Reduced Accumulation of Specific MicroRNAs in Colorectal Neoplasia

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Abstract

Short non-coding RNAs are known to regulate cellular processes including development, heterochromatin formation, and genomic stability in eukaryotes. Given the impact of these processes on cellular identity, a study was undertaken to investigate possible changes in microRNA (miRNA) levels during tumorigenesis. A total of 28 different miRNA sequences was identified in a colonic adenocarcinoma and normal mucosa, including 3 novel sequences and a further 7 that had previously been cloned only from mice. Human homologues of murine miRNA sequences, miR-143 and miR-145, consistently display reduced steady-state levels of the mature miRNA at the adenomatous and cancer stages of colorectal neoplasia.

Introduction

MicroRNAs (miRNAs) are short 20–22 nucleotide RNA molecules that have been shown to regulate the expression of other genes in a variety of eukaryotic systems. In *Caenorhabditis elegans*, miRNAs coordinate the transitions between stages of larval development by regulating the translation of heterochronic genes (1). A specific miRNA in *Arabidopsis* is known to direct the cleavage of transcripts encoding several putative transcription factors (2). The *Drosophila bantam* gene encodes a miRNA that regulates cell proliferation and the proapoptotic gene *hid* (3). More recently, a human miRNA has been shown to regulate the expression of the *Hes1* gene that is involved in retinoic acid-induced neuronal differentiation of NT2 cells (4). These are all examples of how miRNAs provide additional regulatory constraints on the development of higher organisms. In plants, yeast, and *Tetrahymena*, short RNA species also regulate the formation of heterochromatin and maintain genomic stability by suppressing the activity of transposable elements and "silencing" repetitive sequences (5, 6).

miRNAs are formed from larger transcripts that fold to produce hairpin structures and serve as substrates for the Dicer family of RNase III enzymes. They share this process with an experimental system, RNA interference (RNAi), which is used to silence the expression of endogenous genes in eukaryotic cells. The products of Dicer cleavage are short dsRNA molecules, one strand of which is retained in a ribonucleoprotein complex called the RNA-induced silencing complex (RISC; 7). The retained RNA acts as a guide to target this complex to a complementary mRNA sequence which is inactivated either by cleavage or translational interference, depending on the degree of complementarity between the miRNA and its target (8).

Given the fundamental biological processes that are regulated by miRNAs and the knowledge that many of these processes are altered in tumors, it is important to determine whether miRNAs play a role in oncogenesis. An association between B-cell chronic lymphocytic leukemia (B-CLL) and deletions in a region of chromosome 13, which contains the genes for miRNAs miR-15 and miR-16, led Calin *et al.* (9) to show that these miRNAs are either absent, or down-regulated, in a majority of CLL specimens. This study did not, however, detect an association between these same miRNA genes and either colorectal or gastric tumors. The proteins, eIF2C1, eIF2C2, and Hiwi, are members of the Argonaut protein family and are known to associate with the RISC. Argonaut proteins have been identified as likely causes of tumor formation in tissues that are thought to display retarded cellular differentiation (10). Wilms tumors of the kidney may be affected by overexpression of EIF2C1 when correlated with loss of WT1 tumor suppressor activity (10). The chromosome 12 locus that contains the Hiwi gene is linked to development of testicular germ cell tumors and altered levels of Hiwi activity are also associated with these lesions. Hiwi overexpression is also detectable in seminomas (11). While these studies implicate the processes of miRNA/RNAi gene silencing in some aspects of oncogenesis, there are no reports of an association between specific miRNAs and the formation of solid tumors. As a consequence, we have studied changes in the miRNA populations of tumors of the colon. We describe the identification of miRNAs that are expressed and processed in human colorectal mucosa, including several novel sequences. We also describe a subset of miRNAs that consistently fail to accumulate to normal levels in precancerous and cancer tissues.

Results

Identification of Colorectal MicroRNAs

Small RNA fragments (between 18 and 27 bases) in total RNA, purified from both a colonic adenocarcinoma (patient

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#196-Dukes' stage B) and its matched normal mucosa, were size fractionated and cloned (Table 1). The 283 clones from the cancer-derived sample and 257 clones representing normal mucosa were then sequenced. Sequence analysis and comparison with public database nucleotide sequences enabled identification of many of these clones (Table 1) or assignment to a possible genomic origin for the transcripts. From the tumor sample, we have identified 19 clones (Table 2) that correspond to five known human miRNAs (12–15). Four clones are homologous to three known murine miRNA sequences. Two of these murine sequences, miR-143 and miR-145, show tissue-specific patterns of accumulation (16). Two miRNAs from the tumor sample have not been previously reported.

Of the sequences identified from normal tissue, 18 known human miRNAs were represented while 7 others were homologous to previously identified murine miRNAs (16). Three novel human miRNAs were identified in the normal mucosa-derived population.

Following submission to the miRNA registry (17), the novel miRNAs have been named miR-320, miR-321, and miR-200c (Table 2). The mature miR-200c displays a two-base difference to the mature murine miR-200b sequence; however, there is little similarity between their predicted precursor sequences. The human homologues of murine sequences are listed in Table 2.

Accumulation of MicroRNAs in Colorectal Tissues

To confirm that the various sequences accumulate as miRNAs and investigate whether changes in miRNA steady-state levels are associated with neoplastic epithelium, Northern blot analysis was undertaken against a panel of RNAs from four matched colorectal cancer and normal mucosa specimens. Hybridization to RNA blots revealed that most clones show similar steady-state levels of the Dicer-processed mature miRNAs in both normal and cancer tissues (Fig. 1). Where detectable, this was also true for the relative levels of the precursor hairpin pre-miRNAs. However, two different miRNAs, miR-143 and miR-145, exhibited significantly reduced levels of the fully processed miRNA in tumors compared to normal (Fig. 1). This remained true when Northern blot analysis was extended to 12 matched tumor and normal samples (Fig. 2). Decreased levels of miR-143 and miR-145 were also detected in precancerous adenomatous polyps. Interestingly, unlike the Dicer-cleaved fragments, the hairpin precursors of these particular miRNAs are of equal abundance in both normal and neoplastic tissues (Fig. 1). Both of these miRNAs appear to be derived from genomic sequences within 1.7 kb of each other on chromosome 5 (5q32-33). Their gene(s) reside approximately 50 kb from the interleukin 17 gene, within the 1.5-Mb region that is deleted in the myelodysplastic 5q− syndrome (18).

Table 1. Composition of Small RNA Populations Cloned From Colorectal Tissues

RNA Class	Adenocarcinoma	Normal Mucosa
miRNA	25	85
rRNA	147	78
mRNA	36	27
tRNA	10	4
snRNA	6	4
Non-coding cytoplasmic RNA	4	3
mitochondrial	3	5
unknown	52	51
Total	283	257

Note: The number of sequences obtained for each classification of RNA is shown.

MicroRNAs in Cancer Cell Lines

Northern blot analysis was used to determine the levels of mature miRNAs and precursor hairpin molecules in cell lines derived from a variety of cancerous human tissues. The types of cancer represented included: colorectal adenocarcinoma (CaCo-2 and LIM1863 organoids); breast carcinoma (MCF-7 and T47-D); prostate carcinoma (LNCaP); chronic myelogenous leukemia (MEG-01); and cervical carcinoma (HeLa). miRNA levels were also compared with those in a colorectal adenocarcinoma (from patient #100b) and the matched normal mucosa to enable comparison with previous experiments.

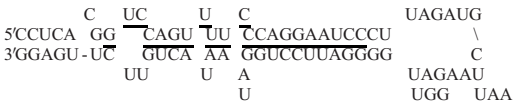
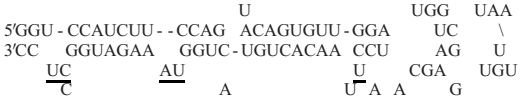
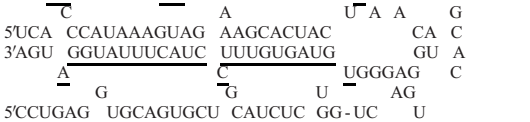
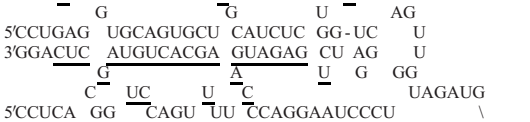
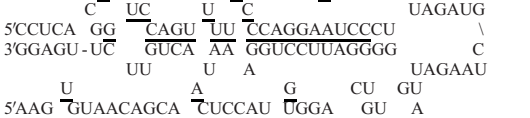
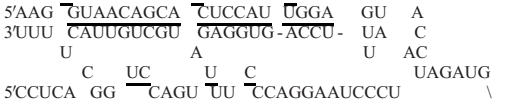
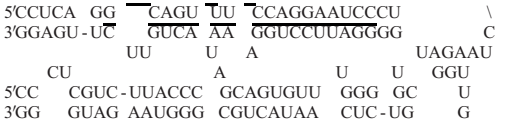
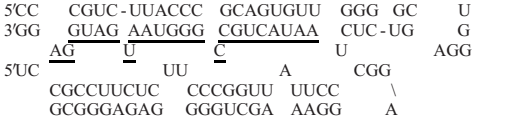
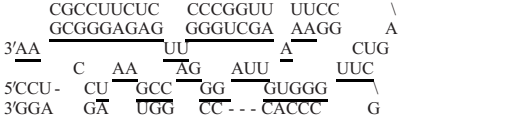
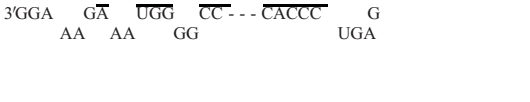
Two probes were used to determine the levels of mature and precursor miR-143. Hybridization with the end-labeled miR-143 antisense oligonucleotide detected elevated levels of mature miR-143 in only the normal mucosa sample (Fig. 3). The same blot, however, displays significant and consistent levels of the ~70-bp precursor pre-miR-143 in each of the cell lines, indicating that the levels of mature miR-143 are controlled by a posttranscriptional mechanism. A miR-143 probe, with higher specific activity, was synthesized from a concatamer of three direct repeats of the miR-143 sequence and was also used to assess the levels of miR-143 on this blot. The second probe confirmed the lack of miR-143 accumulation in each of the cell lines, relative even to the colorectal tumor sample. While this second probe was more efficient at detecting the mature miRNA sequence, it did not detect the pre-miR-143 sequence, consistent with observations from previous experiments (data not shown).

Of the cell lines under study, HeLa cells accumulated mature miR-145 and this was at a level lower than that seen in the colorectal tumor. Faint pre-miR-145 levels were also detected in the colorectal and prostate-derived cell lines indicating, again, that in these tissues, reduced miR-145 levels are due to posttranscriptional processes.

Levels of miR-16 are similar in each of the cell lines and colorectal tissues, with the exception of the leukemia-derived MEG-01 cells, which display less than half of the accumulation seen in other RNA samples. This observation is consistent with the findings of a previous study (9) that reported diminished miR-16 accumulation in a majority of the B-CLL lines tested. Interestingly, the levels of the mature Let 7a-1 miRNA were found to vary between cell lines. Maximal abundance was detected in the HeLa, LNCaP, and colorectal tissues.

In summary, Northern blot analyses show that accumulation of miRNAs, miR-143 and miR-145, is down-regulated in cells derived from breast, prostate, cervical, and lymphoid cancers as well as colorectal tumors. They also indicate that this reduction is due to posttranscriptional processes.

Table 2. Summary of miRNA Information

miRNA	No. Clones (Tumor)	No. Clones (Normal)	Sequence (5′–3′)	Hairpin Precursor	Chromosome	Reference
hsa-miR-15b		1	UAGCAGCACAUCAUGGUUUAC		3	
hsa-miR-141	1	2	UAACACUUUCUGGUAAAGAUGG		12	
hsa-miR-142as		1	UGUAGUGUUUCCUACUUUAUGGA		17	
hsa-miR-143	1	12	UGAGAUGAAGCACUGUAGCUC		5	
hsa-miR-145	2	8	GUCCAGUUUUCCCAGGAAUCC		5	
hsa-miR-194		2	UGUAACAGCAACUCCAUGUGGA		11,1	
hsa-miR-200b		4	UAAUACUGCCUGGUAAUGAUGA		1	
miR-200c	1		AAUACUGCCGGGUAAUGAUGGU		12	
miR-320		2	AAAAGCUGGGUUGAGAGGGCGAA		8	
miR-321	1	1	UAAGCCAGGGAUUGUGGGUUC		16	
Let-7a/b/c	2	5	UGAGGUAGUAGGUUGUAUAGUU			(13)
Let-7f		2	UGAGGUAGUAGAUUGUAUAGU			(13)
miR-10b		2	UACCCUGUAGAACCGAAUUUGUG			(14)
miR-16		3	UAGCAGCACGUAAAUAUUGGCG			(13)
miR-19b		1	AGUUUUGCAGGUUUGCAUCCAGC			(13)
miR-20		1	UAAAGUGCUUAUAGUGCAGGUAGA			(13)
miR-21	14	15	UAGCUUAUCAGACUGAUGUUGA			(13)
miR-22	1	1	AAGCUGCCAGUUGAAGAACUGU			(13)
miR-23		1	AUCACAUUGCCAGGGAUUUCCAAA			(13)
miR-24		4	UGGCUCAGUUCAGCAGGAACAGU			(13)
miR-26a		5	UUCAAGUAAUCCAGGAUAGGC			(13)
miR-26b		2	UUCAAGUAAUUCAGGAUAGGC			(13)
miR-27*		1	UUCACAGUGGCUAAGUUCUGC			(13)
miR-29	1	3	CUAGCACCAUCUGAAAUCGGUU			(13)
miR-97		1	UGUAAACAUCCUUGACUGGAAG			(12)
miR-102		3	UAGCACCAUUUGAAAUCAGUGUU			(12)
miR-192		1	UGACCUAUGAAUUGACAGCCAG			(15)
miR-199b	1	1	CCCAGUGUUUAGACUAUCUGUUC			(14)

Putative Targets for miR-143 and miR-145

Comparison of the sequences for miR-143 and miR-145 with known mRNA sequences has identified several transcripts that may, potentially, serve as targets of miRNA repression (Tables 3 and 4). Several genes encoding components of signal transduction pathways [Raf, Rho GTPase activating protein (RICS), G-protein γ7, HGK] and chromatin-mediated control of gene expression (M96A, SMARCF1) are included amongst the potential mRNA targets. Transcripts for proteins involved in physiological and metabolic processes [ATP1A1, GOLGA2, SMS, TMOD1, NUDT5, PAH, DHFR, methylene-tetrahydrofolate reductase (MTHFR)], as well as the processing of RNAs and proteins (HNRPC, TXNDC4, Erp44), are also represented in the putative targets. A further potential target of miR-145 is the transcript from gene 1 of a tumor-suppressing subchromosomal fragment from chromosome 11p15.5, which displays homology with Rb-associated protein p48 and chromatin assembly factor CAF1 (19). A region within the transcript for the *RICS* gene displays considerable complementarity with miR-145. In this case, identity extends for 17 bp at the 5′ end of the miRNA sequence, which is believed to be the most important region for determining target recognition (20).

Discussion

This study has identified two miRNAs that exhibit reduced levels in precancerous and neoplastic colorectal tissue. This occurs despite the maintenance of constant levels of unprocessed hairpin precursors in both normal and tumor tissues, suggesting that altered transcription is not responsible for reduced miRNA levels. These observations could be due to reduced Dicer-processing activity in the neoplastic cells (unlikely, given the appropriate processing of most other miRNAs) or reduced stability of these specific miRNAs, possibly as a result of diminished retention in a ribonucleoprotein complex. Real-time RT-PCR analyses, using a subset of matched tissue-RNA samples, indicate that Dicer and a known human RISC component, eIF2C2, do not display consistently altered expression between normal and neoplastic cells (data not shown). This suggests that other, as yet unidentified, components of the miRNA/RNAi pathway may be altered during the progressive development of neoplasia. Alternatively, the reduced accumulation of miR-143 and miR-145 may reflect diminished levels of their target sequences in dysplastic and cancer cells. While such an effect on miRNA stability has not been shown, a stoichiometric balance between miRNAs and their regulatory targets has been proposed (21). It is also possible that the altered accumulation of these mature miRNAs reflects early changes in the cellular composition of tumors, compared with normal mucosae. The identification of miRNAs that consistently display reduced steady-state levels in tumors raises the possibility that they, or their targets, may be directly involved in the processes that lead to neoplasia. It remains to be seen whether the observed reduction of these miRNAs is an epiphenomenon or if they do, in fact, play a regulatory role.

Several gene transcripts encoding proteins involved in signal transduction and gene expression have been identified as possible targets for repression by miR-143 and miR-145. These include RAF1 kinase, G-protein γ7, and tumor-suppressing subfragment candidate 1, all of which have been implicated in oncogenesis (17, 22, 23). The RICS protein is believed to interact with β-catenin and to be involved with cytoskeletal organization and signal transduction in neural cells (24). Given the exposure accorded to compartmentalization of β-catenin as a possible cause of colorectal cancer (25), it will be of interest to determine whether the RICS gene shows altered expression in colorectal cancer cells. Allelic variants of the gene encoding MTHFR have been associated with aberrant DNA methylation patterns in colorectal tumors (26). How the observed reduction of miR-143 and miR-145 mature miRNA levels is associated with the translation of these putative targets is still unknown. However, if proven, miRNA-directed control of the expression of these target genes will provide novel insights into possible causes for cancer progression.

The sodium and potassium-dependent ATPase α subunit (ATP1A1), which is responsible for establishing a membrane potential across the intestinal epithelium, is also identified as a likely target for miR-143-directed repression. Physiological studies indicate that Na,K-ATPase pump activity is reduced in premalignant mucosa soon after treatment for experimentally (1,2-dimethylhydrazine)-induced colorectal cancer in mice (27). The steady-state levels of ATP1A1 mRNA are also known to decrease in colorectal tumors (28). Base pairing between miR-143 and ATP1A1 mRNA is predicted to occur within the coding sequence of ATP1A1 (between bases 1245 and 1262 of Genbank ID 806751). Grindstaff *et al.* (29) have reported translational repression of the Na,K-ATPase α1 mRNA when expressed in several epithelial cell lines (including CaCo-2) and have shown that this repression is due to unknown sequences within the coding region. While this highlights the ATP1A1 transcript as a potential target for miR-143 repression, it would follow that the reduced levels of miR-143 detected in CaCo-2 cells may in fact not limit the ability of this miRNA to inhibit ATP1A1 translation. Is it possible that the depletion of the mature miRNA sequences is actually related to their activity in binding and inactivating target mRNAs in the cytoplasm, or does this argue against a role for miR-143-directed ATP1A1 repression? Clearly, more work is required to examine the interactions between miR-143, miR-145, and their potential targets, as well as the mechanism of miRNA-induced translational repression.

Recently, Calin *et al.* (9) reported an association between chronic lymphocytic leukemia and deletion of a section of

Notes to Table 2: Representation of the identified miRNAs in the cloned colonic populations and their sequences are shown. The predicted precursor structures are included for the novel miRNAs and those homologous to murine sequences. Human homologues of murine sequences are identified by a "hsa" prefix. References are included for previously cloned human miRNAs. Underlined bases represent the mature miRNA sequence.

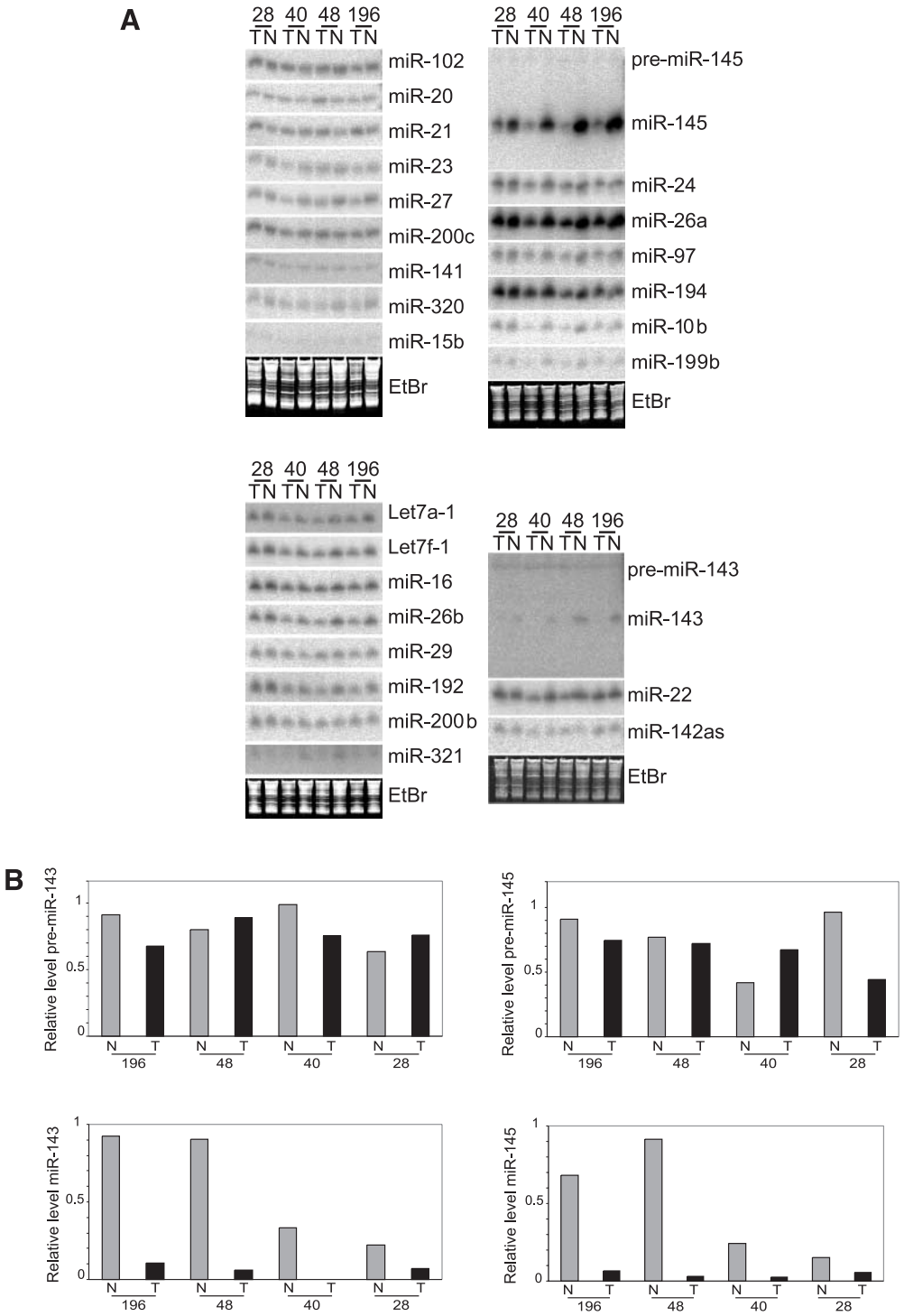


FIGURE 1. Accumulation of miRNAs in colorectal tissues. Representative Northern blot analyses (**A**) of the (~21 nt) RNase-processed miRNAs are shown. Northern blots of total RNA from matched normal mucosa (*N*) and adenocarcinoma (*T*) were probed for the indicated miRNA. The larger RNA bands that represent the precursor (*pre*) miRNA molecules are only shown for clones miR-143 and miR-145. The ethidium bromide-stained gels are included as loading controls. Relative signal intensities for miR-143 and miR-145 precursor and mature sequences in the RNA blots (**B**), normalized to the 5.8S rRNA loading control, are included for comparison of miRNA levels.

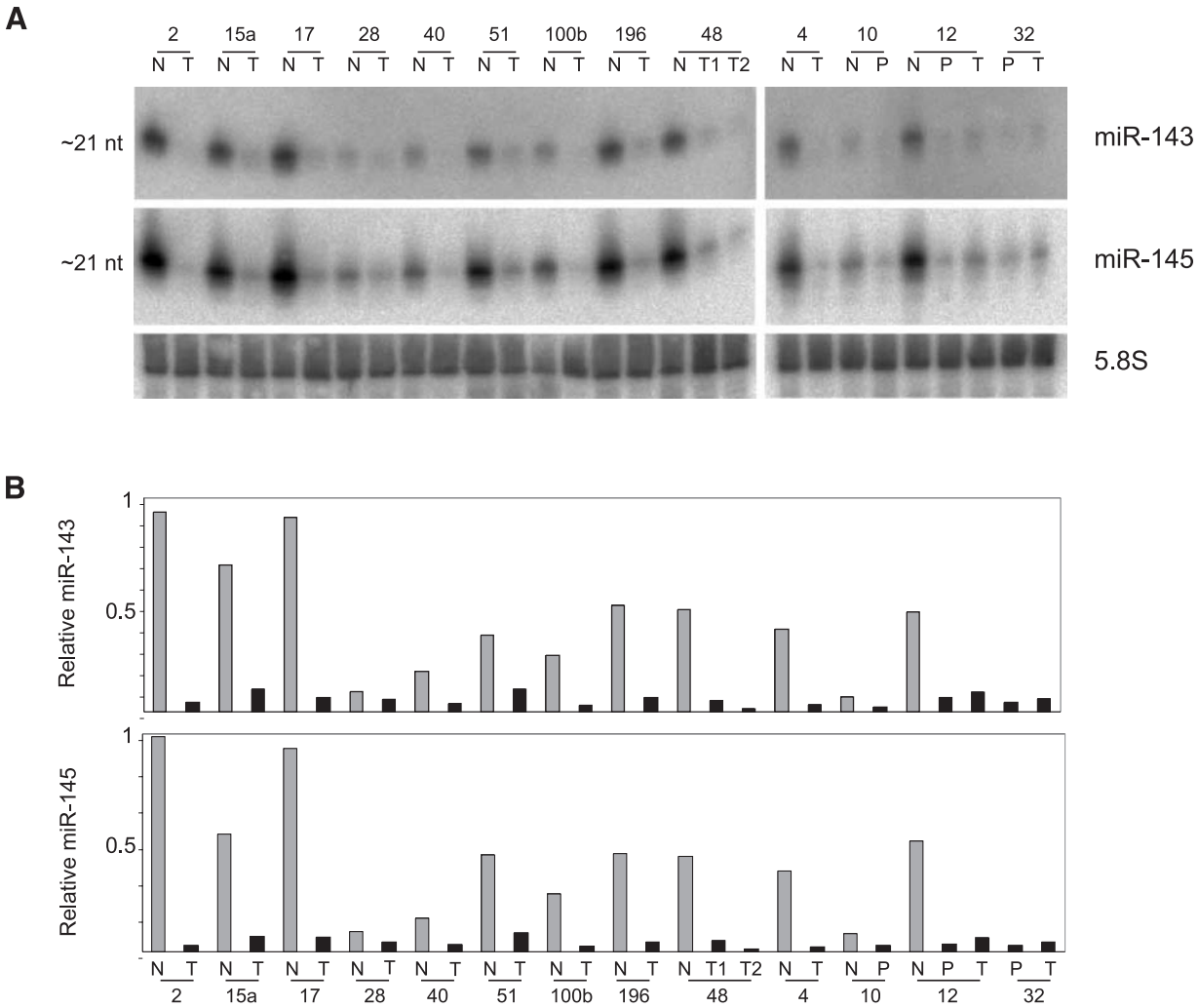


FIGURE 2. Accumulation of miRNAs in colorectal tissues. Northern blot analyses of the (~21 nt) RNase-processed miRNAs, miR-143 and miR-145, are depicted (**A**). Northern blots of total RNA from matched normal mucosa (*N*), adenocarcinoma (*T*), or adenomatous polyps (*P*) were probed for the indicated miRNA. 5.8S rRNA is included as a loading control. Relative signal intensities in the RNA blots (**B**), normalized to the 5.8S rRNA loading control, allow comparison of mature miRNA levels.

chromosome 13 that contains the genes for miR-15 and miR-16. That study showed down-regulation of these miRNAs in 68% of the leukemia lines studied. The same study also examined mutations in the miR-15 and miR-16 genes in gastrointestinal cancers, but failed to detect significant mutations or promoter methylation in these genes, which correlated with neoplasia. The present study has examined miR-16 expression in colorectal tissues and found that the levels of this miRNA do not change in colorectal cancers. Quantification of miR-16 accumulation in cancer cell lines derived from a variety of tissues displayed reduced levels only in the leukemia (chronic myelogenous leukemia) MEG-01 cell line and not in other lines derived from solid tumors.

In summary, this study has identified many miRNAs that are expressed in human colorectal epithelium. Characterization of these miRNAs has shown an association between reduced levels of two specific miRNAs and cancer. The identification of these two miRNAs provides the impetus for validation of their mRNA targets, to determine whether, and how, they directly impact the early processes of tumorigenesis in the colon. It will be interesting to see whether the paradigm shift that non-coding RNAs have created in our understanding of developmental processes will be mirrored in the way that we address cancer biology.

Patients and Methods

RNA Isolation From Tissue Samples and Cell Lines

Colorectal tumors and the corresponding normal mucosae were obtained from fresh surgical resections, following informed consent from patients. All tissues were collected within the framework of the Flinders Tissue Bank, with appropriate approval from the Flinders Clinical Research Ethics Committee. Histopathological classification of adenomas and

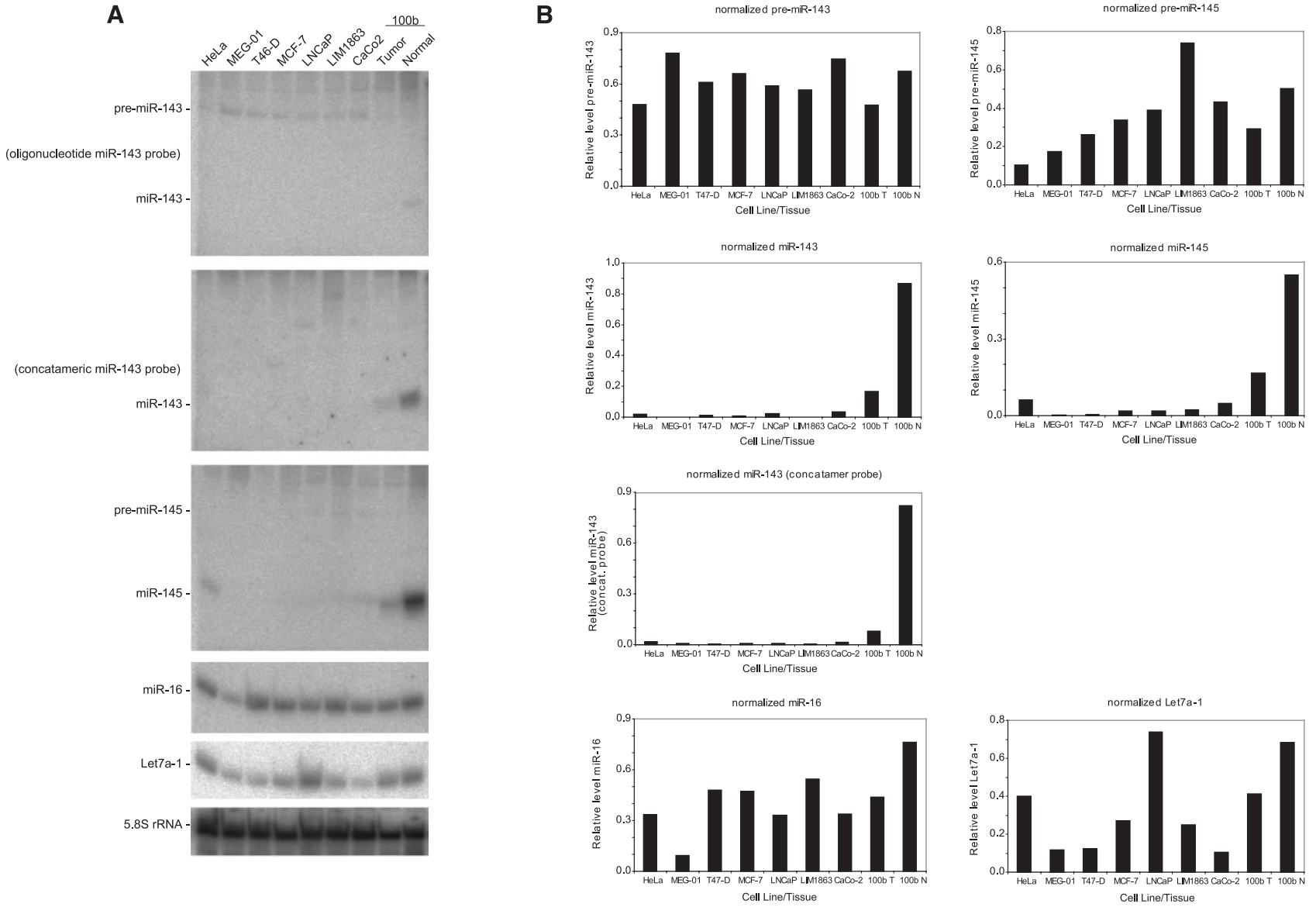


FIGURE 3. Accumulation of miRNAs in cancer-derived cell lines. Northern blot analyses of (21–22 bp) mature miRNA sequences and (~70 bp) pre-miR143 and pre-miR-145 sequences are shown (**A**). RNA blots of total RNA from cancer-derived cell lines were probed for the indicated miRNA. The blots were hybridized with end-labeled oligonucleotide probes to detect mature miRNA sequences. MiR-143 was also detected using a higher specific activity concatameric probe containing three direct repeats of the antisense strand. The concatamer probe did not detect the pre-miR-143 sequence. 5.8S rRNA is included as a loading control. Relative signal intensities in the RNA blots (**B**), normalized to the 5.8S rRNA loading control, allow comparison of miRNA levels.

Table 3. Human Messenger RNAs That Display Imperfect Complementarity With miR-143

Gene Name	Gene Symbol	Genbank ID	miR-143 Homology
Na, K+-ATPase α subunit	ATP1A1	806751	18 bp overlap—72%
Spermine synthase	SMS	21264340	19 bp overlap—68%
M96A Polycomb-like protein	M96	3402196	17 bp overlap—82%
HPK1/GCK-like kinase	HGK	22035605	17 bp overlap—71%
Raf oncogene	RAF1	35841	20 bp overlap—75%
Swi/snf related chromatin regulator	SMARCF1	21264574	15 bp overlap—73%
Tropomodulin 1	TMOD1	18605805	18 bp overlap—61%
Dihydrofolate reductase	DHFR	7262376	19 bp overlap—89%
Methylene-tetrahydrofolate reductase	MTHFR	31566106	17 bp overlap—82%

adenocarcinomas is presented in Table 5. Normal colorectal mucosae were dissected away from underlying tissues. Histopathological examination of normal mucosae confirmed their being unremarkable in every case, except for specimen #12, which displayed melanosis coli. RNA was purified from colorectal tissues using the procedure of Chomczynski and Sacchi (30).

Human cell lines used in this study included the following: colorectal adenocarcinoma lines, CaCo-2, and LIM1863 organoids (31); the prostate metastatic carcinoma line, LNCaP; breast carcinoma lines, MCF-7 and T47-D; chronic myelogenous leukemia line, MEG-01; and the cervical HeLa carcinoma line. LIM1863 was obtained from the Ludwig Institute for Cancer Research (Melbourne, Australia), and all other lines are available through the American Type Culture Collection. Cell lines were maintained in either: RPMI 1640 with 10% fetal bovine serum and L-glutamine (LIM1863, LNCaP, MEG-01, MCF-7, T47-D), or DMEM with 10% fetal bovine serum and added glucose, L-glutamine, sodium pyruvate, and pyridoxine hydrochloride (HeLa, CaCo-2). RNA was isolated from cell lines, using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Cloning of MicroRNAs

miRNAs were cloned, essentially, as described by Elbashir *et al*. (32), except that nucleic acids were electroeluted from acrylamide gel slices using the Biotrap system (Schleicher and Schuell GmbH, Dassel, Germany). Briefly, small RNA fractions of between 18 and 26 bases were size selected on a denaturing polyacrylamide gel. Adapter oligonucleotides, containing *Eco*RI restriction sites, were directionally ligated to the RNA molecules. The adapter-ligated RNA was then amplified by RT-PCR. Concatamerized fragments, containing multimers of religated, *Eco*RI-digested PCR products, between 200 and 650 bp, were size selected on an agarose gel and recovered by electroelution. The concatamers were end-repaired and dA-tailed with Taq DNA polymerase, then cloned into pGEM T-easy (Promega, Madison, WI) or pTOPO (Invitrogen) according to the manufacturers' instructions. Plasmid inserts from the resultant colonies were analyzed by PCR using primers to vector sequences. The nucleic acid sequence of selected inserts was determined (Beckman CEQ8000 system) following treatment of the PCR products with Exonuclease I and Shrimp Alkaline Phosphatase according to the ExoSAP-IT protocol (USB Corporation, Cleveland, OH). The clones created by this procedure contained concatamers of PCR products, which generally represented between two and five independent small RNAs.

Clones were classified as miRNAs if they satisfied the following criteria. (*a*) They were largely represented in the total population of clones identified. (*b*) They were found to correlate to genomic sequences within a region that would fold into a hairpin structure if transcribed. (*c*) They were shown to accumulate as appropriately sized fragments by Northern blot analysis with total colorectal RNA. As many miRNA genes are known to cluster on human chromosomes (12), clones with single representation were also investigated if they corresponded to genomic sequences within close proximity of other miRNA genes and fulfilled criteria *b* and *c*.

Northern Analysis

Total RNA (20 μg) was separated on a 15% denaturing polyacrylamide gel. Loadings were visualized by ethidium bromide staining. The RNA was then transferred to Hybond N+ nylon membrane by semi-dry blotting (OWL Separation Systems, Portsmouth, NH). Probes were generated by T4 Polynucleotide Kinase (New England Biolabs, Beverly, MA) mediated end-labeling of DNA oligonucleotides with [γ-^{32}P]ATP. To increase the specific activity of the miR-143 probe (Figs. 1 and 3), the miRNA sequence was concatamerized as a trimer of direct repeats, then cloned into pGEM T-easy and the insert amplified using PCR with M13 forward and reverse primers. Antisense probes were then synthesized using Taq polymerase-generated linear amplification from the Sephadex G-50-purified PCR products to incorporate multiple [α-^{32}P]dCTP bases. Filter hybridization was performed in QuikHyb Solution (Stratagene, La Jolla, CA) containing 10^6 cpm/ml probe for 1 h, with washes, as per the manufacturers'

Table 4. Human Messenger RNAs That Display Imperfect Complementarity With miR-145

Gene Name	Gene Symbol	Genbank ID	miR-145 Homology
Rho GTPase activating protein	RICS	12802987	17 bp overlap—100%
G-protein γ7	GNG7	3149953	16 bp overlap—94%
Nuclear ribonucleoprotein-C	HNRPC	13097278	16 bp overlap—88%
Thioredoxin containing ER protein	TXNDC4	27478880	11 bp overlap—100%
Nucleoside diphosphate linked moiety X	NUDT5	7657402	16 bp overlap—81%
Endoplasmic reticulum protein p44	ERP44	18873557	15 bp overlap—93%
Golgi autoantigen GM130	GOLGA2	7644349	17 bp overlap—76%
Tumor-suppressing candidate 1	TSSC1	4507702	20 bp overlap—70%
Phenylalanine hydroxylase	PAH	2462721	19 bp overlap—79%

Table 5. Summary of Histopathology Data for Specimens Used in Study

Patient	Cancer Dukes' Stage	Cancer Differentiation Status	Cancer Location	Adenoma Size (mm)	Adenoma Type	Adenoma Location	Adenoma Features
2	A	moderate	rectum				
4	A	moderate	ascending colon				
10				30 × 20 × 20	tubulovillus	ascending colon	focal high-grade dysplasia
12	A	moderate	rectum				
12*				20 × 20 × 22	tubular	rectum	high-grade dysplasia
15a	C	poor	rectum				
17	A	moderate	sigmoid				
28	B	poor	descending colon				
32	A	moderate	sigmoid				
32*				8 × 6 × 5	tubular	descending colon	low-grade dysplasia
40	D	moderate	sigmoid				
48-T1	A	moderate	descending colon				
48-T2	A	moderate	descending colon				
51	D	moderate	ileocaecal junction				
100b	A	moderate	rectum				
196	B	moderate	transverse colon				

Note: Tumors T1 and T2 represent independent primary lesions from the same patient (patient 48). Asterisk indicates independent adenoma collected from the same patient.

recommendations. Filters were analyzed using a Fujifilm-BAS 2500 phoshorimager and signal intensity was quantitated (as photostimulated luminescence/mm^2) using Analytical Imaging Station (version 3.0) software (Imaging Research Inc., Brock University, Ontario, Canada).

miRNA sequences were identified by BLAST (33) comparison to the Genbank and EMBL public nucleotide databases. The secondary structures of putative pre-miRNA hairpins were determined using the Mfold 3.1 algorithm (34). Potential mRNA target sequences were identified by searching the Genbank nonredundant and dbEST databases using BLAST and FASTA (35) algorithms. The miRNA sequences identified in this study have been submitted to the miRNA registry (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml).

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