Tumor suppressors miR-143 and miR-145 and predicted target proteins API5, ERK5, K-RAS, and IRS-1 are differentially expressed in proximal and distal colon

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Submitted 24 June 2014; accepted in final form 12 November 2014

The proximal and the distal colon differ in many respects, including embryological origin, local luminal environment, excretory function, and gene expression. While colonic regional differences in the microbiome, fecal stream composition, transcription factor expression, and DNA promoter methylation are believed to contribute to these differences, microRNAs (miRNAs), a more recently described class of gene regulators, are also likely involved in differential gene expression.

miRNAs are small noncoding RNAs that regulate gene expression by binding to the 3′-untranslated region of mRNA, causing translational inhibition or degradation of mRNA (5). On the basis of intracellular and extracellular cues, miRNAs modulate gene expression to control cellular proliferation, differentiation, and apoptosis (3). miRNAs regulate normal intestinal cell function, and many miRNAs are dysregulated in carcinogenesis, acting as oncogenes when upregulated or unmasking tumor suppressor functions when downregulated (3, 26, 30). As such, expression profiles of miRNAs have been shown to be altered in many tumors, including colon cancer (13, 54, 57).

Most prior studies examining miRNAs in colonic tissue have not distinguished regional variation in miRNA expression, despite known differences in biological function and embryological origins of proximal and distal colon (33, 45, 46, 54). Previous genome-wide gene expression analyses have reported differential gene expression by location in normal colons (17, 38). Furthermore, tumors arising in proximal and distal colon differ in natural history, cell biology, mutation spectrum, and gene expression (2, 8, 9, 39). Because miRNAs regulate an estimated 60% of genes and miRNA profiles in tumors have been shown to differ between proximal and distal colon tumors, we speculated that proximal and distal colon might also differ in miRNA expression (15, 49). Such differences could elucidate region-specific mechanisms of cancer development.

Genes encoding polycistronic miR-143 (miR-143) and miR-145 on chromosome 5 are highly expressed in the colon and downregulated early in colon cancer (32). These miRNAs appear to drive a differentiated cell phenotype, inhibiting growth in transplanted colon cancer cells (60). It seems likely, therefore, that these miRNAs play important roles in normal colonic biology and that their loss contributes causally to neoplastic progression. In this study, we demonstrate that these miRNAs are differentially expressed in proximal and distal colon. Increases in cellular proliferation and decreases in apoptosis have been described in colon cancer development. Therefore, we investigated regulation of these miRNAs, as well as their putative targets, which might control cell turnover and, thereby, contribute to malignant progression with loss of these miRNAs.
RESULTS

Mature miR-143 and miR-145 are differentially expressed in proximal and distal colon. miR-143 and miR-145 expression levels were measured in proximal and distal colon in eight healthy individuals. The mean level of miR-143 was 2.0-fold higher in proximal than distal colon ($P < 0.001$), and miR-143 expression levels in all eight individuals were higher in proximal than distal colon. Similarly, the mean level of miR-145 expression was 1.8-fold higher in proximal than distal colon ($P = 0.03$; Fig. 1). In seven of eight individuals, miR-145 expression levels were higher in proximal than distal colon. In agreement with these real-time PCR findings, in situ hybridization of miR-145 was increased 1.8-fold in proximal compared with distal colonic mucosa (relative intensity: $30,000$ for proximal colon and $17,000$ for distal colon; Fig. 1).

Impact of DNA methylation on miR-143 and miR-145 expression. The DNA methyltransferase inhibitor 5-AZA results in net DNA demethylation. Expression levels of primary transcripts and mature miR-143 and miR-145 were measured...
MiR-143 and MiR-145 in Proximal and Distal Colon

In 5-AZA-treated LoVo, DLD-1, HCT-116, and HT-29 colon cancer cells. As shown in Fig. 2, for MiR-143 there were significant 5-AZA-induced increases in LoVo and DLD-1 cells in primary MiR-143 [33.0-fold (P = 0.02) and 18.5-fold (P = 0.004) with 5 and 10 μM 5-AZA, respectively], in LoVo cells and 8.9-fold (P = 0.002) and 3.7-fold (P = 0.04) with 5 and 10 μM 5-AZA, respectively, in DLD-1 cells] and mature MiR-143 [6.5-fold (P = 0.04) and 5.3-fold (P < 0.001) with 5 and 10 μM 5-AZA, respectively, in DLD-1 cells] and mature MiR-143 [6.5-fold (P = 0.04) and 5.3-fold (P < 0.001) with 5 and 10 μM 5-AZA, respectively, in DLD-1 cells]. There was no change in primary MiR-143 following 5-AZA treatment in HT-29 cells, although there were significant increases in mature MiR-143 [5.4-fold (P = 0.005) and 3.0-fold (P = 0.05) with 5 and 10 μM 5-AZA, respectively]. In contrast, there was no change in primary or mature MiR-143 expression in HCT-116 cells (Fig. 2).

Similarly, primary MiR-145 increased in LoVo and DLD-1 cells [16.1-fold (P = 0.2) and 21.6-fold (P = 0.06) with 5 and 10 μM 5-AZA, respectively], in LoVo cells and 23.6-fold (P = 0.01) and 31.1-fold (P = 0.002) with 5 and 10 μM 5-AZA, respectively, in DLD-1 cells]. However, expression of mature MiR-145 did not change in these cell lines. In HCT-116 cells, treatment with 10 μM 5-AZA downregulated primary MiR-145 expression (0.5-fold, P = 0.01) but did not affect mature MiR-145. In HT-29 cells, there was no change in primary MiR-145, although we noted a significant increase in mature MiR-145 following 5-AZA treatment [2.0-fold (P = 0.02) and 1.7-fold (P = 0.02) with 5 and 10 μM 5-AZA, respectively; Fig. 2].

To further examine the impact of DNA methylation on expression levels of these miRNAs in colon cancer cells, we used direct bisulfite sequencing from these cell lines to assess methylation of the genes coding for MiR-143 and MiR-145 (Fig. 2). In all four cell lines, the overall percentage of DNA methylation was high (63–100%) at the CpG sites. Hence, differences in primary miRNA transcript expression levels following 5-AZA treatment were not directly explained by methylation differences in CpG sites in genes encoding MiR-143 and MiR-145.

To investigate the impact of methylation on differences in MiR-143 and MiR-145 expression between normal proximal and distal colon, we performed sequencing of bisulfite-treated DNA on biopsies from proximal and distal colon in four subjects. As shown in Table 1, there were no differences in methylation between proximal and distal colon for MiR-143 (78.7% vs. 81%, P = 0.39) or MiR-145 (79.5% vs. 83.4%, P = 0.55). It is also important to note the high percentage of DNA methylation at the interrogated CpG sites in normal proximal and distal colon (Fig. 3).

Expression of primary transcripts in proximal and distal colon. As differences in expression of MiR-143 and MiR-145 could not be explained by differences in DNA methylation of genes encoding these miRNAs between proximal and distal colon, we sought to investigate if the regional differences in expression were more likely secondary to differences in miRNA transcription or processing. We measured primary transcripts of MiR-143 and MiR-145 by real-time PCR. As shown in Fig. 1, there were no differences in primary MiR-143 expression between proximal and distal colon (P = 0.8). In contrast to mature MiR-145 expression, which was higher in proximal colon, primary MiR-145 was lower in proximal than distal colon (P < 0.001). These findings suggest that regional differences in mature MiR-143 and MiR-145 are likely secondary to regional differences in miRNA processing, rather than differences in transcription of these miRNAs.

Expression and DNA methylation of MiR-143 and MiR-145 in colon cancer. Because differences in mature MiR-143 and MiR-145 expression between normal proximal and distal colon are likely secondary to differences in processing of the primary transcript, we sought to determine whether expression differences between the mature miRNA and the primary transcript were similar in sporadic colon cancer. Primary MiR-143 was downregulated 4.0-fold (P = 0.03) and primary MiR-145 was downregulated 3.4-fold (P = 0.1) in colon cancer compared with adjacent tissue (n = 11). Similarly, mature MiR-143 was downregulated 4.9-fold (P < 0.001) and MiR-145 was downregulated 4.6-fold (P = 0.009) in colon cancer compared with adjacent tissue, suggesting that decreases in transcription probably contribute to downregulation of mature forms of these miRNAs in colon cancer (Fig. 4).

Bisulfite genomic sequencing was performed on four cancers and adjacent nondysplastic tissue. As shown in Table 2, no significant differences in methylation were observed in colon.
cancers compared with adjacent tissue for miR-143 (73.8% vs. 67.7%, \( P = 0.29 \)) or miR-145 (81.2% vs. 79.7%, \( P = 0.79 \)) (Fig. 5).

Expression of miR-143 and miR-145 target proteins in proximal and distal colon. Since miR-143 and miR-145 are lost early in colon cancer development and premalignant lesions have increased proliferation and decreased apoptosis, we used TargetScan 6.2 (www.targetscan.org), microRNA.org (www.miRNA.org), and miRDB (www.mirdb.org) to examine putative targets of these miRNAs that might regulate cell cycle or survival. In a preliminary analysis in four patients, examination of putative target proteins identified four proteins (IRS-1, API5, ERK5, and K-RAS) with differential expression between proximal and distal colon. To confirm these results, expression of these four proteins was measured by Western blotting in proximal and distal colonic mucosal biopsies from an additional 8 –11 patients. As shown in Fig. 6, these predicted targets demonstrate an inverse pattern of differential expression compared with miR-143 and miR-145, with higher expression levels in distal than proximal colon. Relative ratios of expression in distal colon to expression in proximal colon were 3.2 ± 2.6 for IRS-1 (\( P = 0.04 \)), 2.5 ± 1.0 for K-RAS

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**Fig. 2.** Primary and mature miR-143 and miR-145 expression and DNA methylation in colon cancer cells. A and B: real-time PCR measurement of expression of mature and primary miR-143 and miR-145 following 5 consecutive days of treatment with 0, 5, or 10 µM 5-aza-2’-deoxycytidine (5-AZA). C: primer positions (black boxes) relative to miRNAs (gray boxes) and CpG locations (vertical bars) for sequences of bisulfite-treated DNA around genes encoding miR-143 and miR-145. D: sequencing for the indicated loci in DLD-1, HCT-116, HT-29, and LoVo colon cancer cells after bisulfite conversion and PCR amplification. Each line represents a single clone. Black boxes, methylated CpG sites; gray boxes, unmethylated CpG sites.
Comparison of expression of these proteins for each patient showed higher expression of IRS-1 in 6 of 8 patients, higher expression of K-RAS in 7 of 8 patients, higher expression of API5 in 9 of 11 patients, and higher expression of ERK5 in 8 of 11 patients in distal than proximal colon (Fig. 6).

**DISCUSSION**

While downregulation of miR-143 and miR-145 has been reported in colon cancer, this is the first report describing regional variation of these miRNAs in normal colon (1, 32, 51). Interestingly, ratios of abundance of predicted targets, API5, ERK5, IRS-1, and K-RAS, in distal colon to that in proximal colon showed an inverse relationship to the concentrations of these miRNAs. Although in this report proliferation and antiapoptotic signals were increased in distal colon compared with proximal colon, previous studies have...

**Table 1. Patient characteristics and percent methylation for each sample in methylation analysis of normal distal and proximal colon**

<table>
<thead>
<tr>
<th>Subj No.</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Race</th>
<th>miR-143 Distal</th>
<th>miR-143 Proximal</th>
<th>miR-145 Distal</th>
<th>miR-145 Proximal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>M</td>
<td>Ca</td>
<td>80</td>
<td>84</td>
<td>78.4</td>
<td>65.3</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>M</td>
<td>AA</td>
<td>81.3</td>
<td>73.3</td>
<td>86.3</td>
<td>95.8</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>F</td>
<td>AA</td>
<td>79</td>
<td>80</td>
<td>85</td>
<td>70.6</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>F</td>
<td>AA</td>
<td>83.7</td>
<td>80</td>
<td>84</td>
<td>86.3</td>
</tr>
</tbody>
</table>

Mean 81.0 ± 78.7 (Distal) vs. 83.4 ± 79.5 (Proximal) (P = 0.39) vs. (P = 0.55)

Percent methylation is based on the number of CpG sites methylated of all CpG sites analyzed for all clones (range 10–23/sample). M, male; F, female; Ca, Caucasian; AA, African American.

In contrast, no differences were seen in expression of API5 with transfection of miR-143 (Fig. 6).

**Fig. 3. Sequencing of bisulfite-treated DNA encoding miR-143 and miR-145 in proximal and distal colon.** After bisulfite conversion and PCR amplification, DNA sequencing was performed in 4 subjects for the indicated loci in proximal and distal colon. Each line represents a single clone. Black boxes, methylated CpG sites; gray boxes, unmethylated CpG sites. Ten representative clones for each sample are included.
been inconsistent with respect to regional variation in proliferation rates in normal colon (4, 24, 25, 28, 40, 56). Since homeostatic regulation is complex, however, other mechanisms are undoubtedly also involved in controlling proliferation and apoptosis.

In agreement with other studies in which increased DNA methylation was shown to contribute to downregulation of miR-143 and miR-145 in other cell types, we used 5-AZA to demonstrate that DNA methylation likely contributes to downregulation of these miRNAs in colon cancer cells (14, 53). Our analysis, however, did not demonstrate significant differences in DNA methylation in genes coding for these miRNAs in colon cancers compared with adjacent tissue or in colon cancer cell lines in which expression in the primary transcript of miR-143 and miR-145 was increased following 5-AZA treatment compared with those in which expression was not changed. The finding that both miRNAs increased following 5-AZA treatment in colon cancer cells, despite the absence of differences detected in DNA methylation in colon cancers compared with adjacent tissues, suggests that DNA methylations controlling miRNA expression are occurring at loci remote from those we investigated or in a single cell type that could not be detected given the cellular heterogeneity in human colonic biopsies.

Previous analyses demonstrated differences in DNA methylation between proximal and distal colon (21, 22). Despite the finding that 5-AZA upregulated expression of miR-143 and miR-145 in some colon cancer cell lines, we did not identify changes in methylation of these miRNAs between proximal and distal colon. In fact, the discordance between mature miRNAs and primary transcript expression of these miRNAs in proximal and distal colon suggests that regional differences in expression of mature miR-143 and miR-145 are secondary to differences in miRNA processing. In contrast, the mature and primary transcripts were downregulated in sporadic colon cancer compared with adjacent nondysplastic tissue. These findings suggest that transcriptional dysregulation is contributing to the downregulation of these miRNAs in colon cancer. As mature miR-143 and miR-145 were more significantly downregulated than the primary transcripts in our analysis of colon cancers, however, we hypothesize that alterations in miRNA processing are also contributing to decreases in mature miRNAs in colon cancer.

Several potential targets of miR-143 and miR-145 have been suggested in silico and further supported in cell culture studies (18, 55, 58, 59). In this study, we examined API5, ERK5, IRS-1, and K-RAS, which are reported targets of miR-143 or miR-145 and causally implicated in colon cancer progression. API5 is an antiapoptotic protein that suppresses E2F-dependent apoptosis, an important regulator of tumor growth in colon cancer (11, 34). Upregulation of API5 has not been reported in colon cancer but appears to play an important tumor-promoting role in other cancers (23, 52). ERK5 is a member of the mitogen-activated protein kinase family, which is important in cellular proliferation, including mediation of epidermal growth factor-induced proliferation (20, 35). IRS-1 is involved in cellular proliferation and survival and is upregulated in multiple cancers, including colon cancer. IRS-1 is required for efficient neoplastic transformation induced by β-catenin (43). K-RAS is an upstream activator of ERKs, important transducers of proliferation (19, 47). In the present study, these proteins displayed regional variations consistent with differences in miR-143 and miR-145 expression. In agreement with other studies, we showed that transfection of miR-143 downregulated ERK5, as well as K-RAS, and transfection of miR-145 downregulated IRS-1 in CRC cells (12, 48). We did not observe differences in API5 expression after transfection of miR-143 in HT-29 cells. In a previous analysis, we did demonstrate such a downregulation of API5 with miR-143 transfection in HCT-116 cells, another colon cancer cell line. It is possible that this discrepancy could be related to cell-specific

Table 2. Patient characteristics and percent methylation for each sample in methylation analysis of colon cancer compared with adjacent tissue

<table>
<thead>
<tr>
<th>Subj No.</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Race</th>
<th>Location of Tumor</th>
<th>%Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>64</td>
<td>M</td>
<td>Ca</td>
<td>Left colon</td>
<td>71.7, 78.5</td>
</tr>
<tr>
<td>6</td>
<td>71</td>
<td>F</td>
<td>AA</td>
<td>Left colon</td>
<td>57.2, 73.4</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>M</td>
<td>U</td>
<td>Right colon</td>
<td>76.3, 69.8</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>F</td>
<td>AA</td>
<td>Right colon</td>
<td>65.5, 73.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>67.7, 73.8</td>
</tr>
</tbody>
</table>

See Table 1 footnote for explanation of %methylation. U, unknown.
differences, such as alternative splicing, which might alter the 3’-untranslated region of API5.

There are several potential limitations of this study. 1) While we demonstrated significant differences between proximal and distal colon in a relatively small cohort of normal individuals, larger studies are needed to confirm these differences. 2) The regional variation in miRNAs and proteins identified in this study cannot be extrapolated to other miRNAs and proteins that may be regulated by different mechanisms. 3) Despite demonstrating an association between specific miRNAs and...

Fig. 5. Sequencing of bisulfite-treated genes encoding miR-143 and miR-145 in primary colon cancers and adjacent nondysplastic tissue. After bisulfite conversion and PCR amplification, sequencing was performed for the indicated loci in proximal and distal colon in 4 subjects. Each line represents a single clone. Black boxes, methylated CpG sites; gray boxes, unmethylated CpG sites. Ten representative clones from each sample are included.

Fig. 6. Protein expression levels of targets of miR-143 and miR-145 in proximal and distal colon. A: representative Western blots of apoptosis inhibitor 5 (API5), cyclin-dependent kinase 6 (CDK6), cyclin D2, E2F3, E-cadherin, ERK5, insulin receptor substrate 1 (IRS-1), K-RAS, and β-actin in proximal and distal colon (n = 4). B: quantitative expression levels of API5, ERK5, K-RAS, and IRS-1 in proximal and distal colon for 8–11 subjects. C: Western blots of protein expression levels 48–72 h after transfection of 50 nM mimic of indicated mature miRNA or scrambled probe (Scr) into HCA-7 cells (IRS-1) or HT-29 cells (K-RAS, API5, and ERK5) (n = 3).
proteins in vivo and a potential causal effect in cell culture, we cannot conclude that there is a direct causal relationship in vivo, where miRNA-mRNA interactions are more complex. 4) The methylation analysis may have been limited by the number of patients studied, the heterogeneous cell type sampled in human colonic tissues, and known variation in total methylation among individuals. Although we controlled for inter-individual variability in tissue methylation by comparing regional DNA methylation in the same subject, the degree of methylation may be related to other factors such as age and sex.

Distal colon tumors are more prevalent than proximal colon cancers (7). In contrast, proximal colon cancers present with larger tumor size, higher grade, and worse outcomes than distal colon tumors (7, 16, 31, 50). These findings likely are driven in part by biological differences between normal proximal and distal colon. Indeed, molecular markers of colon cancer are known to vary by colon location (2, 6, 9, 10, 29, 36, 37). These observations may result from different embryological origins of proximal vs. distal colon, as well as regional differences in the colonic microbiome and differences in constituents of the fecal stream, such as secondary bile acids and short-chain fatty acids, and potential carcinogen concentrations in the aqueous phase. Because the miRNAs and proteins examined in this study are potentially implicated in colonic carcinogenesis, their differential expression might contribute to differences in the pathobiology of proximal vs. distal colon cancers and have implications for CRC prevention or treatment.

In conclusion, we have demonstrated differential expression of miR-143 and miR-145 and API5, ERK5, IRS-1, and KRAS, proteins that are putative targets of these miRNAs, in normal proximal and distal colon. These differences may contribute to regional differences in cell turnover in normal colon and site-specific predilections in colonic malignant transformation. Examination of these miRNAs, as well as their putative target transcripts and proteins, within tumors of proximal and distal colon is needed to further explore this hypothesis. Given the differential miRNA expression pattern we observed in proximal vs. distal colon, location should be taken into account in future studies that examine miRNAs in the colon.

GRANTS

This study was funded by National Institute of Diabetes and Digestive and Kidney Diseases Grant K08 DK-09015 (J. Pekow), the Crohn’s and Colitis Foundation of America (J. Pekow), and National Institutes of Health Digestive Diseases Research Core Center Grant P30 DK-42086 and Grant 1R01 CA-164124 (M. Bissonnette).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.P., L.J.J., and M.B. developed the concept and designed the research; J.P., K.M., U.D., F.B., R.M., L.J.J., and M.B. performed the experiments; J.P. prepared the figures; J.P. drafted the manuscript; J.K., M.B., and L.J.J. edited and revised the manuscript; J.P., K.M., U.D., F.B., R.M., L.J.J., and M.B. approved the final version of the manuscript.

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