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Report

Potential mRNA Degradation Targets of *hsa-miR-200c*, Identified Using Informatics and qRT-PCR

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ABSTRACT

Using an anchored oligo(dT) based RT-PCR approach we quantified endogenous expression of ten microRNAs in six cell lines. This identified a miRNA, *miR-200c*, with variable expression, ranging from undetectable in MDA-MB-231 and HT1080 to highly expressed in MCF7. The variable expression provided a model system to investigate endogenous interactions between miRNAs and their computationally predicted targets. As the expression level of the predicted mRNA targets and *miR-200c* in these lines should have an inverse relationship if cleavage or degradation results from the interaction. To select targets for analysis we used Affymetrix expression data and computational prediction programs. Affymetrix data indicated ~3500 candidate mRNAs, absent in MCF7 and present in MDA-MB-231 or HT1080. These targets were cross-referenced against ~600 computationally predicted *miR-200c* targets, identifying twenty potential mRNAs. Expression analysis by qRT-PCR of these targets and an additional ten mRNAs (selected using the prediction program ranking alone) revealed four mRNAs, BIN1, TCF8, RND3 and LHFP with an inverse relationship to *miR-200c*. Of the remainder, the majority did not appear to be degraded (and may be translational targets) or were undetectable in the cell lines examined. Finally, inhibition of *miR-200c* using an anti-miRNA 2'-0-Methyl oligonucleotide (AMO) resulted in an increase in expression of one of the targets, the transcription factor TCF8. These results indicate that a single miRNA could directly affect the mRNA levels of an important transcription factor, albeit in a manner specific to cell lines. Further investigation is required to confirm this in vivo and determine any translational effects.

INTRODUCTION

The identification of microRNAs and their mechanism of action were originally made in *C.elegans*[1] followed by other species including vertebrates. The interpretation of miRNA biogenesis and mode of action has since led to the recognition of a new level of eukaryotic gene regulation.[2,3] The mechanism involves endogenous ~22 nt miRNAs of which there are thought to be several hundred expressed at varying levels and in a tissue specific manner. In eukaryotes, initial expression is in the form of nascent pri-microRNA transcripts, generated by Pol II and capped and polyadenylated.[4] These are then processed into ~70nt pre-miRNAs by the Drosha RNAseIII endonuclease[5] and transported from the nucleus by Ran-GTP/Exportin-5.[6] The pre-miRNAs are next processed by Dicer into the final ~22nt noncoding single stranded mature miRNAs that regulate gene expression.[5] The mature miRNAs are then incorporated into the RNA induced silencing complex (RISC) and targets recognized based on perfect (or almost perfect) complementarity to the mRNA, the result is either cleavage or transcriptional repression.[7-9] The miRNA registry currently contains information on 3424 miRNAs of which 326 are predicted to be human.[10] Additional estimates of eukaryotic miRNAs suggest there may be 500 to 1000 miRNA's in the genome.[11] Identification of microRNAs has involved cDNA cloning, usually following size fractionation[12] or computational algorithms that identify the distinct structural features of the pri-miRNAs[13,14] or their targets.[15] The function of the majority of these miRNAs is currently unknown, with identification of (mRNA) targets also being predicted through computational means.[16] Such algorithms have to account for the majority of animal miRNAs (unlike plant miRNAs) lacking a perfect pairing to their targets and predictions may alter following experimental investigation.[16,17] Computational predictions of miRNA/mRNA interactions do not include the outcome i.e., translational repression or cleavage. Experiments involving transfection of reporter constructs have indicated that in mammals the outcome is often translational repression. However, it has recently been reported that transfected microRNAs can also regulate the mRNA levels of

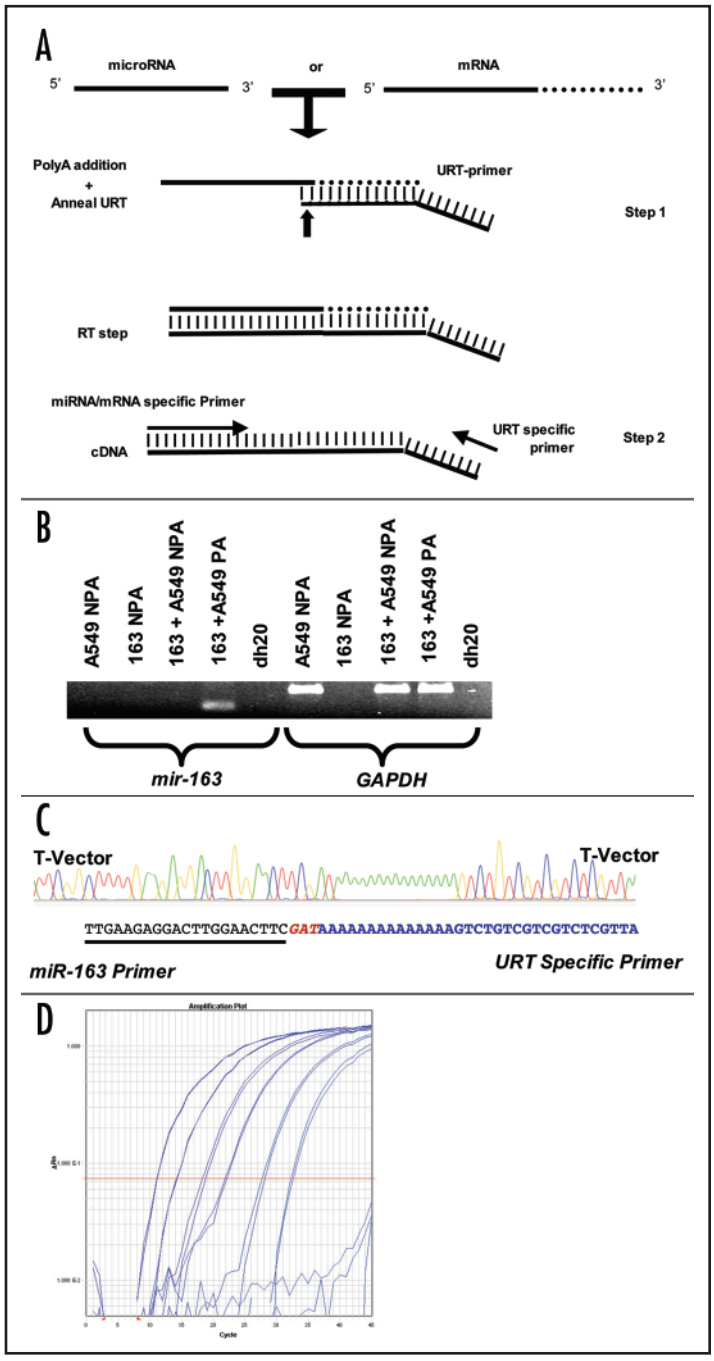

Figure 1. (A) Mature miRNA are first modified through addition of a polyA tail as shown. The universal RT-primer, with variable 1bp anchor (arrow), polyT and unique universal RT primer sequence, then anneals followed by reverse transcription. The resultant cDNA can then be amplified using specific primers for the miRNA and URT sequence. (B) No mir-163 product is generated from either total RNA alone (A549 NPA), oligo alone (163 NPA), or A549 RNA with mir-163 oligo template in the absence of polyA addition (163 + A549NPA), Lanes 1–3. Product is only generated when mir-163 oligo is added to RNA and polyA (PA) tail is added (163 + A549 PA), Lane 4. GAPDH transcript has an endogenous polyA tail and is therefore positive for all A549 cell line RNA extracts, Lanes 6,8,9) with the exception of the negative control, 163 oligo template alone (163 NPA), Lane 7. (C) Amplification product from Lane 4 was cloned and sequenced; a representative trace is shown with sequence of *mir-163* primer underlined. (D) Dynamic range of the URT miRNA amplification assay using a synthetic oligonucleotide for *miR-163*. Serial dilutions ranged from 10^3 to 10^{-3} fM equivalent to between ~10 and 10^6 copies.

a large number of targets.[18] In addition, mature microRNAs (miRNAs) are variably expressed in a manner thought to be specific to cell types, developmental stages and diseases (such as cancer). To examine these predicted interactions in an endogenous cell based system, we adapted a technique[19] to allow for the simultaneous quantitation of miRNA and mRNA expression levels relative to GAPDH (Fig. 1A). A conceptually similar method was recently used for the quantitative analysis of miRNA levels in plants.[20] Initially we examined the expression of ten miRNAs and found several with highly variable expression in cell lines (Fig. 2A). We focused on one of these, *has-miR-200c* as it had a distinct range from highly expressed to undetectable in multiple cell lines. This provided an ideal model system to identify miRNA/mRNAs interactions resulting in degradation. As both the miRNA and its computationally predicted mRNA target are amplified quantitatively, we could look for an inverse relationship in endogenous expression levels.

MATERIALS AND METHODS

Cell lysis and RNA preparation. The cell lines used in this study were from the ATCC (except NHBE) cultured according to recommended conditions. Cell lines; MCF7 & MDA-MB-231(Breast) HT1080 (Fibrosarcoma), A549 (Lung), MALME (Melanoma) and NHBE (Normal Human Bronchial Epithelium). Cell were pelleted at 1000g for 5mins and washed twice in PBS. Total RNA was extracted with Trizol (Invitrogen) according to the manufacturers recommended procedures. Briefly, cell pellets were lysed in 1ml of Trizol reagent and incubated for 5mins at room temperature. Two hundred microliters of chloroform was added mixed and incubated for 2–3 min at room temperature before centrifugation at 12000 g for 15 min at 4°C. The aqueous layer was transferred to a fresh tube, mixed with 500µl of isopropanol and incubated for 10 min at room temperature. Samples were spun at 12000 g for 10min at 4°C and the supernatant removed. The pellet was then washed with 1 ml of 75% EtOH, spun at 7500 g for 5 min and the supernatant removed before drying at room temperature for 10 min. After resuspending in DNase/RNase free water, RNA quantity and quality was determined at 260/280 nm using a spectrophotometer (Nanodrop Technologies).

MicroRNA primer design. The synthetic oligonucleotide mir-163 and the anti-miRNA 2'-0-methyl oligonucleotides were purchased from Integrated DNA Technologies (IDT Skokie, IL). The microRNA sequences used were taken from the miRNA database maintained by the Sanger Institute (accession numbers shown in Table 1) and primers designed with the primer3 program; the respective URLs are shown.

http://microrna.sanger.ac.uk/
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

Modification and amplification of mature miRNA's. Mature miRNAs were first modified by the addition of a polyA tail (additional polyAs are also added to nascent mRNA) (Fig. 1, Step 1) as follows. Total RNA (0.01–1.0 µg) in 12.2 µl of DNase/RNase free water was added to 4 µl of 5x E-PAP buffer, 1.5 µl of 25 nM MnCl and 1.5 µl of 10 mM ATP solution. Then 0.8 µl of *E. coli* Poly (A) Polymerase I (E-PAP) (Ambion Inc. Austin, TX) was added and the sample thoroughly mixed and incubated at 37°C for 1hr.

Reverse transcription. Reverse transcription was performed with 10 µl (500 ng) of the E-PAP treated total RNA using Superscript III reverse transcriptase (Invitrogen Carlsbad, CA) as follows. RNA template was added to a master mix containing 1 µl of 100 µM Universal RT primer, 1 µl of dNTP mix (each base 10 µM) and 1 µl

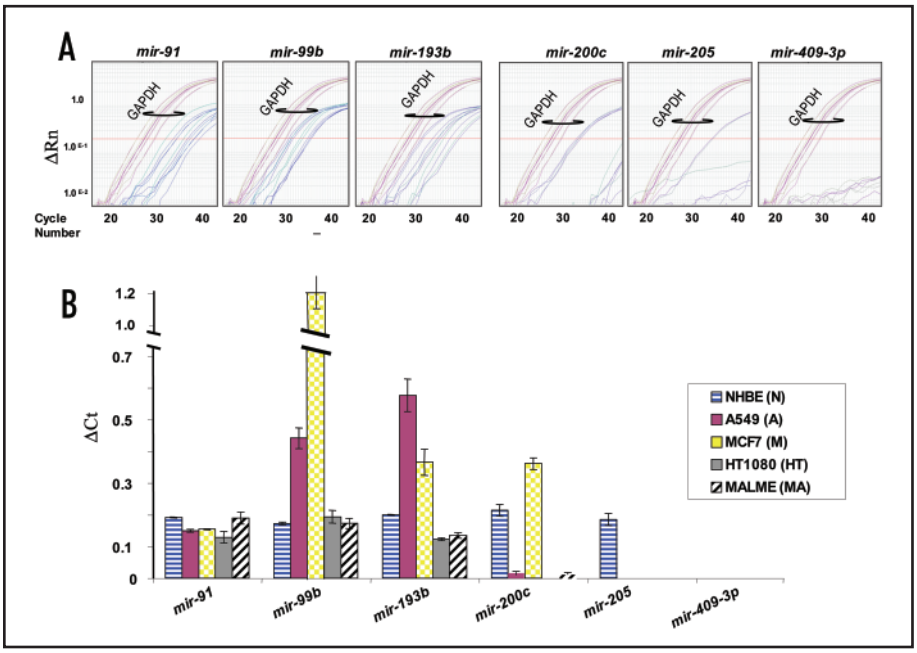

Figure 2. (A) Duplicate amplification plots of six microRNAs, and GAPDH (circled), using RNA extracted from five cell lines. Cycle numbers are shown below, horizontal line indicates (Ct) threshold, ΔRn is the measure of relative fluorescence. Duplicate amplification curves are shown for each miRNA and for GAPDH with Ct represented as a horizontal red line. (B) Amplification plots represented graphically in with the average ΔCt (inverted) plotted for each miRNA relative to GAPDH in the five lines.

Table 1 **Mature miRNA sequences and corresponding primers**

Name	Acc No.	Sequence 5′-3′
ath-miR163	MIMAT0000184	UUGAAGAGGACUUGGAACUUCGAU
mir-163	Primer	TTGAAGAGGACTTGGAACTTC
hsa-miR-200c	MIMAT0000617	UAAUACUGCCGGGUAAUGAUGG
mir-200c	Primer	ATACTGCCGGGTAATGATGG
hsa-miR-154	MIMAT0000452	UAGGUUAUCCGUGUUGCCUUCG
mir-154	Primer	AGGTTATCCGTGTTGCCTTC
hsa-miR-99b	MIMAT0000689	CACCCGUAGAACCGACCUUGCG
mir-99b	Primer	CACCCGTAGAACCGACCTT
hsa-miR-181a	MIMAT0000256	AACAUUCAACGCUGUCGGUGAGU
mir-181a	Primer	ATTCAACGCTGTCGGTGAG
hsa-miR-502	MIMAT0002873	AUCCUUGCUAUCUGGGUGCUA
mir-502	Primer	ATCCTTGCTATCTGGGTGCT
hsa-miR-409-3p	MIMAT0001639	CGAAUGUUGCUCGGUGAACCCCU
mir-409-3p	Primer	CGAATGTTGCTCGGTGAAC
hsa-miR-518b	MIMAT0002844	CAAAGCGCUCCCCUUUAGAGGU
mir-518b	Primer	AAAGCGCTCCCCTTTAGA
hsa-miR-193b	MIMAT0002819	AACUGGCCCUCAAAGUCCCGCUUU
mir-193b	Primer	AACTGGCCCTCAAAGTCC
hsa-miR-205	MIMAT0000266	UCCUUCAUUCCACCGGAGUCUG
mir-205	Primer	CCTTCATTCCACCGGAGT
URT-primer	URT ADAptorPrimer	AACGAGACGACGACAGACTTTTTTTTTTTTTTV
Universal PCR primer	Primer	AACGAGACGACGACAGACTTT

of DNase/RNase-free water. Total volume adjusted to 13 µl with DNase/RNase-free water. The solution was incubated at 65°C for 5 min and then cooled to 4°C. A master mix containing 4µl of 5X first-strand buffer, 1 µl of 0.1 mM DTT, 1 µl DNase/RNase-free water and 1 µl SuperScript III per RT sample was prepared and added to each sample. The samples were incubated at 25°C for 5 min, 50°C for 60 min, followed by 70°C for 15 min. (Fig. 1 Step 2). Typically, the RT reaction was diluted 1:20 and 2µl used in the amplification of miRNAs with the transcript specific forward PCR primers (Table 1) and a universal reverse primer identical to the 18 bp tag added during the RT step.

Cloning of amplification products. Fresh RT-PCR products were cloned using a TA-cloning kit (Invitrogen) according to manufacturer's instructions. Subsequent ligations were transformed into chemically competent DH5α; blue/white selection identified cloned inserts for sequencing.

Robotic systems. Quantitative RT-PCR analysis was performed using an ABI 7900 real-time detection system and SYBER Green Dye (ABI, Foster City, CA). Sequencing was performed on an ABI 3700 DNA analyzer.

Transfection of cell lines with anti-miR oligos (AMOs). Cells were plated at 2 x 10^5 cells /well in a 12 well plate in 800 µl of DMEM media with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin and 1% L-glutamine. After 24 hrs media was removed and replaced with antibiotic free DMEM media containing 2% FCS. Transfections were performed in 12 well plates using lipofectamine 2000 according to manufacture protocol as follows, prepare solution A [2 µl of 100 µM anti-miRNA 2'-0-methyl oligonucleotide + 2 µl pEGFP-C1 (BD Clontech) and 996 µl blank DMEM] and solution B (20 µl lipofectamine 2000 and 980 µl blank DMEM). Incubate these for 5 min at ~25°C before combining and incubating for further 20 min (~25°C). Add 200 µl of combined mixture to each well containing cells, in 800 µl of DMEM and incubate at 37°C. The media is changed the following day, with addition of 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine. After 48 hours incubation the cells are collected, RNA isolated and analyzed.

RESULTS

Quantitative amplification of miRNA and mRNA. In the technique

Table 2. **Predicted mRNA targets based on Affymetrix data (column 1) or computational algorithms (column 2).**

1.	Affymetrix Predictions	2.	Computational
Name	**Brief Description**	**Name**	**Brief Description**
ASXL1	Additional sex combs like 1 (Drosophila)	ATP11C	ATPase, Class VI, type 11C
TCF8	Transcription factor 8 (represses interleukin 2 expression)	NTF3	Neurotrophin 3
ZFPM2	Zinc finger protein, multitype 2 ,	PKD1	Polycystic kidney disease 1
BIN1	Bridging integrator 1, nucleocytoplasmic adaptor protein.	PPM1F	Protein phosphatase 1F (PP2C domain)
CNN3,	Calponin 3, acidic	TCF8	Transcription factor 8
NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)	ZFHX1B	Zinc finger homeobox 1b
NAP1L5	Nucleosome assembly protein 1-like 5	ZFPM2	Zinc finger protein, multitype 2
NIN	Ninein (GSK3B interacting protein)	TARDBP	TAR DNA binding protein
PALM2-AKAP2	Function unknown	RND3	Rho family GTPase 3
PKD1	Polycystic kidney disease 1 (autosomal dominant)	PCTK2	PCTAIRE protein kinase 2
PSAT1	Phosphoserine aminotransferase		
CFL2	Cofilin 2 (muscle)		
DOCK4	Dedicator of cytokinesis 4 involved in regulation of adherens junctions between cells.		
EDG2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2,		
GLIS3	GLIS family zinc finger 3 ,from family of Kruppel-like zincfinger proteins with repressor and activation functions		
HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1.		
LHFP	Lipoma HMGIC fusion partner		
MPP4	Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)		
SRF	Serum response factor		
SYDE1	Synapse defective 1, Rho GTPase, homolog 1		

outlined in Figure 1A, the Universal Reverse Transcription primer (URT-primer) is first annealed using a string of oligo(dT)s with a variable one base pair anchor (arrow, Fig. 1). This is followed by reverse transcription (RT) converting all miRNAs (and mRNAs) into cDNA. Both the miRNA(s) and mRNAs were then amplified using primers specific for each in conjunction with a primer for the unique sequence on the URT tail. Quantitation of the reaction products is achieved using the intercalating dye SYBR green with GAPDH as an internal standard. The sensitivity and dynamic range was initially assessed with a synthetic oligonucleotide based on the sequence of the plant miRNA *ath-mir-163* (Fig. 1B). Cloning and sequencing of the resulting products confirmed no interference from PCR artefacts (Fig. 1C). This demonstrated that the method was specific, sensitive and had a dynamic of between 1.5 x 10^3 and 1.5 x 10^{-3}fM (Fig. 1D).

We then assessed the endogenous levels of ten microRNAs, selected at random from the Sanger Institute database, in several cell lines. Again, a number of the products generated were sequenced, confirming that they originated from the mature miRNA. Having shown that the primers could specifically amplify miRNAs, the expression relative to GAPDH was then quantified. Figure 2A, shows representative amplification plots with the cross threshold (Ct) values for six microRNAs and GAPDH. The ΔCT between each miRNA and GAPDH is also represented graphically in Figure 2B showing the wide variation of expression levels of some of these miRNAs. For example, *mir91, 99b* and *193b* could be detected in most samples at a similar level. However, *mir-99b* is expressed at a significantly higher level in MCF7. In contrast, *mir-205* could only be detected in NHBE and miR-*200c* was not detectable in HT1080 was found at a low level in A549 and NHBE and significantly higher levels in MCF7. Finally, the *mir-409-3*, was undetectable in all five lines but the correct product was generated when RNA extracted from normal blood was examined (data not shown).

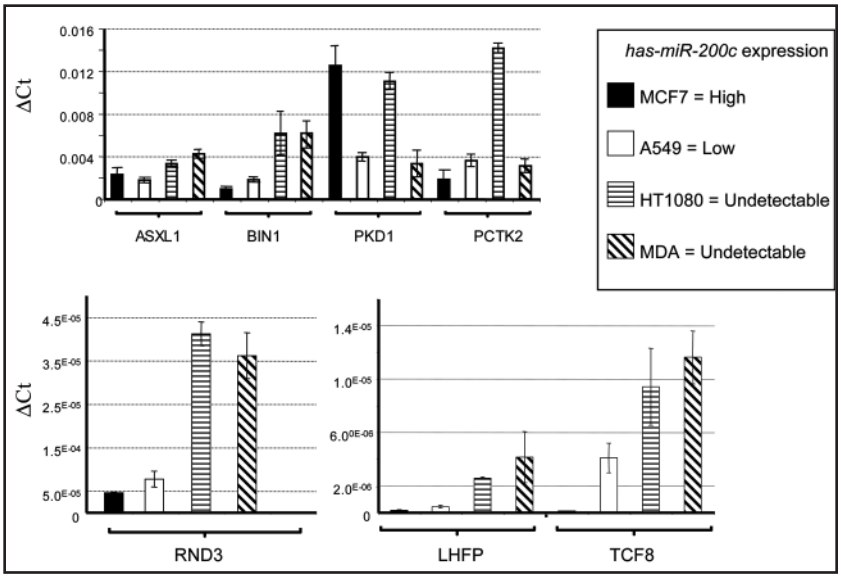

Figure 3. Expression levels of predicted targets of *miR-200c*. In top panel both PKD1 and PCTK2 are expressed in MCF7 cells, in addition expression of PCTK2 is lower in MDA-MB-231 cells that do not express *miR-200c*. In the lower panel both examples have an expression profile that is inversely related to *miR-200c*.

Table 3 **Summary of expression data of targets relative to *miR200c* as assessed by qRT-PCR in four cell lines**

qRT-PCR result. Indicates expression inverse to *miR200c*	qRT-PCR result. expression not inverse to *miR200c*	Below detection level
TCF8	ASXL1	ZFHX1B
*RND3	*PKD1	*PPMIF
LHFP	NAB1	CFL2
BIN1	SRF	NIN
	CNN3	NAP1L5
	*TARDBP	PALM2
	*NTF3	
	*ATP11C	
	*ZFPM2	
	*PTCK2	

*Indicates consensus targets from the prediction programs

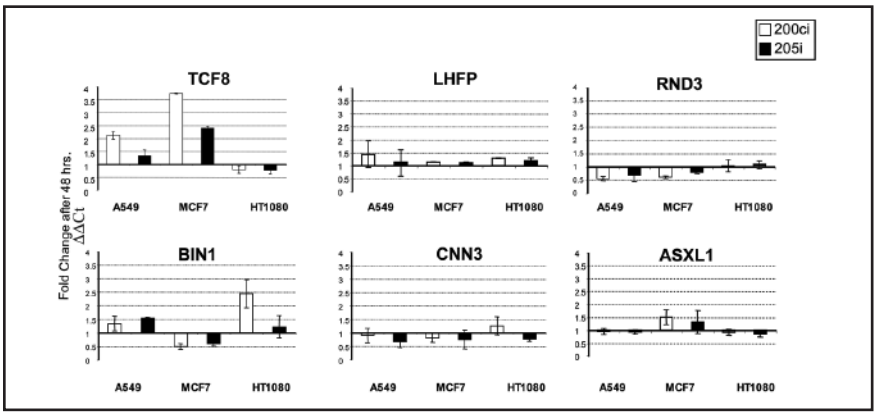

Figure 4. Expression levels of predicted targets following transient transfection of Anti-miRNA 2′-0-Methyl oligonucleotides (AMOs) for *miR-200c* or *miR-205*. Prior to transfection, expression of miR-200c was highest in MCF7, lower in A549 and undetectable in HT1080. The relative levesl of TCF8 increase following treatment with 200ci (200c AMO). However, a non-specific increase is observed after treatment with 205i. (205 AMO). The remaining targets are essentially unchanged in cell lines expressing miR-200c.

Endogenous expression of miRNA and mRNA targets. The varied expression levels of *hsa-miR-200c* provided an ideal cell based system with which to examine endogenous expression of both a miRNA and its predicted targets. In order to identify targets we analyzed Affymetrix expression data for the cell lines MCF7, HT1080 and MDA-MB-231, looking for expression in the latter two but not in the former lines. Approximately 6000 probe sets representing ~3500 genes were classified as present in MCF7 but absent in HT1080 and MDA-MB-231. These results where then cross-referenced against the ~600 predicted targets from online prediction programs (miRBase http://microrna.sanger.ac.uk/ and PicTar, http://pictar.bio.nyu.edu), identifying twenty mRNAs with expression profiles inversely related to *miR-200c*. We then designed primers for these and for an additional ten consensus target predictions as shown in Table 2. Representative results for the levels of a number of these mRNAs is shown in Figure 3 and summarized in Table 3. The majority of targets analyzed were expressed at relatively high levels in MCF7 (in comparison to other cell lines) suggesting no degradation. Of the remainder, many are undetected, unaffected or variably but not inversely expressed in relation to levels of *miR-200c* (Fig. 3). However, the expression analysis did reveal four targets with levels inversely related to *miR-200c*, these were BIN1, TCF8, RND3 and LHFP.

Transient knockdown of miRNA using and anti-miR oligonucleotide. Having shown that there was a correlation between miRNA levels and mRNA levels of some targets, we attempted a knockdown of *miR200c*. To do this anti-miRNA 2'-0-methyl oligonucleotides (AMOs) for *miR-200c* or *miR-205* where cotransfected into cell lines with a plasmid pEGFP-C1 that expressed GFP. The effect on regulation of predicted targets mRNAs was then measured with qRT-PCR (Fig. 4). Of the six targets analyzed, only TCF8 shows an increase in relative mRNA levels following transfection with the anti-*miR-200c* AMO. However, an unrelated increase in expression of TCF8 is also observed following transfection with the AMO for *miR-205*.

DISCUSSION

Currently, the available online prediction programs (e.g., MIRTAR and PICTAR) indicate that there are multiple mRNA targets for each microRNA. In addition, the prediction programs suggest that multiple miRNAs can target each mRNA. This paradigm is further complicated by the potential outcome of such an interaction being either translational repression or degradation. As a result, the majority of predicted microRNA targets remain to be verified. In this report, we have identified variably expressed miRNAs in cell lines, providing an endogenous model system for further investigations. The reported analysis is limited to the identification of mRNA degradation resulting from such interactions. However, further investigation of these cell lines could verify translational repression of targets and enable more accurate prediction algorithms. This may in turn greatly alter the current number of predicted targets for individual miRNAs.

The combination of Affymetrix analysis, prediction programs and qRT-PCR demonstrated a strong correlation between miRNA expression and the mRNA levels of some target. The indications from this analysis (based on mRNA levels) are that a relatively small number of targets are degraded. It is possible however, that the degradation mechanism requires multiple miRNAs interactions, which is beyond the scope of this assay.

A detailed investigation of the 3UTRs did confirm that all of the predicted targets contained a least one 'site' for *miR-200c* (i.e., 6–7 bp match between 3'UTR and 5' end of miRNA). Significantly, only TCF8 and ZFHX1B had multiple target sites for *miR-200c*, each had two perfect 7 bp "seed" matches in addition to other 6 bp matches. Both these transcription factors have similar functions and it is possible that ZFHX1B (which, could not be detected in the cell lines examined) is expressed during development or in other tissue and cell types and may be targeted by *miR-200c* in these situations.

To further validate our findings, we attempted the inhibition of *miR-200c* expression using transient transfection of an anti-miRNA 2'-0-methyl oligonucleotide (AMO) in multiple cell lines. This did result in the significant upregulation of TCF8 in MCF7 cells, which have the highest levels of *miR-200c*. However, a nonspecific increase was also observed in MCF7 when the transient transfection was with

an AMO for *miR-205*. While this does indicate that action of AMOs is nonspecific, it should also be noted that expression levels of the remaining targets was unchanged (Fig. 4).

Our analysis of *miR-200c* may indicate that a single microRNA is capable of specifically regulating one target. Degradation of the other targets may require a complex interplay between multiple microRNAs or may involve translational repression. To better understand these systems it will be necessary to stably express *mir-200c* in cell lines that lack this miRNA. It is hoped that this will overcome the issues regarding the lack of specificity that may prevent the effective downregulation of miRNAs using current techniques.

The identification of variable expression of miRNAs in cell lines presents an ideal opportunity for the clarification of mechanism miRNA/mRNA interactions. Further investigation will then be needed to determine if such interactions occur in vivo.

References

1. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75:843-54.
2. Ambros V. microRNAs: Tiny regulators with great potential. Cell 2001; 107:823-6.
3. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed *RNAs*. Science 2001; 294:853-8.
4. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. Rna 2004; 10:1957-66.
5. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. Nature 2003; 425:415-9.
6. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 2003; 17:3011-6.
7. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev 2004; 18:504-11.
8. Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 1999; 216:671-80.
9. Hammond SM, Bernstein E, Beach D, Hannon GJ. An *RNA*-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 2000; 404:293-6.
10. Griffiths-Jones S. The microRNA Registry. Nucleic Acids Res 2004; 32:D109-11.
11. Berezikov E, Plasterk RH. Camels and zebrafish, viruses and cancer: A microRNA update. Hum Mol Genet 2005; 14:R183-90.
12. Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T. New microRNAs from mouse and human. RNA 2003; 9:175-9.
13. Ohler U, Yekta S, Lim LP, Bartel DP, Burge CB. Patterns of flanking sequence conservation and a characteristic upstream motif for *microRNA* gene identification. RNA 2004; 10:1309-22.
14. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human *microRNA* genes. Cell 2005; 120:21-4.
15. Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 2005; 434:338-45.
16. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003; 115:787-98.
17. Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, Hatzigeorgiou A. A combined computational-experimental approach predicts human microRNA targets. Genes Dev 2004; 18:1165-78.
18. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005; 433:769-73.
19. Hurteau GJ, Spivack SD. mRNA-specific reverse transcription-polymerase chain reaction from human tissue extracts. Anal Biochem 2002; 307:304-15.
20. Shi R, Chiang VL. Facile means for quantifying microRNA expression by real-time PCR. Biotechniques 2005; 39:519-25.