SNAIL Regulates Interleukin-8 Expression, Stem Cell–Like Activity, and Tumorigenicity of Human Colorectal Carcinoma Cells

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BACKGROUND & AIMS: Some cancer cells have activities that are similar to those of stem cells from normal tissues, and cell dedifferentiation correlates with poor prognosis. Little is known about the mechanisms that regulate the stem cell–like features of cancer cells; we investigated genes associated with stem cell–like features of colorectal cancer (CRC) cells. **METHODS:** We isolated colonospheres from primary CRC tissues and cell lines and characterized their gene expression patterns by microarray analysis. We also investigated the biological features of the colonosphere cells. **RESULTS:** Expanded CRC colonospheres contained cells that expressed high levels of CD44 and CD166, which are markers of colon cancer stem cells, and had many features of cancer stem cells, including chemoresistance and radioresistance, the ability to initiate tumor formation, and activation of epithelial-mesenchymal transition (EMT). SNAIL, an activator of EMT, was expressed at high levels by CRC colonospheres. Overexpression of Snail in CRC cells induced most properties of colonospheres, including cell dedifferentiation. Two hundred twenty-seven SNAIL-activated genes were up-regulated in colonospheres; gene regulatory networks centered around interleukin (IL)-8 and JUN. Blocking IL-8 expression or activity disrupted SNAIL-induced stem cell–like features of colonospheres. We observed that SNAIL activated the expression of *IL8* by direct binding to its E3/E4 E-boxes. In CRC tissues, SNAIL and IL-8 were coexpressed with the stem cell marker CD44 but not with CD133 or CD24. **CONCLUSIONS: In human CRC tissues, SNAIL regulates expression of IL-8 and other genes to induce cancer stem cell activities. Strategies that disrupt this pathway might be developed to block tumor formation by cancer stem cells.**

Keywords: Colon Cancer; Tumor Development; Systems Biology; Gene Regulation.

Colorectal cancer (CRC) is one of the most common cancers worldwide. Reevaluating our current knowledge of CRC and developing novel therapeutic strategies is still crucial. Evidence suggests that cancer cells possess characteristics reminiscent of those of normal stem cells.[1] Unveiling the genes responsible for CRC cell stemness and chemoradioresistance should lead to novel therapeutic approaches.

The epithelial-mesenchymal transition (EMT) is a critical process involved in the transdifferentiation of polarized epithelial cells into an invasive mesenchymal phenotype.[2] The EMT also contributes to the acquisition of stem cell traits in breast cancer cells and the generation of cells with features similar to breast cancer stem cells.[3,4] These results have provided a crucial link between metastasis and stem cell properties. One of the major EMT regulators is the zinc-finger transcription factor family protein Snail.[5,6] The critical roles of Snail in CRC have been previously identified; this involves Snail enhancing the degradation of prostaglandin E_2, which promotes cancer progression.[7] Snail also contributes to the down-regulation of E-cadherin and the vitamin D receptor in colon cancer, which results in the failure of vitamin D analogue treatment.[8,9] Expression of Snail correlates with poor patient prognosis in CRC.[10] In breast cancer, Snail promotes stemness and generates mammosphere cells.[3] How Snail induces dedifferentiation is unclear, and whether Snail can introduce similar stemness traits in CRC is also undecided. Because Snail or other EMT transcription factors are nontargetable by current therapeutic approaches, the identification of Snail-regulated genes that can be targeted by drugs may lead to the development of novel therapeutic strategies against EMT-related malignancy.

Materials and Methods

Tissue Collection and Colonosphere Expansion

This study was approved by the Institutional Ethics Committee/Institutional Review Board of the Taipei Veterans General Hospital. A total of 22 primary colorectal specimens from individuals aged 42 to 90 years with disease grade 2 to 3 and American Joint Committee on Cancer stage IIA to IVB were collected during surgery

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; CRC, colorectal cancer; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; IL, interleukin; nAb, neutralizing antibody; qPCR, quantitative polymerase chain reaction.

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(Supplementary Table 1). These samples were immersed in normal saline, brought to the laboratory within 1 hour, and washed 5 times with phosphate-buffered saline containing 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco, Carlsbad, CA). Samples of each solid tissue were then immersed in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA isolation. Other samples from each tissue were immersed in 4% paraformaldehyde (Sigma, St Louis, MO) to allow the preparation of paraffin-embedded blocks. Finally, the remaining samples were minced into small fragments (2 mm^3) for primary culture. These tissue fragments were washed in 100 mmol/L phosphate buffer (pH 7.0) with 6.5 mmol/L dithiothreitol (Sigma) for 15 minutes at room temperature to avoid mucus contamination.[11] After removal of dithiothreitol solution and additional washing with phosphate-buffered saline, the tissue fragments were resuspended in serum-free Dulbecco's modified Eagle medium/F12 (Gibco) supplemented with 500 U/mL penicillin-streptomycin and 1.5 mg/mL type IV collagenase (Sigma) for enzymatic dissociation at 37°C for 1 hour. The cells were mechanically broken apart by pipetting every 15 minutes during digestion. Released cell samples were subjected to fluorescence-activated cell sorter analysis, while the remaining cells were cultured using type I collagen–coated (Sigma) plates in RPMI 1640 or Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin to obtain primary cells. Some released cell samples were cultured in stem cell medium (Dulbecco's modified Eagle medium/F12 supplemented with N_2 Plus Supplement [R&D Systems, Minneapolis, MN], 10 ng/mL recombinant basic fibroblast growth factor [bFGF; PeproTech Asia; Rehovot, Israel], 10 ng/mL epidermal growth factor [EGF; PeproTech Asia], and 1% penicillin-streptomycin) to obtain colonospheres.[12]

To obtain colonospheres from the cancer cell lines, dissociated cells were cultured in stem cell medium for at least 2 weeks.[12] To enhance in vitro epithelial differentiation, suspended colonospheres were collected by centrifugation and cultured in Dulbecco's modified Eagle medium supplemented with 1% fetal bovine serum[13] and the resultant epithelial-like cells were named sphere-derived adherent cells. TrypLE Express (Invitrogen) was used to separate cells from the centrifuge-collected colonospheres and adherent cells to allow cell counting and other experiments.

Miscellaneous Methods

For other methods, please refer to the Supplementary Materials and Methods.

Results

Enhanced Malignancy in CD44$^+$ CRC Colonospheres Expanded by Culture Under Serum-Free Conditions

Self-renewal capability is a major property of stem cells, and spheroid formation is a self-renewal index.[3] Culture under bFGF(+)EGF(+) serum-free conditions has been used to expand stem-like spheroid cells from primary CRC tissue.[14-16] Primary cells derived from the bFGF(+)EGF(+) serum-free culture more faithfully mirror the original gene expression profiles and tumor morphology of the cells.[17] We expanded the cancer stem cells as colonospheres from primary CRC specimens and also assessed the sphere formation ability of 5 CRC cell lines. CRC colonospheres were obtained by expansion from a large subset of specimens (16/22; Supplementary Figure 1*A* and Supplementary Table 1). However, only the HT29 and HCT15 cell lines formed significant spheroid bodies reminiscent of primary colonospheres after 3 weeks of culture (first colonospheres) (Supplementary Figure 1*B*). The expanded primary colonospheres were positive for A33, which is expressed in almost all colonic epithelium irrespective of differentiation status[18,19] (Supplementary Figure 2). These CRC colonospheres originated from a single cell rather than having developed by mere cell aggregation (Supplementary Figure 1*C*).

CD44, CD24, CD166, CD133 and aldehyde dehydrogenase 1 (ALDH1) have all been proposed as markers of CRC cancer stem cells.[20] In this context, the existence of various cancer stem cell populations in isolated colonospheres was evaluated. CD44$^+$, CD24$^+$, CD133$^+$, or CD166$^+$ population was enriched in primary colonospheres (Figure 1*A* and Supplementary Figure 3*B* and *C*, patients 7 and 8). CD44$^+$, CD24$^+$, or CD166$^+$ population was also enriched in HT29-derived or HCT15-derived colonospheres (Figure 1*A* and Supplementary Figure 3*A*). After spheroid cultivation, the CD44$^+$ and CD166$^+$ populations were most consistently enriched among the expanded colonospheres (Figure 1*A*).

To compare relative malignancy between the expanded colonospheres and the putative cancer stem cell populations of sorted and unsorted parental cells (Supplementary Figure 3*E* and *F*), as well as colonospheres derived from various sorted populations, these were subjected into soft agar assay and a spheroid-forming assay. CD44$^+$ cells formed more colonies and spheroids than CD44$^-$ cells. However, double sorting with either for CD24 or CD133 did not further increase the number of malignant cells (Figure 1*B* and *C*). Colonosphere cells formed more colonies and spheroids than CD44$^+$ cells (Figure 1*B* and *C*). Colonospheres from presorted CD44$^+$ or CD133$^+$ parental cells were not more malignant than spheres from nonsorted cells (Figure 1*B* and *C*). Colonospheres also harbored enhanced tumorigenicity in vivo (Supplementary Figure 4*A*). Serum-free culture is therefore one useful approach to expanding malignant CRC stem-like cells.

CRC Spheres Inherit Stem Cell Properties

Because malignant cells harbor more stemness traits,[1] we examined stem cell and differentiation markers in the colonospheres. Sphere-derived adherent cells exhibited epithelial-like morphology on serum exposure (Supplementary Figure 1*D*). Compared with parental cells or sphere-derived adherent cells, the expression levels of various stemness genes in colonospheres were significantly higher, whereas those of the differentiation markers CDX2 and BMP4 were lower (Figure 2*A* and Supplementary Figure 4*B*).