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Effects of the miR-143/-145 microRNA Cluster on the Colon Cancer Proteome and Transcriptome

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Abstract

The miR-143/-145 cluster is greatly reduced in several cancers, including colon cancer. Both miR-143 and miR-145 have been shown to possess antitumorigenic activity with involvement in various cancer-related events such as proliferation, invasion and migration. As the deregulation of the miR-143/-145 cluster is implicated in tumorigenesis, we combined SILAC and microarray analyses to systematically interrogate the impact of miR-143/-145 on the colon cancer proteome and transcriptome. Using SILAC we identified over 2000 proteins after reintroduction of miR-143 and miR-145, in the colon cancer cell line SW480, individually and then, in concert. Our goal was to determine whether these microRNAs function individually or synergistically. The resulting regulated gene products showed evidence of both mRNA destabilization and translational inhibition with a bias towards the former mechanism of regulation. Numerous candidate targets were identified whose expression is attributable to an individual microRNA or whose regulation was more apparent following reintroduction of the miR-143/-145 cluster. In addition, several shared targets of miR-143 and miR-145 were identified. Overall, our results indicate that the summed effects of individually introduced microRNAs produce distinct molecular changes from

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Supplemental Table S1. Information on FFPE patient samples.

Supplemental Table S2. qRT-PCR oligonucleotides (5'-3').

Supplemental Table S3. Terminology for microRNA cluster gene targets.

Supplemental Table S4. qRT-PCR expression profiling.

Supplemental Table S5. SRM transitions for differential protein quantification validation.

Supplementary Table S6. Previously validated miR-143 and miR-145 targets.

Supplemental Figure S1. miR-143 and miR-145 expression level comparison.

Supplemental Figure S2. Comparison of detected protein between samples.

Supplemental Figure S3. Cumulative distribution analysis.

Supplemental Figure S4. Cumulative distribution analysis for contrary cluster member.

Supplemental Figure S5. Cumulative distribution analysis for unrelated microRNAs.

Supplemental Figure S6. Target prediction algorithm performance.

Supplemental Figure S7. Pathway overrepresentation analysis using Reactome.

the consequences of the assembled cluster. We conclude that there is a need to investigate both the individual and combined functional implications of a microRNA cluster.

Keywords

SILAC; microRNA; miR-143; miR-145; Colon cancer

Introduction

MicroRNAs are small (~22 nucleotide) endogenous activators of the RNA interference (RNAi) pathway. These products of non-coding genes interact with the RNAi machinery to sequester, destabilize or degrade target mRNA, resulting in translational inhibition. MicroRNAs have seed regions of approximately 7 nucleotides (position 2–8 from the 5' end of the mature microRNA) and transcripts with sufficient base-pair matching (generally a 7–8 nucleotide match) to the microRNA are targeted for repression ².

The 1516 registered human microRNAs (miRBase, release 18) can be grouped into families and clusters based on sequence and genomic relatedness, respectively. MicroRNA family members are composed of multiple monocistronic microRNAs that have primary sequence similarity with the same seed. There is extensive mRNA target overlap by family members presumably because the seed sequence contributes significantly to mRNA target specificity. MicroRNAs with the same seed are often thought to have redundant functions as well ³.

MicroRNAs that are closely distributed in the genome, usually consecutively located within 10kb of each other, are considered to belong to one microRNA cluster ⁴. Clusters of microRNAs are transcribed coordinately as a polycistron that is processed to produce the individual members resulting in consistent co-expression ⁵. MicroRNAs originating from a single cluster will often display corresponding sequence homology, and therefore overlapping targets. For example, miR-15a and miR-16, comprising the miR-15a-miR-16 cluster, have homologous seed sequences and thus possess the same targets ⁶. However, for microRNAs in a cluster that do not share homology, their individual and combined functionality is less clear. Phenotypic studies of the six microRNAs that make up the human miR-17–92 cluster indicate that two microRNAs have antiangiogenic properties not observed with manipulation of the other four ⁷. MicroRNA genomic coordination resulting in their coordinated transcription may provide an internal mode for functional coordination exerted by microRNA clusters ^{8, 9}. However, functional implications of the clustering of microRNAs remain unclear.

While proper microRNA function is critical for the health of normal tissues, it has been found that many microRNAs reside in fragile regions of the genome and are often altered in expression in the progression of cancer ¹⁰. Expression of miR-143 and miR-145 are significantly reduced in colon cancer compared to normal colon tissue ^{11, 12}. In particular, colon cancer is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women. There will be an estimated 100,000 new cases and 50,000 deaths from colon cancer in the United States in 2012 ¹. These two microRNAs are located in a cluster in the 5q32 chromosomal region, and do not share sequence homology (Fig. 1a). Several mRNAs have been shown to be direct targets of miR-143 ^{13, 14} or miR-145 ^{15–17} in colon cancer. These two microRNAs have also been investigated in breast cancer ¹⁸, gastric cancer ¹⁹, non-small cell lung cancer ²⁰ and prostate cancer ^{21, 22} with a systematic study of miR-143 in pancreatic cancer ²³. However, there are most likely additional unconfirmed targets as there are hundreds of computationally predicted targets for these two microRNAs ^{24–29}.

The identification and confirmation of microRNA targets remains a challenge since the base matching criteria is rather promiscuous and microRNAs are estimated to impact one-third of all human genes²⁴. Given that those primary targets then interact with downstream targets, expression of a single microRNA can have ramifications on the expression of hundreds of gene products. Previous studies have examined the effect of adding or subtracting a single microRNA on the global transcriptome and proteome, revealing hundreds of transcripts and proteins subtly, but significantly altered in expression^{30, 31}. The high-throughput nature of gene expression microarrays has been successful in identifying microRNA targets. However, this approach is likely to overlook the targets regulated through translational repression mechanisms. Therefore, protein expression changes accompanying manipulation of microRNA expression are also necessary. SILAC (Stable Isotope Labeling with Amino Acids in Cell Culture) quantification has been proven an accurate method for mass spectrometry-based proteomics³².

In this study, we were the first to perform a global analysis of the effect of a microRNA cluster and its individual members on the transcript and proteome using a combined and complimentary SILAC and microarray approach. Our results indicate that examining microRNA cluster members individually can identify novel targets. However, the functional implications of a microRNA cluster may not be apparent until the individual members are analyzed in concert, demonstrating the challenging nature of deciphering the widespread functions of individual microRNAs and microRNA clusters.

Experimental Procedures

Cell culture and tissue sections

Cell lines were maintained as previously described³³. Formalin-Fixed Paraffin-Embedded tissue sections were obtained from the South Bend Medical Foundation (<http://www.sbmf.org/>) with IRB approval. Details on patient samples are provided in Supplementary Table S1. Total RNA, including miRNA, was extracted from the FFPE tissue sections using the RNeasy FFPE kit (Qiagen, Germantown, MD) following the manufacturer's instructions with minor revisions. Normal human colon RNA isolated postmortem from a donor (used for comparison with RNA from cell lines) was purchased from Ambion (Applied Biosystems, Foster City, CA).

MicroRNA mimic oligo transfections

SW480 cells were grown in SILAC RPMI 1640 media containing either naturally occurring isotopes of arginine and lysine or heavy arginine ($^{13}\text{C}_6$ $^{15}\text{N}_4$) and lysine ($^{13}\text{C}_6$ $^{15}\text{N}_2$) (Isotec, Sigma). At 50–60% confluence, SW480 cells were transfected with 50 nM miR-143, miR-145 or 25 nM miR-143 and 25 nM miR-145 miRIDIAN microRNA mimic oligos (Thermo Scientific) using Lipofectamine RNAiMAX (Invitrogen). Cells grown in heavy SILAC medium were mock transfected and used as a negative control. For transfection validation studies and analysis of mRNA, the microRNA mimic oligos (25 nM or 50 nM) was added to individual wells in a six-well plate in 750 μL of serum-free RPMI and complexed with 10.5 μL transfection reagent in 750 μL of serum-free RPMI for 30 min at ambient temperature. Next, SW480 cells (180,000 cells/well) were added in 1500 μL RPMI supplemented with 20% FBS. The final mixture was incubated at ambient temperature for 45 min before being placed in an incubator in 5% CO_2 at 37°C. For proteomic analysis, transfections were conducted in 10-cm plates and all reagent amounts were scaled accordingly. All transfections were conducted in triplicate. Twenty-four and forty-eight hours post transfection, the six-well plates were harvested for microRNA (miRNeasy) and mRNA (RNeasy) (Qiagen), respectively. The microRNAs were harvested for transfection validation (qRT-PCR) and the extracted mRNA was used for expression profiling

(microarray) and RTPCR validation of target genes. Seventy-two hours after transfection, the 10-cm plates were harvested with Complete Lysis-M Reagent kit (Roche Diagnostics, Indianapolis, IN) with 1X Complete Protease Inhibitor (Roche). The total protein concentration was determined for each sample using a BCA protein assay kit (Thermo Scientific) and bovine serum albumin standards (Thermo Scientific) and mixed in a 1:1 (light: heavy) ratio.

Preparation of Mass Spectrometric Samples

Lysates (pooled triplicate biological replicates) were resolved by a NuPAGE SDS-PAGE system (Invitrogen) (4–12% acrylamide, Bis-Tris with MOPS running buffer) and stained with Colloidal Blue staining kit (Invitrogen). Gel lanes were excised into 8 sections and cut into 2 mm-wide pieces and subjected to in-gel tryptic digestion. Gel pieces were washed/dehydrated three times in 50mM ABC /50mM ABC + 50% ACN. Cysteine bonds were reduced with 10mM DTT for 1hr at 56°C and alkylated with 55mM IAA for 20min at room temperature in the dark. Following subsequent wash/dehydrate cycles, the samples were dried 20min in a MiVac sample concentrator (Genevac Inc, New York, NY) and incubated overnight with 12.5ng/μL trypsin in 25mM ABC at 37°C. Peptides were extracted twice in 50μL 50% ACN/45% water/5% formic acid (FA) (Optima LS/MS, Fischer Scientific, Fair Lawn, NJ). The combined volumes were concentrated and desalted with C18 ZipTips (Millipore, Billerica, MA) according to manufacturer's instructions. The desalted peptide volume was lyophilized and resuspended in 0.1% FA.

Mass Spectrometry analysis

Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS) was performed on a nanoAcquity ultra performance LC system (100 μm × 100 mm C18 BEH column) (Waters, Milford, MA) coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were eluted using a binary solvent system with 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) with the following linear gradient: 10–40% B in 50 min, then washed at 85% B for 5 min and equilibrated with 1% B for 10 min at a 1200 nL/min flow rate. The LTQ Orbitrap Velos was equipped with a nano-electrospray ion source (Thermo Fisher Scientific) and operated with a source voltage, 1.8 kV, source current, 100 μA and capillary temperature, 300°C. Full scan MS spectra (m/z 395–1900, resolution of 60000 at m/z 400) were acquired in the Orbitrap. Automatic gain control was set to 5×10^5 ions and a maximum fill time of 500 ms. The eight most intense multiply charged ions were selected for fragmentation by CID in the ion trap with automatic gain control set to 1×10^4 ions and a maximum fill time of 100 ms. Fragmentation was carried out at a normalized collision energy of 35%, activation $q=0.25$, activation time of 10 ms on ions above the 3000 selection threshold. The precursor isolation width was set to 4 m/z and precursors were set in an exclusion list for 45 s after 1 repeat count. All samples were run in triplicate.

Mass spectrometric data analysis

The mass spectrometric raw data was analyzed with the MaxQuant software (version 1.2.0.18)³⁴. The allowed mass deviation of the precursor ion was set to 7 ppm and 0.5 Da for the fragment ion. Search of the MS/MS spectra against a decoy database based on the reverse sequence database concatenated with the forward Uniprot human database (73,448 entries) and combined with common contaminants was performed using the Andromeda search engine (version 1.2.0.14)³⁵. Enzyme specificity was set as C-terminal to lysine and arginine with a maximum of two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification and N-terminal protein acetylation, methionine oxidation and N-terminal glutamine deamination as variable modifications. The labeling state of paired peptides was determined in advance via MaxQuant to allow separate database searches

where each SILAC state was set as a fixed modification with $^{13}\text{C}_6$ - $^{15}\text{N}_2$ -lysine and $^{13}\text{C}_6$ - $^{15}\text{N}_4$ -arginine set as heavy labels. The false discovery rate (FDR) was set to 0.01 for peptides and proteins. Proteins identified were required to contain a "unique or razor" peptide with a minimum length of six amino acids for identification ("Unique or razor" is a setting in MaxQuant). Protein groups were reported for proteins identified from the assignment of the same peptides. Relative peptide and protein quantification were performed automatically by MaxQuant with "Re-quantify" and "Filter labeled amino acids" features enabled. MaxQuant reported the median of all peptide ratios for a protein ratio with the protein ratios normalized so that the median of all ratios is zero. The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche using the following hash: XVhNvyT6tEc5rZdCxaS+9MqRZhmP4mgjvT64dNAIFEXPTaC4OrGmjvwaE6NznxRGF bEhX1HaYLhfm1150/jRBIG67JIAAAAAAAAgNQ==

SRM analysis

SRM method generation and sample quantification was performed with Skyline v1.0³⁶. SRM detection was performed on a 5500 QTRAP (AB SCIEX, Concord, Ontario, Canada) running in triple quadrupole (QqQ mode). Peptide separation was carried out on a HPLC 2D NanoLC Ultra (Eskigent) equipped with a Acquity BEH C18 (100 μm $\times$ 100mm) column (Waters) using a linear 90 min gradient from 3% to 31% solvent B followed by an organic wash and equilibration step (solvent A: 0.1% formic acid (Optima) in 3% ACN; solvent B: 0.1% formic acid 97% ACN) at a flow rate of 600 nL/min. The mass spectrometer was operated in positive ion mode with a curtain gas of 20.0, an interface heater temperature of 150.0 and an ionspray voltage of 2350.0. A dwell time of 12–25 ms was used depending on the number of transitions measured per run. Collision energies (CE) were calculated using the formula $\text{CE} = 0.029 * (\text{precursor } m/z) + 2.0$ for doubly charged precursor ions and $\text{CE} = 0.05 * (\text{precursor } m/z) + 6.0$ for triply charged precursor ions. All samples were analyzed in two technical replicates. SRM responses were transformed with Savitzky-Golay smoothing prior to integration with the Skyline software. Samples were normalized to an endogenous protein to account for sample variability before determining the light: heavy ratios. Supplemental table S5 contains the peptide sequences and corresponding transitions used for quantification.

Microarray analysis

Total mRNA samples with RIN 8.0 were analyzed via Affymetrix GeneChip Human Exon ST 1.0 arrays (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Microarray analysis was performed in biological triplicate. The Affymetrix data files were processed using the Bioconductor software package with mean fluorescence intensities derived from a log₂ transformation of the data and normalization using the quantile normalization method. The data was subjected to Student's t-test using a p-value of 0.05 and the Benjamini and Hochberg false discovery rate multiple testing correction³⁷.

Quantitative Real-Time PCR

Gene expression analysis was performed as previously described³³. MicroRNA expression analysis was performed using TaqMan microRNA assays (Applied Biosystems, Foster City, CA) for miR-16 (used for microRNA expression level normalization), miR-143 and miR-145. Forty ng of total microRNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and 3 μL of RT primer. The 15 μL reactions were incubated in a thermocycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and held at 4°C. Quantitative PCR was performed with a real-time PCR system, StepOne-Plus (Applied Biosystems). The 20 μL reactions consisting of 10 μL TaqMan 2x PCR master mix, 1 μL probe mix, 1.33 μL diluted RT product and 7.67 μL nuclease-free

water were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. All samples were analyzed in triplicate wells. A list of qRT-PCR primers is provided in supplemental table S2.

Terminology

We are using the following terminology to describe microRNA cluster gene targets (Supplemental Table S3). MicroRNA targets detected in our experimental data have been termed detected. Predicted will refer to computationally predicted targets for an individual microRNA. The term unique will be used to describe targets that have been detected for the first time in our experimental dataset; these targets may also be predicted. Targets experimentally shown to be repressed by both microRNAs when expressed singularly are referred to as shared targets. Clustered targets describe microRNA targets that have been detected as repressed only when both microRNAs are expressed together.

Results

MiR-143 and miR-145 profiling in colon cancer

We profiled the expression levels of miR-143 and miR-145 in 5 human colon cancer cell lines (DLD-1, HCT116, HT29, SW480, SW620) and 6 pairs of patient-matched formalin-fixed, paraffin-embedded (FFPE) colon tissue samples (normal colon mucosa and Stage II biopsy from each patient) to validate that miR-143 and miR-145 are downregulated in colon cancer (Fig. 1b–c). These cell lines represent adenocarcinoma and carcinoma tumors with microsatellite instability (DLD-1 and HCT 116) and chromosomal instability (HT-29 and SW480) as well as metastatic colon cancer (SW620), showing the broad loss of miR-143 and miR-145 in colon cancer³⁸. The expression levels of this cluster of microRNAs are also highly regulated ($r^2=0.873$, $P<0.0001$), consistent with the notion that these microRNAs are co-expressed and transcribed as a single primary microRNA (pri-miRNA) (Fig. 1d). Furthermore, profiling miR-143 and miR-145 levels in 356 human colon tissue samples from three publicly available microRNA microarrays (GSE30454, GSE18392, GSE28364) revealed strong positive correlation between miR-143 and miR-145 expression ($n=74$, $R^2=0.759$, $p<0.001$; $n=146$, $R^2=0.899$, $p<0.001$; $n=136$, $R^2=0.790$, $p<0.001$, respectively) reinforcing the co-expression of these clustered microRNAs and resulting in a roughly 1:1 stoichiometric ratio in vivo. (Supplemental figure S1).

As a normal colon cell line is not readily available, it is not feasible to reduce the expression of these microRNAs in a normal colon cell line to study their effects. Therefore, in order to determine the effects of the miR-143/-145 cluster in colon cancer, we reintroduced the microRNAs into a cancerous cell line. We transfected SW480s, an archetypal human colon adenocarcinoma cell line, with microRNA mimic oligos for miR-143 and miR-145, individually as well as in concert. Colon cancer cell lines have been shown to recapitulate the miR-143 and miR-145 expression patterns observed in primary tumors, making cell lines a suitable model for functional analysis of these microRNAs³⁹. We selected SW480 because it has chromosomal abnormalities commonly seen in colon cancer including: gain of 7, 8q, 13q, 20q and loss of 4q, 8p, 17p, 19q⁴⁰. As the miR-143/-145 cluster is expressed at low levels in normal colon tissue, we hypothesized that miR-143/-145 overexpression in an established colon cancer cell line would decrease the expression of the associated targets. Over expression of miR-143 and/or miR-145 was verified by qRT-PCR 24 hours post transfection. After careful optimization, the concentration of transfected microRNA mimic resulted in microRNA expression levels comparable to those detected in normal colon mucosa (Fig. 1e). We then employed these transfection conditions for microarray analyses and quantitative proteome profiling following reintroduction of miR-143, miR-145 or miR-143/-145. An overview of our experimental design is depicted in Fig. 2.

Quantitative protein and mRNA profiling

To identify miR-143/-145 targets we used SILAC and GeLC-MS/MS to quantify the expression changes among microRNA transfected samples and control transfected samples. The mass spectrometric analyses were performed using three pooled biological replicate transfections and triple technical replicate LC-MS/MS runs (refer to "Experimental Procedures" for details). The resulting datasets were analyzed in MaxQuant with the integrated Andromeda search engine. Table 1 lists the results of the database search including spectra, peptides and proteins identified and quantified in the three independent microRNA reintroduction SILAC experiments. Between 13,192 and 13,643 peptides were identified per experiment mapping to a combined 2,772 proteins. There were 247,807 to 260,785 SILAC pairs identified per experiment. These SILAC pairs lead to an average of 1965 quantified proteins for the three experiments, for a combined 2,416 proteins quantified. Supplemental figure S2 illustrates the significant overlap between the three independent proteomic data sets (miR-143, miR-145, miR-143/145) on the level of identification and quantification. The overall median sequence coverage and coefficient of variation were 16.1% and 16.5%, respectively, with a median ratio count of 9 peptides. Proteins with PEP (posterior error probabilities) greater than 0.01 were excluded from further analysis. Relative changes in protein expression were determined by the ratio of the intensity from microRNA-transfected samples to control transfected samples. We also performed gene expression profiling to detect mRNA changes following miR-143, miR-145 or miR-143/-145 reintroduction using Affymetrix GeneChip Human Exon 1.0 ST arrays. The mean ratios for the three independent biological replicate microarrays for each condition were used to draw conclusions.

MicroRNA reintroduction revealed no significant change in expression for the majority of proteins. However, following the transfection of miR-143, miR-145 or miR-143/-145, a handful of proteins were detected and quantified with significant abundance changes (log2-fold change -0.5 and 0.5). Similarly, the distribution of mRNA ratios showed mild changes following microRNA overexpression for a subset of transcripts (Fig. 3).

Protein and mRNA changes accompanying reintroduction of miR-143 and/or miR-145

A cumulative distribution analysis of proteins and mRNAs with the respective miR-143 or miR-145 seed sites in their 3' untranslated regions (UTRs) revealed a shift toward repression when compared to gene products without microRNA seed sites (Fig. 4a–d). Analysis of gene products with seed sites from the alternative microRNA in the cluster whose expression remained unaltered revealed a less substantial or lack of a shift towards repression (Supplementary Fig. S3). The subtle bias toward lower expression seen when predicted miR-145 targets were enriched in the miR-143 dataset may be attributable to the overlap of predicted miR-143 and miR-145 targets based on computational microRNA target prediction algorithms (Supplementary Figure S4). Furthermore, we performed analysis on several microRNAs that are also misexpressed in colon cancer, but unrelated to the miR-143/-145 cluster. The predicted targets of the tumor suppressor microRNAs (miR-1 and miR-365) and the oncogenic microRNAs (miR-21 and miR-31) did not yield a shift towards repression at either the protein or the mRNA level (Supplemental Fig. S5). These results indicate that the reduced protein expression and transcriptional repression are dependent on the presence of microRNA seed-matching binding sites of the particular microRNA reintroduced.

We also analyzed whether transcript and protein changes following miR-143/-145 reintroduction could be specifically attributed to expression of both miR-143 and miR-145. Cumulative distribution analysis showed reduced protein and mRNA expression for gene products with miR-143 and miR-145 seed sites in their 3'-UTRs compared to gene without

seed sites (Fig. 4c–d). These results suggest that the experimental data is consistent with both miR-143 and miR-145 being reintroduced.

Evaluation of Target Prediction Algorithm Performance

Numerous computational target prediction algorithms, based on seed complementarity to the 3'-UTR and favorable microRNA-target duplex thermodynamics, have been established. We have evaluated the efficacy of several computational methods for identifying potential genomic binding sites based on the degree of repression of computationally predicted microRNA targets seen in our experimental microarray and proteomic profiling results. A comparison of the predictions from DIANA microT²⁵, miRanda²⁶, miRDB²⁷, PicTar²⁸, PITA²⁹ and TargetScan²⁴ revealed that those from miRDB and TargetScan agreed best with our experimental data. Predictions from these algorithms yield the greatest degree of repression in the experimental data. (Supplemental Fig. S6a) These particular algorithms are based on site conservation and include messages with 7–8mer 3'-UTR binding sites. Even though miRDB and TargetScan targets were most accurate, numerous predicted targets were unresponsive in our experimental results. Enhanced algorithm performance was attained when taking into account prediction rank, the presence of 8mer seed sites and messages with multiple sites based on more repressed mean log2-fold changes (Supplemental Fig. S6b–d).

Translational repression versus mRNA destabilization

By analyzing gene expression changes at the transcript and proteome level, the contribution of translational repression and mRNA destabilization can be examined to determine which mechanism is the dominant scenario in microRNA gene regulation. Previous efforts have yielded differing conclusions, reporting that the dominant mode of target regulation occurs at the protein level²³, via mRNA destabilization^{31, 41} or through co-regulation⁴². Pervasive mRNA destabilization is indicated by mRNA reduction that consistently mirrors protein reduction as evidenced by a diagonal line (slope 1.0) and strong correlation (R^2) resulting from plotting protein versus mRNA ratios. Translational inhibition is suggested by a negative y-intercept from the resulting linear regression analysis. Our experimental data indicates mRNA destabilization as the dominant mode of regulation with a cohort of genes undergoing translational repression.

Plotting protein changes as a function of mRNA changes indicated a strong positive correlation for transcripts containing 3'-UTR seed sites and a weaker correlation for genes without a cognate microRNA seed site ($R^2 = 0.486$ and 0.161 , $P < 0.0001$ and $P < 0.0001$, respectively) for the miR-145 protein: mRNA correlation. Similar results were seen in the miR-143 ($R^2 = 0.5369$ and 0.0835 , $P < 0.0005$ and $P < 0.0001$, respectively) and miR-143/-145 ($R^2 = 0.4154$ and 0.0471 , $P < 0.0001$ and $P < 0.0001$, respectively) datasets (Fig. 5). Additionally, the correlation was stronger for 8mer seed sites ($R^2 = 0.561$, $P < 0.0001$) compared to 7mer seed sites ($R^2 = 0.124$, $P < 0.0001$) and when considering multiple seed sites per message ($R^2 = 0.731$, $P = 0.015$) versus a single site ($R^2 = 0.3902$, $P < 0.0001$) as seen in the miR-145 dataset. The miR-143 and miR-143/-145 datasets showed similar trends. The increased correlation between protein and mRNA changes for predicted targets supports mRNA destabilization of the genes.

If protein changes were accounted for based on mRNA destabilization alone, the data points would fall along the diagonal with a y-intercept of zero based on a linear regression of a protein versus mRNA changes scatter plot. However, the experimental data points are scattered around the origin with additional points extending into quadrants III and I. The resulting linear regressions yield statistically significant negative y-intercepts and origin-centered y-intercepts for the genes with seed sites. This indicates that both mRNA destabilization and translational repression are contributing mechanisms to microRNA gene

regulation. The protein-mRNA correlations with significant negative y-intercepts suggest a cohort of genes were depressed at the protein level with little or no change at the mRNA level. Several detected targets under regulation at the translational level were identified for miR-143 and miR-145. The presence of a cohort of genes undergoing translational repression indicates the need for further studies to reveal proteins that were not detected by SILAC, but mechanistically regulated through translation inhibition. Although there is evidence for both mRNA destabilization and translation-only repression, proteins with enhanced repression seem to derive from messages with detectable decreases in transcript expression. As the degree of protein inhibition increases, the average mRNA repression also increases. Overall, for genes undergoing more robust repression, mRNA destabilization seems to be the pervasive mechanism for regulation.

Genomic and proteomic identification of targets

Candidate targets were identified based on differential regulation at the mRNA and/or protein level. Numerous novel independent miR-143 and miR-145 targets were detected following the introduction of these two microRNAs individually (Fig. 5a–b). We also observed differential regulation for previously confirmed miR-143 (ANP32B, ERK5, GMPSP, KRAS) and miR-145 (BIRC2, DFF45, FSCN1, NRAS, RTKN, YES1) targets, which serve as positive controls for our experimental results. Based on the independent targets of miR-143 and miR-145, there is overlap between the lists of genes targeted, indicating a cohort of shared targets (Fig. 5c). Although miR-143 and miR-145 are not homologous, these two microRNAs may still have shared targets. MicroRNAs are known to bind to the mRNA 3'-UTR and the median human mRNA 3' UTR is 700 nucleotides. Therefore, it should not be surprising that microRNAs with differing seed sequences could target the same gene. For example, the putative shared targets TPM3 and HIPK2 each have two miR-143 and two miR-145 binding sites within their 3'UTRs. Interestingly, additional targets of the miR-143/-145 cluster were detected following transfection of both members (Fig. 5d). These cluster targets do not display corresponding regulation when the individual microRNAs comprising the cluster were transfected.

Expression Profiling Validation

We used qRT-PCR to confirm the microarray results of select transcripts with a wide range of altered expression changes. Agreement in the direction of change in expression between both microarray and qRT-PCR was evaluated for 15 genes (Supplemental Table S4). Overall, a significant correlation of $R^2=0.689$ was observed (Pearson correlation test, $p<0.001$) with correlations for the individual genes ranging from $R^2=0.185$ to $R^2=0.964$.

We carried out a targeted validation strategy, selected-ion reaction monitoring (SRM), to verify expression differences for detected proteins. We were able to confirm the differential expression for 16 proteins with consistency in direction and magnitude (Fig. 6). The peptide sequences and corresponding transitions used for quantification can be found in supplemental table S5.

Inverse correlation of microRNA and target expression

Expression profiles for pairs of microRNA and mRNA that are inversely correlated indicate microRNA-target relationship as microRNA-induced mRNA degradation would result in a negative association between microRNA and target mRNA expression levels. Using RT-PCR we analyzed ARF6 mRNA expression in 6 normal colon tissues and 6 colon tumor tissues (Fig. 7). We found that ARF6 expression is upregulated in the tumor samples compared to the normal tissue ($p=0.030$) while the expression of miR-143 ($p<0.001$) and miR-145 ($p<0.001$) are downregulated in the same samples. Overexpression of

miR-143/-145 lead to reduced levels of ARF6 on both the protein and mRNA levels, supporting the role of ARF6 as a novel miR-143/-145 target.

Pathway analysis

Pathway overrepresentation evaluation was performed using Reactome⁴³ to determine the biological processes strongly enriched in the repressed gene targets following overexpression of miR-143, miR-145 and miR-143/145 (Supplemental Fig. S7). Reintroduction of miR-143/145 revealed several pathways enriched for repressed targets that were not significant when the individual microRNAs were transfected including cell junction organization, DNA repair and muscle contraction.

Discussion

In the present study, we performed proteomic and transcriptomic profiling of human colon cancer cells with SILAC and microarray analyses following microRNA transfections to reveal the complex functionality of a microRNA cluster. Among the downregulated proteins and mRNAs, we identified 150 candidate targets of the miR-143/-145 cluster. Of these, 19 are unique shared miR-143 and miR-145 targets with an additional two previously validated shared targets, FSCN1⁴⁴ and MDM2⁴⁵ (Sup Table S6 for more information on previously detected targets), also detected.

These shared targets suggest that miR-143 and miR-145 function, in part, synergistically to impact gene expression. One such candidate is the oncogene CDH17, a member of the cadherin superfamily. Cadherins function as calcium dependent cell adhesion proteins and whose dysregulated expression is associated with tumor formation and metastasis⁴⁶. CDH17 has also been shown to be involved in proliferation by activating Wnt signaling⁴⁷. CDH17 was repressed by more than 2-fold on the protein and mRNA level following transfection of miR-143 or miR-145. The miR-143 targets PLEC and CLDN3 and the miR-145 targets CTND1, PODXL and F11R are also involved with aspects of cell adhesion phenomena and cell junction organization. Determination of these targets provides support for not only coordinated gene expression regulation but also coordinated biological function between clustered microRNA members.

In addition to the miR-143 and miR-145 targets previously validated in the literature and supported by our experimental data, our analysis discovered 49 miR-143 targets and 110 miR-145 targets predicted by TargetScan providing support for the detection of biological targets by our methodology.

As very few demonstrations of a cluster of non-family microRNAs targeting the same genes, our results represent a novel dataset to explore individual and combined microRNA functionality. Most importantly, our results indicate that the collective expression of all the members of a microRNA cluster can impart changes in gene expression not evident when the members are expressed singularly. For example, ARF6, ADP-ribosylation factor-6, plays a role in endosomal membrane traffic and actin remodeling that contributes to cell-surface-associated activities such as cell adhesion, migration and invasion^{48, 49}. ARF6 was not significantly regulated following individual transfection of miR-143 or miR-145. However, ARF6 was repressed by more than 2-fold on the protein level after miR-143/-145 reintroduction. Additionally, the cluster target PNX, paxillin, was detected as repressed only when both microRNAs comprising the cluster were expressed together. This multidomain adaptor protein plays a role in transducing adhesion and growth factor signals to elicit changes in cell migration, cell spreading and gene expression⁵⁰. For microRNA clusters, the whole cluster seems to be different than the sum of the parts. Our results indicate that a

cluster of microRNAs needs to be studied both on the individual level and in concert in order to gain a complete understanding of targets and regulatory roles.

Evaluation of the biological consequences reveals miR-143 and miR-145 to play distinct, yet complementary, roles in colon cancer. Pathway enrichment analysis of the downregulated gene targets of miR-143 and miR-145 point to diverse biological processes being strongly overrepresented. Interestingly, the combined expression of the miR-143/145 cluster resulted in additional enriched pathways that were not significant when the individual microRNAs were reintroduced. These results indicate complementary roles for miR-143 and miR-145 where individual microRNAs comprising a cluster can be deployed to mediate distinct roles and overlapping function depending on their targets emphasizing the regulatory complexity in pathways involving microRNA clusters.

In conclusion, our results show that proteomic expression profiling, coupled with microarray analysis, is an excellent strategy to systematically investigate a microRNA cluster to identify targets and mechanisms of action. Future experiments will be performed to distinguish between primary and secondary targets. In addition, functional assays will demonstrate the impact of microRNA mediators on cancer-related processes and begin to unravel the complex regulatory network surrounding the miR-143/-145 microRNA cluster.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. miR-143 and miR-145 expression levels in colon cancer

(A) Non-homologous miR-143-145 cluster with 2 members located on chr 5q32-33. (B) qRT-PCR results for miR-143 and miR-145 expression level in six formalin-fixed, paraffin-embedded (FFPE) normal colon and patient matched colon tumor tissue samples (C) qRT-PCR results for miR-143 and miR-145 expression levels in five human colon cancer cell lines. (D) Scatter plot of miR-145 versus miR-143 levels in cell lines and FFPE samples with linear regression line ($R^2 = 0.873$, $P < 0.0001$, $n = 17$). (E) qRT-PCR results for miR-143 and miR-145 expression levels in normal colon mucosa and SW480's after overexpression of 50nM miR-143 or miR-145. Data represented as median $\pm$ S.D. (B, C, E).

Figure 2. Experimental Design

SW480 cells were plated and transfected with miR-143, miR-145, or miR-143/-145 or remained untreated in light media (naturally occurring isotopes of arginine and lysine) or were plated and mock transfected (negative control) in heavy media ($^{13}\text{C}_6$, $^{15}\text{N}_4$ -arginine; $^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine). Total RNA and proteins were harvested 48 and 72 hours post transfection, respectively and prepared for microarray analysis or mass spectrometry analysis.

Figure 3. Fold change ratio distributions

(A) Histogram analysis of protein expression ratios (log₂-fold change between -2.0 and 2.5) for overexpression of miR-143; (B) miR-145; (C) miR-143/145. (D) Histogram analysis of mRNA expression ratios (log₂-fold change between -2.5 and 2.5) for overexpression of miR-143; (E) miR-145; (F) miR-143/145. Genes with fold change ratios -0.5 and 0.5 are indicated in color.

Figure 4. Correlation of protein and mRNA changes

(A, B, C) Scatter plots of protein versus mRNA expression changes following overexpression of miR-143, miR-145 and miR-143/145 with seed-matching sites (blue) and genes without seed sites (grey). The linear regression and Pearson's correlation coefficient are shown for genes with predicted seed sites and those without seed matches. (D) Scatter plot of predicted target expression changes for all three biological conditions. (E) Scatter plot of predicted target expression changes for all three biological conditions with specific genes labeled.

Figure 5. Detected candidate targets

Expression ratios (log₂ fold change) for candidate miR-143 (A), miR-145 (B), shared miR-143 and miR-145 (C) and cluster (D) targets. Targets regulated at the protein and/or mRNA level are in blue or green with mRNA regulated targets in red or orange.

Figure 6. Comparison of measurement techniques

SRM (yellow) and MaxQuant (blue) data correlation of microRNA targets at the protein level. Data is displayed as log₂ (ratio) represented as median ± S.D.

Figure 7. Inverse relationship between microRNA and target expression levels

(A) Box plot showing statistically significant down-regulation ($p < 0.001$) of miR-143 expression levels in colon tumor samples (primary, $n=6$; cell line, $n=5$) compared to normal colon tissue (primary, $n=6$). (B) Box plot displaying statistically significant ($p < 0.001$) down-regulation of miR-145 expression levels in colon tumor samples (primary, $n=6$; cell line, $n=5$) compared to normal colon tissue (primary, $n=6$). (C) Box plot showing statistically significant ($p = 0.030$) ARF6 up-regulation in colon tumor samples ($n=6$) compared to normal colon tissue ($n=6$).

Summary of the number of proteins identified and quantified as a result of the SILAC MS data

Table 1

	MS spectra	MSMS spectra	Identification [%]	Peptides	Proteins identified	SILAC pairs	SILAC pairs ID [%]	Proteins quantified
miR-143	72838	246640	32.61	13643	2260	258860	50.73	1999
miR-145	71218	258223	30.24	13192	2008	260785	49.21	1969
miR-143/145	67284	254848	28.79	13381	2073	247807	48.34	1927