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

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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.

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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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