Bioinformatics Methods for Studying MicroRNA and ARE-Mediated Regulation of Post-Transcriptional Gene Expression

Richipal Singh Bindra, New Jersey Institute of Technology, USA

Jason T.L. Wang, New Jersey Institute of Technology, USA

Paramjeet Singh Bagga (corresponding author), Ramapo College of New Jersey, USA

ABSTRACT

MicroRNAs (miRNAs) are short single-stranded RNA molecules with 21-22 nucleotides that are known to regulate post-transcriptional expression of protein-coding genes involved in most of the cellular processes. Prediction of miRNA targets is a challenging bioinformatics problem. AU-rich elements (AREs) are regulatory RNA motifs found in the 3' untranslated regions (UTRs) of mRNAs; they play dominant roles in the regulated decay of short-lived human mRNAs via specific interactions with proteins. In this paper we review several miRNA target prediction tools and data sources, as well as computational methods used for the prediction of AREs. Then we discuss the connection between miRNA- and ARE-mediated post-transcriptional gene regulation. Next we present a data mining method for identifying the co-occurrences of miRNA target sites in ARE containing genes. Finally we conclude the paper by pointing out some directions for future research.

Keywords: Apriori Algorithm, AU-Rich Element, Computational Biology, MicroRNA, MicroRNA Target Prediction

INTRODUCTION

The expression of protein-coding genes is regulated via a variety of mechanisms. While regulation of gene transcription is central to the cellular metabolism, post-transcriptional control of gene expression has also gained significant attention in the recent years. Post-transcriptional processes like translational regulation and mRNA turnover are fairly complex mechanisms and are being recognized as equally if not more sophisticated than the transcriptional regulation of gene expression.

Several *cis*-acting signals are known to regulate mRNA stability. In the 3' untranslated regions, AU-rich elements (AREs) play dominant roles in the regulated decay of short-lived human mRNAs via specific interactions with proteins. While some of these proteins promote ARE-mediated mRNA degradation (AMD) (Stoecklin et al., 2002), others delay it (Peng, Chen, Xu, & Shyu, 1998). The mRNAs containing AREs generally code for proteins that are involved in transient processes such as cellular proliferation, stress response or development, which require delicate but strict regulation. The expression of these genes requires stringent and prompt controls which can be best achieved at the post-transcriptional level (Barreau, Paillard, & Osborne, 2005).

The post-transcriptional expression of a large number of metazoan genes is also influenced by microRNAs (miRNAs) which have received significant attention recently as important regulators of translation and destabilization of mRNAs. For reviews, see: (Bushati, & Cohen, 2007; Filipowicz, Bhattacharyya, & Sonenberg, 2008; Bartel, 2009; Zhang, & Su, 2009). The impact on gene expression is mediated by annealing of the miRNAs to complementary segments of the targeted mRNAs, especially in the 3' untranslated regions (UTRs). Approximately 30-50% of the human protein-coding genes appear to be regulated by miRNAs (John et al., 2004; John, Sander, & Marks, 2006; Filipowicz, Bhattacharyya, & Sonenberg, 2008; Friedman, Farh, Burge, & Bartel, 2009). Since miRNAs are considered to be involved in almost all types of cellular processes, an alteration in their own expression could lead to human diseases (Esquela-Kerscher, & Slack, 2006; Kloosterman, & Plasterk, 2006; Chang, & Mendell, 2007; Rodriguez et al., 2007). Therefore, investigating the mechanism of miRNA-mediated regulation of gene expression is critical to the understanding of cellular function and the diseases associated with altered miRNA function.

Several studies in the recent years have suggested that miRNAs can regulate post-transcriptional gene expression by targeting ARE-mediated mRNA turnover [reviewed in (von Roretz, & Gallouzi, 2008; Asirvatham, Magner, & Tomasi, 2009)]. For example, expression of cytokine genes could be regulated via miRNA targeting of proteins involved in AMD (Asirvatham, Gregorie, Hu, Magner, & Tomasi, 2008). Although more than 3,000 human genes are predicted to contain AREs (Bakheet, Williams, & Khabar, 2006), what proportion of these *cis*-acting elements require interactions with miRNAs or miRNPs for mRNA destabilization is not clear.

Owing to a lack of full length complementarity between the ~22 nucleotide miRNAs and their corresponding target sites in the animal mRNAs, accurate prediction of miRNA binding sites has been challenging, especially when attempted at a genomic scale. Prediction of miRNA targeted AREs in the 3'UTR is even trickier because the ARE-complementary region may not be in the 5'-region of the miRNA. It has been widely accepted that successful target recognition in animal mRNAs is dependent on pairing with bases in the 5'-region of the miRNAs (Bartel, 2004; Brennecke, Stark, Russell, & Cohen, 2005; Mendes, Freitas, & Sagot, 2009). This principle has been adopted by several miRNA target prediction tools.

In this article we review well-known methods for the prediction of AREs and miRNA target sites on the mRNAs, and discuss a bioinformatics approach for genome wide investigation of the correlation between miRNA target sites and AREs.

2 AU-RICH ELEMENTS

In this section we first discuss the biology of AREs. We then review some computational methods for ARE prediction.

2.1 Biology of AREs

AU-**R**ich **E**lements (AREs) are regulatory RNA motifs found in the 3' untranslated regions of mRNAs (Bakheet, Frevel, Williams, Greer, & Khabar, 2001). These *cis*-acting elements are commonly found in the mRNAs that are known to undergo rapid degradation through the 3'-5' exosome pathway following deadenylation. However, depending upon their interaction with proteins and cellular conditions, AREs can also help stabilize mRNAs (Ford, Watson, Keene, & Wilusz, 1999; Anderson et al., 2000; Mobarak et al., 2000). Loss of regulated stability in ARE-

mRNAs has been associated with disease (Khabar, 2005). In addition to mRNA turnover, AREs are known to regulate other post-transcriptional processes such as mRNA export and translation (Wilusz, Wormington, & Peltz, 2001; Espel, 2005). Roughly 3,700 human genes with diverse functions are known to code for ARE-containing mRNAs (Halees, El-Badrawi, & Khabar, 2008). Majority of these genes, such as growth factors and oncogenes, are involved in transient processes and therefore require strict expression control (Khabar, Bakheet, & Williams, 2005).

ARE sequences can range from 50 to 150 bases in length. A pentamer, 'AUUUA', which can be found in multiple repeats amidst U-rich regions in the 3'UTR, is considered as the identifying motif of the ARE (Chen, & Shyu, 1994). However, a nonamer 'UUAUUUAUU' is minimally needed for destabilization of the mRNA and, therefore, is considered the functional ARE motif (Zubiaga, Belasco, & Greenberg, 1995). AREs can be categorized into multiple classes [reviewed in (Barreau, Paillard, & Osborne, 2005)]. Extensions of the pentamer are at the core of the first two classes which differ on the basis of the repetition pattern of this sequence motif. Class II has been further sub-divided into clusters based on the number of pentamer repeats. The third class of AREs, although U rich, is not known to contain a well-defined sequence motif.

ARE-mediated regulation of post-transcriptional gene expression requires interactions with cellular proteins. Analysis of ARE-binding proteins (ARE-BPs) has gained significant attention since alteration in their expression can lead to cancer (Audic, & Hartley, 2004). Most AREs can bind to multiple proteins (Barreau, Paillard, & Osborne, 2005). Overall interactions of an ARE containing mRNA with ARE-BPs could be complex and eventually result in either degradation or destabilization of the mRNA. Some of these proteins, such as Tristetraprolin (TTP), Butyrate Response Factor (BRF1) and KH-type Splice Regulatory Protein (KSRP), are known to decrease the mRNA stability (Stoecklin et al., 2002; Gherzi et al., 2004; Barreau, Paillard, & Osborne, 2005; Cao, Deterding, & Blackshear, 2007). On the other hand, members of the *Hu* family of ARE-binding proteins that are related to ELAV, generally stabilize the mammalian mRNAs (Barreau, Paillard, & Osborne, 2005). For example, HuR, which is present during cellular proliferation, has been found to bind to mRNAs of all three ARE classes (Peng, Chen, Xu, & Shyu, 1998; Barreau, Paillard, & Osborne, 2005). AUF1, which was one of the first identified functional ARE-BP, is known to enhance or inhibit mRNA decay rate depending upon its own alternative expression, cell type and the class of targeted AREs (Xu, Chen, & Shyu, 2001; Sarkar, Xi, He, & Schneider, 2003; Lal et al., 2004; Raineri, Wegmueller, Gross, Certa, & Moroni, 2004).

Interactions between ARE-BPs and the mRNA decay enzymes result in rapid mRNA degradation. Mammalian AMD is dependant on deadenylation, which is followed by 3'-5' degradation by a multimeric complex of exonucleases called the exosome [reviewed in (Wilusz, & Wilusz, 2004; Barreau, Paillard, & Osborne, 2005)]. Degradation of mRNA can be enhanced by direct interaction of exosome components to either AREs (Mukherjee et al., 2002), or ARE-BPs like KSRP and TTP (Chen et al., 2001). Several ARE-BPs are also capable of influencing translation of mRNAs. For example, while TIA-1 is known to inhibit translation of TNF-alpha and Cox-2 mRNAs (Piecyk et al., 2000; Dixon et al., 2003), Hel-N1, a member of the *Hu* family, can increase translation of GLUT1 mRNAs (Jain et al., 1995). On the other hand, HuR itself can increase or down-regulate translation (Galban et al., 2003; Katsanou et al., 2005). The mechanism of ARE-dependent regulation of translation requires further investigation.

2.2 Computational Approaches for ARE Prediction

In order to understand the biological role of AREs in regulating the functioning of transient transcriptome, it would be essential to delineate the genes that contain these *cis*-acting elements.

Computational strategies for genome-wide identification of AREs are full of challenges. While the pentamer discussed above forms the core of majority of AREs, the minimal functional ARE is a nonamer with many variations. Attempts have been made to search mammalian genomes for AREs using computational approaches and to classify them (Bakheet, Frevel, Williams, Greer, & Khabar, 2001; Bakheet, Williams, & Khabar, 2003; Bakheet, Williams, & Khabar, 2006; Halees, El-Badrawi, & Khabar, 2008). While very useful databases of ARE containing transcripts have been established as the result of these efforts, these collections are not expected to represent the entire repertoire of AREs in the trancriptome.

Bakheet and coworkers (Bakheet, Frevel, Williams, Greer, & Khabar, 2001), used a list of known 57 ARE containing mRNAs as a training set for computation of the ARE motif. This analysis resulted in a 13-bp pattern WWWUAUUUAUWWW which was then used to search CDS and 3'UTR sequences of 13,057 human mRNA sequences from NCBI-GenBank (Karsch-Mizrachi, & Ouellette, 2001). The 897 ARE-mRNAs thus identified were curated in a web accessible database, named ARED 1.0 (http://brp.kfshrc.edu.sa/ARED/). AREs in this database were clustered in 5 groups based on the subsequence patterns and phylogenic relationship. ARED has been updated several times successively. All the versions are accessible at the same URL. The second generation database, named ARED 2.0, was established in 2003 (Bakheet, Williams, & Khabar, 2003). It contained 1,500 non-redundant human full-length ARE-mRNAs. Several inconsistencies of the first version were also removed.

Khabar and co-authors (Khabar, Bakheet, & Williams, 2005) focused on complementing the ARED 1.0 and 2.0 databases (discussed above) with ARE-mRNAs that were initially missed because of inadequate 3'UTRs, partial sequence information, or complexities involving alternatively expressed isoforms. Two similar computational approaches were used to search for AREs in the validated 3' ends of human ESTs. The pentamer ATTTA motif was at the core of search patterns for mining and prediction. The first approach searched 3.4 million human ESTs and resulted in 673 known mRNA records and 26 novel alternatively spliced transcripts containing AREs. A total of 199 ARE-genes and 140 novel ARE-mRNAs were identified. The second approach used an updated version of dbEST with approximately 5.5 million records and involved ARE EST clusters with two or more 3' ends. This experiment resulted in 1,096 ARE-mRNAs including more than 350 novel alternative ARE-mRNA forms. Their combined efforts from both the experiments identified over 1,500 human ARE genes including those which are alternatively spliced, alternatively polyadenylated, or with variant 3'UTRs.

ARED 3.0 (Bakheet, Williams, & Khabar, 2006) contains over 4,000 human ARE-mRNAs. Transcript sequences from NCBI databases: RefSeq (Pruitt, Tatusova, & Maglott, 2007), GenBank, including dbEST (Boguski, Lowe, & Tolstoshev, 1993), were screened with a new refined pattern WWWT(ATTTA)TTTW as the ARE motif for the third version of the database. Eventually, all the versions of the databases were combined, along with ARED 4.0, to generate an ARED integrated database of ARE-containing human mRNAs (Halees, El-Badrawi, & Khabar, 2008). This expanded and updated database contains 3,700 ARE-genes and 6,153 RefSeq transcripts. Another ARED organism database (Halees, El-Badrawi, & Khabar, 2008), which is obtained from the analysis of mRNAs in ENSEMBL (Hubbard et al., 2007), contains human, mouse and rat AREs.

3 MicroRNAs

After describing the biology of AREs and computational approaches for ARE prediction, we now turn to microRNAs. In this section we first discuss the biology of microRNAs. We then review several microRNA target prediction tools and online data sources.

3.1 Biology of MicroRNAs

MicroRNAs (miRNAs) have emerged as a large class of noncoding RNAs which can regulate important biological processes in eukaryotes and other species. Since they have been the subject of many reviews recently (Filipowicz, Bhattacharyya, & Sonenberg, 2008; Asirvatham, Magner, & Tomasi, 2009; Bartel, 2009; Zhang, & Su, 2009; Fabian, Sonenberg, & Filipowicz, 2010; Newman, & Hammond, 2010; Siomi, & Siomi, 2010), we will provide only a brief overview of miRNA biogenesis and function in this article.

MicroRNAs are short singe-stranded RNA molecules with 21-22 nucleotides that are known to regulate post-transcriptional expression of protein-coding genes. More than one thousand miRNAs are thought to be expressed in the humans (Filipowicz, Bhattacharyya, & Sonenberg, 2008). Metazoan miRNAs are known to be differentially expressed in different tissues, especially during development and differentiation (Sempere, Sokol, Dubrovsky, Berger, & Ambros, 2003; Biemar et al., 2005; Wulczyn et al., 2007).

Human miRNA genes, which are located in the introns, intergenic regions or other locations, are mostly transcribed by RNA pol II. The primary transcript, a long pri-miRNA, which could be hundreds of bases long, gets cleaved by a multiprotein complex containing an RNAse III enzyme called Drosha in the nucleus. The resulting ~70 nucleotide double stranded stem-loop pre-miRNA is then transported to the cytoplasm with the help of a protein complex containing Exportin 5. The cytoplasmic RNAse III, Dicer, which recognizes the double stranded stem-loop, then cleaves the pre-miRNA to release the double stranded ~22 nucleotide miRNA intermediate. One of the miRNA strands is then incorporated into the RNA-induced silencing complex (RISC). The miRISC subsequently targets 3'UTRs for miRNA:mRNA interactions. The overall impact of this interaction in animals generally is modulation of translation or mRNA destabilization. These mechanisms don't seem to involve cleavage of the mRNAs in animal systems. Biogenesis of miRNAs in plants is somewhat similar to that in animals. However, plant miRNAs are generally known to cleave the target mRNAs.

Over 50% of the human protein-coding genes are thought to be under an evolutionary pressure to maintain miRNA binding sites in their 3'UTRs (Friedman, Farh, Burge, & Bartel, 2009). Therefore, a wide range of biological processes are likely to be influenced by miRNA targeting. For example, miR-155 influences immune response in mice and has been predicted to regulate a wide spectrum of genes including cytokines and transcription factors (Rodriguez et al., 2007). MicroRNAs are thought to affect growth, morphogenesis, apoptosis and cell to cell signaling. Therefore, alteration in miRNA function can lead to disease (Esquela-Kerscher, & Slack, 2006; Kloosterman, & Plasterk, 2006; Chang, & Mendell, 2007; Rodriguez et al., 2007). Understanding the mechanism of their interactions with target mRNAs, regulatory proteins and involvement in molecular pathways is essential.

Several models for regulation of translation and mRNA decay by miRNAs are reviewed in (Filipowicz, Bhattacharyya, & Sonenberg, 2008). However, the more we learn about these

complex pathways, more questions are raised. The mechanistic details of many steps are still sketchy and require more research.

3.2 Computational Methods for MicroRNA Target Prediction

In this subsection we first review some miRNA target prediction tools. We then survey four online databases, which store the miRNA target information along with the genes in which miRNA target sites occur, as well as their location information in 3'UTR regions.

3.2.1 MicroRNA Target Prediction Tools

Plant miRNAs are almost entirely complementary to their respective pairing sites in the plant mRNAs (Rhoades et al., 2002) (Figure 1). However, animal miRNAs have only about 7-8 base-pair partial complementarity of their 5' end to the target sites in the animal mRNAs (Brennecke, Stark, Russell, & Cohen, 2005). While it is easier to search for miRNA target sites by looking for complementary sequences in the plant mRNAs (Rhoades et al., 2002), this approach generates many false positives in animals (Bartel, 2009). Therefore, genome-wide miRNA target prediction in metazoans has been challenging. Several methods also use partial complementarity of the 3' end of miRNAs in addition to the 5' end. However, the miRNA target prediction methods that rely solely on pairing of the 5'- and 3'-ends of the miRNAs still result in false positives. The accuracy of predictions can be improved by consideration of mRNA secondary structure, positioning within the 3'UTR, proximity to other miRNA target sites, site conservation and sequence context (Grimson et al., 2007; Bartel, 2009; Mendes, Freitas, & Sagot, 2009).

Figure 1. Illustration of the binding between a miRNA and an mRNA (a) in plants and (b) in animals

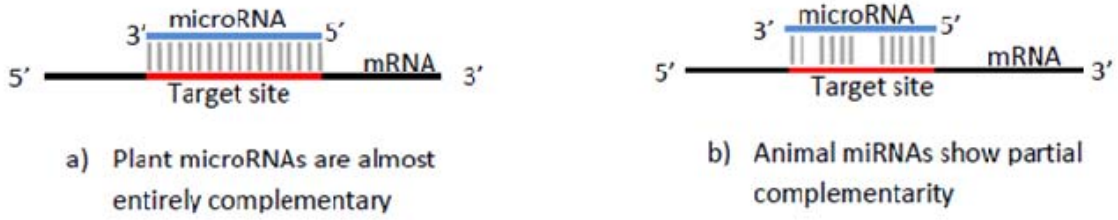


Table 1 summarizes some widely used miRNA target prediction tools. TargetScan (Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003) adopts thermodynamics-based modeling of miRNA:mRNA interactions. Given a miRNA conserved in various organisms, this tool uses comparative sequence analysis to predict miRNA targets conserved across multiple genomes. Specifically, the tool takes a seed-match approach where a seed is defined as a 7-nt segment containing positions 2-8 of a mature miRNA. The TargetScan algorithm consists of the following steps: (i) Search the UTRs of interest for perfect Watson-Crick complementarity (called "seed match") to bases 2-8 of a miRNA (called "miRNA seed"). (ii) Extend each seed match allowing wobble G:U pairs. (iii) Optimize base-pairing to 35 bases. (iv) Assign a folding free energy value to each miRNA:target site. (v) Assign a Z score to each miRNA:mRNA binding. (vi) Sort the UTRs and rank them. (vii) Repeat the process for other organisms. TargetScan identified 451 putative miRNA target sites in 400 distinct genes; the false positive rate is estimated to be 31% for human, mouse and rat and 22% for pufferfish.

Table 1. Summary of the miRNA target prediction tools

Name	URL	Availability	Reference
TargetScan	http://targetscan.org/	Online	(Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003)
TargetScanS	http://targetscan.org/	Online	(Lewis, Burge, & Bartel, 2005)
RNAHybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	Download	(Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004)
DIANA-microT	http://diana.cslab.ece.ntua.gr/microT/	Online	(Maragkakis et al., 2009)
miRanda	http://www.microrna.org/microrna/home.do	Download	(Betel, Wilson, Gabow, Marks, & Sander, 2008)
PicTar	http://pictar.mdc-berlin.de	Online	(Lall et al., 2006)
MicroTar	http://tiger.dbs.nus.edu.sg/microtar/	Download	(Thadani, & Tammi, 2006)
mirWIP	http://146.189.76.171/query	Online	(Hammell et al., 2008)

TargetScanS (Lewis, Burge, & Bartel, 2005) is a simplified version of TargetScan; it includes two more species (dog and chicken). In the new algorithm, the authors improved the target prediction of miRNA by relaxing the score and rank cutoffs. Furthermore, the requirement of the 7-nt match to the seed region was relaxed to a 6-nt (2-7 nucleotides) match followed by an additional 3' match of an adenosine anchor at position 1. High conservation at downstream of a seed match was observed and was usually a conserved A, which can pair to the first nucleotide U of a miRNA. This algorithm does not take into account the thermodynamic stability of pairing, and the presence of multiple complementary sites per UTR. TargetScan and TargetScanS have

low false positive rates, but due to the requirement of strict complementarity some targets having 3' compensatory sites are likely to be missed.

RNAHybrid is an extension to the RNA secondary structure (RSS) algorithm (Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004). RSS uses dynamic programming along with an artificial linker between a miRNA and its potential binding site. However, as per (Stark, Brennecke, Russell, & Cohen, 2003) there are some issues with this approach; the linker may lead to artifacts. RNAHybrid, on the other hand, directly predicts binding sites in target mRNAs; the algorithm finds energetically most favorable hybridizations of a small RNA (miRNA) to a large RNA (mRNA). RNAHybrid does not allow intramolecular hybridizations and multi-loops; the algorithm calculates the minimum free energy of a hybridization of a miRNA and an mRNA. The time complexity of the algorithm is linear with respect to the length of the miRNA.

DIANA-microT (Maragkakis et al., 2009) uses the following features: binding site structural accessibility, nucleotide composition flanking the binding sites or proximity of one binding site to another within the same 3'UTR. The tool groups miRNA driver sequences with presumptive MREs (miRNA recognition elements). The tool first dynamically divides the 3'UTR sequence into 9-nt sections, and then scores these sections with miRNA driver sequences and identifies the one with the highest score. The tool uses Watson-Crick or wobble (G:U) matching, which generates 7-mers, 8-mers and 9-mers, each with a wobble base pair. Second, the tool uses RNAHybrid (Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004) to calculate free binding energy values. Third, the tool finds MREs and checks their sequence conservation, based on sequence alignment. A MRE score is calculated separately for each miRNA, and each binding category. The scores of the MREs are combined through a weighted sum to get the final score. The authors indicated that their tool was found to achieve the highest precision reaching 66%.

The miRanda tool uses dynamic programming to search for maximal local complementarity alignments, corresponding to a double-stranded anti-parallel duplex (John et al., 2004; Betel, Wilson, Gabow, Marks, & Sander, 2008). It uses weighted sum match and mismatch scores for Watson-Crick base pairs. Wobbles (G:U) are allowed but much less scored than perfect pairing; especially favored are pairings between 2-8 nucleotides; gaps are penalized. In the final score the presence of 3' pairing is incorporated along with the free energy of the miRNA:mRNA duplex. Less conserved sites are excluded from prediction. The tool uses the PhastCons conservation score to filter out less conserved anticipated sites. In addition, a conservation of the miRNA:mRNA relationship is considered which depends on (i) conservation of the mature miRNA; (ii) conservation of the relevant parts of the mRNA; and (iii) the presence of a homology between the miRNA and the relevant parts of the mRNA.

PicTar (Krek et al., 2005; Lall et al., 2006) identifies potential targets by using a perfect 7-mer seed match as Watson-Crick or G:U pairing at positions 1-7 or 2-8 from the miRNA 5' end. Imperfect seed pairs are admitted but are scored poorly. A free energy calculation of the miRNA:mRNA duplex is performed and the results are further filtered; the sites are then checked for conservation. These potential targets are then scored using a hidden Markov model (maximum likelihood score), which assigns probabilities to 3'UTR subsequences as a binding site for a miRNA. The results are scored for each individual species and are combined to get the final score, the outcome of which is the final prediction of miRNA target sites. The false positive rate estimate is 30%, and each vertebrate miRNA has about 200 target transcripts.

MicroTar (Thadani, & Tammi, 2006) assumes that (i) miRNA target specificity is caused by 7-mer seed sequences, and (ii) if miRNA:mRNA duplex development is energetically favorable targets are functional (Thadani, & Tammi, 2006). MicroTar does not depend on evolutionary

conservation. The tool calculates the minimum free energy for each molecule of mRNA using mfold (Zuker, 2003). The energy obtained is denoted as G_1. Next, the tool performs a seed search within mRNA 3'UTRs for Watson-Crick or wobble (G:U) complementary to a seed sequence. This step yields several seed matches, where the seed matches are again folded using cofold (Bernhart et al., 2006). The energy obtained is denoted as G_2. The output of these two steps is a list of seed matches. If miRNA:mRNA heterodimer is energetically more stable than free mRNA (i.e. $G_2 < G_1$) then the seed matches reflect functional targets. To decide the importance of each match of miRNA:mRNA, statistic analysis is done, which results in the final list of miRNA targets.

The mirWIP tool (Hammell et al., 2008) uses RNAHybrid (Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004) with modifications, to find an initial set of miRNA targets in organisms *C. elegans* and *C. Briggsae*. The tool adopts several liberal filters. For example, a limit was set for the hybrid minimal free energy (MFE) of the duplex, referred to as the hybrid interaction energy. Some heuristics were implemented, including (i) up to three G:U wobble pairs in a 6-mer beginning at miRNA position 2 were allowed; (ii) up to one bulge on the mRNA side of the duplex in a 7-mer beginning at miRNA position 2 was allowed; and (iii) perfect 6, 7, and 8-mers beginning at miRNA position 3 were allowed. Scoring of the targets was done based on three features: seed topology (S), structural accessibility (A), and total interaction energy (E). A site score was calculated as $S \times A \times E$. This gave an initial set of conserved *C. elegans* miRNA binding sites which were further analyzed using related information and scores.

3.2.2 Online Data Sources

Table 2 summarizes some well-known miRNA target databases. The miRBase database (Griffiths-Jones, Saini, van Dongen, & Enright, 2008) is an online repository with a searchable interface. It stores microRNA nomenclature, sequence data, annotation and target predictions, which allows download of files in various formats including MySQL Dump and FASTA. The miRBase database uses miRanda along with a pipeline for prediction of miRNA targets. The database (release 10.0) contains 5,071 miRNA loci from 58 species, expressing 5,922 unique mature miRNA sequences. The current release 15.0 has over 3,000 new hairpin sequences and more than 4,000 new mature sequences. The web site link is http://www.mirbase.org/.

TarBase 1.0 (Sethupathy, Corda, & Hatzigeorgiou, 2006) stores information of miRNA targets in human, mouse, fruit fly, worm, and zebrafish. The database is functionally linked to Gene Ontology (GO) and UCSC Genome Browser. There are more than 550 target site entries in total, where each target site has information of binding miRNA, the gene in which it occurred, and the location information in the 3' UTR region. TarBase 5.0 (Papadopoulos, Reczko, Simossis, Sethupathy, & Hatzigeorgiou, 2009), which is an updated and extended version of TarBase 1.0, contains data extracted from more than 200 scientific papers and has over 1,300 miRNA target site entries. This version not only contains all the information from the older version, but has additional information such as (i) specific cell lines, (ii) cell-type-specific expression of the gene, (iii) developmental or pathological events that a specific miRNA is involved in, and (iv) HGNC symbols as defined by HUGO. This database is functionally linked to other databases including ENSEMBL, HUGO, UCSC and SwissProt. TarBase5.0 can be accessed at http://microrna.gr/tarbase.

Table 2. Summary of the online data sources

Name	URL	Availability	Reference
miRBase	http://www.mirbase.org/	Online	(Griffiths-Jones, Saini, van Dongen, & Enright, 2008)
TarBase	http://microrna.gr/tarbase	Online	(Sethupathy, Corda, & Hatzigeorgiou, 2006; Papadopoulos, Reczko, Simossis, Sethupathy, & Hatzigeorgiou, 2009)
miRNAMap	http://miRNAMap.mbc.nctu.edu.tw/	Online	(Hsu et al., 2006; Hsu et al., 2008)
miRGator	http://genome.ewha.ac.kr/miRGator/	Online	(Nam, Kim, Shin, & Lee, 2008)

The miRNAMap database (Hsu et al., 2006) is a collection of known miRNA genes, putative miRNA genes, known miRNA targets and putative miRNA targets in human, mouse, rat and other metazoan genomes. It provides cross links to many other biological databases. The miRNAMap database has two versions. Version 1.0 (Hsu et al., 2006) uses known miRNAs from miRBase version 6.0 together with the miRanda target prediction tool and has no filtering criteria. Version 2.0 (Hsu et al., 2008) adopts miRBase version 9.2 for known miRNAs. It uses three target prediction tools, namely miRanda, RNAHybrid and TargetScan. It houses experimentally verified miRNAs and miRNA target genes for human, mouse, rat and other metazoan genomes. In addition, version 2.0 uses three criteria to reduce the false positive rate of prediction of miRNA targets along with Q-PCR miRNA profiling of 224 human miRNAs. It contains more than 2,400 miRNAs in total, of which about 540 are in Homo sapiens (hsa) with 6,750 conserved genes in hsa. The miRNAMap database can be accessed online at http://miRNAMap.mbc.nctu.edu.tw/.

The miRGator database (Nam, Kim, Shin, & Lee, 2008) integrates gene expression data, target prediction, functional analysis and genome annotation in a single repository. For miRNA target prediction, three algorithms are used, which include miRanda, PicTar and TargetScanS. The miRGator database provides a statistical enrichment test of miRNA targets in functional categories like GO function, GenMAPP and KEGG pathways, and various diseases. Search can be performed based on the functional categories or expression profiling. The miRGator database supports the exploration of human and mouse genomes and can be accessed online at http://genome.ewha.ac.kr/miRGator/.

4 DISCUSSION

Is there a connection between miRNA- and ARE-mediated post-transcriptional gene regulation? As discussed above, mRNA turnover and regulation of translation, both biologically important post-transcriptional processes, can be significantly influenced by interactions of regulatory RNA binding proteins with *cis*-regulatory elements in 3'UTRs of the mRNAs. Do ARE- and miRNA-mediated gene regulatory pathways intersect at any point? Is there a relationship between them?

Both of the pathways have been found to share some components. For example, human Dicer, a protein normally involved in miRNA maturation, and an Argonaute (AGO) protein, which is part of the miRNA-induced silencing complex (miRISC), are also required for ARE-mediated mRNA decay in HeLa cells (Jing et al., 2005). Mammalian KSRP, an ARE-BP that promotes AMD, is found in the Dicer and Drosha complexes and is involved in regulating the biogenesis of miRNAs (Trabucchi et al., 2009). ARE-BPs like TTP (tristetraprolin), AUF1 and *Hu* family members could be targeted by miRNAs. In fact, TTP has been shown to interact indirectly with miR16 through the AGO family members for destabilization of TNF-alpha mRNA (Jing et al., 2005).

Over 200 genes of miR16 targets contain AU-rich sequences in their 3'UTRs (Asirvatham, Gregorie, Hu, Magner, & Tomasi, 2008). Grimson and coworkers have found with the help of experimental and bioinformatics studies that efficacy of the miRNA target sites is substantially affected by local AU sequence context (Grimson et al., 2007). Presence of miRNA target sites in the AU-rich regions of the 3'UTRs would presumably keep the sites accessible to regulatory proteins. Therefore, it is not unreasonable to speculate that miRNA target sites may overlap AREs or occur in their vicinity in at least a subset of mRNAs.

Asirvatham and coworkers have found that in three genes (SOCS2, AGO1, and RARB), potential miR16 target sites were associated with 3'UTR AREs (Asirvatham, Gregorie, Hu, Magner, & Tomasi, 2008). In fact, miR16, a human miRNA which contains a sequence complementary to the 3'UTR ARE, has been shown to be required for ARE-mediated turnover of the TNF-alpha mRNA (Jing et al., 2005), raising the possibility that miRNAs can also bind to AREs. However, since the ARE-complementary sequence of miRNAs doesn't seem to be large enough for efficient ARE-targeting, the association may require assistance from other proteins. That is why perhaps miR16 targeting to the ARE sequences requires assistance from tristetraprolin (TTP), an ARE binding protein (Jing et al., 2005).

MicroRNAs can be involved in destabilization or stabilization of ARE-mRNAs. MicroRNA-4661 has been found to upregulate IL-10 expression by binding to its 3'UTR ARE and interfering with TTP-mediated AMD (Ma et al., 2010). The IL-10 ARE sequence was found to be complementary to the seed sequence of miR-4661. Ma and coworkers also discovered miR-4661 targets on many other ARE-mRNAs and found some of them to be down regulated in a gene reporter system (Ma et al., 2010). Several factors, including the type of gene and the cells in which they are expressed, may determine how miRNA targeting will impact ARE-mediated mRNA turnover.

Cooperation between miRNAs and ARE-BPs can also regulate TNF-alpha mRNA translation. The miR369-3p sequence has two complementary binding sites within the 34 nucleotide ARE in the 3'UTR of TNF-alpha mRNA, and has been shown to be responsible for translational up- or down-regulation (Steitz, & Vasudevan, 2009). This miRNA is responsible for directing ARE-BPs, fragile X mental retardation related protein 1 (FXR1) and AGO2, to the ARE

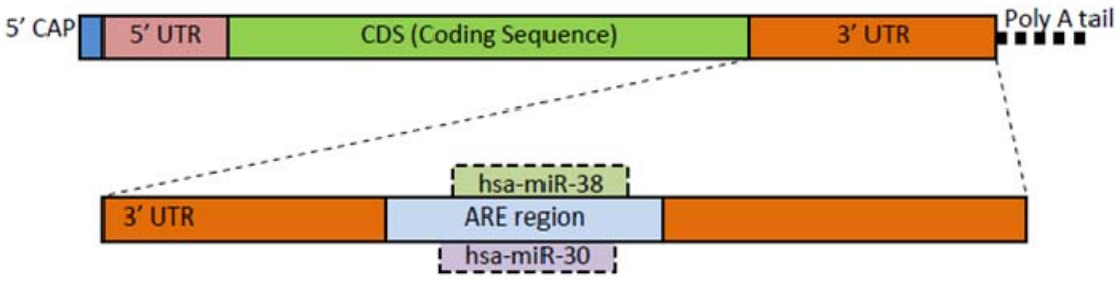
for translational up-regulation.

Understanding the relationship between miRNA and ARE-mediated regulated gene expression requires detailed investigation. As discussed above and reviewed in (von Roretz, & Gallouzi, 2008; Asirvatham, Magner, & Tomasi, 2009), emerging evidence points towards links between the two pathways. The observed cooperation involves sharing of protein factors as well as participation of co-located *cis*-elements. One could speculate that miRNA target sites and AREs may have co-evolved along with the pathways. Studying the genome-wide distribution patterns of ARE and miRNA target site co-occurrences in the 3'UTRs can help develop a better picture of these two post-transcriptional regulation pathways and their relationship with each other.

We have conducted an experimental study to gain a better understanding of the association between AREs and miRNA target sites. We selected the miRNA target prediction tool, DIANA-microT, surveyed in Section 3, and the database of miRNA targets predicted by DIANA-microT, which can be downloaded from the website http://diana.cslab.ece.ntua.gr/microT/. The dataset contains information of miRNA target sites and their known locations in 3'UTR regions of human genes, as well as the ENSEMBL ID and Gene ID of each sequence. Another dataset we selected was ARED downloaded from the website http://brp.kfshrc.edu.sa/ARED/. This website contains flat files of ARED database and cluster groups, where the ARED files are in GenBank flat file view (i.e. nucleotide sequence with annotation).

We used the Apriori algorithm (Agrawal, Imielinski, & Swami, 1993) to find association rules between miRNA target sites in ARE-containing genes. Apriori is a well-known data mining algorithm which generates association rules from a given set of transactions. We modeled the ~3200 ARE containing genes in the ARED database as transaction records and items in the transactions by the miRNA target sites and AREs from the datasets we selected. We found that whenever a hsa-miR-30 target site occurs, a hsa-miR-38 target site and an ARE co-occur with a support of 1.3% and confidence of 37.3% (Figure 2). In Figure 2, *cis*-acting elements, including two miRNA target sites and an ARE, both found in the 3'UTR region of an mRNA sequence, can potentially regulate post-transcriptional gene expression. Overlapping elements may suggest cooperation between the two pathways. Other findings include: whenever a hsa-miR-32 target site occurs, a hsa-miR-36 target site and an ARE co-occur with a support of 1% and confidence of 16.5%; whenever a hsa-miR-41 target site occurs, a hsa-miR-88 target site and an ARE co-occur with a support of 1.3% and confidence of 12.1%; whenever a hsa-miR-51 target site occurs, a hsa-miR-52 target site and an ARE co-occur with a support of 1.2% and confidence of 29.5%; whenever a hsa-miR-54 target site occurs, a hsa-miR-55 target site and an ARE co-occur with a support of 2.5% and confidence of 22.7%.

Figure 2. Illustration of the co-occurrence of two miRNA target sites and an ARE region



5 CONCLUSION

AU-rich elements have been mapped to a huge diversity of genes that are involved in important biological processes like development, growth, RNA metabolism, transcriptional regulation, cell to cell signaling, stress response and immune defense. Understanding the mechanisms of ARE and miRNA-mediated mRNA turnover and translational control is vital to unraveling the complexities of post-transcriptional gene expression. Although several computational methods for miRNA target prediction have been developed, it may not be possible to accurately identify AREs as targets. Most miRNA target prediction methods have shortcomings. While some programs can miss legitimate targets, many of them are known to have high false-positive rates. Besides, miRNAs may require assistance from sequence specific RNA binding proteins to bind to their targets. Therefore, studying the role of miRNAs in ARE-mediated mRNA decay will likely require evaluation of miRNA associations with other proteins.

We surveyed here a number of miRNA target prediction tools and online databases of miRNA target sites. We also provided an overview of AREs and available online databases of AREs. We discussed the correlation between AREs and miRNA target sites, and identified co-occurrences between some miRNA target sites in ARE containing genes using a data mining method. Future work includes further analysis of the co-occurrences between AREs and miRNA target sites to understand their biological significance, as well as their role in post-transcriptional gene regulation.

REFERENCES

Agrawal, R., Imielinski, T. & Swami, A.N. (1993) Mining association rules between sets of items in large databases. In *Proceedings of the 1993 ACM SIGMOD International Conference on Management of Data*, 207-216.

Anderson, K.D., Morin, M.A., Beckel-Mitchener, A., Mobarak, C.D., Neve, R.L., Furneaux, H.M., Burry, R. & Perrone-Bizzozero, N.I. (2000) Overexpression of HuD, but not of its truncated form HuD I+II, promotes GAP-43 gene expression and neurite outgrowth in PC12 cells in the absence of nerve growth factor. *J Neurochem, 75*, 1103-1114.

Asirvatham, A.J., Gregorie, C.J., Hu, Z., Magner, W.J. & Tomasi, T.B. (2008) MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. *Mol Immunol, 45*, 1995-2006.

Asirvatham, A.J., Magner, W.J. & Tomasi, T.B. (2009) miRNA regulation of cytokine genes. *Cytokine, 45*, 58-69.

Audic, Y. & Hartley, R.S. (2004) Post-transcriptional regulation in cancer. *Biol Cell, 96*, 479-498.

Bakheet, T., Frevel, M., Williams, B.R., Greer, W. & Khabar, K.S. (2001) ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res, 29*, 246-254.

Bakheet, T., Williams, B.R. & Khabar, K.S. (2003) ARED 2.0: an update of AU-rich element mRNA database. *Nucleic Acids Res, 31*, 421-423.

Bakheet, T., Williams, B.R. & Khabar, K.S. (2006) ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic Acids Res*, *34*, D111-114.

Barreau, C., Paillard, L. & Osborne, H.B. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res*, *33*, 7138-7150.

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, *116*, 281-297.

Bartel, D.P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell*, *136*, 215-233.

Bernhart, S.H., Tafer, H., Muckstein, U., Flamm, C., Stadler, P.F. & Hofacker, I.L. (2006) Partition function and base pairing probabilities of RNA heterodimers. *Algorithms Mol Biol*, *1*, 3.

Betel, D., Wilson, M., Gabow, A., Marks, D.S. & Sander, C. (2008) The microRNA.org resource: targets and expression. *Nucleic Acids Res*, *36*, D149-153.

Biemar, F., Zinzen, R., Ronshaugen, M., Sementchenko, V., Manak, J.R. & Levine, M.S. (2005) Spatial regulation of microRNA gene expression in the Drosophila embryo. *Proc Natl Acad Sci U S A*, *102*, 15907-15911.

Boguski, M.S., Lowe, T.M. & Tolstoshev, C.M. (1993) dbEST--database for "expressed sequence tags". *Nat Genet*, *4*, 332-333.

Brennecke, J., Stark, A., Russell, R.B. & Cohen, S.M. (2005) Principles of microRNA-target recognition. *PLoS Biol*, *3*, e85.

Bushati, N. & Cohen, S.M. (2007) microRNA functions. *Annu Rev Cell Dev Biol*, *23*, 175-205.

Cao, H., Deterding, L.J. & Blackshear, P.J. (2007) Phosphorylation site analysis of the anti-inflammatory and mRNA-destabilizing protein tristetraprolin. *Expert Rev Proteomics*, *4*, 711-726.

Chang, T.C. & Mendell, J.T. (2007) microRNAs in vertebrate physiology and human disease. *Annu Rev Genomics Hum Genet*, *8*, 215-239.

Chen, C.Y. & Shyu, A.B. (1994) Selective degradation of early-response-gene mRNAs: functional analyses of sequence features of the AU-rich elements. *Mol Cell Biol*, *14*, 8471-8482.

Chen, C.Y., Gherzi, R., Ong, S.E., Chan, E.L., Raijmakers, R., Pruijn, G.J., Stoecklin, G., Moroni, C., Mann, M. & Karin, M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell*, *107*, 451-464.

Dixon, D.A., Balch, G.C., Kedersha, N., Anderson, P., Zimmerman, G.A., Beauchamp, R.D. & Prescott, S.M. (2003) Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. *J Exp Med*, *198*, 475-481.

Espel, E. (2005) The role of the AU-rich elements of mRNAs in controlling translation. *Semin Cell Dev Biol*, *16*, 59-67.

Esquela-Kerscher, A. & Slack, F.J. (2006) Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer, 6*, 259-269.

Fabian, M.R., Sonenberg, N. & Filipowicz, W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem, 79*, 351-379.

Filipowicz, W., Bhattacharyya, S.N. & Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet, 9*, 102-114.

Ford, L.P., Watson, J., Keene, J.D. & Wilusz, J. (1999) ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. *Genes Dev, 13*, 188-201.

Friedman, R.C., Farh, K.K., Burge, C.B. & Bartel, D.P. (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res, 19*, 92-105.

Galban, S., Martindale, J.L., Mazan-Mamczarz, K., Lopez de Silanes, I., Fan, J., Wang, W., Decker, J. & Gorospe, M. (2003) Influence of the RNA-binding protein HuR in pVHL-regulated p53 expression in renal carcinoma cells. *Mol Cell Biol, 23*, 7083-7095.

Gherzi, R., Lee, K.Y., Briata, P., Wegmuller, D., Moroni, C., Karin, M. & Chen, C.Y. (2004) A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. *Mol Cell, 14*, 571-583.

Griffiths-Jones, S., Saini, H.K., van Dongen, S. & Enright, A.J. (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res, 36*, D154-158.

Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P. & Bartel, D.P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell, 27*, 91-105.

Halees, A.S., El-Badrawi, R. & Khabar, K.S. (2008) ARED Organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. *Nucleic Acids Res, 36*, D137-140.

Hammell, M., Long, D., Zhang, L., Lee, A., Carmack, C.S., Han, M., Ding, Y. & Ambros, V. (2008) mirWIP: microRNA target prediction based on microRNA-containing ribonucleoprotein-enriched transcripts. *Nat Methods, 5*, 813-819.

Hsu, P.W., Huang, H.D., Hsu, S.D., Lin, L.Z., Tsou, A.P., Tseng, C.P., Stadler, P.F., Washietl, S. & Hofacker, I.L. (2006) miRNAMap: genomic maps of microRNA genes and their target genes in mammalian genomes. *Nucleic Acids Res, 34*, D135-139.

Hsu, S.D., Chu, C.H., Tsou, A.P., Chen, S.J., Chen, H.C., Hsu, P.W., Wong, Y.H., Chen, Y.H., Chen, G.H. & Huang, H.D. (2008) miRNAMap 2.0: genomic maps of microRNAs in metazoan genomes. *Nucleic Acids Res, 36*, D165-169.

Hubbard, T.J., Aken, B.L., Beal, K., Ballester, B., Caccamo, M., Chen, Y., Clarke, L., Coates, G., Cunningham, F., Cutts, T. *et al.* (2007) Ensembl 2007. *Nucleic Acids Res, 35*, D610-617.

Jain, R.G., Andrews, L.G., McGowan, K.M., Gao, F., Keene, J.D. & Pekala, P.P. (1995) Hel-N1, an RNA-binding protein, is a ligand for an A + U rich region of the GLUT1 3' UTR. *Nucleic Acids Symp Ser*, 209-211.

Jing, Q., Huang, S., Guth, S., Zarubin, T., Motoyama, A., Chen, J., Di Padova, F., Lin, S.C., Gram, H. & Han, J. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell*, *120*, 623-634.

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C. & Marks, D.S. (2004) Human microRNA targets. *PLoS Biol*, *2*, e363.

John, B., Sander, C. & Marks, D.S. (2006) Prediction of human microRNA targets. *Methods Mol Biol*, *342*, 101-113.

Karsch-Mizrachi, I. & Ouellette, B.F. (2001) The GenBank sequence database. *Methods Biochem Anal*, *43*, 45-63.

Katsanou, V., Papadaki, O., Milatos, S., Blackshear, P.J., Anderson, P., Kollias, G. & Kontoyiannis, D.L. (2005) HuR as a negative posttranscriptional modulator in inflammation. *Mol Cell*, *19*, 777-789.

Khabar, K.S. (2005) The AU-rich transcriptome: more than interferons and cytokines, and its role in disease. *J Interferon Cytokine Res*, *25*, 1-10.

Khabar, K.S., Bakheet, T. & Williams, B.R. (2005) AU-rich transient response transcripts in the human genome: expressed sequence tag clustering and gene discovery approach. *Genomics*, *85*, 165-175.

Kloosterman, W.P. & Plasterk, R.H. (2006) The diverse functions of microRNAs in animal development and disease. *Dev Cell*, *11*, 441-450.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M. *et al.* (2005) Combinatorial microRNA target predictions. *Nat Genet*, *37*, 495-500.

Lal, A., Mazan-Mamczarz, K., Kawai, T., Yang, X., Martindale, J.L. & Gorospe, M. (2004) Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. *Embo J*, *23*, 3092-3102.

Lall, S., Grun, D., Krek, A., Chen, K., Wang, Y.L., Dewey, C.N., Sood, P., Colombo, T., Bray, N., Macmenamin, P. *et al.* (2006) A genome-wide map of conserved microRNA targets in C. elegans. *Curr Biol*, *16*, 460-471.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P. & Burge, C.B. (2003) Prediction of mammalian microRNA targets. *Cell*, *115*, 787-798.

Lewis, B.P., Burge, C.B. & Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, *120*, 15-20.

Ma, F., Liu, X., Li, D., Wang, P., Li, N., Lu, L. & Cao, X. (2010) MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. *J Immunol*, *184*, 6053-6059.

Maragkakis, M., Alexiou, P., Papadopoulos, G.L., Reczko, M., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Simossis, V.A. *et al.* (2009) Accurate microRNA target prediction correlates with protein repression levels. *BMC Bioinformatics*, *10*, 295.

Mendes, N.D., Freitas, A.T. & Sagot, M.F. (2009) Current tools for the identification of miRNA genes and their targets. *Nucleic Acids Res*, *37*, 2419-2433.

Mobarak, C.D., Anderson, K.D., Morin, M., Beckel-Mitchener, A., Rogers, S.L., Furneaux, H., King, P. & Perrone-Bizzozero, N.I. (2000) The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. *Mol Biol Cell*, *11*, 3191-3203.

Mukherjee, D., Gao, M., O'Connor, J.P., Raijmakers, R., Pruijn, G., Lutz, C.S. & Wilusz, J. (2002) The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *Embo J*, *21*, 165-174.

Nam, S., Kim, B., Shin, S. & Lee, S. (2008) miRGator: an integrated system for functional annotation of microRNAs. *Nucleic Acids Res*, *36*, D159-164.

Newman, M.A. & Hammond, S.M. (2010) Emerging paradigms of regulated microRNA processing. *Genes Dev*, *24*, 1086-1092.

Papadopoulos, G.L., Reczko, M., Simossis, V.A., Sethupathy, P. & Hatzigeorgiou, A.G. (2009) The database of experimentally supported targets: a functional update of TarBase. *Nucleic Acids Res*, *37*, D155-158.

Peng, S.S., Chen, C.Y., Xu, N. & Shyu, A.B. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *Embo J*, *17*, 3461-3470.

Piecyk, M., Wax, S., Beck, A.R., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M. *et al.* (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. *Embo J*, *19*, 4154-4163.

Pruitt, K.D., Tatusova, T. & Maglott, D.R. (2007) NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res*, *35*, D61-65.

Raineri, I., Wegmueller, D., Gross, B., Certa, U. & Moroni, C. (2004) Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference. *Nucleic Acids Res*, *32*, 1279-1288.

Rehmsmeier, M., Steffen, P., Hochsmann, M. & Giegerich, R. (2004) Fast and effective prediction of microRNA/target duplexes. *RNA*, *10*, 1507-1517.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. & Bartel, D.P. (2002) Prediction of plant microRNA targets. *Cell*, *110*, 513-520.

Rodriguez, A., Vigorito, E., Clare, S., Warren, M.V., Couttet, P., Soond, D.R., van Dongen, S., Grocock, R.J., Das, P.P., Miska, E.A. *et al.* (2007) Requirement of bic/microRNA-155 for normal immune function. *Science*, *316*, 608-611.

Sarkar, B., Xi, Q., He, C. & Schneider, R.J. (2003) Selective degradation of AU-rich mRNAs promoted by the p37 AUF1 protein isoform. *Mol Cell Biol*, *23*, 6685-6693.

Sempere, L.F., Sokol, N.S., Dubrovsky, E.B., Berger, E.M. & Ambros, V. (2003) Temporal regulation of microRNA expression in Drosophila melanogaster mediated by hormonal signals and broad-Complex gene activity. *Dev Biol*, *259*, 9-18.

Sethupathy, P., Corda, B. & Hatzigeorgiou, A.G. (2006) TarBase: A comprehensive database of experimentally supported animal microRNA targets. *RNA*, *12*, 192-197.

Siomi, H. & Siomi, M.C. (2010) Posttranscriptional regulation of microRNA biogenesis in animals. *Mol Cell*, *38*, 323-332.

Stark, A., Brennecke, J., Russell, R.B. & Cohen, S.M. (2003) Identification of Drosophila microRNA targets. *PLoS Biol*, *1*, E60.

Steitz, J.A. & Vasudevan, S. (2009) miRNPs: versatile regulators of gene expression in vertebrate cells. *Biochem Soc Trans*, *37*, 931-935.

Stoecklin, G., Colombi, M., Raineri, I., Leuenberger, S., Mallaun, M., Schmidlin, M., Gross, B., Lu, M., Kitamura, T. & Moroni, C. (2002) Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. *Embo J*, *21*, 4709-4718.

Thadani, R. & Tammi, M.T. (2006) MicroTar: predicting microRNA targets from RNA duplexes. *BMC Bioinformatics*, *7 Suppl 5*, S20.

Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A.D., Filipowicz, W., Ramos, A., Gherzi, R. & Rosenfeld, M.G. (2009) The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature*, *459*, 1010-1014.

von Roretz, C. & Gallouzi, I.E. (2008) Decoding ARE-mediated decay: is microRNA part of the equation? *J Cell Biol*, *181*, 189-194.

Wilusz, C.J., Wormington, M. & Peltz, S.W. (2001) The cap-to-tail guide to mRNA turnover. *Nat Rev Mol Cell Biol*, *2*, 237-246.

Wilusz, C.J. & Wilusz, J. (2004) Bringing the role of mRNA decay in the control of gene expression into focus. *Trends Genet*, *20*, 491-497.

Wulczyn, F.G., Smirnova, L., Rybak, A., Brandt, C., Kwidzinski, E., Ninnemann, O., Strehle, M., Seiler, A., Schumacher, S. & Nitsch, R. (2007) Post-transcriptional regulation of the let-7 microRNA during neural cell specification. *Faseb J*, *21*, 415-426.

Xu, N., Chen, C.Y. & Shyu, A.B. (2001) Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover. *Mol Cell Biol*, *21*, 6960-6971.

Zhang, R. & Su, B. (2009) Small but influential: the role of microRNAs on gene regulatory network and 3'UTR evolution. *J Genet Genomics*, *36*, 1-6.

Zubiaga, A.M., Belasco, J.G. & Greenberg, M.E. (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol Cell Biol*, *15*, 2219-2230.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, *31*, 3406-3415.