SNAI2 Modulates Colorectal Cancer 5-Fluorouracil Sensitivity through miR145 Repression

Victoria J. Findlay1,2, Cindy Wang3, Lourdes M. Nogueira1, Katie Hurst3, Daniel Quirk3, Stephen P. Ethier1,2, Kevin F. Staveley O’Carroll2,3,4, Dennis K. Watson1,2,5, and E. Ramsay Camp2,3,4

Abstract

Epithelial-to-mesenchymal transition (EMT) has been associated with poor treatment outcomes in various malignancies and is inversely associated with miRNA145 expression. Therefore, we hypothesized that SNAI2 (Slug) may mediate 5-fluorouracil (5FU) chemotherapy resistance through inhibition of miR145 in colorectal cancer and thus represents a novel therapeutic target to enhance current colorectal cancer treatment strategies. Compared with parental DLD1 colon cancer cells, 5FU-resistant (5FUr) DLD1 cells demonstrated features of EMT, including >2-fold enhanced invasion (P < 0.001) and migration, suppressed E-cadherin expression, and 2-fold increased SNAI2 expression. DLD1 and HCT116 cells with stable expression of SNAI2 (DLD1/SNAI2; HCT116/SNAI2) also demonstrated EMT features such as the decreased E-cadherin as well as significantly decreased miR145 expression, as compared with control empty vector cells. On the basis of an miR145 luciferase promoter assay, we demonstrated that SNAI2 repressed activity of the miR145 promoter in the DLD1 and HCT116 cells. In addition, the ectopic expressing SNAI2 cell lines demonstrated decreased 5FU sensitivity, and, conversely, miR145 replacement significantly enhanced 5FU sensitivity. In the parental SW620 colon cancer cell line with high SNAI2 and low miR145 levels, inhibition of SNAI2 directly with short hairpin sequence for SNAI2 and miR145 replacement therapy both decreased vimentin expression and increased in vitro 5FU sensitivity. In pretreatment rectal cancer patient biopsy samples, low miR145 expression levels correlated with poor response to neoadjuvant 5FU-based chemoradiation. These results suggested that the SNAI2:miR145 pathway may represent a novel clinical therapeutic target in colorectal cancer and may serve as a response predictor to chemoradiation therapy. Mol Cancer Ther; 13(11); 1–14. ©2014 AACR.

Introduction

5-Fluorouracil (5FU)-based neoadjuvant chemoradiation therapy has become standard care for patients with advanced rectal cancer, resulting in decreased local recurrence rates, improved tolerance of prescribed therapy, improved organ preservation, and fewer treatment-related complications (1). However, with current preoperative treatment strategies, complete pathologic response only is observed in approximately 20% of cases with about 40% of the tumors demonstrating no pathologic response (2). Molecular mechanisms resulting in therapeutic resistance in colorectal cancer are poorly understood. Therefore, investigating molecular pathways associated with chemoradiation resistance should provide insight into tumor cell survival mechanisms and identify novel targets to improve colorectal cancer response to therapy.

Epithelial-to-mesenchymal transition (EMT)-associated phenotypes, including enhanced motility and invasion, have been well-characterized in cancer cells (3–5). Recently, mediators of EMT also have been associated with enhanced cellular survival (6, 7). Similarly, an inverse relationship between the tumor suppressor miR145 and EMT has been described in breast cancer cells (8, 9). Although loss of E-cadherin has been linked with chemotherapy resistance in colorectal cancer, only recently have EMT pathways been identified as mediators of colon cancer chemotherapy resistance (10). In addition, miR145 also has been linked with 5FU chemotherapy sensitivity in gastric cancer (11). Therefore, we hypothesized that the EMT transcriptional mediator SNAI2 may mediate 5FU resistance through inhibition of miR145.
expression in colorectal cancer and, thus, constitutes a novel axis that can be targeted to reverse chemoresistance.

Extending this concept further, expression of EMT-associated genes has been associated with cancer stem cell (CSC)–like phenotypes in multiple organ systems including colon cancer (10, 12, 13). The stem cell concept is centered on a hierarchical theory for cancer development suggesting that only specific undifferentiated cancer cells, the tumor-initiating cells, have the ability to self-renew, propagate, and differentiate leading to cancer growth and progression (14). CSCs have demonstrated resistance to therapy and are predictive of poor patient prognosis (12, 15, 16). Recently, investigators demonstrated that ectopic expression of SNAI1 in colorectal cancer cell lines resulted in tumor-initiating properties and increased tumor growth (10). Therefore, therapeutically blocking EMT-mediated pathways may also serve to target the tumor-initiating cancer cells improving the benefit of conventional antineoplastic therapies.

Materials and Methods

Cell lines and culture conditions

Human colon cancer cell lines (DLD1, HCT116, LS174T, HT29, and SW620) were obtained from ATCC, cultured according to ATCC recommendations, and maintained at 37°C with 5% CO2. Stocks were immediately generated and stored in liquid nitrogen. Cells were only cultured up to 25 passages before being replaced from low-passage stocks. Mycoplasma-negative cultures were ensured by PCR testing before the investigations (Stratagene, #302108). Cells were monitored throughout the course of these studies and demonstrated consistent morphology and doubling time. 5FU-resistant DLD1 colon cancer cell lines were kindly provided by Dr. Bingliang Fang (University of Texas, MD Anderson Cancer Center, Houston, TX) and maintained in 50 μmol/L 5FU confirming 5FU resistance (17). 5FU-resistant DLD1 cell lines were not otherwise authenticated.

SNAI2 stable cell lines

For the generation of DLD1 and HCT116 cells overexpressing SNAI2 (DLD1/SNAI2, HCT/SNAI2), an SNAI2 or empty pCMV-3Tag-1 (kanamycin-resistant) vector was transfected into cells using Lipofectamine 2000 according to the manufacturer’s recommendations (Invitrogen; ref. 18). Stable transfected pools of cells were selected in medium containing G418 (400 μg/mL; Invitrogen). SNAI2 was amplified from gDNA using SNAI2-specific primers as previously reported (18).

Reagents

5FU was purchased from the pharmacy at the Medical University of South Carolina (Charleston, SC). The following antibodies were used for immunofluorescent (IF) staining: mouse anti-E-cadherin antibody (Zymed Laboratories); goat anti-rabbit horseradish peroxidase-conjugated, horse anti-mouse antibodies, and Draq 5 dye (Cell Signaling Technology, Inc.); and Alexa Fluor488-conjugated antibodies specific for rabbit and mouse IgG (Molecular Probes, Inc.). The following antibodies were used for Western blot analyses: E-cadherin (BD Biosciences); SnaI2, vimentin, Ki67, Sox2, Myc, and Nanog (Cell Signaling); actin (Sigma-Aldrich); and GAPDH (Santa Cruz).

Western blot analysis

Cells were suspended in RIPA protein lysis buffer (pH 7.4), containing 20 mmol/L sodium phosphate, 150 mmol/L sodium chloride, 1% Triton X-100, 5 mmol/L EDTA, 5 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin, 1 μg/mL leupeptin, and 500 μmol/L Na3VO4. Protein concentration was quantified using Bio-Rad protein assay (Bio-Rad Laboratories). SNAI2 was amplified from gDNA using SNAI2-specific primers as previously reported (18). Stable transfected pools of cells were selected in medium containing G418 (400 μg/mL; Invitrogen). SNAI2 was amplified from gDNA using SNAI2-specific primers as previously reported (18).

Reverse transcription PCR

Total RNA from cultured cells was extracted using the RNeasy Plus Mini Kit (Qiagen). Total RNA (0.8μg) was reverse transcribed in a 20 μL reaction using iScript (Bio-Rad). Real-Time PCR was performed with 5 μL of a 1:16 dilution cDNA for cell line samples using the UPL monocomode probes in the Roche LightCycler 480 machine (Roche Diagnostics). The conditions for all genes were preincubation at 95°C for 10 minutes, followed by 55 cycles of denaturation at 95°C for 15 seconds and amplification/extension at 60°C for 30 seconds; after cycle completion, cooling was held for 30 seconds at 40°C. Triplicate reactions were run for each cDNA sample. Data were normalized to GAPDH and confirmed with biologic replicate samples. Sequences for gene-specific primers and probe numbers are provided in Table 1.

MicroRNA analysis

RNA was extracted as described above using the RNeasy Plus Mini Kit from Qiagen. Total RNA (100 ng) was reverse transcribed using miR145 or RNU6B-specific primers and the Applied Biosystems reverse transcription kit per the manufacturer’s instructions. Real-time PCR (qPCR) was performed with 1 μL of reverse-transcribed cDNA using the TaqMan Assay from Applied Biosystems as per the manufacturer’s instructions on the Roche LightCycler 480. Data were normalized to RNU6B as described above.

Transwell migration and invasion assay

Cells were seeded into the upper chamber of a Transwell insert precoated with 5 μg/mL fibronectin for
migration or a BD Matrigel invasion chamber for invasion in serum-free medium at a density of 50,000 cells per well (24-well insert; 8-μm pore size; BD Biosciences). A medium containing 10% FBS was placed in the lower chamber to act as a chemoattractant, and cells were further incubated for the indicated time points. Nonmigratory cells were removed from the upper chamber. The migratory cells remaining on the lower surface of the insert were stained using Diff-Quick dye (Dade Behring, Inc.). Cells were enumerated as the average number of cells counted in 5 random microscope fields in 3 independent inserts.

Morphologic and immunofluorescence analysis

Cells were seeded and grown as monolayers onto sterile, confocal glass coverslip culture dishes (35 mm; MatTek Corp.), coated with 5 μg/mL fibronectin, and allowed to attach overnight. Cells then were fixed for 10 minutes with 3.7% formaldehyde, washed 3 times with 1× PBS, permeabilized with 0.1% Triton X-100, and blocked in 2% BSA in 1× PBS for 1 hour at room temperature. E-Cadherin primary antibody was incubated overnight at 4°C with 1% trichloroacetic acid (TCA) followed with staining at room temperature for 30 minutes in 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. After 4 washes with 1% acetic acid, the protein-bound dye was extracted with 10 mmol/L unbuffered Tris base (19). The plates were read at 560 nm. The half maximal inhibitory concentration (IC50) values were calculated by nonlinear regression analysis using GraphPad Prism version 5.0 software (GraphPad Software, Inc.).

Cell viability/trypan blue assay

In the experiments assessing the impact of miR145 on chemotherapy sensitivity, cells (1 × 105) were initially seeded in 12-well plates. After 24-hour incubation, cells were transfected with miR145 and scrambled control plasmid DNA, using X-tremeGENE HP DNA transfection reagent (Roche). At 24 hours posttransfection, cells were incubated with 5FU or vehicle alone. At 72 hours post-5FU treatment, cells were trypsinized, stained with trypan blue, and counted using a hemocytometer.

Sphere-forming assay

The ability to form spheres in 96-well ultra-low-attachment plate was evaluated as described previously (10). The culture medium consisted of DMEM/F12 supplemented with 1× B27 (Life Technologies), 20 ng/mL EGF, and 20 ng/mL basic FGF (Invitrogen) and penicillin-streptomycin. After 7 days of incubation, the total number of spheres greater than 50 μm in diameter was quantified by counting under light microscopy.

Luciferase promoter assay

The luciferase reporter plasmid containing the putative 1.4-kb miR145 promoter in pGL3 basic vector (Promega) was generously provided by Dr. Yin-Yuan Mo (University of Mississippi, Oxford, Mississippi; ref. 20). Luciferase assays were carried out in DLD/SNAI2, HCT/SNAI2, and empty vector stably transfected cells. Cells were transfected with miR145 promoter and Renilla luciferase plasmids in 12-well plates. The cells were lysed for luciferase assay 48 hours after transfection. Luciferase assays were performed using dual luciferase assay kit (Promega) according to the manufacturer’s protocol.

Patients

We identified 15 patients with T3-T4 and/or N1 primary rectal cancers treated with neoadjuvant chemoradiation therapy from the Hollings Cancer Center (HCC;
Medical University of South Carolina, Charleston, SC tumor registry after obtaining Institutional Review Board (IRB) approval. From the medical records, we obtained patient demographics, staging procedures, and treatment strategies. Treatment response was evaluated and graded by pathology as standard procedure. Tumor regression grading was used to quantitate response to therapy (21).

Tissue samples and pathologic evaluation
Pre-treatment rectal cancer biopsies were obtained from the HCC biorepository. For optimal tissue sampling, our gastrointestinal pathologist examined the available paraffin-embedded tumor blocks and evaluated specimens for viable tumor and necrosis. Thick 10-um sections were obtained from the identified tumor sections with the most representative viable tumor. An additional hematoxylin and eosin (H&E)-stained slide was obtained adjacent to the analyzed section and examined by our pathologist to confirm the presence of adequate tumor tissue for analysis. RNA was extracted from the patient tumor samples by TRIzol (Invitrogen). RNA was processed for miR145 and RNU6B as described above.

Statistical analysis
Statistical analyses were performed using the Student t test for paired data. P < 0.05 was considered significant. The patient data were analyzed using Graph Pad Prism Software.

Results
5FU-resistant DLD1 colon cancer cell line display EMT-related phenotypes and enhanced migration and invasion
The 5FU-resistant DLD1 (5FUr DLD1) cells demonstrated a >100-fold increase in 5FU resistance as compared with parental DLD1 cells (Fig. 1A). Phase-contrast microscopy revealed a marked altered cellular morphology in the 5FUr DLD1 cells with spindle shape, pseudopodia, and intercellular space/scattering, suggesting the loss of cell–cell adhesions in the 5FUr DLD1 cells as compared with parental DLD1 cells (Fig. 1B). These changes were suggestive of an EMT-like phenotype and implied that the resistant cells had transitioned to a mesenchymal state. On the basis of these observations, we performed chemokinese migration and invasion assays with Boyden transwell migration chambers using 10% FBS as a chemoattractant. At 10 hours after plating, the 5FUr DLD1 cells demonstrated significantly greater cell migration than parental DLD1 cells (Fig. 1C, 1.7-fold; P = 0.013). Similarly, the 5FUr DLD1 cells also demonstrated increased invasion at 24 (4.2-fold) and 48 hours (3.4-fold) after plating relative to the parental DLD1 cells (Fig. 1D, P < 0.001). Over this same time period, there was no difference in cell proliferation between the parental and 5FUr DLD1 cells (data not shown).

Altered expression of EMT markers in 5FU-resistant DLD1 cells
Decreased E-cadherin expression and loss of cell surface membrane-localized E-cadherin are fundamental changes observed with EMT (3, 4, 22). On the basis of the morphologic changes observed in the 5FUr DLD1 cells, we hypothesized that E-cadherin expression would be reduced in the 5FU-resistant cells. Western blot analysis indicated that the 5FUr DLD1 cells expressed reduced E-cadherin protein relative to parental DLD1 cells (Fig. 2A). Immunofluorescence revealed that 5FUr DLD1 cells had both decreased E-cadherin expression and loss of cell surface membrane-bound E-cadherin as compared with parental DLD1 cells (Fig. 2B). The EMT alterations observed in the 5FUr DLD1 colon cancer cells are consistent with prior investigations in pancreatic cancer cells and other colon cancer cells with acquired 5FU chemotherapy resistance, demonstrating loss of an epithelial cell phenotype and a gene expression pattern more consistent with mesenchymal cells (23, 24).

Pathways leading to EMT are regulated by a family of transcriptional factors including SNAI1, Zeb1, and SNAI2, which directly inhibit E-cadherin transcription (3, 4). To explore further the molecular mechanism driving the changes in 5FUr DLD1 cells, we investigated the expression of E-cadherin, SNAI1, SNAI2, and Zeb1 by qPCR (Fig. 2C). Confirming the reduced E-cadherin protein expression demonstrated in the 5FUr DLD1 cells, E-cadherin mRNA was significantly reduced by about 60% in 5FUr DLD1 cells as compared with the parental cells (Fig. 2C). Zeb1 expression was not significantly different from the parental cells, and SNAI1 expression was reduced (P < 0.001). However, the 5FUr DLD1 cells demonstrated a 2.7-fold increase in SNAI2 expression relative to the parental cells (P < 0.01), suggesting a possible molecular mechanism contributing to the observed changes in E-cadherin expression in 5FU-resistant cells.

5FU sensitivity correlates with expression of EMT markers and miR145
To determine whether the EMT status of colorectal cancer cell lines is related to the response to 5FU treatment, we performed Western blot analysis for E-cadherin, vimentin, and SNAI2 in a panel of colorectal cancer cell lines (Fig. 3A). We observed reduced levels of E-cadherin protein and an increase in both vimentin and SNAI2 protein (and RNA) expression in the SW620 cell line as compared with the other colorectal cancer cell lines (Fig. 3A and B; ref. 25). The increased levels of SNAI2 and vimentin combined with the decrease in E-cadherin in the SW620 cell line correlated with increased resistance to 5FU treatment as compared with the other cell lines tested (Fig. 3C), suggesting that EMT features are associated with resistance to 5FU chemotherapy in colorectal cancer cells.

miRNAs have been shown to play a pivotal role in colorectal cancer, and miR145 has been previously linked with 5FU sensitivity and inversely correlated with EMT
Therefore, we measured levels of miR145 expression in our panel of colorectal cancer cell lines to assess the relationship between SNAI2, miR145 and 5FU chemosensitivity. We observed that miR145 levels were significantly reduced in the SW620 (5FU-resistant) cells that demonstrated high SNAI2 expression than in DLD1 and HCT116 cell lines (Fig. 3D), suggesting miR145 as a mediator of 5FU sensitivity in these cells. The HCT116 cell line was the most 5FU-sensitive cell line assessed and expressed the highest level of miR145, further supporting the correlation of miR145 expression and 5FU sensitivity.

SNAI2 impairs response to 5FU chemotherapy and induces tumor-initiating cell properties

On the basis of the association of EMT with 5FU resistance and our observation of increased SNAI2 expression in the 5FU DLD1 cells, we investigated SNAI2 as a direct mediator of colorectal cancer chemotherapy (5FU) sensitivity. Previously, our research group established that the SNAI2-overexpressing DLD1 (DLD1/SNAI2) cells demonstrated molecular changes and phenotype consistent with enhanced EMT (18). In addition, we established an SNAI2-overexpressing HCT116 (HCT/SNAI2) cell line to similarly assess 5FU sensitivity. To confirm SNAI2 overexpression, we demonstrated that SNAI2 expression was increased at the protein (Fig. 4A and B) in both SNAI2 overexpressing cell lines. We assessed 5FU chemotherapy sensitivity using the SRB cytotoxicity assay after 96 hours of 5FU treatment. The estimated IC50

Figure 1. 5FU-resistant DLD1 colorectal cancer cells have properties consistent with EMT. A, 5FU-resistant (5FUr DLD1; gray square) and parental DLD1 (black diamond) were treated with increasing concentrations of 5FU for 96 hours, and cell viability was assessed by SRB staining and reported as percentage viability. ***, $P < 0.001$ versus DLD1. B, representative bright-field confocal images of 5FUr DLD1 cells as compared with the parental DLD1 cell line, demonstrating increased spindle shape (fat arrow), pseudopodia (dashed arrow), and intercellular space/scattering (thin arrow) in the 5FUr DLD1 cells. C, quantification of chemotactic migration of 5FUr DLD1 and parental DLD1 cells. Columns, average number of cells migrating per high-powered field measured after 10 hours (black bars) and 24 hours (gray bars). **, $P < 0.05$ versus DLD1. D, quantification of chemotactic invasion of 5FUr DLD1 and parental DLD1 cells. Columns, average number of cells invading per high-powered field measured after 24 hours (black bars) and 48 hours (gray bars). ***, $P < 0.001$ versus DLD1.
for the empty DLD1 cells was 4.3 μmol/L as compared with 339.7 μmol/L for the DLD1/SNAI2 cells (Fig. 4C), providing further support for the notion that increased expression of SNAI2 contributes to 5FU resistance in colorectal cancer cells. Similarly, the HCT/SNAI2 cells demonstrated resistance to 5FU cytotoxicity (Fig. 4D).

Recent investigations have highlighted an association between EMT alterations and induced CSC-like properties (10, 13). Furthermore, reports have demonstrated that this CSC-like population, also termed tumor-initiating cells, is resistant to therapy (12, 15, 16). We investigated the ability of SNAI2 to increase CSC-like properties as a potential mechanism contributing to chemotherapy resistance. Prior studies have demonstrated the ability of tumor-initiating cells to establish clonal spheroid formation in low-adherence conditions without serum (26). Compared with empty vector control cells, DLD/SNAI2 and HCT116/SNAI2 cells increased spheroid formation,
suggesting that SNAI2 enhances the tumor-initiating cell population (17.4% ± 4.3% vs. 6.9% ± 4.8% in HCT116/SNAI2 vs. EV, P < 0.05; 80.5% ± 6.2% vs. 47.5% ± 5.7% in DLD1/SNAI2 vs. EV, P < 0.05; Fig. 4E and F).

SNAI2 represses miR145 promoter activity and miR145 expression in colorectal cancer cells

On the basis of the inverse correlation between the expression of miR145 and SNAI2 in the parental colorectal cancer panel of cell lines and the previous reports demonstrating an association between miR145 expression and 5FU sensitivity (Fig. 3A and D; ref. 11), we decided to investigate SNAI2-mediated negative regulation of miR145 as a potential mechanism associated with 5FU resistance. Expression of SNAI2 mRNA was confirmed in the 2 SNAI2-overexpressing cell lines (Fig. 5A and B). The DLD1/SNAI2 cell demonstrated a significant decrease (64%) in miR145 expression when compared with the empty vector control cells (Fig. 5C). Similarly, miR145 expression was reduced in the HCT/SNAI2 cell pools [HCT/SNAI2 (1) and HCT/SNAI2 (2)] by 72% and 55%, respectively, when compared with the empty vector control cells (Fig. 5D). In the HCT116/SNAI2 cell pools, the relative reduction in miR145 expression correlated with degree of ectopic SNAI2 expression in the 2 HCT/SNAI2 cell pools that were assessed, supporting evidence of a molecular repression of miR145 by SNAI2 (Fig. 5B and D). All other experiments involving the HCT/SNAI2 cells were performed only with the highest SNAI2-expressing cell pool [HCT/SNAI2 (1)].
In the miR145 promoter region, we identified the SNAI2 consensus binding site core sequence CA(C/G)(C/G)TG with the upstream CT-rich region (Fig. 5E; refs. 3, 4). To determine whether SNAI2 represses the miR145 promoter, we transfected the ectopic SNAI2-expressing colorectal cancer cell lines with the luciferase reporter plasmid containing the putative miR145 promoter (Fig. 5F; ref. 27). In both the DLD/SNAI2 and HCT/SNAI2 cell lines, miR145 promoter activity was suppressed (by 38% and 60%, respectively) when compared with empty vector–transfected controls (Fig. 5G and H).

Prior reports have demonstrated that miR145 directly targets critical stem cell transcription factors (28–30). We decided to further explore the inverse relationship between SNAI2 and miR145 by investigating the expression of established miR145 target stem cell transcription factors including nanog, myc, Klf4, Sox2, and Oct4 (28–30). Initially, we examined expression in the colorectal cancer cell panel and observed that of the factors examined, nanog and myc, seemed to be more highly expressed in the aggressive SW620 (high SNAI2 expression) cell line when compared with the other cell lines (Supplementary Fig. S1). We also observed an increase in nanog in the 5FUr DLD1 when compared with the parental DLD1 cells (Fig. 2C) and in the DLD1/SNAI2 cells when compared with the empty vector controls (Fig. 4A). In the HCT116/SNAI2 cells, myc was significantly increased as compared with control cells (Fig. 4B). The findings of both increased spheroid formation and expression of CSC transcription factors in the ectopic SNAI2-expressing cell lines support
the correlation between SNAI2 expression and increased tumor-initiating population.

**Inhibition of SNAI2 restores miR145 expression and sensitivity to 5FU**

We assessed the impact of SNAI2 silencing on miR145 expression and 5FU sensitivity using short hairpin directed toward SNAI2 (shSNAI2) in the SW620 cell line (Fig. 6A). As opposed to the ectopic expression of SNAI2, SNAI2 inhibition resulted in an about 1.5-fold increased miR145 expression (Fig. 6B). In addition, we observed by Western blotting that the inhibition of SNAI2 decreased vimentin (Fig. 6C). As opposed to the increase in CSC transcription factors observed with ectopic SNAI2 expression, SW620 cells transfected with shSNAI2 demonstrated decreased expression of nanog and myc (Fig. 6C). Along with the observed molecular changes with shSNAI2, we demonstrated an increased sensitivity to 5FU in the SW620 cells with inhibition of SNAI2, when compared with the scrambled control (Fig. 6D).

**miR145 expression restores sensitivity to 5FU and augments CSC transcription factor expression**

miRNAs are currently very attractive as novel therapeutics and are being actively pursued in clinical development; therefore, we sought to determine whether...
miR145 could reverse the effects of SNAI2 expression. To assess whether the SNAI2:miR145 axis is functional in our cell model and to determine whether expression of miR145 can diminish the SNAI2-mediated resistance to 5FU chemotherapy, we transiently overexpressed miR145 in our DLD1/SNAI2 cells using an miR145 expression plasmid (Fig. 7A). In a cell viability assay, we observed an increased sensitivity to 5FU in the DLD/SNAI2 cells transfected with miR145, as compared with scrambled control DLD/SNAI2 cells treated with 5FU (Fig. 7B). Compared with the scrambled control-transfected DLD1/SNAI2 cells, miR145 transfection enhanced 5FU cytotoxicity as demonstrated by an additional 26.4% reduction in cell viability. In addition, we observed that transfection of miR145 alone, in the absence of 5FU treatment, resulted in significantly decreased DLD/SNAI2 cell survival (Fig. 7B). This is similar to that observed by other groups who showed that miR145 reduced cellular proliferation and tumor growth (31).

In the 5FU-resistant parental SW620 cells with high SNAI2 expression, transient transfection of miR145 (Supplementary Fig. S2A) resulted in a decrease in vimentin protein and SNAI2 mRNA expression (Fig. 7C and Supplementary Fig. S2B). Furthermore, similar to SNAI2 inhibition, miR145 downregulated expression of the stem cell transcription factors including nanog and myc in the SW620 cells (Fig. 7C and Supplementary Fig. S2B). Importantly, the expression of miR145 in SW620 resulted in an increase in sensitivity to 5FU when compared with scrambled control cells (Fig. 7D), similar to the effect observed in the miR145-transfected DLD/SNAI2 cells (Fig. 7B).

Given the relative 5FU resistance observed in the HT29 cells, we elected to assess the impact of miR145 restoration in this cell line (Supplementary Fig. S2C). Similar to the molecular changes observed in the SW620 cells, miR145 restoration resulted in an about 75% and 65% reduction in nanog and myc, respectively (Fig. 7E). miR145 restoration also increased HT29 5FU sensitivity as demonstrated by an additional 32% reduction in cell viability (Fig. 7F). These findings suggest a similar molecular and functional downstream effect of miR145 restoration even in the absence of SNAI2 expression.
miR145 expression predicts response to chemoradiation in human colorectal cancer tumor samples

Patients with stage II and III rectal cancer are routinely treated with neoadjuvant 5FU-based chemoradiation to downstage tumors before surgery. Tumor regression grading as an assessment of treatment response is an effective surrogate marker of long-term survival and recently was demonstrated as an effective benchmark for oncologic outcomes in this subset of patients (2, 21, 32). To assess whether miR145 would serve as a biomarker of response in these patients, we extracted RNA from 15 pretreatment rectal cancer biopsy specimens. Using pathologic tumor regression grade, we compared tumors demonstrating greater than 50% response (grades III and IV) with tumors demonstrating less than 50% response (grades I and II). We observed significantly higher levels of miR145 in patients who responded well to treatment, as compared with patients who demonstrated a poor response to therapy (Fig. 8A). Together, these data support the existence of an SNAI2:miR145 axis as a mechanism of therapeutic response in patients with colorectal cancer (Fig. 8B) and provide a strong rationale for the development of an miR145-targeted therapeutic.

Discussion

Growing evidence has linked EMT with CSC properties, enhanced cancer cell survival, and resistance to conventional antineoplastic therapies (6, 10, 13, 15).
Although elevated expression of E-cadherin transcriptional mediators has been linked with chemotherapy resistance in colorectal cancer, only recently have EMT pathways been characterized as mediators of colon cancer chemotherapy resistance (10, 33). EMT pathways make attractive therapeutic molecular targets that have the potential to enhance current antineoplastic strategies and potentially target the tumor-initiating cells. However, the clinical effectiveness of targeting EMT pathways and transcription factors remains challenging. Defining the molecular mediators driving the therapeutic resistance associated with expression of EMT and CSC transcription factors may identify novel therapeutic targets.

Prior reports have demonstrated an inverse relationship between miR145 expression and EMT (8, 9, 28, 34), and in this report, we demonstrate a novel SNAI2:miR145 axis that mediates cancer cell survival associated with 5FU chemotherapy response in colorectal cancer. Previously, miR145 has not been described as a direct target of SNAI2. Our group demonstrated that SNAI2 repressed miR145 promoter activity and, thereby, inhibited miR145 expression. Ectopic expression of SNAI2 also significantly enhanced resistance to 5FU. Conversely, SNAI2 inhibition or miR145 replacement rescued sensitivity to 5FU in SNAI2-expressing cancer cells. We further demonstrated that miR145 expression is associated with neoadjuvant 5FU-based chemoradiation response in patients with rectal cancer, highlighting the clinical relevance of our findings. Collectively, our data highlight the potential of miR145 replacement as a novel molecular-targeted strategy to enhance current colorectal cancer therapy.

Associated with the impact on therapeutic response, the SNAI2:miR145 axis also influenced tumor-initiating cell properties and expression of critical stemness transcription factors, including nanog and myc. Nanog has proven to be central in maintaining both embryonic and CSC abilities, suggesting that nanog may serve as a key gatekeeper to maintaining pluripotency (35–39). Recently, investigators have reported a strong association with EMT-associated gene expression and CSCs (12, 13, 40). Interestingly, as opposed to SNAI2, which inhibited miR145 and upregulated nanog and myc, miR145 replacement suppressed expression of both nanog and myc. The ability of the SNAI2:miR145 axis to augment expression of stemness factors highlights a possible novel mechanism of action for SNAI2 and a further therapeutic rationale for miR145 replacement therapy.

Future studies will address the impact of the SNAI2:miR145 axis on CSCs and the functional properties associated with them.

The clinical implications of our findings suggest that our treatments may result in the development of more aggressive cancer cells, perhaps even generating CSCs as suggested by the development of EMT and the expression of nanog in the chronically treated 5FU colorectal cancer cells. In a recent report supporting the association between CSCs and resistance to therapy, a chemoresistant population of colorectal cancer cells expressed CSC markers and phenotype (15). As well, the presence of CSCs in human tumor specimens has correlated with a poor prognosis across many organ systems (12, 41, 42). The close relationship between expression of EMT-associated genes, repression of the tumor suppressor miR145, and CSCs may further fuel the motivation to develop EMT molecular targets.

Conclusions

In summary, the dynamic process of EMT serves to enhance tumor progression by increasing cellular mobility and invasion and improving cellular survival. SNAI2-mediated pathways may represent a novel clinical therapeutic target in colorectal cancer, as well as serve as a molecular predictor of response to chemoradiation therapy in colorectal cancer. In the future, identifying tumors with elevated EMT markers also may help dictate appropriate therapy. This is particularly applicable to neoadjuvant therapy for patients with advanced rectal cancer.
where individualized treatment strategies based on molecular markers may enhance current practices and outcomes.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: V.J. Findlay, K.F. Staveley O'Carroll, D.K. Watson, E.R. Camp

Development of methodology: V.J. Findlay, L.M. Nogueira, E.R. Camp

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc): V.J. Findlay, K. Hurst, E.R. Camp

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.J. Findlay, C. Wang, K. Hurst, K.F. Staveley O'Carroll, D.K. Watson, E.R. Camp

**Grant Support**

The study was supported by NIH grant 5R1K08CA142904 (E.R. Camp), Hollings Cancer Center translational Research Award (E.R. Camp), and American Cancer Society: Institutional Research Grant (E.R. Camp).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 10, 2014; revised July 24, 2014; accepted September 6, 2014; published OnlineFirst September 23, 2014.

**References**


Molecular Cancer Therapeutics

SNAI2 Modulates Colorectal Cancer 5-Fluorouracil Sensitivity through miR145 Repression


Mol Cancer Ther Published OnlineFirst September 23, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0207

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/09/24/1535-7163.MCT-14-0207.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.