

# Identification of microRNAs regulating expression of vimentin gene and miR-17-3p targets beyond vimentin

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## Introduction

MicroRNAs (miRNAs) are a group of small non-coding RNA sequences that are approximately 22 nucleotides in length. They play a significant role in regulating gene expression by binding to protein-coding regions of mRNAs, thereby repressing translation or degrading the mRNA altogether, depending on the degree of complementarity between the two sequences [1].

MiRNAs affect a variety of key cellular processes, such as cellular differentiation [1] and proliferation [2]. Aberrant miRNA levels have been noted in a number of diseases, including several types of cancer, where miRNAs have demonstrated the ability to act as tumor suppressors and oncogenes [3].

Presently, there are only five miRNAs linked with prostate cancer, three as oncomiRs and two as tumor suppressors. Prostate cancer is the second leading cause of cancer deaths for men in the United States and the most frequently diagnosed malignant tumor [4]. Studies have indicated that levels of vimentin, an intermediate filament with a key role in providing structural support for organelles in the cytosol [5], are correlated with prostate tumor growth [6]. Additionally, vimentin affects cell motility, a contributing factor to the development of metastatic phenotypes. One study comparing three genetically related sublines demonstrated higher vimentin levels in the metastatic and highly tumorigenic cell line than in both the slightly tumorigenic and non-metastatic cell lines [6].

A number of interactions between miRNAs and their target mRNA regions have been identified, but the mechanisms behind many miRNA target recognitions have yet to be determined. An investigation into possible miRNAs affecting vimentin expression yielded a correlation with miR-17-3p, a member of the miR-17-92 cluster, which was shown to bind to the 3'-UTR of the vimentin mRNA. Laser Capture Microdissection (LCM) analysis of RNA extracted from human prostate tumor samples confirmed the lack of miR-17-3p in normal glandular epithelium or stroma, indicating the role of miR-17-3p as a tumor suppressor. Unlike some of the other members of the miR-17-92 cluster, miR-17-3p has yet to be investigated in depth [7].

As the role of miR-17-3p appears to be significant, one of the goals of the current investigation is to identify other targets of miR-17-3p in hopes of elucidating the repertoire of mRNAs regulated by miR-17-3p. This study also aims to locate other miRNAs that affect the expression of vimentin.

The most common means of detecting miRNA targets is through the use of bioinformatic tools to predict potential candidates, followed by the implementation of laboratory techniques to investigate the actual roles of the predictions [4]. One of the main obstacles encountered in the identification of microRNAs and their corresponding targets is the reliability of current computational prediction programs. For example, the

proven interaction between miR-17-3p and vimentin is not predicted by either PicTar or TargetScan, two commonly used prediction tools in miRNA research.

Although over ten computational approaches are currently available, very few of them produce overlapping results [8]. This is undoubtedly due to variability between the sets of rules employed by the prediction programs regarding the likelihood of a miRNA-gene interaction. Thus, the third goal, and necessarily the first step to be accomplished, is to develop a novel program to make better predictions of both miRNA targets and target genes. If it betters the performance of current computational tools, this new program will hopefully prove instrumental in the development of an extensive miRNA expression signature for prostate cancer.

### Progress: Summer 2009

During the summer of 2009, a preliminary program was developed and successfully predicted the interaction between miR-17-3p and vimentin. The program, which is currently at approximately 200 lines of code, has three requirements for outputted predictions: perfect seed region binding, a total of 14 base pairs between the miRNA and target gene sequences, and a binding energy of  $\Delta G < -24$  kcal/mol. The binding energy was arbitrarily determined in order to narrow down the set of predictions to a manageable size.

The two main classes of miRNA targets are one consisting of target sites showing at least perfect complementarity to the seed region of the miRNA and the second consisting of those with limited base pairing to the 5' end of the miRNA but extensive base pairing to the 3' end to compensate [8]. The program being developed combines the two types of target sites in hopes of obtaining the best predicted interactions. In other words, seed region binding is necessary, as well as extensive base pairing to the 3' end of the miRNA.

A total of four programs were developed. The first takes a file with all human miRNA sequences known to date and a second file with the 3' UTRs of all human genes. Taking one miR sequence and one 3' UTR at a time, the program checks first for perfect seed region binding and then the presence of eight other bonds between the remaining upstream bases, allowing for loops to form in the miRNA. Below is an example of the occurrence of such a loop:

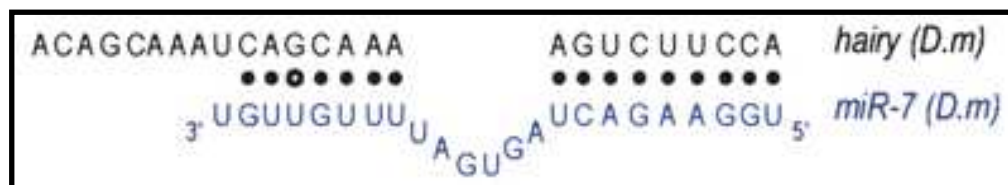


Figure 1. Interaction between miR-7 and target gene with perfect seed region binding, one loop in the miRNA sequence, and extensive base pairing to the 3' end of the miR [9].

The second program takes the set of predictions from the first program and passes it through RNAhybrid, a program that calculates the minimum free energy of hybridization between the miR and target gene. A sample of the program output is shown below:

microRNA	miR sequence	Gene name	Gene sequence	Starting nt	Ending nt	Matches	Binding energy
Homo sapiens miR-17*	ACUGCAGUGAAG	Vimentin	GCTTTCAAGTGCCT	1430	1452	19	-32.8 kcal/mol
Homo sapiens let-7b	UGAGGUAGUAGG	Vimentin	GAAACAGCTTCACT	765	789	18	-24.6 kcal/mol
Homo sapiens miR-1184	CCUGCAGCGACU	Vimentin	CAGCTTTCAAGTGC	1428	1452	17	-24.6 kcal/mol
Homo sapiens miR-1275	GUGGGGGAGAG	Vimentin	CACAATTGCCTCTC	272	291	15	-34.7 kcal/mol
Homo sapiens miR-509-3-5p	UACUGCAGACGU	Vimentin	GCTTTCAAGTGCCT	1430	1453	18	-24.4 kcal/mol

Figure 2. Sample output from program filtering out interactions with free energies of hybridization greater than -24 kcal/mol.

The third and fourth programs take the final output and calculate the average number of genes targeted by one miRNA and the average number of miRNAs targeted by one gene. Subsequent comparison of these averages to corresponding values of existing programs is one way to assess of the validity of the current program.

In addition to developing the program, Western blots were done to test select proteins predicted to be targeted by miR-17-3p for which antibodies were readily available: RAB8B, PPP2CA, and PPIA. Differences in protein expression were noted between P69, a normal and non-tumorigenic subline, M12, which is highly tumorigenic and metastatic, and M12 with overexpression of miR-17-3p. Targets of miRNAs should theoretically decrease in expression following an increase of the miRNA. In fact, the protein levels appeared to either remain constant or increase upon overexpression of miR-17-3p in M12 cells. The results confirmed the need for the creation of an improved computational tool.

In addition to vimentin, E-cadherin, a key epithelial marker whose expression should theoretically decrease with metastasis, was investigated. Using the intersection of PicTar, TargetScan, and RNA22, one miRNA was found to target E-cadherin: miR-9. Using the current program under development, miR-9 was predicted to target four different regions of the 3' UTR of E-cadherin.

### Goals for Academic Year

There is a considerable amount of work to be done with the program in the coming school year. First, the program as it stands will need to be measured against other tools. Most likely, additional features will need to be considered for implementation into the program.

Once the program is complete, the top predictions will be tested in the lab depending on availability of equipment and materials at Vanderbilt University. Results from laboratory testing will hopefully lead to a better idea of the roles of vimentin and other key proteins, not to mention factors (i.e. miRNAs) affecting the expression of said proteins in the prostate tumor microenvironment.

### Plans for Academic Year

Evaluation of the program will include calculating specificity and sensitivity values and comparing the results to those obtained from other programs. In addition, the averages of the number of targets for one miRNA and of the number of miRNAs targeting one gene will be used to determine whether more stringent rules regarding possible interactions need to be imposed.

Many additions to the program have been investigated, such as accounting for loops in the target gene, as opposed to solely in the miRNA sequence. Another

possibility is to give priority to those predictions with relatively less complex duplex structures, such as fewer or smaller loops, and those interactions in which the miRNA is predicted to target more than one region of the 3' UTR, as was predicted with miR-9. The decision as to which factors will be incorporated into the program will be based on previous studies, so much of this process will involve literature research.

Once the program is completed, predicted interactions will be tested in the lab. First, the target protein region will be fused to the luciferase reporter gene. Luciferase activity will be observed after transfection into both M12 cells lacking miR-17-3p and M12 cells with a stable over-expression of miR-17-3p. The results should indicate a reduction in reporter gene activity in the presence of miR-17-3p. To confirm that the results are due to the binding of miR-17-3p, a mutation will be made in either the target sequence or the seed region of the miRNA. If miR-17-3p binding is in fact the cause of reduction in gene expression, there should be no difference in luciferase activity between the M12 and M12+miR-17-3p cells. Second, a Western blot will be used to measure and compare endogenous expression of the predicted targets in M12 and M12+miR-17-3p, depending on antibody availability. It is expected that protein levels should decrease in M12+miR-17-3p cells. Third, q-RT-PCR will be used to quantify mRNA levels for the specific protein targets and to determine whether miR-17-3p is interfering through mRNA degradation or by repressing translation. The results will be compared to proteomic analyses of human prostate tumors.

## References

1. Porkka, K.P., et al., *MicroRNA expression profiling in prostate cancer*. *Cancer Res*, 2007. **67**(13): p. 6130-5.
2. Negrini, M., M.S. Nicoloso, and G.A. Calin, *MicroRNAs and cancer--new paradigms in molecular oncology*. *Curr Opin Cell Biol*, 2009. **21**(3): p. 470-9.
3. Calin, G.A. and C.M. Croce, *MicroRNA-cancer connection: the beginning of a new tale*. *Cancer Res*, 2006. **66**(15): p. 7390-4.
4. Shi, X.B., C.G. Tepper, and R.W. White, *MicroRNAs and prostate cancer*. *J Cell Mol Med*, 2008. **12**(5A): p. 1456-65.
5. Fuchs, E. and K. Weber, *Intermediate filaments: structure, dynamics, function, and disease*. *Annu Rev Biochem*, 1994. **63**: p. 345-82.
6. Zhang, X., et al., *Inhibition of vimentin or beta1 integrin reverts morphology of prostate tumor cells grown in laminin-rich extracellular matrix gels and reduces tumor growth in vivo*. *Mol Cancer Ther*, 2009. **8**(3): p. 499-508.
7. Zhang, X., et al., *MicroRNA-17-3p Reverts Morphology of Prostate Tumor Cells Grown in Laminin-rich ECM Gels and Reduces Tumor Growth in vivo*. *Journal of Cellular and Molecular Medicine*, 2009: p. 31.
8. Rajewsky, N., *microRNA target predictions in animals*. *Nat Genet*, 2006. **38** **Suppl**: p. S8-13.
9. Kiriakidou, M., et al., *A combined computational-experimental approach predicts human microRNA targets*. *Genes Dev*, 2004. **18**(10): p. 1165-78.