MicroRNAs Indirectly Regulate Other MicroRNAs in Ovarian Cancer Cells

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ABSTRACT

MicroRNAs (miRNAs) are a class of regulatory RNAs that control the expression of genes critical to cell function. Ectopic expression of miRNAs has been shown to result in genome-wide changes in patterns of gene expression. While the reasons for these global alterations in gene expression patterns have been attributed to the ability of miRNAs to target multiple genes, and/or to induce indirect effects downstream of target genes, the molecular basis of indirect effects of miRNA regulation remains poorly understood. In this study, we demonstrate the potential of miRNAs to regulate other miRNAs. Using miRNA microarray analysis, we show that over 70 different miRNAs are differentially expressed (≥1.4 fold, FDR≤5%) in human ovarian cancer cells after transfection with a single miRNA (miR-7). We present evidence that a major component of miR-7 induced changes in levels of miRNAs is the indirect consequence of miR-7 mediated alterations in levels of protein coding genes (*e.g.*, transcription and splicing factors) that exert trans-regulatory control on miRNAs.

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1. INTRODUCTION

MicroRNAs (miRNAs) are a conserved class of small RNAs that can regulate gene expression by altering the translation and/or mRNA stability of target genes (Bartel, 2004; Fabian et al., 2010). Since miRNAs are differentially expressed in cancer and other diseases (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Farazi et al., 2011; Lee and Dutta, 2009; Visone and Croce, 2009; Wiemer, 2007), they may have clinical potential as diagnostic, prognostic and/or therapeutic agents (Garofalo and Croce, 2011; Wahid et al 2010). However, the molecular causes and consequences of perturbations in levels of miRNAs in cancer cells can be varied and complex (Baek et al., 2008; Lim et al., 2005; Selbach et al., 2008; Shahab et al., 2011). Thus, the potential clinical impact that miRNAs will ultimately have in cancer medicine rests heavily upon our ability to understand the basis of this complexity. We report here that perturbations in levels of a single miRNA can not only induce significant changes in levels of mRNAs, but also significantly modulate levels of other miRNAs. We discuss the potential significance of miRNA-miRNA regulation in cancer cells and present evidence from global gene expression and protein analyses for molecular mechanisms that may contribute to the process.

2. MATERIALS AND METHODS

2.1 Cell Culture and miRNA/siRNA Transfections

Cell culture conditions and miRNA/siRNA transfection procedures were carried out as described previously (Dickerson et al., 2010). Briefly, approximately 12h before transfection, cells (duplicates or triplicates per transfection, 1.5×10^5 per well) were seeded on six-well plates in growth medium RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10 % v/v heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech), 10 mM HEPES buffer (Mediatech), penicillin (100 U/ml), and streptomycin (100 µg/mL) and allowed to adhere overnight at 37°C in a 5 % CO_2 atmosphere. The following day after washing the wells with PBS and replacing the growth medium with Opti-MEM (Invitrogen), cells were transfected with the miRNA or siRNA [hsa-miR-7 miRIDIAN mimic (cat # C-300546-07), miRIDIAN miRNA mimic negative control #1 (miR-NC; cat # CN-001000-01, is a *C.elegans* miRNA, cel-miR-67, with minimal sequence identity in humans), ON-TARGET *plus*siRNARELA (cat # J-003533-06) or ON-TARGET *plus* non-targeting siRNA (siNC, cat # D-001700-01) (Thermo Fisher Scientific, Lafayette, CO)] using Lipofectamine 2000 transfection agent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions at a final concentration of 25nM. All transfections were carried out in triplicate. The cells were incubated with the reduced serum transfection medium (Opti-MEM, Invitrogen) for 4 hours, washed and then allowed to grow in growth medium (RPMI 1640) for 44 hours before collecting RNA or protein. Transfection efficiency was estimated from the relative knock-down of EGFR/RELA, based on recommendations by the reagent manufacturer (Thermo Fisher Scientific).

2.2 Quantitative (Real-time) PCR

For miRNA qPCR, total RNA was extracted from HEY cells using the mirVana miRNA isolation kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The RNA (10 ng) was then converted to amplified cDNA for qPCR by using the TaqMan miRNA Reverse Transcription Kit and miRNA specific primers (Applied Biosystems) following manufacturer's protocols. TaqMan miRNA assays (Applied Biosystems) were

conducted following manufacturer's protocol (20 µl total volume) forhsa-miR-198, hsa-miR-628-3p, hsa-miR-149, hsa-miR-10b, hsa-let-7f, hsa-miR-503, hsa-miR-218, hsa-miR-224,and for RNU6B. Specificity of TaqMan miRNA assays is supported by publications from the manufacturer (Chen et al., 2007; Liang et al., 2007; Tavazoie et al., 2008) as well as subsequent literature from independent groups (Huang et al., 2011; Shibata et al., 2011; Wilson et al., 2011).

For mRNA qPCR, total RNA (1-5µg) was extracted from cells, subjected to DNase treatment (Qiagen, Valencia, CA) and converted to cDNA using the Superscript III First Strand synthesis system (Invitrogen). cDNA was then purified using the Qiagen PCR purification kit following manufacturer's instructions. TaqMan gene expression assays were conducted following manufacturer's protocol for *MGC16121, FSTL1, GAPDH, RELA,* and *SRSF1*. qPCR experiments were also carried out for the *EGFR* and *GAPDH* genes using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The sequence specific primers used for *EGFR* are described in (Micallef et al., 2009) and *GAPDH* primers are described in (Koppelstaetter et al., 2005). All reactions were optimized with non-template controls, and -RT (no reverse transcriptase) controls prior to the experiment. Specificity of TaqMan gene expression assays is supported by thousands of independent citations (*e.g.,* (Bergmann et al., 2011; Colazzo et al., 2011; Hevir et al., 2011; Lossos et al., 2004).

All qPCR experiments were carried out using at least 3 technical replicates and 2-3 independent biological replicates on the CFX96 Real Time PCR detection system (Bio-Rad). For each targetexpression values were normalized to an endogenous control (RNU6B/GAPDH). RNU6B is a previously established control for human ovarian samples (*http://www.ambion.com/techlib/tn/151/3.html; http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_044972.pdf*). GAPDH was chosen as endogenous control because it displays minimal change between HEY cells transfected with miR-NC and miR-7 (Shahab et al., 2011). Relative fold change of target RNA level between transfection groups was determined by the ΔΔCtmethod. Statistical significance was determined using the pair-wise fixed reallocation randomization test in the Relative Expression Software Tool (REST 2008)(Pfaffl et al., 2002).

2.3 Immunoblotting

Immunoblots for EGFR, RELA/NFkB, SRSF1 and β-Actin were performed as described previously (Blackburn et al., 2009) with the following modifications. The blots were incubated for 2 hours with goat anti-rabbit IgG (1:2000) linked to Fluorescein (FITC; Southern Biotech, Birmingham, AL), or with donkey anti-mouse IgG (1:5000) linked to Phycoerythrin (PE; Southern Biotech, Birmingham, AL) secondary antibodies at room temperature. Primary anti-EGFR and anti-Actin antibodies were described in (Blackburn et al., 2009). Primary anti-RELA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA; sc-372) and primary anti-SRSF1 antibody was purchased from Millipore (Billerica, MA; 06-1363). Bands were visualized using a Typhoon 9400 Imager (GE Healthcare, Piscataway, NJ) followed by densitometric analysis using the ImageQuant TL Software (GE Healthcare). Statistical significance of difference between average levels from at least three replicates quantified by densitometry was determined using a 2-tailed unpaired Student's t-test.

2.4 RNA Isolation for miRNA Microarray

Low molecular weight RNA was isolated from HEY cells (two replicates per treatment using the mirVana miRNA isolation kit according to the manufacturer's instructions (Applied Biosystems). The quantity and size of miRNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). MiRNAs (200 μg/sample) were labeled with the GenisphereFlashTag HSR Biotin RNA labeling kit (Genisphere, Hatfield, PA) followed by hybridization with GeneChip miRNA Arraychips (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. The chips were washed and then scanned with a GeneChip Scanner 3000 (Affymetrix). Raw data in the form of CEL files were produced by AffymetrixGeneChip Operating System (GCOS) software.

2.5 RNA Isolation for Whole Genome Microarray

Total RNA for whole genome microarray was isolated using the RNeasy Mini RNA isolation kit (Qiagen) followed by DNAse treatment according to manufacturer's instructions. The integrity of the RNA was verified using an Agilent 2100 Bioanalyzer (RIN 8.0-9.8); Agilent Technologies). mRNAs were converted to double stranded cDNA and amplified using the Applause 3'-Amp System (NuGen, San Carlos, CA). This cDNA was then biotin labeled and fragmented by using the Encode Biotin Module (NuGen). The labeled cDNA was hybridized to Affymetrix HG-U133 Plus 2.0 oligonucleotide arrays and analyzed with a GeneChip Scanner 3000 (Affymetrix). Raw data in the form of CEL files were produced by AffymetrixGeneChip Operating System (GCOS) software.

2.6 Microarray Data Analysis

miRNA microarray data were analyzed using miRNA QC Tool software (Affymetrix). Normalization was performed using the default workflow of the software. The log_2 transformed expression values were then normalized across samples by Z-score calculations using Spotfire DecisionSite for Microarray Analysis (DSMA). Probesets with "Absent" call in all groups were removed from analysis. Probeset intensities were filtered with DSMA to include only those probesets with a fold change ≥1.4. Differentially expressed probesets were identified using the SAM algorithm at a threshold 5% false discovery rate (FDR) correction. Sequence of each differentially expressed miRNA probeset was then used to find the corresponding human miRNA using the BLASTN algorithm (*http://www.mirbase.org/search.shtml*) with E-value cut-off of 10. In each case, only the human miRNA with the highest score and lowest E-value was considered. If two miRNAs had the same high score, then both were included. Sequences with 1<E-value<10 were included in the table (see results) but were excluded from further analysis because of their "poor homology". Sequences with E-value >10 for human miRNAs are ignored and marked with "N/A" under the 'homolog' columns in results. Genomic locations of miRNA paralogs were obtained from the miRBase registry as well. In the miR-7 transfection experiment these probesets correspond to 71 unique mature human miRNAs (excluding miR-7), 3 sequences with very poor (BLASTN E-value >1) homology to human miRNAs, 2 snoRNAs, 1 vault RNA, 1 tRNA, 1 KIAA1407 RNA fragment, and some sequences that we could not map to the human genome (E-value >10).

mRNA microarray data were analyzed using Expression Console (Affymetrix) and R (www.r-project.org). Normalization was performed using MAS 5.0, and PLIER (Expression Console) and GCRMA algorithm (R). The log_2 transformed expression values from MAS5.0 were

analyzed for Affymetrix "Present/Absent" calls using Spotfire DecisionSite for Microarray Analysis (DSMA). Probe sets with "Absent" call in all groups were removed from analysis. Average probe set intensities for each group were calculated based on the $\log_2$ transformed values from PLIER and then filtered with DSMA to include only those probe sets with a fold change ≥1.3. The t-test p-value for each probe set was calculated from the $\log_2$ transformed values after GCRMA normalization using DSMA. Finally, differentially expressed probesets were identified using a threshold fold change ≥ 1.3, t-test p-value <0.05 and "Present/Marginal" call in at least one sample. These three different filtering approaches were used based on recommendations from a recent publication (Mieczkowski et al., 2010) and the combination of all three was used to achieve the most stringent filtering.

Raw data from a previous miR-7 transfection paper (Webster et al., 2009) were downloaded from GEO (Gene Expression Omnibus; *http://www.ncbi.nlm.nih.gov/geo/*) and processed in a similar fashion. All microarray data are MIAME compliant and have been submitted to GEO under the accession nos. GSE29129 (miRNA) and GSE27431 (mRNA).

2.7 Identification of Transcription Factors

Transcription factors were identified by searching for the Gene Ontology (GO) biological process annotation "regulation of transcription" for every probeset differentially expressed after miR-7 transfection.

2.8 miRNA Target Download

The miRNA target prediction file based on miRanda-mirSVR was downloaded from *www.microrna.org* (August 2010 release) (Betel et al., 2008;Enright et al., 2003; John et al., 2004). Information about the prediction algorithm, parameter settings and raw data source is available at the above link.

2.9 Identification of Putative NF-κBRegulated miRNAs

The coordinates of the putative NF-κB binding sites identified by ChIP-seq in the ENCODE project (Birney et al., 2007) were downloaded from the UCSC table browser (*http://genome.ucsc.edu*) based on the NCBI36/hg18 assembly of the human genome for each miRNA using a window of ±10 kb around the locus of each pre-miRNA corresponding to the differentially expressed miRNAs following miR-7 transfection.

2.10 Identification of Putative SRSF1 Regulated miRNAs

SRSF1 binding sites identified by (Sanford et al., 2009) using genome-wide cross-linked immunoprecipitation and sequencing (CLIP-seq) were downloaded from the authors website (with authors permission; *http://sanfordlab.mcdb.ucsc.edu/Sanford_Lab/Datasets.html*). For miRNAs embedded in known transcriptional units the SRSF1 sites were searched within the genomic coordinates of the host transcript. For intergenic miRNAs, length of the pri-miRNA was assumed to be ~4 kb on average with the transcription start site located ~2 kb upstream and the poly-A signal located ~2 kb downstream of the pre-miRNA, and thus the SRSF1 sites were searched within ±2 kb around each pre-miRNA, based on the average lengths of pri-miRNAs described by (Saini et al., 2007). Coordinates of coding transcriptional units and pre-miRNAs were obtained from the UCSC genome browser (*http://genome.ucsc.edu*) based on the NCBI36/hg18 assembly of the human genome.

2.11 BLAST Alignment of miRNAs

Sequences of miRNA probesets obtained from the Affymetrix annotation file corresponding to the differentially expressed miRNAs following miR-7 transfection were searched for significant complementarity to miR-7 using NCBI nucleotide BLAST algorithm (http://blast.ncbi.nlm.nih.gov) with the following parameter settings. Under program selection, optimize for "more dissimilar sequences" was selected, and under algorithm parameters, "automatically adjust parameters for short input sequences" was selected.

3. RESULTS

3.1 Ectopic Expression of miR-7 Alters the Levels of Endogenous microRNAs

To study the effect of changes in levels of a single miRNA on expression levels of other miRNAs in cancer cells, we transfected a well-characterized ovarian cancer cell line (HEY) with a miRNA (miR-7) previously shown to be significantly up-regulated in ovarian and other cancers relative to normal (Chou et al., 2010; Jiang et al., 2010; Kefas et al., 2008; Reddy et al., 2008; Saydam et al., 2010; Shahab et al. 2011; Veerla et al., 2009; Webster et al., 2009; Wyman et al., 2009). Using small RNA sequence datasets generated from a number of ovarian cell lines and tissue samples (Creighton et al. 2011, Gene Expression Omnibus, GSE 16709), we determined that the endogenous level of miR-7 in HEY cells is relatively low and generally representative of miR-7 levels in other OC cell lines and primary OC tumors (Figure S1).

To assess the biological effectiveness of our transfection, we monitored changes in levels of EGFR, a validated target of miR-7 (Kefas et al., 2008; Webster et al., 2009). Since it has previously been demonstrated that EGFR is highly expressed in HEY cells (Dickerson et al., 2010), we expected levels of *EGFR* mRNA and protein to be significantly reduced after a successful miR-7 transfection. Our results confirm that levels of *EGFR* mRNA and protein are both significantly reduced after miR-7 transfection (Figure 1) relative to a negative control (miR-NC).

To evaluate the effect of miR-7 transfection on global miRNA expression profiles, we isolated total RNA from cells transfected with either miR-7 or a negative control miRNA (miR-NC) 48h after transfection and monitored changes in levels of miRNA expression by microarray analysis. The results demonstrate that 259 distinct miRNA probesets corresponding to 71 mature miRNAs (excluding miR-7) were significantly altered in expression (31 up-regulated; 40 down-regulated) in the miR-7 transfected cells relative to controls (Figure 2, Table S1).

Eight of the miRNAs displaying significant changes in expression by microarray (hsa-miR-198, hsa-miR-628-3p, hsa-miR-149, hsa-miR-10b, hsa-let-7f, hsa-miR-503, hsa-miR-218 and hsa-miR-224) were randomly selected for confirmatory quantitative (real-time) PCR (qPCR) analysis. The results were uniformly consistent with the microarray results (Figure S2 A-H).

3.2 The Majority of Genes in Which Differentially Expressed miRNAs are Embedded Do Not Display Correlated Changes in Expression
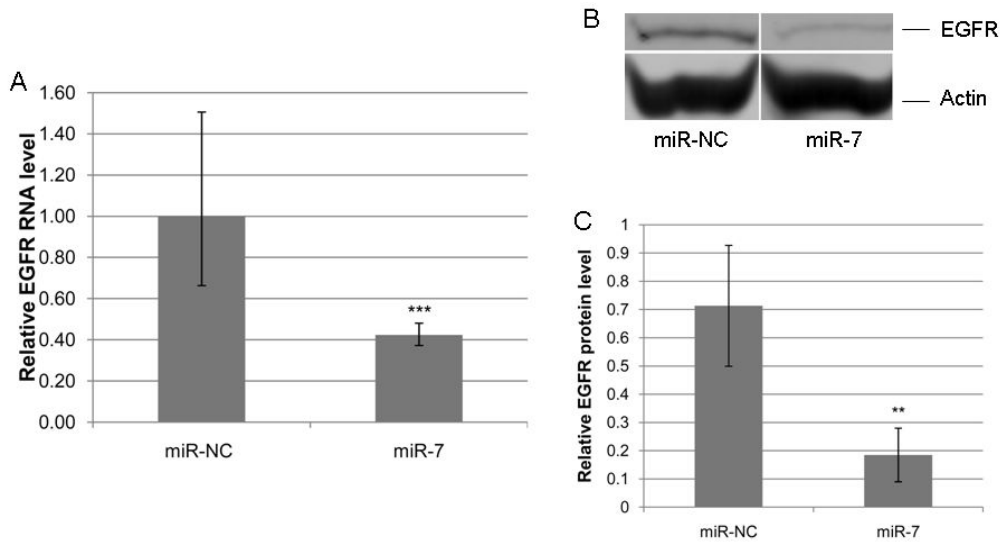
Figure 1. Confirmation of successful transfection of miR-7 into HEY cells.

Successful transfection of miR-7 into HEY cells is confirmed by measuring the level of EGFR RNA by qPCR (A) and protein by Western blot analysis (B and C). In both cases EGFR expression is down-regulated significantly (** p<0.01; *** p<0.001) after miR-7 transfection compared to negative control (miR-NC) transfection. Bar graphs in C show the relative intensity of blots in B. EGFR expression is normalized to GAPDH used as endogenous control (A), or β-Actin used as loading control (C). Error bars reflect standard deviation of the means calculated from 3 independent replicate samples.

Human miRNAs have been mapped to exons and introns of protein coding and non-protein coding genes (*i.e.,* embedded in genes), as well as to intergenic regions of the genome (Kim and Nam, 2006). Some embedded miRNAs have been shown to display changes in expression levels that positively correlate with those of their host genes. Such correlated patterns of expression are consistent with the hypothesis that embedded miRNAs and their host genes are under the same and/or coordinated regulatory controls (Baskerville and Bartel, 2005;Rodriguez et al., 2004). In other cases, changes in patterns of embedded miRNAs do not correlate with those of their host genes (Bargaje et al., 2010) indicating that at least some embedded miRNAs are under independent regulatory control (Fujita and Iba, 2008; Monteys et al., 2010; Ozsolak et al., 2008).

Of the 71 miRNAs differentially expressed after miR-7 transfection, 50 were found to map to annotated genes (55 including paralogs) and 21 (25 including paralogs) map to intergenic regions (Figure 3, Table S2). To determine if changes in miRNA levels are correlated with changes in mRNA levels of the 50 genes in which miRNAs are embedded, we compared changes in miRNA profiles with changes in the mRNA levels of host genes in miR-7transfected HEY cells (Table S3).We found that 9 of the 50 host genes were significantlydifferentially expressed (fold change≥1.3, p<0.05) in miR-7 transfected cells relative to controls (Tables 1 and S3, Figure S2 I-J). Eight of these nine genes displayed changes in expression positively correlated with 13 embedded miRNAs while one of the genes displayed a change in expression inversely correlated with a single embedded miRNA (Table 1). The majority of genes (41/50) hosting differentially expressed miRNAs did not display a significant change in expression in miR-7 transfected cells relative to controls (Table S2).
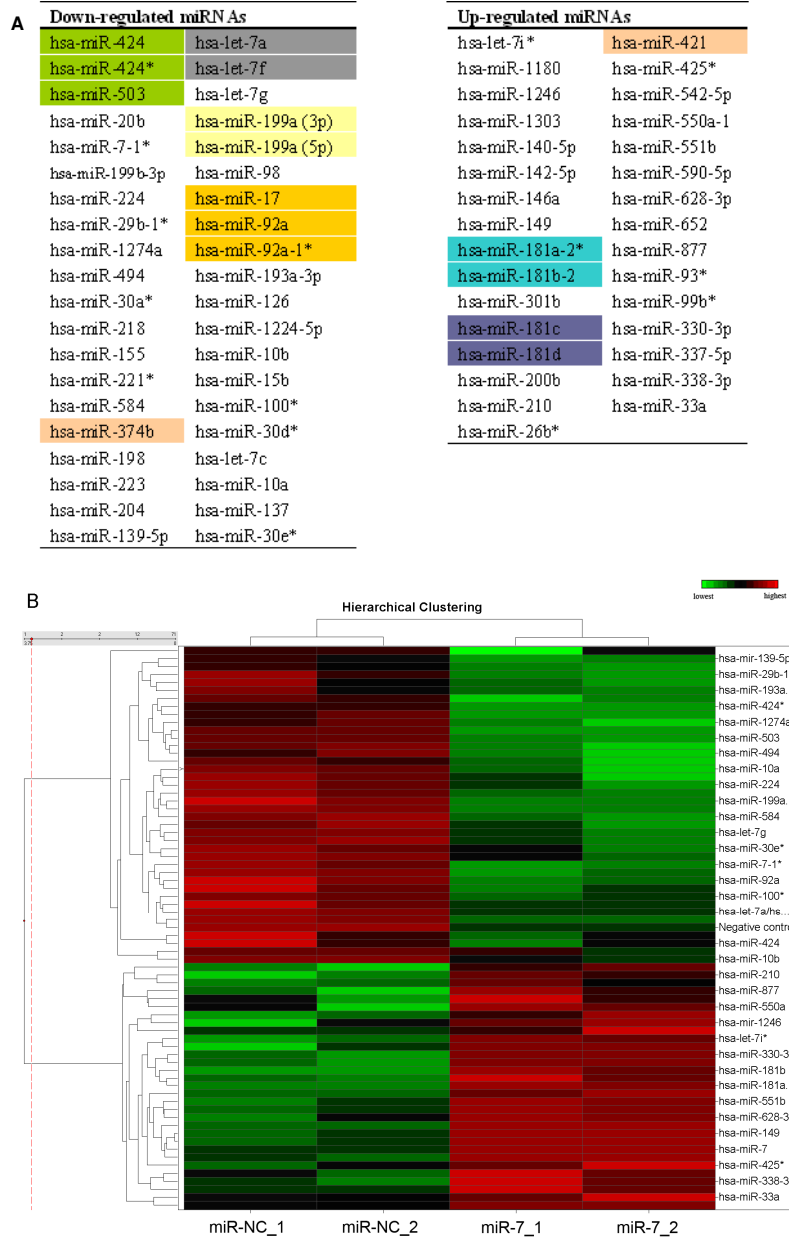
A

Down-regulated miRNAs	
hsa-miR-424	hsa-let-7a
hsa-miR-424*	hsa-let-7f
hsa-miR-503	hsa-let-7g
hsa-miR-20b	hsa-miR-199a (3p)
hsa-miR-7-1*	hsa-miR-199a (5p)
hsa-miR-199b-3p	hsa-miR-98
hsa-miR-224	hsa-miR-17
hsa-miR-29b-1*	hsa-miR-92a
hsa-miR-1274a	hsa-miR-92a-1*
hsa-miR-494	hsa-miR-193a-3p
hsa-miR-30a*	hsa-miR-126
hsa-miR-218	hsa-miR-1224-5p
hsa-miR-155	hsa-miR-10b
hsa-miR-221*	hsa-miR-15b
hsa-miR-584	hsa-miR-100*
hsa-miR-374b	hsa-miR-30d*
hsa-miR-198	hsa-let-7c
hsa-miR-223	hsa-miR-10a
hsa-miR-204	hsa-miR-137
hsa-miR-139-5p	hsa-miR-30e*

Up-regulated miRNAs	
hsa-let-7i*	hsa-miR-421
hsa-miR-1180	hsa-miR-425*
hsa-miR-1246	hsa-miR-542-5p
hsa-miR-1303	hsa-miR-550a-1
hsa-miR-140-5p	hsa-miR-551b
hsa-miR-142-5p	hsa-miR-590-5p
hsa-miR-146a	hsa-miR-628-3p
hsa-miR-149	hsa-miR-652
hsa-miR-181a-2*	hsa-miR-877
hsa-miR-181b-2	hsa-miR-93*
hsa-miR-301b	hsa-miR-99b*
hsa-miR-181c	hsa-miR-330-3p
hsa-miR-181d	hsa-miR-337-5p
hsa-miR-200b	hsa-miR-338-3p
hsa-miR-210	hsa-miR-33a
hsa-miR-26b*	

Figure 2. MicroRNAs differentially expressed after miR-7 transfection in HEY cells.
A. List of differentially expressed (fold change≥1.4 and FDR≤5%) miRNAs following transfection of hsa-miR-7 into HEY cells compared to miR-NC transfected cells. There were 40 different human miRNAs significantly down-regulated and 31 miRNAs significantly up-regulated (excluding miR-7) after miR-7 transfection. MicroRNAs mapping to the same transcriptional unit are shaded with the same color (See Table S2 for additional details) B. Hierarchical clustering of significantly differentially expressed miRNAs between cells transfected with either miR-7 or miR-NC. The two negative control samples are on the left and the two miR-7 transfected samples are on the right. Selected IDs of the human miRNAs homologous to thesequences specified by the probesets are shown on the right side of the heat map. [Note some of the miRNA names have an asterisk (*) indicating miRNA star strand].
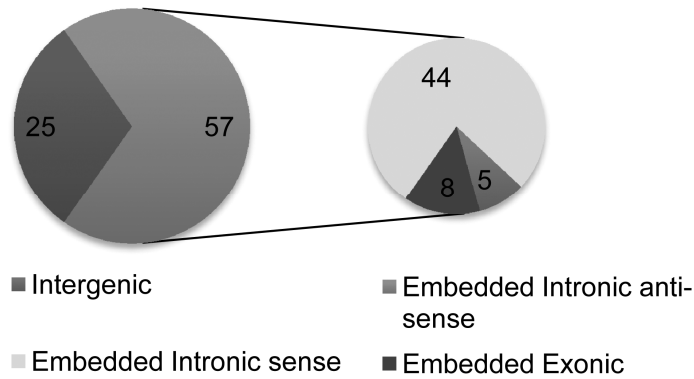
Figure 3. Genomic location of differentially expressed miRNAs between miR-7 transfected and negative control transfected HEY cells

Table 1. Significantly differentially expressed embedded miRNA-mRNA pairs

miRNA	mRNA/ncRNA	Host gene detection method
miRNAs positively correlated in expression with host genes		
hsa-let-7c	**C21orf34** (IN)	Microarray
hsa-miR-139-5p	PDE2A (IN)	Microarray
hsa-miR-198	**FSTL1** (EX-3'UTR)	Microarray and qPCR
hsa-miR-30a*	C6orf155 (IN)	Microarray
hsa-miR-424	MGC16121 (EX)	Microarray and qPCR
hsa-miR-424*	MGC16121 (EX)	Microarray and qPCR
hsa-miR-503	MGC16121 (EX)	Microarray and qPCR
hsa-miR-155	MIR155HG (EX-ncRNA)	Microarray
hsa-miR-181a-2*	MIR181A2HG (IN-ncRNA)	Microarray
hsa-miR-181b-2	MIR181A2HG (IN-ncRNA)	Microarray
hsa-miR-17	MIR17HG (IN-ncRNA)	Microarray
hsa-miR-92a-1	MIR17HG (IN-ncRNA)	Microarray
hsa-miR-92a-1*	MIR17HG (IN-ncRNA)	Microarray
miRNAs inversely correlated in expression with host genes		
hsa-miR-550a-2	AVL9 (IN)	Microarray

The pie-chart on the left shows the proportion of differentially expressed miRNAs that are either embedded in known genes or intergenic. The pie-chart on the right shows that the embedded miRNAs can be classified further based on whether they come from intronic, exonic or anti-sense regions. miRNAs that can be either from intron or exon are counted under both categories (scored twice, resulting in the number 57). The raw numbers used for generating the pie-charts are also shown.

3.3 miR-7 Regulates Expression of Some miRNAs by Altering the Level of the Transcription Factor RELA/NF-κB

We found that transfection of miR-7 into HEY cells results in significant changes in the expression levels of 1368 genes including genes known to encode transcription factors previously linked with cancer onset and progression (Table S3; (Karin and Greten, 2005)). Since miRNAs are under transcriptional regulatory control (Krol et al., 2010), transcription factor genes regulated by miR-7 may exert indirect regulatory effects on other miRNAs. To explore this possibility, we selected RELA (v-relreticuloendotheliosis viral oncogene homolog A, aka, Nuclear factor NF-kappa-B p65 subunit) one of the transcription factor coding genes that was significantly differentially expressed after miR-7 transfection in HEY cells (this study) (Table 2) and previously in A549 lung cancer cells ((Webster et al., 2009), see also Table S4). Moreover, RELA is a component of the well-studied transcription factor NF-κB that is a regulator of miRNA expression (Mott et al., 2010; Pacifico et al., 2010; Zhou et al., 2009). Significant down-regulation of *RELA* in miR-7 transfected HEY cells was confirmed by qPCR and immunoblot assays (Figure 4).

We found that 17 (68%) of the 25 intergenic miRNAs and 38 (69%) of our 55 embedded miRNAs (Figure 5A; Tables S2 and S5) map to within 10 kb of experimentally validated
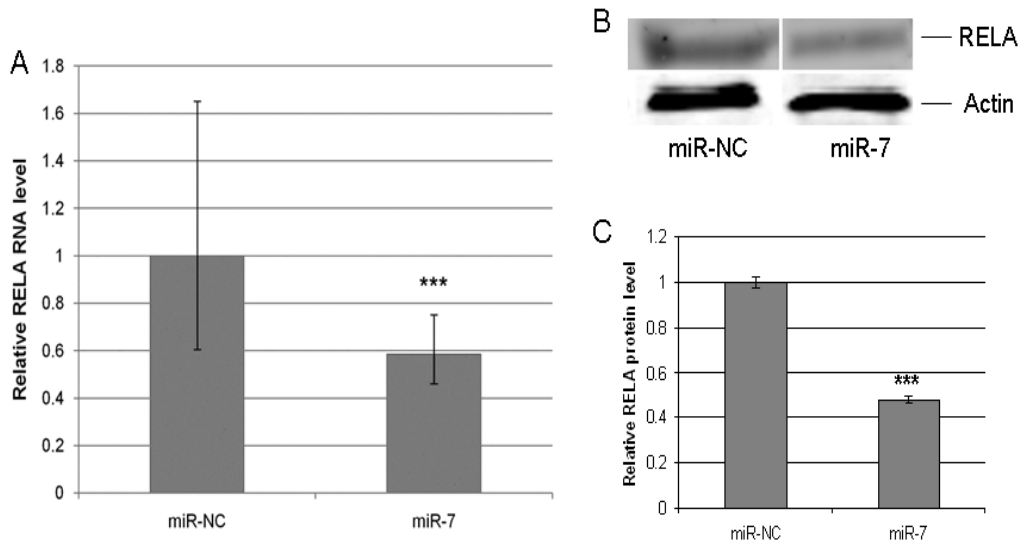

Figure 4. Transfection of miR-7 down-regulates RELA/NF-κB in HEY cells

RELA expression levels in Hey cells 48 hours after transfection with miR-7 or miR-NC are analyzed by qPCR (A) and Western blot (B and C). In both cases RELA expression is down-regulated significantly (*** p<0.001) after miR-7 transfection compared to negative control (miR-NC) transfection. Bar graphs in C show the relative intensity of blots in B. RELA expression is normalized to GAPDH used as endogenous control (A), or β-Actin used as loading control (C). Error bars reflect standard deviation of the means calculated from 3 independent replicate samples.

Table 2. Differentially expressed transcription factors following miR-7 transfection

Gene Symbol	miR7-miR-NC	miR7tgts_miRanda	ENCODE ChIP-seq Data
BACH1	-0.45255		
CNOT6	-0.60309	x	
CNOT8	-1.26668	x	
CREBL2	0.433388		
IFT57	0.664064		
IRF2BP2	-0.43606		
KLF3	-0.60824		
KLF4	-1.06432	x	
LITAF	-1.52641		
NOTCH2	-0.7116	x	
PBX1	1.134572	x	
PPM1A	-0.52971		
RELA	-1.08425	x	E
SETD8	-1.07335	x	
SP1	-0.42641	x	E
TAF4B	-0.54876		
TLE4	-0.61978		
UBTF	0.614296		
VGLL4	-0.72098		
ZBTB20	0.399861		
ZNF512	-0.49876		
ZNF580	0.457916		

Listed are the 22 transcription factors identified as being significantly differentially expressed (1.3 fold change, p<0.05) in both the miR-7 transfected HEY cells (this study) and previously in the A549 lung cancer cells (Webster et al 2009). Differences between the log2 signal values for each gene in HEY cells transfected with either miR-7 or miR-NC is given in the 'miR7-miR-NC'. Of the 22 transcription factors, only 8 have miRanda predicted miR-7 binding sites (marked with 'x') and only 2, RELA and SP1 have ChIP-seq data showing genome-wide binding sites (marked with 'E').

(ChIP-seq) RELA/NF-κB binding sites (Birney et al., 2007). To determine if this distribution is greater than expected by chance, we randomly selected 70 human miRNAs from the 718 listed in the UCSC browser and asked how many are located within 10kb of experimentally validated (ChIP-seq) RELA/NF-κB binding sites. After repeating this process 100,000 times, we found that the results displayed a normal distribution with a mean of 43 (Figure S3). The observed number of miRNAs mapping within 10 kb of experimentally validated (ChIP-seq) RELA/NF-κB binding sites, 55 (17 + 38 = 55), is significantly greater than 43 (p < 0.003) indicating an enrichment of miRNAs within 10 kb of RELA/NF-κB binding sites (Figure S2).

Nearly half (33 out of 71) of the miRNAs that were differentially expressed in our miR-7 transfection experiment have previously been reported to be altered when NF-κB is induced or inhibited in other cellular contexts (Gao et al., 2010; Kluiver et al., 2007; Mott et al., 2010; Pacifico et al., 2010; Wang et al., 2009; Wang et al., 2010; Zhou et al., 2009). Collectively,

these findings suggest that at least some of the changes in miRNA levels observed after miR-7 transfection into HEY cells are likely mediated by the RELA/NF-κB transcription factor.
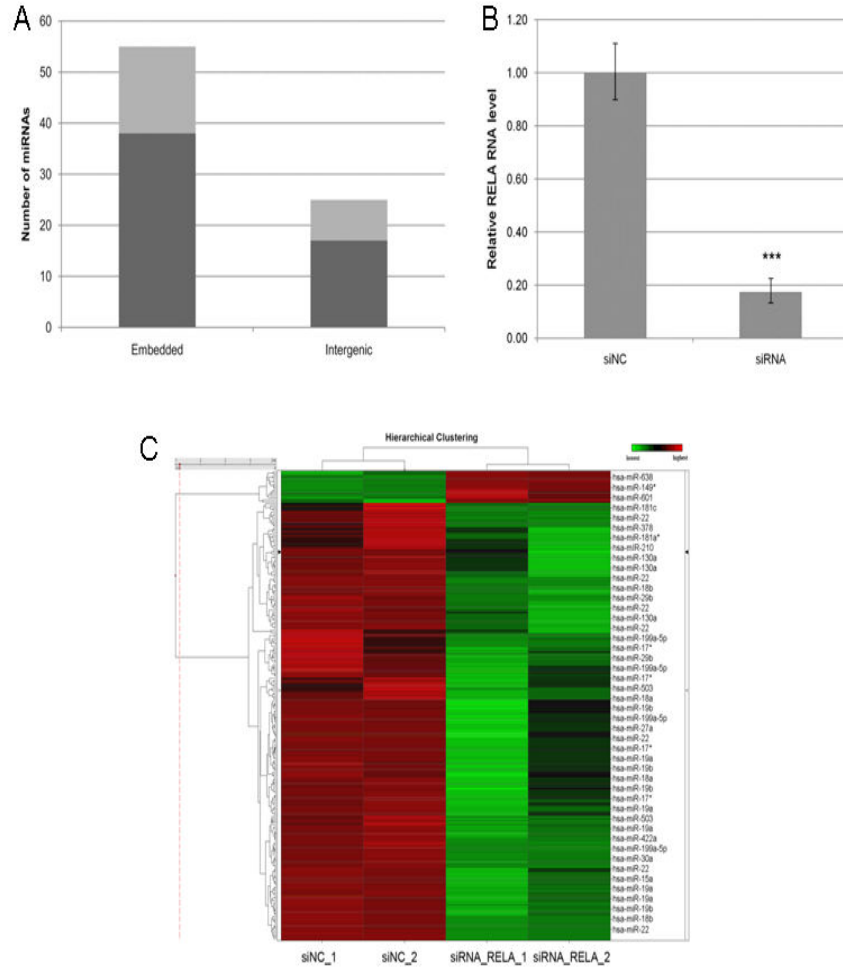

Figure 5.miR-7 regulates expression of some miRNAs by altering the level of RELA.
A. Number of embedded and intergenic differentially expressed miRNAs with (dark grey) or without (light grey) NF-κB binding sites within ±10 kb. The number of differentially expressed miRNAs (fold change ≥1.4, FDR ≤5%) following miR-7 transfection that are either embedded in coding/non-coding genes or intergenic is represented by the height of the bars. B. qPCR confirmation of successful down-regulation of RELA expression 48 hours following transfection of HEY cells with RELA siRNA or siNC.
C. Significantly differentially expressed (fold change ≥1.4 and FDR ≤5%) miRNAs in HEY cells following transfection with RELA siRNA or siNC were used for hierarchical clustering of samples. The two samples transfected with siNC are on the left and the two transfected with RELA siRNAare on the right. Some of the IDs of the human miRNAs homologous to the sequences specified by the probesets are shown on the right side of the heat map (See also Table S6). (Note that due to the Affymetrix multiple probe design, some microRNAs are listed more that once) The relative expression of RELA in(B) was measured using GAPDH as endogenous control. Error bars reflect standard deviation of the means calculated from 3 replicate samples.   *** p<0.001.

To further investigate this hypothesis, we transfected HEY cells with *RELA*siRNA or a negative control siRNA and collected total RNA (48 hrs post-transfection) for microarray analysis (AffymetrixGeneChip miRNA array). Knock down of RELA expression was confirmed by qPCR (Figure 5B). The results of the microarray analysis demonstrated that 234 miRNA probesets corresponding to 51 known human miRNAs (Figure 5C; Table S6)

Table 3. Significantly differentially expressed miRNAs altered by both miR-7 transfection and knock-down by RELA siRNA

Mature miRNAs altered by miR-7 transfection and RELA knock-down
hsa-miR-210
hsa-miR-503
hsa-miR-99b*
hsa-miR-181c
hsa-miR-146a
hsa-miR-193a-3p
hsa-miR-199a-5p

miRNAs transcribed from the same pre-miRNA

RELA knock down	miR-7 transfection
hsa-miR-29b(-1)	hsa-miR-29b-1*
hsa-miR-140-3p	hsa-miR-140-5p
hsa-miR-149*	hsa-miR-149
hsa-miR-17*	hsa-miR-17
hsa-miR-199a-5p	hsa-miR-199a-3p, hsa-miR-199a-5p
hsa-miR-30a	hsa-miR-30a*
hsa-miR-30e	hsa-miR-30e*

miRNAs transcribed from the same cluster

Cluster	RELA knock down	miR-7 transfection
miR-17~92	hsa-miR-17*	hsa-miR-17
miR-17~92	hsa-miR-18a*	hsa-miR-92a(-1)
miR-17~92	hsa-miR-18a	hsa-miR-92a-1*
miR-17~92	hsa-miR-19a	
miR-17~92	hsa-miR-19b(-1)	
miR-18~106	hsa-miR-18b	hsa-miR-20b
miR-18~106	hsa-miR-19b(-2)	hsa-miR-92a(-2)
miR-15-16	hsa-miR-16(-2)	hsa-miR-15b
let-7c, miR-99a	hsa-miR-99a	hsa-let-7c
miR-181a-1, miR-181b-1	hsa-miR-181a*	hsa-miR-181b(-1)
miR-130b, miR-301b	hsa-miR-130b	hsa-miR-301b
miR-424~542	hsa-miR-503	hsa-miR-424, hsa-miR-424*, hsa-miR-503
miR-181c, miR-181d	hsa-miR-181c	hsa-miR-181c, hsa-miR-181d

The lists of miRNAs that were significantly differentially expressed (fold change ≥1.4, FDR ≤ 5%) after either miR-7 or RELA siRNA transfection were compared and the miRNAs that were altered in both, had the same pre-miRNA, or that originated in the same cluster are listed. miRNA names followed by parenthesis containing a number refers to a corresponding paralogous miRNA (for e.g., hsa-miR-19b(-2) refers to hsa-miR-19b originating from hsa-miR-19-2).

were significantlydifferentially expressed (fold change ≥1.4, FDR ≤5%) after *RELA*siRNA transfection relative to controls. Seven of the miRNAs displaying changes in expression after miR-7 transfection displayed similar changes in expression after transfection with *RELA*siRNA (Table 3). Twenty additional miRNAs that displayed significant changes in expression after *RELA*siRNA transfection either originate from the same pre-miRNA transcript or are clustered within thesame locus (<10 kb) with 18 miRNAs displaying significant changes in expression after miR-7transfection (Table 3). While exogenous expression of miR-7 in HEY cells is expected to modulate the expression a number of genes in addition to RELA (see above), our findings are consistent with the hypothesis that a significant number of the changes in miRNA levels observed after miR-7 transfection into HEY cells are likely mediated by RELA.

3.4 miR-7 May Regulate Expression of miRNAs by Altering Levels of the Splicing Factor SRSF1 (SF2/ASF)

Another possible example of a gene regulated by miR-7 that may, in turn, exert indirect regulatory effects on other miRNAs is SRSF1 (serine/arginine-rich splicing factor 1). Wu et al. (2010) recently reported that SRSF1 (SF2/ASF), a gene involved in the splicing of pre-mRNAs (Ge and Manley, 1990; Krainer et al., 1990; Zhou et al., 2002), can form a negative feedback circuit with miR-7. Over-expression of SRSF1 in HeLa cells was shown to result in significant alterations in levels of several (40) miRNAs including a significant elevation in levels of miR-7. Wu et al. (2010) went on to show that elevation in levels of miR-7 results in the down-regulation of SRSF1 (a predicted target of miR-7) indicating a negative feed-back loop.

Since our microarray results (confirmed by qPCR and Western blot analyses, Figure 6) indicate that SRSF1 is significantly down-regulated in miR-7 transfected cells, it is possiblethat at least some of the miR-7 induced changes in levels of miRNAs in these cells may have been mediated by SRSF1. Consistent with this hypothesis, we found that 12 of the 40 miRNAsreported by Wu et al. (2010) to display significant changes in levels of expression in HeLa cells after SRSF1 over-expression also display significant changes in expression after miR-7 transfection in HEY cells (Table 4). In addition, we found that nine additional miRNAs differentially expressed after miR-7 transfection map to primary transcripts containing eightof the differentially expressed miRNAs detected in the Wu et al. (2010) study (Tables 4 and S2).

To further explore the possibility that some of the miR-7 induced changes in miRNA levels in transfected HEY cells may have been mediated by SRSF1, we overlaid the positions of experimentally validated (CLIP-seq or cross-linked immunoprecipitation and sequencing) SRSF1 binding sites (Sanford et al., 2009) with the genomic locations of miRNAs displayingsignificant changes in levels of expression after miR-7 transfection. The results indicate that SRSF1 binding sites are located within the pri-miRNAs of 22 miRNAs (20 embedded, 2 intergenic) displaying significant changes in expression after miR-7 transfection (Table S7). Collectively, these findings are consistent with the hypothesis that some of the miR-7 induced changes in miRNA levels in transfected HEY cells are likely mediated by miR-7 induced changes in levels of SRSF1.
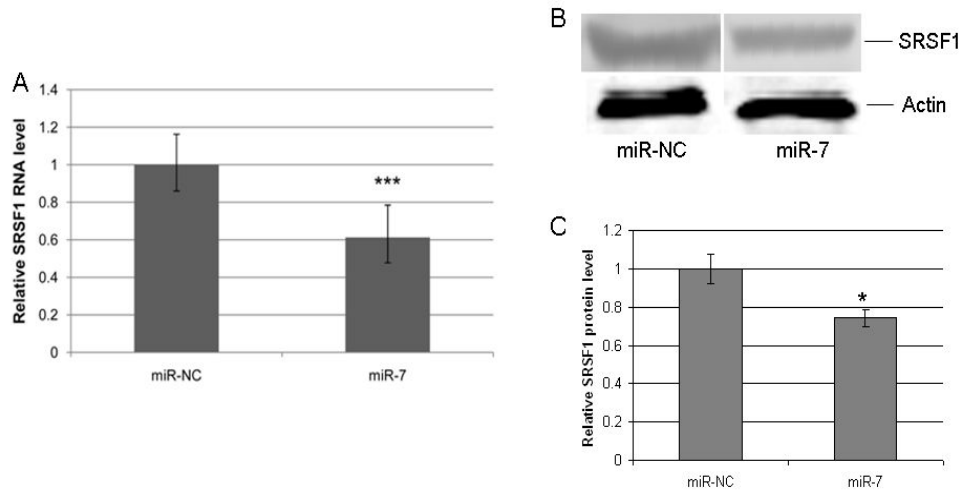
Figure 6. Transfection of miR-7 down-regulates SRSF1 in HEY cells.
SRSF1 expression levels in Hey cells 48 hours after transfection with miR-7 or miR-NC are analyzed by qPCR (A) and Western blot (B and C). In both cases SRSF1 expression is down-regulated significantly (* P<0.05; *** p <0.001) after miR-7 transfection compared to negative control (miR-NC) transfection. Bar graphs in C show the relative intensity of blots in B. SRSF1 expression is normalized to GAPDH used as endogenous control (A), or β-Actin used as loading control (C). Error bars reflect standard deviation of the means calculated from 3 independent replicate samples.

Table 4. Significantly differentially expressed miRNAs altered by both miR-7 transfection and SRSF1 induction

miRNAs altered in SRSF1 induction	miRNAs altered in miR-7 transfection
hsa-let-7c	**hsa-let-7c**
hsa-miR-1246	**hsa-miR-1246**
hsa-miR-137	**hsa-miR-137**
hsa-miR-140-5p	**hsa-miR-140-5p**
hsa-miR-155	**hsa-miR-155**
hsa-miR-210	**hsa-miR-210**
hsa-miR-224	**hsa-miR-224**
hsa-miR-33a	**hsa-miR-33a**
hsa-miR-551b	**hsa-miR-551b**
hsa-miR-181c	**hsa-miR-181c**, hsa-miR-181d
hsa-miR-374b	**hsa-miR-374b**, hsa-miR-421
hsa-miR-92a	**hsa-miR-92a**, hsa-miR-92a-1*, hsa-miR-17
hsa-miR-221	hsa-miR-221*
hsa-miR-29b	hsa-miR-29b-1*
hsa-miR-7	hsa-miR-7-1*
hsa-miR-191	hsa-miR-425*
hsa-miR-125a-5p	hsa-miR-99b*

*The miRNAs that were significantly differentially expressed in either HEY cells after miR-7 transfection (fold change ≥1.4, FDR ≤5%) (this study) or in HeLa cells (previously reported by Wu et al. (2010) following SRSF1 induction (fold change ≥1.5, FDR ≤1%, criterion used in the) were compared and the miRNAs that were altered in both or had the same putative pri-miRNA are listed. Mature miRNAs differentially expressed after both miR-7 transfection and SRSF1 induction are written in **bold** letters.*

4. DISCUSSION

Previously it has been shown that a single miRNA can alter the expression of hundreds of genes (Baek et al., 2008; Lim et al., 2005; Selbach et al., 2008). While some of these changes can be attributed to canonical 3' UTR miRNA targeting, the vast majority (~70-80%) of genes altered by a miRNA may be indirectly regulated (Chen et al., 2011) (Shahab et al. 2011). In this study, we employ global gene expression analyses to explore the possibility that one mechanism that may be contributing to these "off-target" effects is miRNA-miRNA regulation.

Our experimental approach was to transfect a well-characterized ovarian cancer cell line (HEY) with a single miRNA and to monitor the effect on the expression levels of other miRNAs and mRNAs by microarray analysis. While miRNA regulatory effects operating exclusively at the level of translation will go undetected in our approach, recent studies indicate that nearly all regulatory changes induced by mammalian miRNAs involve modulations in levels of mRNAs (Guo et al., 2010). Thus, we believe that a majority of miR-7 induced regulatory changes are likely being detected by our approach.

We selected miR-7 for use in our transfection experiments because levels of this miRNA previously have been shown to be significantly changed in ovarian (Shahab et al., 2011; Wyman et al., 2009) and other cancers (Chou et al., 2010; Foekens et al., 2008; Jiang et al., 2010; Kefas et al., 2008; Reddy et al., 2008; Veerla et al., 2009; Webster et al., 2009) relative to normal tissue. Gene expression profiling by microarray revealed that transfection of miR-7 into HEY cells resulted in significant changes in expression levels of 71 miRNAs and 1368 mRNAs.

In principle, miRNAs may regulate expression of other miRNAs either directly or indirectly. For example, it has been proposed that if miRNAs share significant sequence complementarity with other miRNAs, the resulting nucleotide pairing could serve as the basis of direct miRNA-miRNA regulation (Lai et al., 2004). We found that among 71 miRNAs differentially expressed in our study, only 3 displayed even partial (38-62%, Figure 7) Watson-Crick complementarity to miR-7 indicating that miRNA-miRNA pairing is unlikely to be a significant mechanism used by miR-7 to regulate other miRNAs in our study.

The primary mechanism by which miRNAs regulate expression of mRNA-encoding genes is through translational inhibition and associated mRNA degradation (Djuranovic et al., 2011; Fabian et al., 2010). While this mechanism is presumably not applicable to miRNAs and other untranslated RNAs, there have been reports of miRNAs directly serving as activators (Majid et al., 2010; Place et al., 2008) and repressors (Kim et al., 2008) of transcriptional initiation. It is certainly possible that miRNAs may directly influence the expression of other miRNAs by regulating or co-regulating promoters of miRNA genes and/or protein coding genes in which miRNAs are embedded. However, current evidence suggests that a direct role of miRNAs in promotional control occurs relatively infrequently (Tang et al., 2008; Tang and Zen, 2011). Thus, we believe it is more likely that miRNAs may modulate the expression of other miRNAs indirectly by regulating transcription factors or other regulatory proteins that, in turn, exert direct regulatory control on miRNA expression.

```
hsa-miR-7    3' UGUUGUUUUAGUGAUCAGAAGGU 5'
                  |  |||||||  :  :   |
hsa-miR-7-1*  5' CAACAAAUCACAGUCUGCCAUA 3'
OR
hsa-miR-7    3' UGUUGUUUUAGUGAUCAGAAGGU 5'
                  ||||||              |     :|
hsa-miR-7-1*  5' CAACAAAUCACAGUCUGCCAUA 3'



hsa-miR-7    3' UGUUGUUUUAGUGAUCAGAAGGU 5'
                     || |||||||||  |:|
hsa-miR-224     5' CAAGUCACUAGUGGUUCCGUU 3'



hsa-miR-7    3' UGUUGUUUUAGUGAUCAGAAGGU 5'
                  |    ||||  |||
hsa-miR-92a     5' UAUUGCACUUGUCCCGGCCUGU 3'
```

Figure 7. Alignment of three differentially expressed miRNAs with partial complementarity to miR-7.

Sequences of three miRNAs (hsa-miR-7-1, hsa-miR-224 and hsa-miR-92a) are aligned with miR-7 based on BLAST alignment. Vertical straight lines (|) represents Watson-Crick complementarity and the colon (:) symbol represents GU wobble.*

We observed significant changes in expression of 188 transcription factor coding genes after miR-7 transfection. Forty-nine of these, including *RELA/NF-κB*, are predicted targets of miR-7 regulation (Table S3). We selected RELA/NF-κB for further investigation because this transcription factor has been previously implicated in miRNA regulation (Mott et al., 2010; Pacifico et al., 2010; Zhou et al., 2009) and was also found to be down-regulated in a lung cancer cell line following miR-7 transfection (Webster et al., 2009). We found that siRNA knockdown of *RELA* resulted in a significant change in expression of 51 miRNAs, many of which were also significantly differentially expressed after miR-7 transfection. These results coupled with our finding that ~70% of the miRNAs differentially expressed after miR-7 transfection map to within 10 kb of RELA/NF-κB binding sites suggest that this transcription factor plays a significant indirect role in miR-7 induced changes in miRNA expression.

Further support for the hypothesis that miR-7 induced changes in the expression levels of protein coding regulatory genes may play a significant role in affecting changes in levels of miRNAs comes from our analysis of another direct target of miR-7 regulation, the splicing factor SRSF1. We found that SRSF1 binding sites are located within the pri-miRNAs of 25 miRNAs that displayed significant changes in expression after miR-7 transfection into HEY cells. This result coupled with previous findings implicating SRSF1 in the regulation of miR-7 in HeLa cells (Wu et al., 2010) suggests that SRSF1 may also be playing a role in miR-7 induced changes in the expression of miRNAs in HEY cells.

5. CONCLUSION

Once considered "junk DNA," a large component of the non-protein coding human genome is now known to encode untranslated RNAs that play important, albeit diverse, roles in gene

regulation (Eddy, 2001; Mattick, 2007). Among the most intensively studied of these regulatory molecules are short (~22 nucleotides) regulatory RNAs, called miRNAs. This class of small regulatory RNAs inhibits the translation and/or facilitates the degradation of their targeted messenger RNAs (Fabian et al., 2010). The fact that miRNAs are aberrantly expressed in many cancers has made them attractive candidates for potential use in cancer therapy (Garofalo and Croce, 2011). However, the clinical utility of miRNAs in cancer therapy rests heavily upon our ability to understand and accurately predict the consequences of fluctuations in levels of miRNAs within the context of complex tumor cells. While the regulatory effects of miRNAs in tumor cells is known to be complex (Farazi et al., 2011; Lee and Dutta, 2009; Shahab et al., 2011) the molecular basis of this complexity is only beginning to be explored (Chang et al., 2007; Farazi et al., 2011; Hatley et al., 2010; He et al., 2005; He et al., 2007; Huang et al., 2009).

In this paper, we present evidence from global gene expression analyses that a significant component of this complexity may involve the ability of miRNAs to regulate other miRNAs indirectly through the action of trans-regulatory proteins. Our findings underscore the complexity of miRNA-mediated regulation in cancer cells and the necessity of better understanding the basis of this complexity if we are to fully realize the therapeutic potential of this versatile class of regulatory RNAs.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

SUPPLEMENTARY INFORMATION IS AVAILABLE AT THE WEBSITE OF *BRITISH JOURNAL OF MEDICINE AND MEDICAL RESEARCH.*