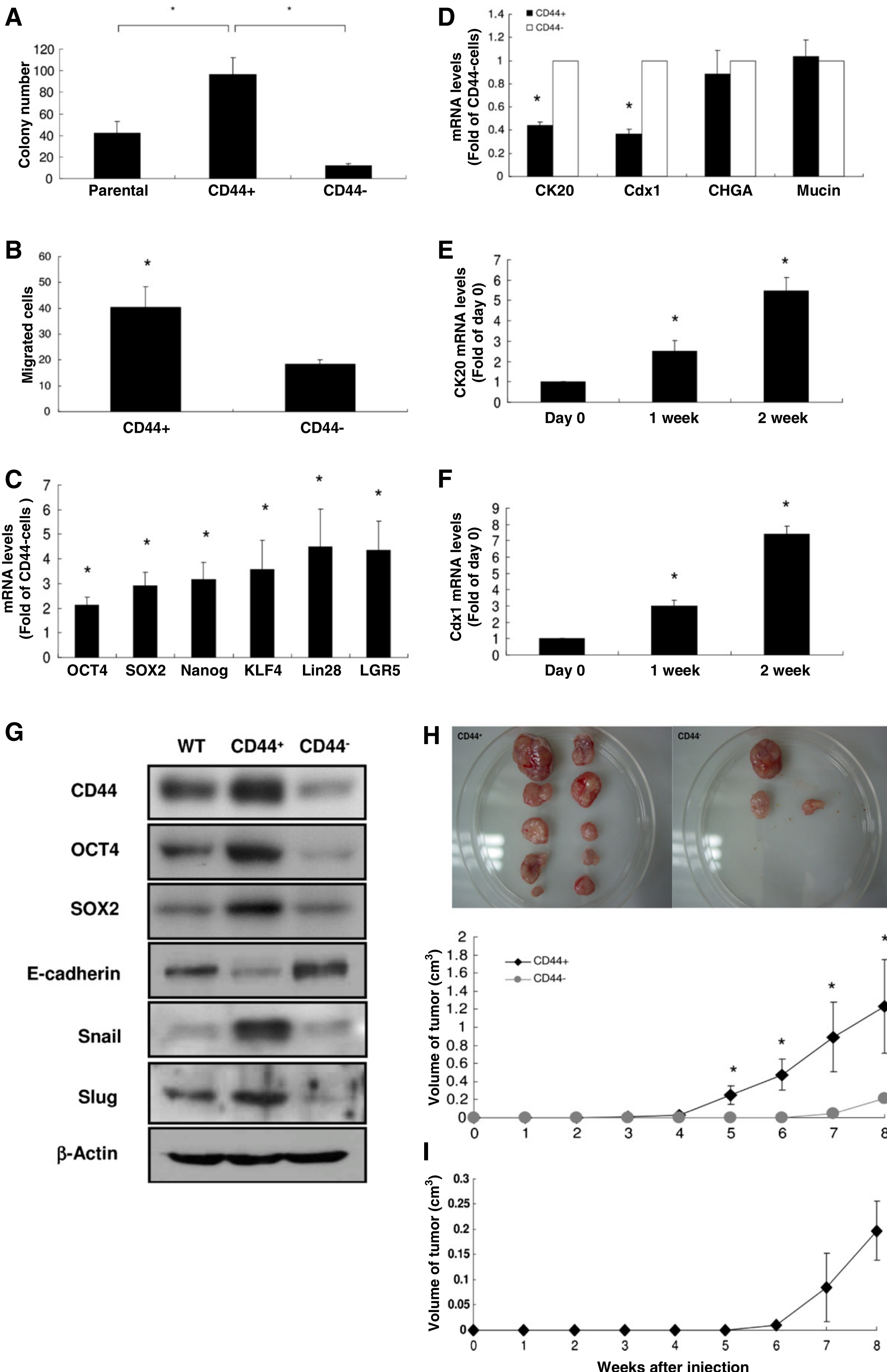

A
Colony number
Parental
CD44+
CD44-
B
Migrated cells
CD44+
CD44-
C
mRNA levels
(Fold of CD44-cells)
OCT4
SOX2
Nanog
KLF4
Lin28
LGR5
D
mRNA levels
(Fold of CD44-cells)
CD44+
CD44-
CK20
Cdx1
CHGA
Mucin
E
CK20 mRNA levels
(Fold of day 0)
Day 0
1 week
2 week
F
Cdx1 mRNA levels
(Fold of day 0)
Day 0
1 week
2 week
G
WT
CD44+
CD44-
CD44
OCT4
SOX2
E-cadherin
Snail
Slug
β-Actin
H
CD44+
CD44-
Volume of tumor (cm3)
CD44+
CD44-
I
Volume of tumor (cm3)
Weeks after injection

Table 1 Incidence of tumor growth.

Subpopulation	Cell numbers injected		
	10^5	10^4	10^3
CD44⁺	10/10	10/10	4/5
CD44⁻	4/10	3/10	ND

Note: Higher incidence of tumor growth was observed in CD44⁺ cells. ND means "not determined".

Dasatinib (a specific inhibitor for c-Src kinase), for another 24 h. While the miR-203 levels in HCT-15 cells were significantly decreased by incubating the cells with HA, this reduction could be fully restored by the addition of PP2 or Dasatinib (Fig. 4A), suggesting that c-Src is the mediator of the HA/CD44 signaling that downregulates miR-203 expression in these cells. In addition, HA treatment could also stimulate the expression of several EMT activators (Fig. 4B) as well as the nuclear accumulation of Snail protein (Fig. 4C). Because Snail has been shown to repress miR-203 expression (and vice versa) in MCF-7 human breast cancer cells (Moes et al., 2012), we examined whether Snail had a similar effect in HCT-15 cells. Indeed, a drastic downregulation of miR-203 was found in a Snail-overexpressing stable clone generated from HCT-15 cells (Fig. 4D). The abovementioned observations strongly suggest that miR-203 downregulation, triggered by the HA/CD44 interaction in HCT-15 cells, is likely through a c-Src-stimulated Snail expression and/or its nuclear accumulation.

Direct binding of Snail to the E-box elements of the miR-203 promoter in HCT-15 cells

Recent studies have indicated that an EMT-activator ZEB1, promotes the stemness-maintenance of cancer stem cells by suppressing stemness-inhibitory microRNAs by downregulating the activities of their promoters (Wellner et al., 2009). Several predicted E-box elements are located upstream of the transcription start site of miR-203 (Fig. 5A), a stemness inhibitor. To determine whether Snail directly regulates the expression of miR-203, two luciferase reporter constructs driven by either a wild-type or an E-box-mutated miR-203 promoter were transfected with or without a Snail-overexpressing vector into 293T cells. Our reporter assays showed that Snail suppressed the activity of the wild-type miR-203 promoter more efficiently than that of the E-box mutant promoter (Fig. 5B), further supporting the theory that the E-box is crucial for Snail-mediated repression of miR-203 promoter activity. To examine whether Snail interacts in situ with the E-box elements in the promoter region of miR-203, chromatin immunoprecipitation (ChIP) assays were performed in the CD44⁺ subpopulation of HCT-15 cells using an anti-Snail antibody. Our results showed that PCR amplification using the anti-Snail-mediated precipitates from CD44⁺ cells as the template and a pair of oligonucleotide primers specific for the miR-203 promoter region containing an E-box gave rise to a specific amplicon with an expression level that was much higher than those derived from CD44⁻ cells (Fig. 5C). The above results were subsequently verified by qPCR analysis (Fig. 5D). Next, we asked whether the interaction between Snail and the E-box element on miR-203 promoter was affected by HA/CD44 signaling. CD44⁺ HCT-15 cells were cultured on dishes coated with or without HA for 24 h, followed by the treatment with or without PP2 for another 24 h. ChIP results clearly showed that the binding of Snail to the E-box element in the miR-203 promoter was markedly increased when cells were grown on HA-coated dishes. Interestingly, this interaction appeared to be significantly reduced by the addition of PP2 (Fig. 5E), reiterating the fact that c-Src kinase activity is crucial for HA/CD44-modulated miR-203 expression. More significant differences between treatments could be observed when qPCR was applied (Fig. 5F). These results verify that the HA/CD44 interaction enhances the direct binding of Snail to the promoter region of miR-203. Collectively, our data strongly suggest that Snail negatively regulates miR-203 expression by binding directly to the E-box of its promoter following HA/CD44 interaction.

MiR-203 functions as a stemness-inhibitor in HCT-15, HT-29 and HCT-116 cells

To further investigate the effects of miR-203 on Co-CSC properties, stable clones derived from HCT-15 and HT-29 cells overexpressing this microRNA (M1 and M2) were selected after retroviral infection. Interestingly, the CD44⁺ population was markedly decreased in miR-203-overexpressing clones (Fig. 6A). Additionally, the numbers of colonies formed in

Figure 1 CD44⁺ HCT-15 cells display some stem-like properties. (A) Soft agar colony-forming assays were conducted by seeding parental, CD44⁺ and CD44⁻ HCT-15 cells on 0.6% agarose at 1×10^4 cells/well in six-well plates. After 2 weeks of culturing, colonies were stained with crystal violet and counted under a microscope. (B) The migration abilities of CD44⁺ and CD44⁻ HCT-15 cells were analyzed by transwell migration assay. Cells (5×10^4) were seeded in the upper chambers, and 16 h later those that had migrated to the lower surface of the filters were stained with crystal violet and then counted by microscopy. Quantitative RT-PCR was performed to analyze the RNA levels of several typical markers of embryonic stem cells (Oct4, Sox2, Nanog, Klf4 and Lin28) and intestinal stem cells (Lgr5) (C) or differentiated colonic cells (CK20, CDX1, CHGA and Mucin) (D) in CD44⁺ and CD44⁻ HCT-15 cells. CD44⁺ cells were cultivated in medium containing 10% FBS for 0, 7 and 14 days before total RNAs were isolated. The expression of CK20 (E) and CDX1 (F) genes in undifferentiated and differentiated CD44⁺ cells was analyzed by real-time RT-PCR. (G) The expression levels of EMT-related proteins and pluripotency factors in CD44⁺ and CD44⁻ cells were analyzed by Western blotting. Data were analyzed by ANOVA with the LSD post hoc test or t-test (*$P < 0.05$) and representing the mean ± SD of three independent experiments. Equal numbers of CD44⁺ and CD44⁻ cells (1×10^4, H) and (1×10^3, I) were mixed, individually, with Matrigel and then injected subcutaneously into nude mice. Tumor growth was monitored on a weekly basis (lower panel H, I) and data indicate the mean tumor size ± SD of 10 tumors per group. *$P < 0.05$ when compared with the tumors grown from the CD44⁻ cells. The tumors shown were collected from animals 8 weeks after they were transplanted with 1×10^4 CD44⁺ (left in upper panel, H) or CD44⁻ (right in upper panel, H).

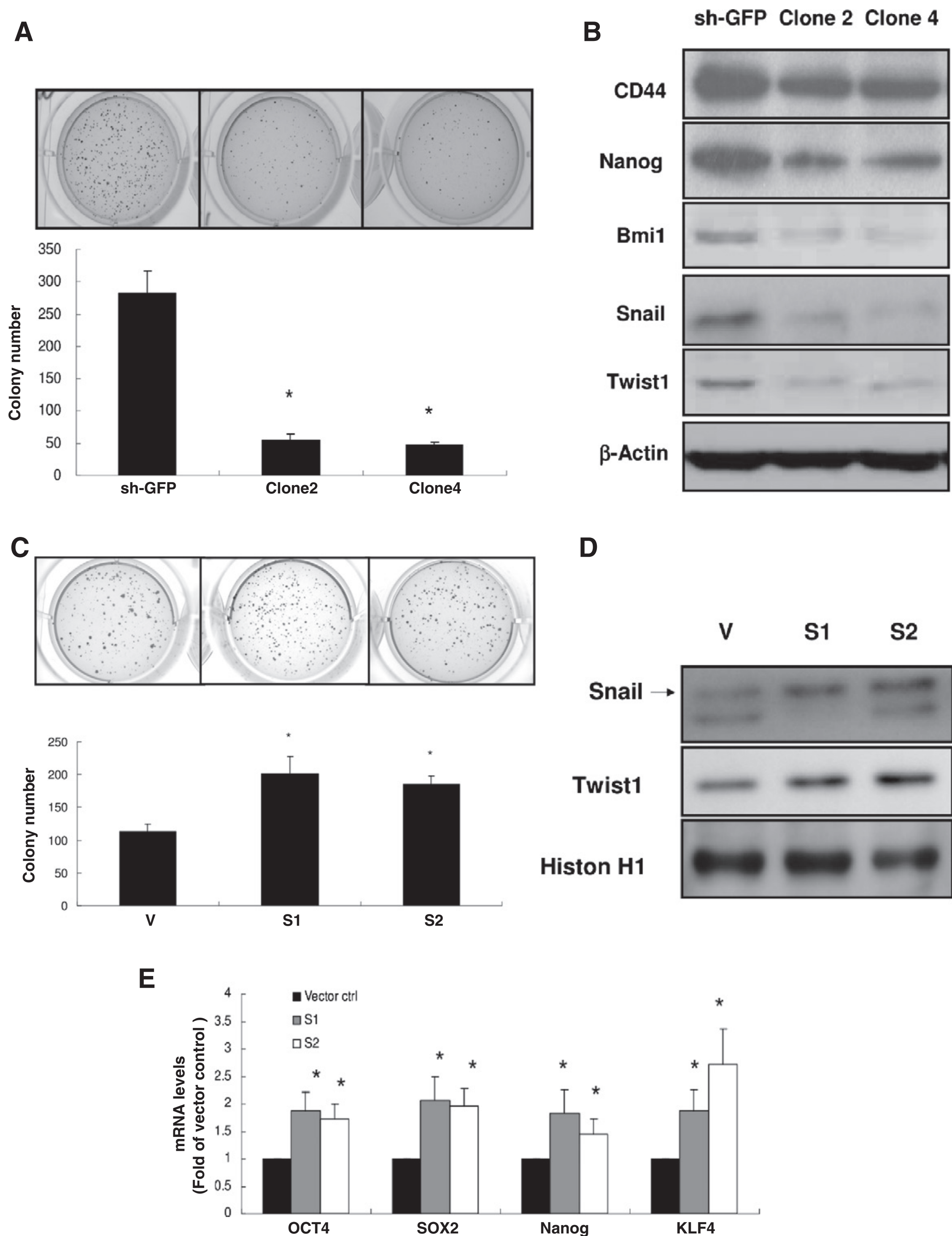


Figure 2 CD44 levels affect several stem-like properties in HCT-15 cells. Two stable transfectants (clones 2 and 4) and one control clone (sh-GFP) were established by expressing CD44 and GFP shRNAs, respectively, via the lentiviral infection of HCT-15 cells. (A) Cells (1×10^4/well) from sh-GFP and clones 2 and 4 were each seeded on 0.6% agarose in a six-well plate. Two weeks later, crystal violet staining was performed and the number of colonies was counted by microscopy. (B) Whole cell lysates prepared from the aforementioned three clones were subjected to Western blotting with antibodies against the indicated proteins. CD44-overexpressing clones (S1 and S2) and the vector-control clone (V) derived from HCT-15 cells were generated as described in Materials and methods. (C) Soft agar colony formation abilities of the CD44-overexpressing S1 and S2 clones derived from HCT-15 cells. (D) Nuclear Snail and Twist1 expression levels were analyzed by Western blotting. (E) Total RNAs were extracted from clones S1 and S2 and the vector control cells. Real-time RT-PCR was performed to measure the mRNA levels of the indicated genes. Data representing the mean ± SD of three independent experiments were analyzed by t-test (*$P < 0.05$).

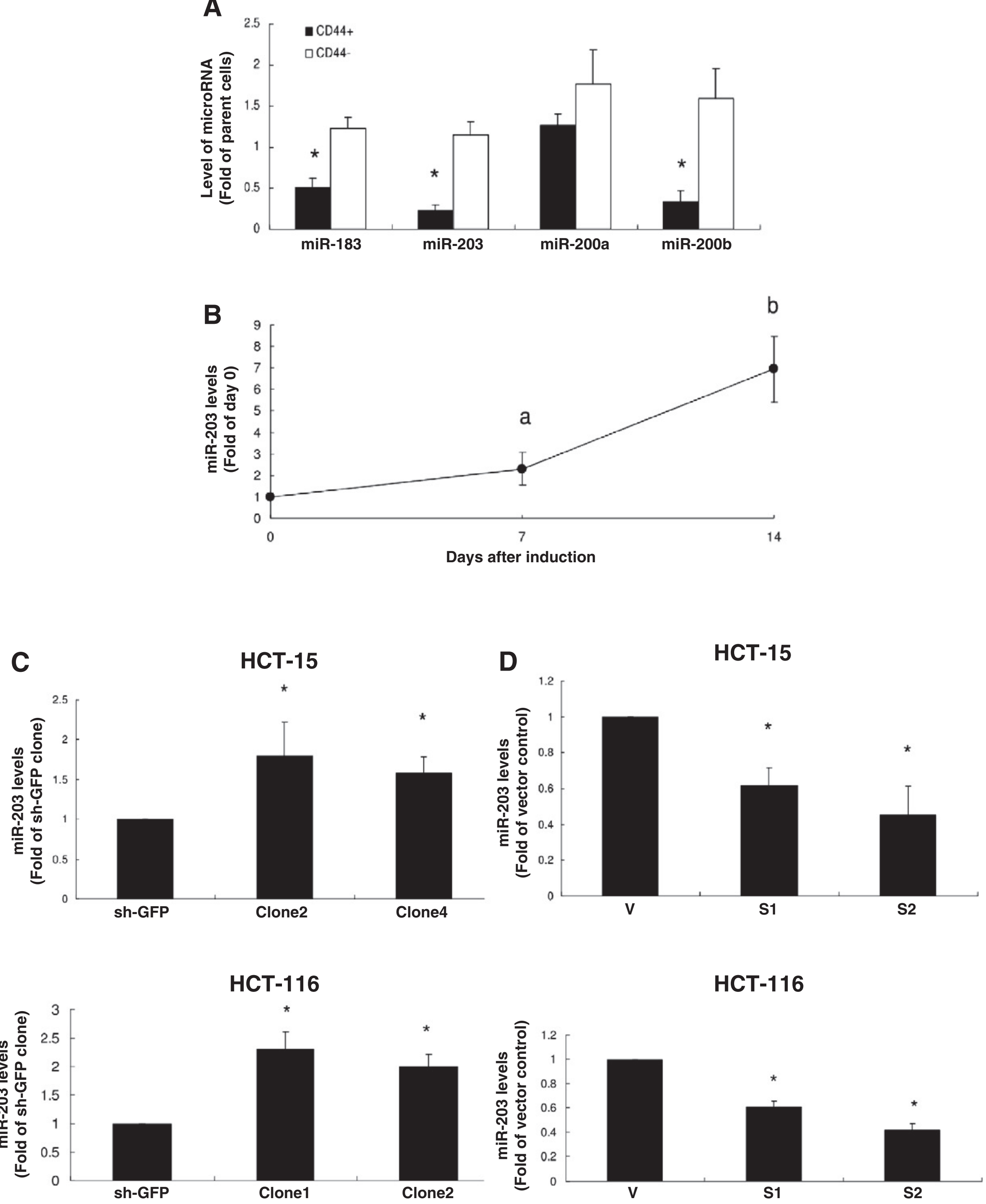


Figure 3 miR-203 expression levels in HCT-15 and HCT-116 cells are negatively correlated with CD44 levels. (A) CD44$^+$ and CD44$^-$ HCT-15 cells were purified by magnetic separation, and the expression levels of miR-200 family members were compared in these two populations by qRT-PCR analysis. (B) Total RNAs were isolated from CD44$^+$ cells cultured in medium containing 10% FBS for 0, 7 and 14 days and miR-203 levels were determined by qRT-PCR. Similar analyses were used to determine miR-203 expression levels in (C) the stable CD44-knockdowned clones (2 and 4) and the sh-GFP clone as well as in (D) the stable CD44-overexpressing clones (S1 and S2) and vector-infected clone derived from both HCT-15 and HCT-116 cells. Data representing the mean ± SD of three independent experiments were analyzed by ANOVA with the LSD post hoc test or t-test (*$P < 0.05$).

soft agar and spheroids were also dramatically reduced in miR-203-overexpressing transfectants (Figs. 6B and C). To examine the effect of miR-203 upregulation on the in vivo tumorigenesis of Co-CSCs, CD44$^+$ HCT-15 cells infected without or with retro-miR-203 were transplanted into nude mice as described above and tumor growth was monitored. As

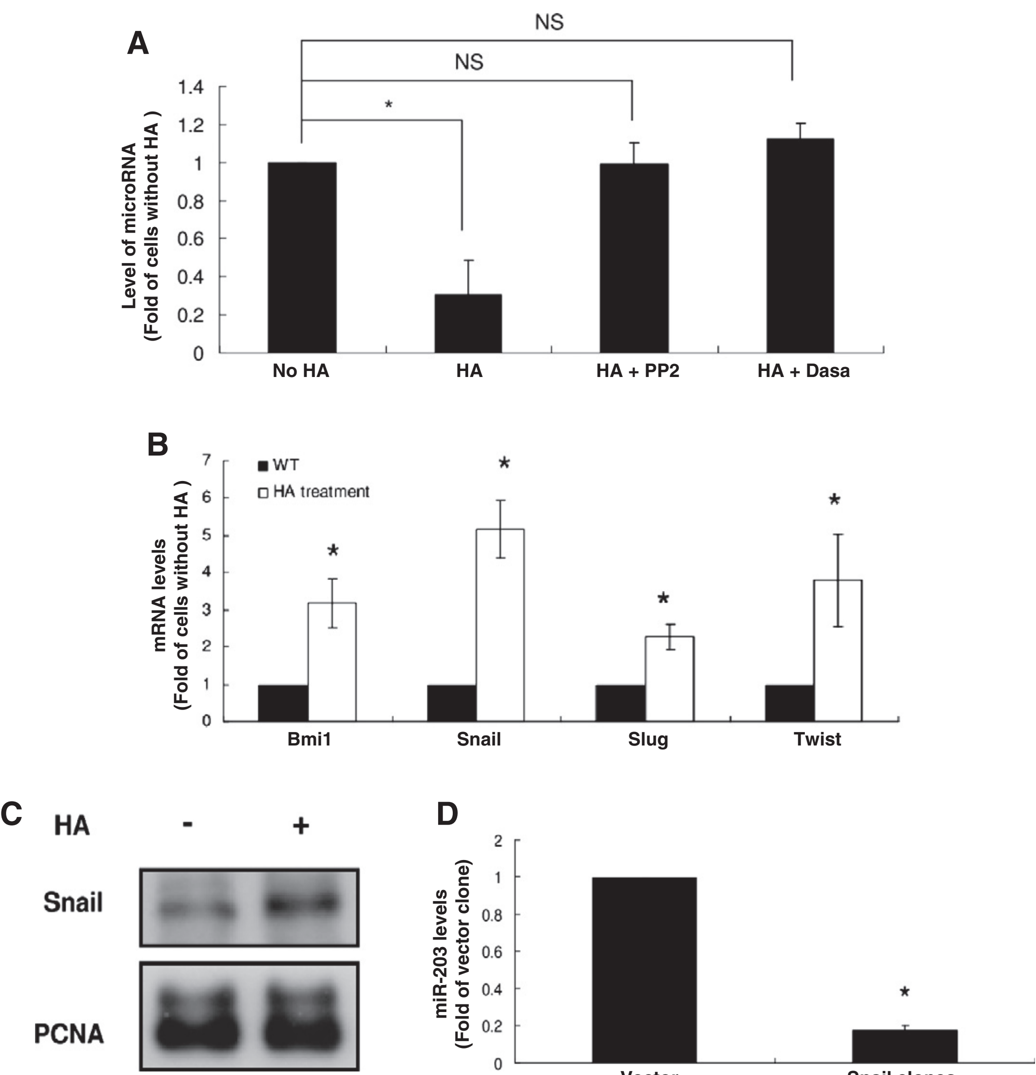


Figure 4 Hyaluronan–CD44 interaction regulates the expression of miR-203 and several EMT-related genes in HCT-15 cells. (A) HCT-15 cells were seeded onto dishes coated with or without hyaluronan (HA, 100 μg/ml). Twenty four hours later, cells were treated without or with PP2 (10 μM) or Dasatinib (2 μM) for another 24 h before total RNAs were isolated. Quantitative RT-PCR analysis was performed to examine the expression levels of miR-203. (B) RNA levels of several EMT-related genes were determined by real-time RT-PCR with total RNAs prepared from HCT-15 cells cultured on HA-coated or uncoated dishes for 24 h. (C) Nuclear fractions prepared from CD44^{+} cells cultured for 24 h on HA-coated or uncoated dishes were subjected to Western blotting with an anti-Snail antibody. (D) Total RNAs were used to assess the miR-203 expression levels of the Snail-overexpressing stable clone and the vector-control clone derived from HCT-15 cells by qRT-PCR. Data representing the mean ± SD of three independent experiments were analyzed by t-test (*P $<$ 0.05).

can be seen, both tumor incidence and growth rate of these cells were significantly diminished by miR-203 upregulation (Fig. 6D). In the meantime, we wanted to know whether the stemness of Co-CSCs could be enhanced by knocking down miR-203 expression. Because of the extremely low transfection efficiency (regardless of the reagents used) observed in HCT-15 cells and the fact that a variety of Co-CSC properties found in CD44^{+} HCT-15 cells were also detected in CD44^{+} HCT-116 cells (Supplementary Fig. 6), CD44^{+} HCT-116 cells were used to analyze the effects of the transient knockdown of miR-203 on the stemness of human colon cancer cells. To no surprise, after introducing a miR-203 antagomir into HCT-116 cells, protein levels of markers such as CD44, ALDH, Nanog and Sox2 were markedly increased (Fig. 6E). In addition, spheroids generated in defined medium from the miR-203 antagomir-transfected HCT-116 cells were more resistant to differentiation (i.e., cell spreading) induced by 1% FCS (Fig. 6F). Taken together, these results suggest that the Co-CSC properties of HCT-15, HT-29 and HCT-116 cells are greatly affected by miR-203.

Discussion

High tumorigenicity has been found in a minor cell population present in a wide variety of primary tumors as well as

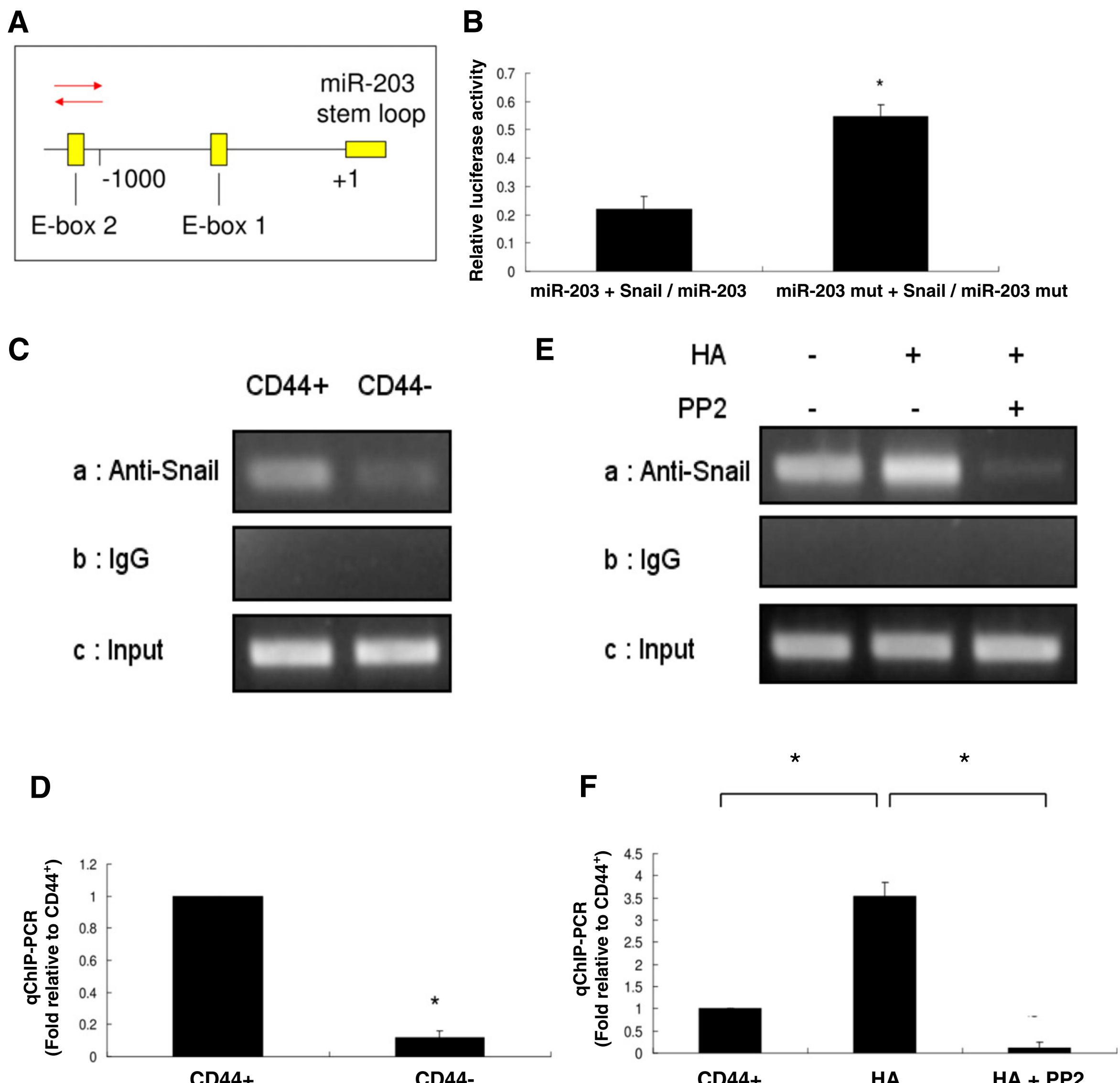
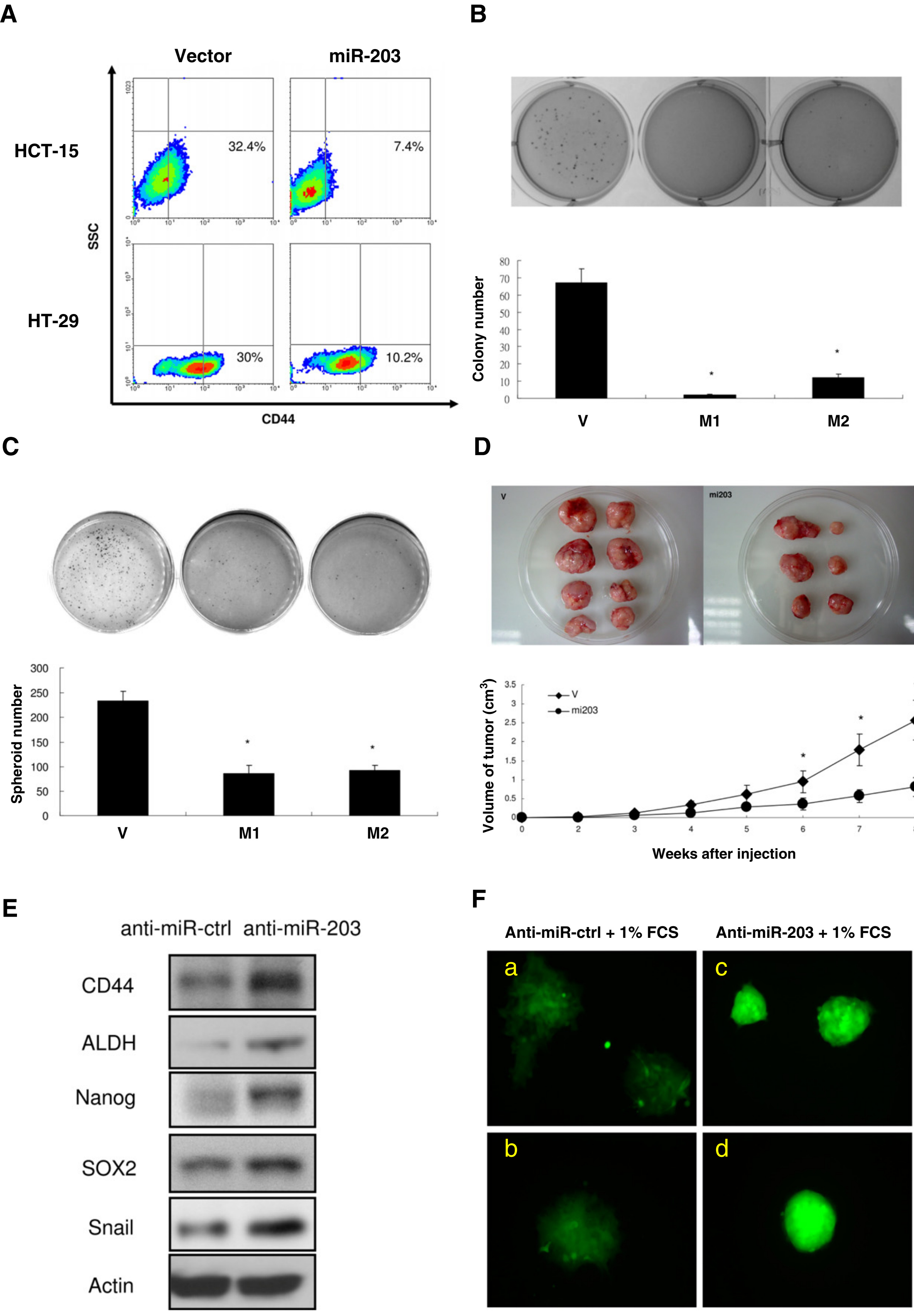


Figure 5 In situ interaction between Snail and the miR-203 promoter in HCT-15 cells. (A) Schematic showing of the upstream promoter region of the human miR-203 gene with two putative Snail-binding sites (E-boxes 1 and 2). The region amplified in chromatin-immunoprecipitation (ChIP) assay is marked with a red arrow. (B) The reporter gene, driven either by the wild-type or the E-box 2-mutated miR-203 promoter was co-transfected with a Snail-expressing or an empty vector into 293T cells. The relative influences of Snail on the wild-type and E-box-mutated miR-203 promoters were estimated using the methods described in Materials and methods. The ChIP assay was performed using genomic DNAs that were immunoprecipitated by the Snail antibody from the $CD44^+$ and $CD44^-$ HCT-15 cells as templates for regular (C) or real-time (D) PCR amplification using the primer set specific to the flanking regions of E-box 2 of the miR-203 promoter. (a, The immunoprecipitates pulled down by anti-Snail antibody; b, The immunoprecipitates pulled down by the IgG isotype control antibody; c, Total input immunoprecipitates). (E) The ChIP assay was performed as described above except for the use of genomic DNAs prepared from $CD44^+$ cells seeded onto HA coated or uncoated dishes and treated with or without PP2 as described in Fig. 4. (F) qPCR was performed to quantify the binding levels of Snail to the miR-203 promoter. Data representing mean ± SD of three independent experiments were analyzed by t-test (*$P < 0.05$).

established tumor cell lines. These so-called cancer stem cells (CSCs) or tumor-initiating cells (TICs) can be isolated and/or enriched by using specific surface and/or intracellular markers, serum-free culturing, and dye exclusion methods. However, neither a common method nor a universal marker is currently available for identifying such a population from all cancers.

CD133 has been used to identify various normal stem cells and CSCs. In fact, two recently published reports describe the isolation of colon cancer stem cells (Co-CSCs) using CD133 as a marker. The first publication showed that a small proportion (1 in 262) of $CD133^+$ cells from primary colon cancers has a much higher tumorigenic potential in immunodeficient mice (Ricci-Vitiani et al., 2007). In addition to


A
Vector
miR-203
HCT-15
32.4%
7.4%
SSC
HT-29
30%
10.2%
CD44
B
Colony number
V
M1
M2
C
Spheroid number
V
M1
M2
D
v
mi203
Volume of tumor (cm3)
Weeks after injection
E
anti-miR-ctrl
anti-miR-203
CD44
ALDH
Nanog
SOX2
Snail
Actin
F
Anti-miR-ctrl + 1% FCS
Anti-miR-203 + 1% FCS
a
b
c
d

reporting a similar finding in vivo, the other study also demonstrated that $CD133^+$ cells isolated from primary tumors formed spheres and could be maintained in an undifferentiated state in serum-free conditions (O'Brien et al., 2007). Although $CD133^+$ cells isolated from the HCT-15 human colon cancer line had a higher colony-forming ability than both the parental and $CD133^-$ cells (data not shown), they did not exhibit higher in vivo tumorigenicity after being cultivated in a defined medium for one month (Supplementary Fig. 2A and B). One possible explanation for this finding is that the purified $CD133^+$ cells may gradually lose their stemness, and therefore their tumorigenicity, when cultured in a serum-free medium supplemented with EGF, bFGF and N2.

Recent studies also demonstrated that Co-CSCs could be isolated using CD44 as a single selection marker (Chu et al., 2009; Du et al., 2008). On the other hand, an earlier work showed that the $EpCAM^{high}/CD44^+/CD166^+$ cells are Co-CSCs (Dalerba et al., 2007). In support of the observations made by Chu and Du, we found that freshly isolated $CD44^+$ HCT-15 cells exhibited a much higher soft agar colony-forming ability and tumor growth after xenograft than their $CD44^-$ counterparts (Fig. 1). These findings indicated that CD44 is a better marker than CD133 for selecting a stem cell-like population from HCT-15 cells. Although CD44 had been previously shown to participate in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis (Misra et al., 2011), the functional aspects of this molecule in generating and/or maintaining CSCs have not yet been explored extensively.

Accumulating evidence has shown that CD44 is not only a marker for Co-CSCs but also a critical factor for cancer initiation (Du et al., 2008). Interestingly, a recent study demonstrated that the induction of EMT stimulates cultured breast cells to adopt stem cell characteristics (Mani et al., 2008). Notably, our CD44 knockdown (Figs. 2A and B) and overexpression experiments (Figs. 2C and D) demonstrated that CD44 is important for maintaining the CSC properties of HCT-15 cells by regulating the EMT activators including Bmi1, Twist and Snail. However, the precise roles of Bmi1 and Twist in establishing and/or maintaining the stemness of $CD44^+$ HCT-15 cells remain to be elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs which regulate gene expression in a post-transcriptional manner. A recent study reported that ZEB1, another EMT activator, can directly suppress the transcription of miR-141, miR-200c, and miR-203 by interacting with their respective promoter regions. Moreover, miR-200c, miR-203 and miR-183 cooperate to suppress the expression of polycomb repressor Bmi1 which plays a role in maintaining the stemness of cancer cells and mouse embryonic stem (ES) cells (Wellner et al., 2009). Another report has shown that hyaluronan (HA)/CD44 interaction can activate c-Src which subsequently stimulates the transcriptional activity of Twist to promote miR-10b production, leading to tumor progression (Bourguignon et al., 2010). Our results demonstrated that miR-203 was significantly decreased in freshly isolated $CD44^+$ HCT-15 and HCT-116 cells (Figs. 3A and Supplementary 6C). Consistently, miR-203 levels were dramatically decreased in CD44-overexpressing transfectants (Fig. 3D) and were increased in CD44-knockdowned clones (Fig. 3C) generated from the aforementioned two cell lines. Therefore, CD44 might play a critical role in regulating miR-203 expression in Co-CSCs.

As described above, hyaluronan (HA) is a component of the extracellular matrix of mammalian cells that specifically interacts with its receptor, CD44, to activate c-Src-dependent signaling. This c-Src signaling promotes cell migration and tumor progression (Taher et al., 1996). According to our results, HA treatment triggered a decrease in miR-203 levels (Fig. 4A) as well as increases in total Snail protein and its nuclear accumulation (Fig. 4C) in $CD44^+$ HCT-15 cells. In addition, a drastic reduction in miR-203 expression was found in a Snail-overexpressing clone (Fig. 4D). Together, these findings suggest that Snail is responsible for the HA/CD44-mediated downregulation of miR-203 and such a speculation was also supported by our ChIP results, which clearly showed a direct interaction between Snail and the E-box-containing region of the miR-203 promoter (Fig. 5). Indeed, Snail-mediated repression of miR-203 has recently been reported in other types of cancer cells (Moes et al., 2012; Wellner et al., 2009). In fact, a double-negative miR-203/Snail feedback loop has been proposed in breast cancer cells because a mesenchymal-to-epithelial transition (MET) was observed in these cells as a result of the miR-203-mediated

Figure 6 The upregulated expression of miR-203 in HCT-15 and HT-29 cells abolishes some of their Co-CSC properties, and the transient knockdown of miR-203 expression results in appearance of some SC characteristics in HCT-116 cells. (A) The vector-control and miR-203-overexpressing stable clones (M1 and 2) derived from HCT-15 and HT-29 cells were established by retroviral infection. The percentage of $CD44^+$ population was quantified by flow cytometry. (B) Soft agar colony-formation assay or (C) sphere culture was carried out using clones derived from HCT-15 and HT-29 cells. After crystal violet staining, the numbers of colonies or spheroids were counted respectively using Colony 1.1 software. (D) Purified $CD44^+$ HCT-15 cells were infected with either the control (vector-ctrl) or miR-203 retroviruses. Forty-eight hours after puromycin selection, equal numbers of cells (1×10^4) from each were mixed, individually, with Matrigel and then injected subcutaneously into nude mice (n = 10 for each type). Tumor growth was monitored on a weekly basis and data indicate the mean tumor size ± SD of tumors per group. $^*P < 0.05$ when compared with the tumors grown from the vector-control clone. The tumors shown were collected from animals 8 weeks after they were transplanted with miR-203-overexpressing (right in the upper panel) or vector-control cells (left in the upper panel). (E) Total lysates were prepared from HCT-116 cells after they were transfected respectively with the miR-203 antagomir or control miR (ctrl-miR) for 48 h. Western blotting was performed to examine the levels of the indicated proteins using antibodies against CD44, ALDH, Nanog, Sox2 and Snail as probes. (F) Spheroids derived from the GFP-expressing HCT-116 cells cultured in a defined medium were transfected with 5 nM ctrl-miR (a, b) or the miR-203 antagomir (c, d) and cultured in media containing 1% FCS. Twenty-four hours later, pictures were taken under a fluorescent microscope (400×) and the differentiation of the spheroid cells was estimated by the extent of their spreading.

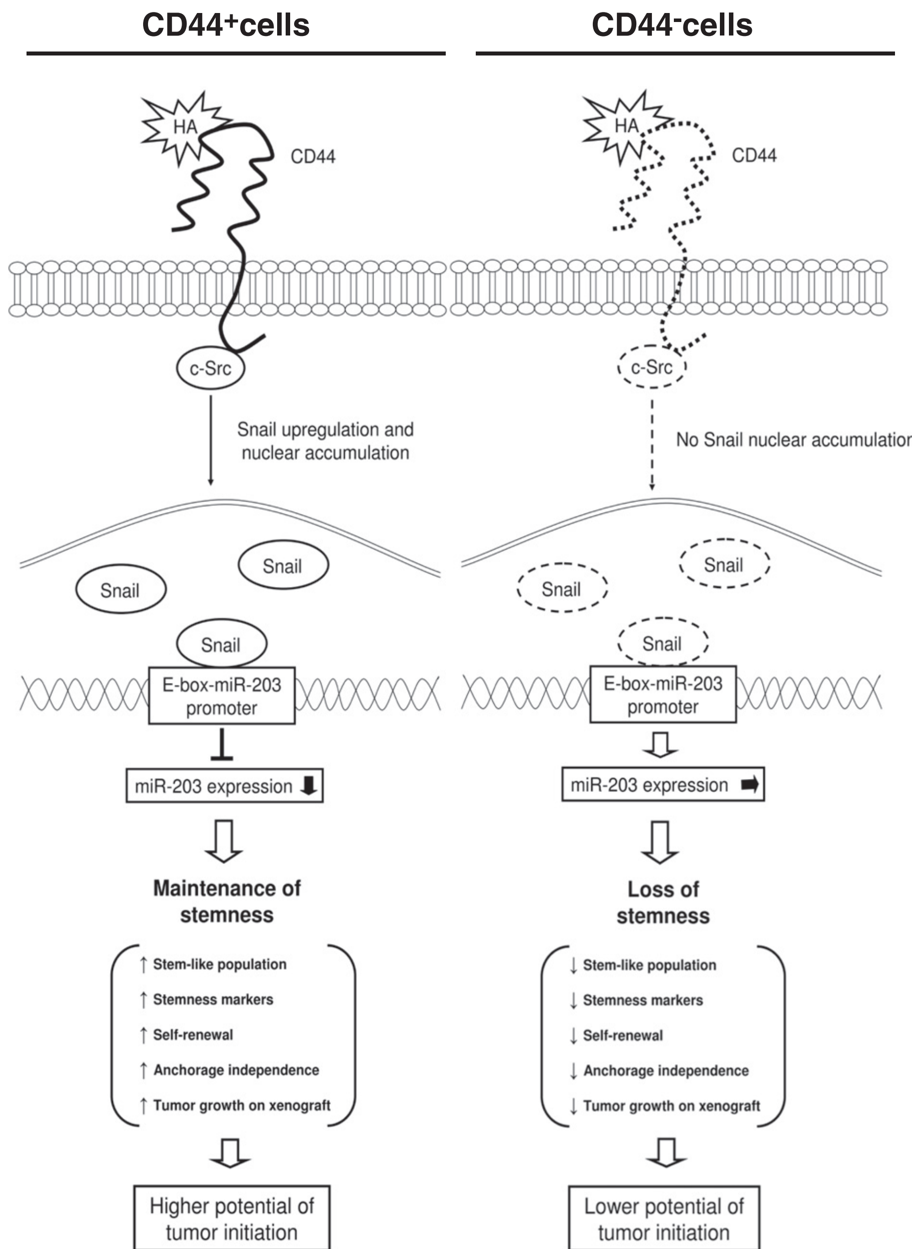


Figure 7 A proposed model for the maintenance of stemness of HCT-15 and HCT-116 human colon cancer cells by HA/CD44 signaling. Initially, HA interacts with CD44, resulting in c-Src activation, which in turn upregulates Snail expression. Subsequently, the increased Snail molecules translocate into the nucleus and bind with the E-box elements of the miR-203 promoter, hence repressing its expression. Once the expression of this microRNA was markedly reduced, not only was the expression of several stemness markers elevated but also certain stemness properties (i.e., self-renewal and anchorage-independent growth) were greatly increased, leading to a superior tumor initiation potential.

suppression of Snail (Moes et al., 2012). It will be interesting to examine whether this type of loop operates in CD44+ HCT-15 cells and to identify the downstream targets of miR-203 responsible for maintaining the stemness of the aforementioned cell population.

Several studies have demonstrated that the expression of Snail could be regulated by both transcriptional and post-transcriptional mechanisms. For example, the TGFβ/Smad and TNFα/NF-κB pathways transcriptionally induce Snail expression via direct binding of the activated factors to the Snail promoter (Barbera et al., 2004; Vincent et al., 2009). On the other hand, receptor tyrosine kinase signaling by molecules such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) increases Snail stability by suppressing the activity of GSK-3β (De Craene et al., 2005). Even though our results suggest the involvement of c-Src in Snail-mediated downregulation of miR-203 triggered by the HA/CD44 interaction (Figs. 5C and D), the precise mechanism responsible for

the activation of Snail by HA/CD44 signaling remains to be investigated.

In conclusion, CD44 seems to be a better marker than CD133 for isolating stem cell-like population from both HCT-15 and HCT-116 cells. In addition, the functional importance of this molecule for stemness maintenance (e.g., increased spheroid and soft agar colony formation, upregulation of stem cell marker genes and downregulation of the stemness inhibitory miRNAs) of Co-CSCs derived from HCT-15 cells has been demonstrated (Fig. 7). We will next dissect the detailed CD44 signaling pathway responsible for establishing the stemness of CRC cells. Understanding this pathway will help to clarify the involvement of CD44 signaling, EMT inducers and microRNAs in a complicated network that leads to the development of Co-CSCs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.09.011.

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