

Down-regulation of SNAIL suppresses MIN mouse tumorigenesis: Modulation of apoptosis, proliferation, and fractal dimension

Hemant K. Roy,¹ Patrick Iversen,² John Hart,³ Yang Liu,⁴ Jennifer L. Koetsier,¹ Young Kim,⁴ Dhanajay P. Kunte,¹ Madhavi Madugula,¹ Vadim Backman,⁴ and Ramesh K. Wali¹

¹Department of Internal Medicine, Evanston Northwestern Healthcare Research Institute, Evanston, Illinois;

²AVI BioPharma, Corvallis, Oregon; ³Department of Pathology, University of Chicago Hospitals, Chicago, Illinois; and ⁴Biomedical Engineering Department, Northwestern University, Evanston, Illinois

Abstract

Objectives: Emerging evidence implicates the SNAIL family of transcriptional repressors in cancer development; however, the role of SNAIL in colorectal cancer has not been established. To investigate the importance of SNAIL in colorectal carcinogenesis, we examined the phenotypic and cellular consequences of SNAIL down-regulation in the MIN mouse. **Methods:** Twenty-eight male MIN mice were randomized to treatment with an antisense phosphorodiamidate morpholino oligomer (AS-PMO) to SNAIL, saline, or a scrambled sequence control for 6 weeks. Tumors were scored and the molecular/cellular effects of anti-SNAIL treatment were evaluated through immunohistochemical analysis of the uninvolved intestinal mucosa for SNAIL and E-cadherin levels along with rates of apoptosis and proliferation. Furthermore, microarchitectural alterations were determined through measurement of fractal dimension. **Results:** In the uninvolved mucosa, SNAIL AS-PMO treatment moderately decreased SNAIL protein when compared with saline-treated animals (immunohistochemistry scores 3.0 ± 0.8 versus 2.1 ± 0.6 , respectively; $P = 0.01$) with a concomitant increase in E-cadherin expression (1.8 ± 0.6 versus 2.4 ± 0.5 ; $P < 0.05$). Anti-SNAIL PMO, but not scramble control,

resulted in a significant decrease in both total tumor number and incidence of tumors > 2 mm (22% and 54%, respectively; $P < 0.05$). Furthermore, this was accompanied by an increased apoptosis rate (2-fold), decreased proliferation (3-fold), and normalization of the fractal dimension in the uninvolved intestinal mucosa. **Conclusions:** We show, for the first time, that SNAIL overexpression is important in intestinal tumorigenesis. While this PMO regimen afforded modest SNAIL suppression and hence tumor reduction, this provides compelling evidence for the role of SNAIL overexpression in colonic neoplasia. [Mol Cancer Ther 2004;3(9):1159–65]

Introduction

Significant progress has been made in understanding the molecular pathogenesis of colorectal cancer. Stepwise inactivation of tumor suppressor genes is critical for the progression through the stages of colon carcinogenesis. For instance, mutational events such as truncation in the adenomatous polyposis coli (APC) protein initiate most colorectal cancers, whereas p53 mutations frequently govern the transition from the large adenomatous polyp to an invasive carcinoma (1). On the other hand, epigenetic silencing is responsible for the loss of several important tumor suppressor genes including *E-cadherin*, *p16INKa*, and *hMLH-1* (2). This frequently occurs at the transcriptional level, most commonly either by promoter hypermethylation or through elaboration of transcriptional suppressor proteins (2). Recently, the SNAIL family of transcriptional repressors has been shown to be overexpressed in a wide variety of human malignancies including breast (3), hepatocellular (4), gastric (5), skin (6), and oral (7) carcinomas. This family of proteins (which includes SNAIL, SLUG, and SNAIL-3) bind E-boxes, motifs found in the promoters of many tumor suppressor genes (8).

Although the role of SNAIL in many malignancies is well established, studies on colon carcinogenesis are minimal. Several reports suggest that SNAIL message is increased in most human colorectal cancer cell lines (9). This is supported by our preliminary report that SNAIL immunoreactivity was detected in 78% of human colorectal cancer (10). Although the mechanisms of SNAIL overexpression remain unclear, several molecular events known to increase SNAIL levels (e.g., AKT activation and epidermal growth factor signaling; refs. 11, 12) have been shown to occur early during colon carcinogenesis (13, 14). Taken together, this provides strong evidence that SNAIL is up-regulated during neoplastic transformation of the colon. However, the biological significance of SNAIL overexpression in colorectal cancer remains to be determined. Several tumor

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Requests for reprints: Hemant K. Roy, Department of Internal Medicine, Evanston Northwestern Healthcare Research Institute, 1001 University Place, Evanston, IL 60201. Phone: 847-570-7683; Fax: 847-570-8011. E-mail: h-roy@northwestern.edu

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suppressor genes important in colon carcinogenesis (*APC*, *p53*, and *E-cadherin*) contain E-boxes and thus could be susceptible to the effects of SNAIL overexpression (15, 16). Suppression of E-cadherin expression has been used as a prototypical marker of SNAIL effect (9). E-cadherin loss has been implicated in both the initiation and the promotional phases of colon carcinogenesis. Previous studies on mechanisms of E-cadherin loss in colorectal cancer have focused exclusively on promoter hypermethylation and have not addressed potential contribution of SNAIL overexpression (17). In breast cancers, SNAIL overexpression can provide either a synergistic or a complementary mechanism for E-cadherin suppression (3, 18). However, to date, no studies have shown the biological significance of SNAIL in colorectal cancer.

To elucidate the role of SNAIL in colon carcinogenesis, we investigated the consequences of SNAIL inhibition on the phenotype of MIN mouse, a well-validated model of colonic neoplasia (19). This model spontaneously develops intestinal adenomas (predominantly small bowel) as a result of germ line truncation in *APC* (which is also the initiating genetic event in most human colorectal cancers). For these experiments, we used antisense phosphorodiamidate morpholino oligomer (AS-PMO), which selectively inhibits SNAIL protein translation. Our primary end point was tumor number. Additionally, we evaluated intermediate biomarkers of colon carcinogenesis including, from a cellular perspective, intestinal epithelial apoptosis and proliferation along with the microarchitectural variable, fractal dimension.

Materials and Methods

Phosphorodiamidate Morpholino Oligomer

PMOs were designed/manufactured by AVI BioPharma (Corvallis, OR; ref. 20). A 19-bp sequence that corresponded to the mouse *SNAIL* gene AUG translation start site was targeted (5'-GGAGCGCGGCATGGCC-3'). Purity was >95% as determined by reverse-phase high-performance liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. The scrambled sequence was 5'-GTCGTAGATCGCTATCTGACC-3'.

MIN Mice

All animal studies were done in accordance with the Institutional Animal Care and Use Committee of Evanston Northwestern Healthcare Research Institute (Evanston, IL). Our primary focus was to establish whether SNAIL PMO (10 µg/g body weight i.p.) would alter tumor number when compared with saline controls. To document that the alteration of the tumor number by the SNAIL AS-PMO was attributable to SNAIL inhibition and not to nonspecific effects of the PMO, we treated MIN mice with a PMO of equal length but random sequence (scramble control) and compared tumor number versus saline controls. A total of 28 male 9- to 10-week-old C57BL6 *APC*^{min} mice (The Jackson Laboratory, Bar Harbor, ME) were randomized to the treatment groups as above. Animals received daily in-

jections five times a week for 6 weeks. Two hours prior to sacrifice, these mice (~15 weeks old) received BrdUrd (50 mg/kg) to label cells in S phase. Animals were euthanized and intestines were removed, rinsed with PBS, and examined under magnification by three independent observers blinded to treatment group. Aliquots were fixed in formalin and embedded in paraffin for immunohistochemical analysis.

Immunohistochemistry

Formalin-fixed uninjured small intestinal tissues from MIN mice were examined by immunohistochemistry to determine cellular and molecular consequences of SNAIL inhibition. We focused exclusively on the small bowel mucosa given the predilection for neoplasia at this site (>90% of adenomas in the small intestine). Paraffin-embedded sections (4 µm), mounted on Vectabond-coated Superfrost slides (Vector Laboratories, Burlingame, CA), were deparaffinized by heating at 60°C for 30 minutes, hydrated by a graded series of ethanol rinses, and washed in PBS. Unmasking of the antigen epitope was accomplished by pressure microwaving (NordicWare, Minneapolis, MN) the sections in an antigen unmasking solution (Vector Laboratories) for 15 minutes. Endogenous peroxidase activity was quenched by 1% H₂O₂ and nonspecific binding was blocked with 5% horse serum in PBS. Sections were incubated with the appropriate primary antibody overnight at 4°C at dilutions of 1:50 for anti-BrdUrd (Zymed, San Francisco, CA), 1:50 for anti-cleaved caspase 3 (Asp¹⁷⁵, Cell Signaling Technology, Beverly, MA), and 1:100 for anti-E-cadherin and anti-SNAIL (both from Santa Cruz Biotechnology, Santa Cruz, CA). After washing in PBS, the samples were incubated with biotinylated secondary antibodies followed by development of avidin-biotin peroxidase complex according to the manufacturer's recommendations (using the Vectastain Elite ABC kit, Vector Laboratories). For the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) apoptosis assay, we used the ApopTag assay kit (Intergen Co., Purchase, NY) according to the manufacturer's directions as we have reported previously (21). All slides were scored by a gastrointestinal pathologist blinded to treatment group with the SNAIL/E-cadherin scored on a four-point scale as described previously (13), whereas the TUNEL/BrdUrd/activated caspase 3 were quantitated as percentage of epithelial cells positive from ~10 villi per section.

Fractal Dimension

Fractal dimension can be used to characterize tissue organization (22–24). It has been shown that fractal dimension of tissue microarchitecture changes early in the process of carcinogenesis (19). Fractal dimension was assessed on fresh intestinal tissue (within 1 hour of sacrifice) through four-dimensional light-scattering fingerprinting. Our advanced light-scattering instrument used to obtain four-dimensional light-scattering fingerprinting readings has been described previously in detail (25, 26). Specifically, fractal dimension was calculated through evaluation of the angular distributions ($\Delta I(\theta)$ at 550 nm) of the scattered light and subjected to Fourier transformation.

This yielded the two-point mass density correlation function $C(r) = \rho(r)\rho(r' + r)$, where $\rho(r)$ is a local mass density at point r , which is proportional to the concentration of intracellular solids, such as proteins, lipids, and DNA (24, 26, 27). Thus, mass correlation between local tissue regions separated by distance r is quantified by $C(r)$. For example, in a perfect solid, $C(r)$ is a constant. On the other hand, for an object composed of randomly distributed material, $C(r)$ vanishes rapidly with distance. We found that $C(r)$ closely approximated a power law for r ranging from ~ 1 to $50 \mu\text{m}$. This power law density correlation functions is characteristic of a fractal-like organization. The general form of such $C(r)$ is r^{D-3} , where D is called fractal dimension (23). We obtained D from the linear slopes of $C(r)$ in the linear regions of log-log scale.

Results

SNAIL AS-PMO Down-Regulated SNAIL Protein and Increased E-Cadherin in the Uninvolved Mucosa

We first evaluated the ability of SNAIL AS-PMO to suppress SNAIL protein translation in the uninvolved small intestinal epithelium by immunohistochemistry. We noted that SNAIL AS-PMO caused a modest but significant decrease in SNAIL protein levels (Fig. 1A). Next, we evaluated E-cadherin levels as a marker of the functional consequences of SNAIL inhibition. There was a notable increase in E-cadherin immunoreactivity with the SNAIL AS-PMO treatment (Fig. 1B). Moreover, the magnitude of

the induction of E-cadherin (1.8 ± 0.6 in control to 2.4 ± 0.5 in the SNAIL AS-PMO; $P = 0.03$) corresponded well with SNAIL inhibition (3.0 ± 0.8 in control to 2.1 ± 0.6 in the SNAIL AS-PMO; $P = 0.01$; Fig. 1C).

SNAIL PMO Decreases Intestinal Tumorigenesis in the MIN Mouse

The total intestinal tumor number, the most robust variable of neoplastic risk, was significantly decreased in MIN mice treated with PMO when compared with saline-treated mice (20.1 ± 4.6 versus 25.9 ± 3.0 , respectively; $P < 0.05$) as shown in Fig. 2. This was most pronounced in the proximal small bowel and colon. Tumor size was not rigorously measured; however, we noted that a higher proportion of mice in the control group harbored tumors >2 mm in diameter when compared with animals treated with SNAIL AS-PMO (83% versus 38%, respectively; $P < 0.05$). Treatment of MIN mice with scramble control failed to alter tumor number when compared with matched saline-treated animals, arguing that the SNAIL AS-PMO inhibition of tumorigenesis was attributable to specifically decreasing SNAIL protein levels. Furthermore, SNAIL AS-PMO had a highly statistically significant decrease in tumors when compared with scramble PMO ($P < 0.005$).

SNAIL PMO Inhibited Proliferation and Induced Apoptosis

Given the importance of the induction of proliferation and inhibition of apoptosis in regulating neoplastic transformation, we investigated the effect of SNAIL AS-PMO on these critical cellular variables. Proliferation was assayed through immunohistochemical determination of BrdUrd

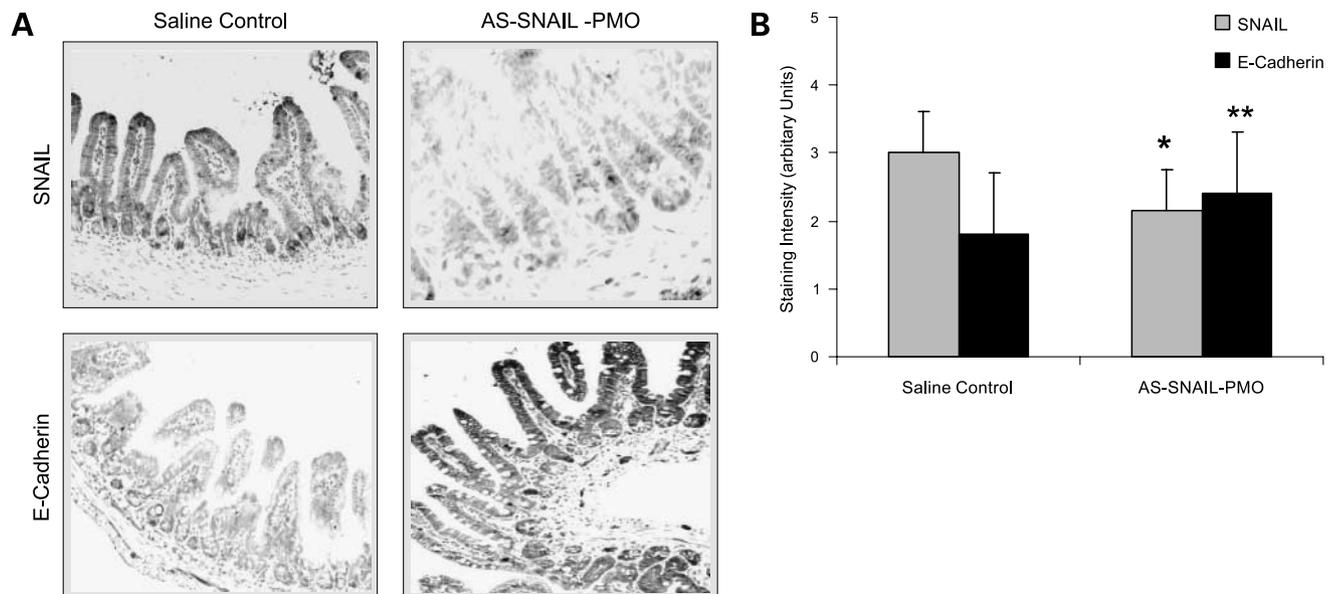


Figure 1. Effect of antisense SNAIL oligonucleotide (AS-SNAIL-PMO) on SNAIL and E-cadherin protein levels in the MIN mice. MIN mice were treated with saline or antisense SNAIL oligonucleotide and subjected to immunohistochemical analysis as described in Materials and Methods. **A**, representative immunohistochemistry. *Top panels*, SNAIL expression in intestinal tissue from control MIN mice in comparison with MIN mice treated with SNAIL AS-PMO. *Bottom panels*, representative sections probed with an anti-E-cadherin antibody in the two groups. Negative controls (identically treated sections, except for omission of primary antibody incubation) manifested no immunoreactivity (data not shown). **B**, immunohistochemical scoring. Compared with control, SNAIL AS-PMO therapy caused a significant decrease in SNAIL expression (*, $P = 0.01$) with a corresponding increase in E-cadherin expression (**, $P = 0.03$). *Columns*, mean ($n = 12$); *bars*, SD.

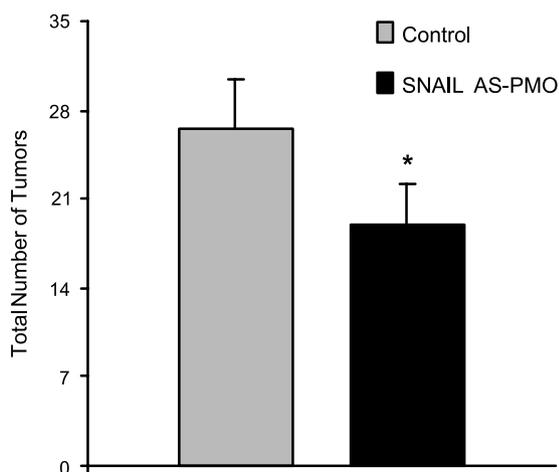


Figure 2. Effect of SNAIL inhibition on the intestinal tumorigenesis in the MIN mouse. Treatment with SNAIL AS-PMO for 6 weeks (see Materials and Methods) resulted in a 22% reduction in intestinal tumor number in 14–15-week-old MIN mice. Columns, mean ($n = 22$); bars, SD. *, $P < 0.05$ compared with control saline-treated mice. There were 30.7 ± 4.5 tumors per scramble PMO-treated animals that did not significantly differ from their matched saline-treated control but was markedly greater than SNAIL AS-PMO-treated mice ($P < 0.005$).

incorporation. BrdUrd is nucleotide analogue that gets incorporated in the DNA that is being synthesized, thus labeling proliferating cells. Pronounced nuclear staining was noted in $18.1 \pm 5.5\%$ of the villus cells in the saline-treated MIN mouse (Fig. 3A). This was dramatically decreased to $5.1 \pm 2.7\%$ in MIN mice that received that anti-SNAIL PMO treatment (Fig. 3B). There was a corre-

sponding decrease in the proliferative zone, defined as a ratio of the height of labeled cell to total villus height (from $52.2 \pm 10.3\%$ in saline-treated animals versus $32.4 \pm 10.4\%$ in mice treated with SNAIL AS-PMO; $P < 0.05$; Fig. 3B). Comparable alterations were also noted in the crypts of the small intestine (data not shown).

We next evaluated apoptosis in the uninvolved small bowel through assessment of cleaved caspase 3 expression, the most accurate immunohistochemical technique for evaluating apoptosis in the small intestine (28). We noted prominent cytoplasmic staining in the small bowel in MIN mice (Fig. 4A). In MIN mice randomized to anti-SNAIL PMO, there was a marked increase (doubling) in number of cells expressing this marker of apoptosis (Fig. 4B). This was confirmed by a TUNEL assay, which showed that treatment with SNAIL AS-PMO resulted in a marked increase in nuclear staining consonant with the DNA fragmentation of apoptosis (3.6 ± 1.6 versus 5.2 ± 1.4 ; $P < 0.05$; Fig. 4A and B).

SNAIL AS-PMO Normalizes Intestinal Fractal Dimension

To assess microarchitectural consequence of SNAIL inhibition, we measured fractal dimension, a robust well-validated early harbinger of neoplastic transformation in the colon (29). Our group has reported previously that fractal dimension of the colonic uninvolved mucosa increases early in experimental colon carcinogenesis, preceding all previous conventional biomarkers of colorectal cancer (25). As shown in Fig. 5, fractal dimension of the uninvolved mucosa was evaluated using four-dimensional light-scattering fingerprinting analysis. When MIN mice were compared with age-matched C57BL6 mice that were wild-type

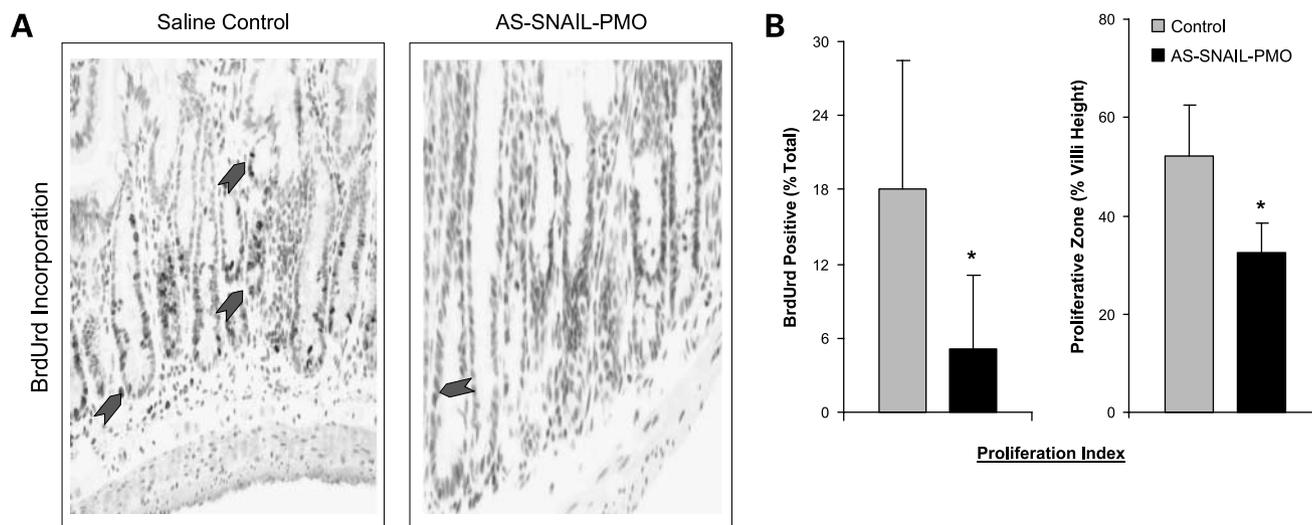


Figure 3. Suppression of uninvolved mucosal cellular proliferation by SNAIL AS-PMO. Epithelial proliferation was measured by BrdUrd incorporation. The antigen labeling was expressed as percentage of positive epithelial cells per whole villi. **A**, representative immunohistochemistry. Prominent nuclear BrdUrd staining was noted in epithelial cells. Specificity was assured by the lack of detectable immunoreactivity on negative controls (identically treated sections, except for omission of primary antibody incubation; data not shown). **B**, quantitation of immunohistochemical scores showing that treatment with SNAIL AS-PMO resulted in a significant decrease in both proliferating cell number and a corresponding decrease in the proliferative zone, defined as a ratio of the height of labeled cell to total villus height. Columns, mean ($n = 16$); bars, SD. *, $P < 0.05$.

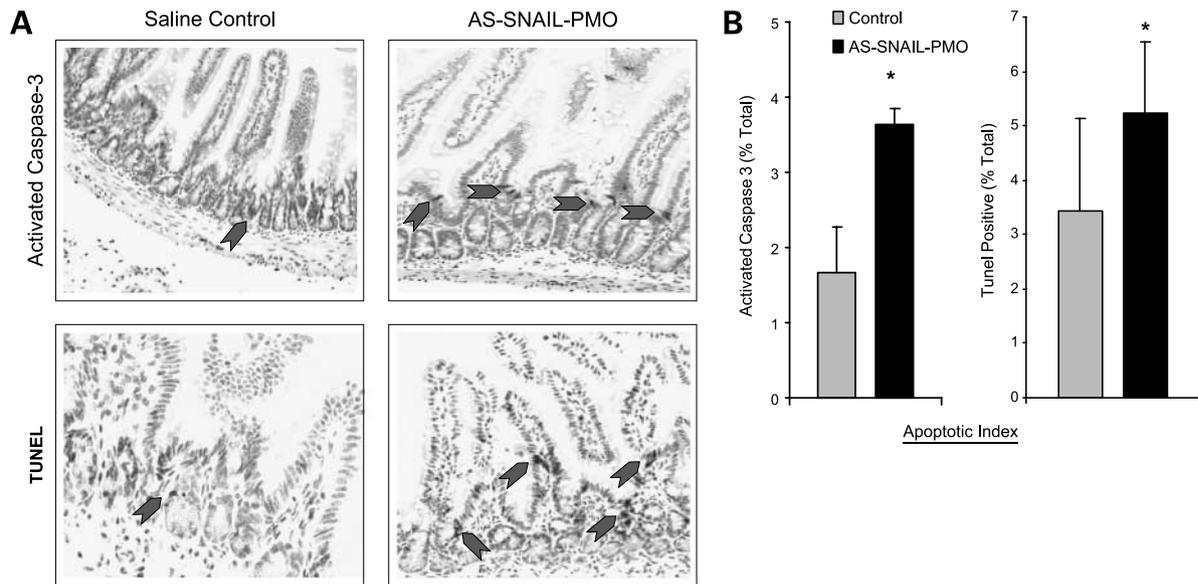


Figure 4. Antisense SNAIL oligonucleotide (AS-SNAIL-PMO) treatment resulted in increase apoptosis as gauged by cleaved caspase 3 immunohistochemistry and TUNEL assay. **A**, representative sections evaluated for cleaved caspase 3 and TUNEL. Cleaved caspase 3 was predominantly cytoplasmic staining, whereas positive TUNEL cells were characterized by nuclear staining (arrows). **B**, scoring of caspase 3 and TUNEL staining indicated that treatment with the SNAIL AS-PMO significantly increased epithelial apoptosis in the uninvolved small intestine as determined by both methodologies. Columns, mean ($n = 10$); bars, SD. *, $P < 0.05$.

at the APC locus, there was a dramatic increase in the fractal dimension noted ($P < 0.00001$). Treatment with anti-SNAIL PMO suppressed this elevated fractal dimension of MIN mice to values approaching that in wild-type mice, suggesting normalization of microarchitectural structures.

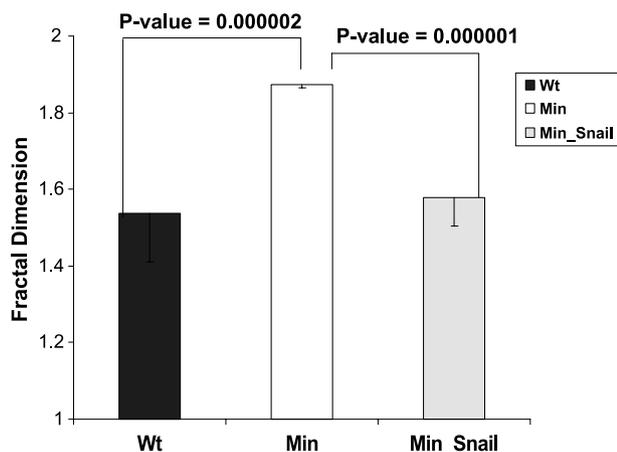


Figure 5. Fractal dimension in the uninvolved MIN mouse mucosa was determined through four-dimensional light-scattering fingerprinting analysis of uninvolved intestinal tissue within 1 hour of sacrifice (see Materials and Methods). Fractal dimension was significantly increased in the ~15-week-old MIN mice (Min) when compared with age-matched mice that were wild-type for APC loci (Wt). This change was almost completely reversed by treatment of MIN mice with antisense SNAIL oligonucleotide (Min_Snail), which was highly statistically significant ($P < 0.001$). Columns, mean ($n = 20$); bars, SD.

Discussion

We show that targeted down-regulation of SNAIL resulted in a significant suppression of intestinal tumorigenesis in the MIN mouse. This provides the first evidence that SNAIL is biologically important in this model of colorectal carcinogenesis. Although the decrease in tumor number by anti-SNAIL PMO treatment was relatively modest, this was proportional to both the decrease in SNAIL protein and the increase in E-cadherin protein resulting from AS-PMO regimen. It is probable that higher doses or more prolonged treatment with the antisense oligonucleotides would have achieved more dramatic tumor suppression. However, the present data provide compelling evidence for the role of SNAIL in the development of colorectal neoplasia.

Previous studies that employed antisense oligonucleotides to dissect gene function have been complicated by the potential for non-sequence-specific cellular effects (30). These are largely attributable to the polyanionic nature of these first-generation (generally phosphorothioate oligodeoxynucleotides) molecules. To overcome this potential confounding factor, we used a PMO formulation for our anti-SNAIL therapy (30, 31). In these molecules, the charged phosphodiester internucleotide linkage of phosphorothioate oligodeoxynucleotides is replaced by an uncharged phosphorodiamidate. In addition, the deoxyribose sugar is substituted by a six-membered morpholino ring. These modifications render the PMO nonionic, thereby not allowing nonspecific binding to proteins that have been problematic with phosphorothioate oligodeoxynucleotides (31). Moreover, PMOs do not form G-quartets, another mechanism through which phosphorothioate oligodeoxynucleotides have exerted nonspecific antiproliferative

effects (31). Previous studies with PMO formulation have indicated that their effects seem to be exclusively related to targeting the gene of interest by preventing protein translation through steric interference with pre-mRNA splicing and ribosomal assembly (30, 31). Thus, the use of PMO formulation allows us to confidently attribute the effects of treatment to specifically targeting the *SNAIL* gene. We confirmed that the effects of our PMO decreased SNAIL protein. Furthermore, the specificity was supported by the observation that our scramble sequence control had no effect on intestinal tumorigenesis.

To understand the cellular basis for the antineoplastic effects of SNAIL PMO, we evaluated enterocyte apoptosis and proliferation. Inhibition of apoptosis is a critical early cellular event enabling the otherwise short-lived epithelial cells to accumulate the genetic events requisite for neoplastic transformation (32). This is accompanied by an increase in proliferation thereby allowing expansion of the initiated clone of cells (33). MIN mice that received SNAIL AS-PMO had a marked decrease in proliferation as assessed by BrdUrd incorporation score and size of proliferative zone. There was a concomitant increase in the mucosal apoptosis rate as gauged by increased cleaved caspase 3 and TUNEL assay. Thus, the SNAIL PMO reversed the cellular events required for tumorigenesis in the MIN mouse.

Our data clearly showed that treatment with anti-SNAIL PMO resulted in modulation in cellular events that are requisite for intestinal tumorigenesis. However, emerging evidence suggests that alterations in the microscale/nanoscale structure of the cell actually precedes the changes in apoptosis and proliferation (25). Fractal dimension has emerged as one of the most robust measures of micro-architecture and is increasingly being employed by pathologists and cancer biologists (29, 34). This variable reflects the complexity and space-filling nature of an object and, when applied to a single cell, represents organization at the suborganelle level (22). In colon carcinogenesis, assessment of fractal dimension both can be used to identify adenomatous tissue and may serve as one of the earliest harbingers of neoplastic transformation in the histologically normal mucosa (29). However, the lack of practical methodology to assess fractal dimension has stymied utilization of this variable. We have recently developed a new generation of light-scattering technology, four-dimensional light-scattering fingerprinting, which affords a robust tool to measure fractal dimension. Using this technology, we have reported that, in the azoxymethane-treated rat model of colon carcinogenesis, the fractal dimension elevation preceded all conventional cellular and morphologic biomarkers (25). In the present study, we observe, by using four-dimensional light-scattering fingerprinting, that the elevation fractal dimension in the uninvolved mucosa of the MIN mouse (when compared with wild-type controls) was mitigated by SNAIL PMO treatment. Indeed, there was nearly complete normalization of fractal dimension (with values approaching that of wild-type mice). This suggests that targeting SNAIL may allow reversal of some of the fundamental events in intestinal tumorigenesis.

From a molecular perspective, there are many candidate genes that modulate apoptosis/proliferation and contain SNAIL regulatory elements; of these, *E-cadherin* is the best established. The importance of E-cadherin is underscored by the demonstration that knocking out this cell adhesion molecule in a MIN mice variant markedly increased intestinal tumor number (35). Loss of E-cadherin has been shown to allow translocation of β -catenin from the plasma membrane (where it is bound to E-cadherin) to the nucleus, thereby allowing activation of genes critical in apoptosis and cell cycle regulation (*c-myc*, *cyclin D1*, etc.; Ref. 36). We show that anti-SNAIL PMO treatment was able to induce E-cadherin expression in the uninvolved mucosa. Thus, regulation of E-cadherin may be central for the antineoplastic effects of the anti-SNAIL PMO, although the potential role of other E-box-containing tumor suppressor genes remains to be determined.

By using the MIN mouse, we are able to examine the effects of SNAIL AS-PMO on early events in intestinal neoplasia (19). However, because it is predominantly a model of small intestinal adenomas rather than frank colorectal cancer, extrapolation regarding events involved in invasion/metastasis is not possible. Several lines of evidence from other malignancy types suggest that SNAIL may play a critical role in these later phases of carcinogenesis. For instance, SNAIL overexpression in breast cancer and hepatocellular cancers connotes a poor prognosis (3, 37). Furthermore, in breast cancer, the aggressiveness of estrogen receptor-negative tumors is thought to be mediated by SNAIL overexpression (38) potentially through the control of epithelial-mesenchymal transition. The role of SNAIL in colorectal cancer progression is supported by both our observation that MIN mice treated with SNAIL AS-PMO had a dramatically lower incidence of larger tumors and our previous report that human colorectal cancers with a metastatic potential were more likely to be SNAIL positive (10).

In conclusion, we show, for the first time, that SNAIL is important for development of intestinal tumorigenesis in the MIN mouse. SNAIL inhibition resulted in alterations in several cellular variables associated with neoplastic transformation (apoptosis, proliferation, and fractal dimension). Once SNAIL AS-PMO optimization has been conducted, future studies will be better able to address the relative contribution of SNAIL on tumor initiation and progression. Nevertheless, this study provides compelling evidence for the hypothesis that SNAIL modulation has therapeutic potential in the prevention/treatment of colorectal cancer.

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