# Analysis of microRNA functions

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## Chapter outline



**Methods in Cell Biology, ISSN 0091-679X, https://doi.org/10.1016/bs.mcb.2018.10.005**

## Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that repress the translation and reduce the stability of target mRNAs in animal cells. Post-transcriptional regulation mediated by miRNAs is a highly conserved mechanism utilized by organisms throughout phylogeny to fine tune gene expression. We document the approaches used to study the function of a single miRNA and miRNA regulation of biological pathways in the sea urchin embryo. The protocols that are described include selection of miRNA inhibitors, test of miRNA direct targets, and the use of target protector morpholinos to evaluate the impact of miRNA inhibition on its targets. Using the described techniques and strategies, the sea urchin researcher will be able to validate a miRNA's direct targets and evaluate how inhibition of the miRNA affects developmental processes. These results will contribute to our understanding of the regulatory roles of miRNAs in development.

## 1 Introduction

MicroRNAs (miRNAs) are short non-coding RNAs that typically bind to the 3′ untranslated region (3′UTR) of their target mRNAs to repress translation (Bartel, 2009). This mechanism of post-transcriptional regulation has been shown to be important in diverse organisms, from protostomes to deuterostomes (Campo-Paysaa, Sémon, Cameron, Peterson, & Schubert, 2011; Rödel, Gilles, & Averof, 2013) to non-bilaterians (Moran, Praher, Fredman, & Technau, 2013). Since the sea urchin has a well-annotated genome, and the majority of their miRNA families consist of a single member, it is an excellent model to use to study miRNA function (Kadri, Hinman, & Benos, 2011; Song et al., 2012; Wheeler et al., 2009). By using the techniques and approaches described below, progress has been made in understanding the function of miRNAs in development (Stepicheva, Nigam, Siddam, Peng, & Song, 2015; Stepicheva & Song, 2015).

## 2 Approaches

### 2.1 Identify single miRNA functions

#### *2.1.1 Use bioinformatics to find gene targets of a single miRNA*

Typically, a mature miRNA is 22–25 nt long, but extensive complementarity between the miRNA and target mRNA is rare in animal cells. Thus, bioinformatic identification of miRNA targets is challenging. The complementarity between the target mRNA and the 7–8 nucleotides (nt) at the 5′ end of the mature miRNA, known as the seed sequence, is the most likely predictor of miRNA:target interaction (Bartel, 2009). If complementarity exists between the target mRNA and the miRNA 3′ region (positions 13–16 of the mature miRNA), this can compensate for non-perfect pairing at the seed region (Riffo-Campos, Riquelme, & Brebi-Mieville, 2016). Some of these target prediction programs are TargetScan (Agarwal, Bell, Nam, & Bartel, 2015), miRanda (Enright et al., 2003) and DIANA tools (Paraskevopoulou et al., 2013; Reczko, Maragkakis, Alexiou, Grosse, & Hatzigeorgiou, 2012). These prediction

algorithms take into account complementarity of the seed sequence to the target mRNA, the thermodynamic stability of the interaction, as well as the conservation of the interaction in other species (Riffo-Campos et al., 2016). Between 15% and 80% of the miRNA:mRNA interactions do not follow the canonical rules of seed sequence complementarity, making the predictive ability of these algorithms low (Bartel, 2009; Seok, Ham, Jang, & Chi, 2016). This, along with the fact that the prediction programs are based on mammalian, fly and nematode databases, makes these difficult to use for the sea urchin researcher. Thus, currently searching sequence complementary to miRNA's seed sequence within a target mRNA remains the best approach in predicting sea urchin miRNA targets. Importantly, the direct regulation of miRNAs against a target mRNA will need to be validated.

#### 2.1.2 Biotinylated miRNA pull-down

A systems biology approach to identify gene targets by a single miRNA is the use of a biotinylated pull-down assay. This has been performed in cell cultures (Tan & Lieberman, 2016), but has the potential to be utilized in sea urchin embryos. Biotin is conjugated to the miRNA of interest and microinjected into the zygotes. The biotinylated miRNA is incorporated into the RNA induced silencing complex (RISC) and binds to its target mRNAs in the embryo. Target mRNAs within the RISC with biotinylated miRNA are isolated by binding to streptavidin coated beads, followed by RNA sequencing. In order to ensure specificity, it is important to include an appropriate control, such as embryos that have been injected with a miRNA with scrambled sequence or a miRNA that is not present in the sea urchin (Awan et al., 2018; Tan & Lieberman, 2016).

### 2.2 Identify miRNAs that regulate a specific pathway

#### 2.2.1 Use bioinformatics to find miRNAs targets within a pathway

A signaling pathway is chosen by the researcher and all the genes of interest in that pathway are identified. The set of genes will be used to bioinformatically identify miRNA target sites within the 3′UTR by searching for perfect complementary sequence to the particular miRNA seed sequence. The list of known *S. purpuratus* miRNAs and their seeds is available (Song et al., 2012). Most miRNA target sites are located in the 3′UTR but could also be located throughout the entire transcript (Fang & Rajewsky, 2011). Any putative targets identified using these *in silico* techniques must be validated using reporter assays.

## 3 Techniques used in studying miRNA perturbations

### 3.1 Inhibitors of miRNA

#### 3.1.1 Sequence selection

Most of the specificity of miRNA:mRNA target binding is due to Watson-Crick base pairing, specifically between nucleotides 2–8 of the miRNA seed sequence (Bartel, 2009). Typically, the reverse complement of the mature miRNA, 21–25 nt long, is

used to generate the inhibitor; however, some researchers use 8-mers that will bind specifically to the seed sequence (Stenvang, Petri, Lindow, Obad, & Kauppinen, 2012). This allows the inhibition of families of miRNAs that contain the same seed sequence. With all miRNA inhibitors, it is important to include controls to ensure that it is the inhibition of the specific miRNA that is causing the phenotype of interest. The two main controls that are utilized are a scramble control or an inhibitor of a miRNA not found in the sea urchin.

### 3.1.2 Selection of nucleotide modifications

Modifications to the nucleotides are made to prevent nuclease activity and increase binding affinity. Increasing binding affinity increases potency of inhibition, allowing a lower dose to be used (Lennox & Behlke, 2010). The common modifications are to the ribose moiety and to the phosphodiester linkage.

**a.** *Morpholino anti-sense oligonucleotides (MASOs)* (GeneTools, LLC, Philomath, OR)

In these modified oligonucleotides, a six-membered morpholine ring replaces the five-membered ribose ring. This alteration prevents degradation by nucleases and slightly increases binding affinity (Stenvang et al., 2012).

**b.** *2′ Fluoro, 2′-O-Methyl, 2′O-Methoxyethyl ribose modifications*

These are other modifications to the ribose ring of the nucleotide that result in resistance to nuclease activity, and slight increase in binding affinity. These modifications, although highly efficient, are more cytotoxic at higher concentrations than locked nucleic acids (see below) (Davis, Lollo, Freier, & Esau, 2006; Meister, Landthaler, Dorsett, & Tuschl, 2004).

**c.** *Locked nucleic acids (LNAs)* (Exiqon/Qiagen, Germantown, MD)

This is the gold standard miRNA inhibitor used in the field. These modified oligonucleotides alter the ribose by introducing a 2′-O, 4′C-methylene bridge, which forces the furanose ring to adopt a C3′-endo conformation, resulting in increased binding affinity to its complementary sequence, as well as increased resistance to nucleases (Vester & Wengel, 2004). This increased binding affinity allows for more potent inhibition of miRNA activity, allowing a lower dose of LNA to be used (Lennox & Behlke, 2010). A more recent development has been the replacement of the phosphodiester backbone with a phosphorothioate (PS) linkage, which increases resistance to nuclease activity; however, this modification does decrease the binding affinity, so stability must be weighed against potency. This is available from Exiqon/Qiagen known as the Power Inhibitor™. LNA™ oligonucleotides contain one or more LNA™ nucleosides and can be custom or predesigned, depending on application and availability (Naguibneva et al., 2006; Stepicheva & Song, 2015). It is most cost effective if one can identify a miRNA inhibitor available from Exiqon/Qiagen to avoid a custom design fee. Search for homologous miRNA from other species and make sure the sequence of the sea urchin miRNA is the same as the one from another species.

### 3.1.3 Introduction of miRNA inhibitors into sea urchin embryos

Microinjection is utilized to introduce miRNA inhibitors into sea urchin zygotes as described previously (Cheers & Ettensohn, 2004; Stepicheva & Song, 2014, 2015).

**a.** Reconstitute the lyophilized inhibitor to stock solutions of 100–200 μM, according to the manufacturer's instructions. Empirically determine the concentration of inhibitor to be used, by testing a range of inhibitor concentrations (i.e. 3–30 μM). The goal is to establish dose-response phenotypes. The inhibitor concentration that results in 30–50% normal embryos may be the appropriate concentration. It is important that the corresponding negative control result in at least 80% normal embryos. Of note is that the recent batches of Exiqon/Qiagen negative controls (A and B) are more toxic to the embryos than the miRNA inhibitor itself. Thus, it is recommended that you use different negative controls, such as a miRNA inhibitor against another species' miRNA.

**b.** Use RNAse-free injection solution consisting of 20% glycerol, a final concentration of 2 mg/mL Texas Red (Thermo Fisher Scientific, catalog # D1863), and miRNA inhibitor or negative control of the same concentration. Texas Red is used to mark injected embryos and can be replaced with a different fluorescent dextran. It is important to centrifuge the solution for at least 15 min at 21,000 × *g* prior to injections to prevent clogging of the needle. Keep the injection solution on ice and use RNAse-free injection needles. RNAse-free needles are made by incubating the injection needles in a sterile 50 mL conical tube with filtered methanol:HCl (9:1 by volume) for no more than 10 h (this prevents the filament of the injection needles from melting). Rinse the injection needles with filtered methanol twice. The injection needles are then drained as much as possible and dried overnight in a vacuum or a lyophilizer. Keeping an RNAse free environment is critical and extra precautions should be taken by wearing gloves and using forceps when holding the needle to put the injection solution into it.

**c.** Use a miRNA mimic (Exiqon/Qiagen, Germantown, MD) to test the specificity of the miRNA inhibitor. The miRNA mimic correspond to a specific miRNA's sequence and are co-injected with the inhibitor at a 1:1 molar ratio into sea urchin zygotes. It is recommended that the miRNA mimic you use has the same modification as your miRNA inhibitor. For example, if you used a miRNA inhibitor with locked nucleic acid (LNA) chemistry, you would use a miRNA mimic with the same chemistry. The amount of mimic used may need to be determined empirically. Alternatively, synthetic double stranded RNA Dicer substrates can be designed from Integrated DNA Technologies (Idtdna.com; IDT, Coralville, IA) (Kim et al., 2005; Rose et al., 2005). In our experience, miRNA mimic from Exiqon/Qiagen is more effective in rescuing miRNA inhibitor induced phenotypes than dsRNA Dicer substrate (Stepicheva & Song, 2015).

#### 3.1.4 Test efficiency of miRNA inhibition

Perform fluorescent *in situ* hybridization (FISH) against the perturbed miRNA to ensure efficient decrease of the miRNA. For miRNA FISH, use a LNA detector probe (miRCURY LNA miRNA Detection Probe, Exiqon/Qiagen) for your corresponding miRNA. The LNA miRNA probe is required to ensure high binding affinity and specificity, because of the short length of the miRNA.

**a.** The FISH procedures are based on previously published protocol with modifications (Sethi, Angerer, & Angerer, 2014). Perform pre-hybridization in hybridization buffer containing 70% formamide for 3 h at 50 °C without equilibration steps.
**b.** Incubate embryos with 0.1 ng/μL miRNA or scrambled miRNA negative control probes for 4 days at 50–60 °C. The optimal temperature would need to be empirically determined.
**c.** After hybridization, treat embryos with MOPS buffer washes, blocking, and incubation with the anti-digoxigenin-POD Fab fragment (Roche Diagnostics, Indianapolis, IN). Use TSA-Plus Fluorescein system (PerkinElmer, Waltham, MA) to detect the fluorescence signal.
**d.** Acquire images with a confocal microscope. Since the inhibition is occurring due to the inability of the miRNA to bind to its target, no actual reduction in the amount of miRNA would be detected, so a difference will not be observed in PCR or sequencing-based methods. The expectation is to observe a visible and significant decrease of the level of miRNA in the presence of miRNA inhibitor compared to the control embryos.

### 3.2 Validation of microRNA direct regulation

#### 3.2.1 Cloning of luciferase reporter constructs

For generating luciferase reporter constructs, the 3′UTR is cloned from cDNA or synthesized as a gBlock DNA fragment (Integrated DNA Technologies, Inc., Coralville, IA).

#### 3.2.2 Luciferase vector construction

Generate *Renilla* luciferase vector (*rLuc*) by PCR amplification of the rLuc coding sequence from the psiCHECK dual-luciferase vector (Promega, Madison, WI). This *rLuc* PCR fragment is cloned into a generic vector containing either Sp6 or T7 RNA polymerase binding sites for *in vitro* transcription. For example, previously the *rLuc* was subcloned into the pSp6 plasmid (Gustafson & Wessel, 2010) to generate the SupLuc (Stepicheva et al., 2015; Stepicheva & Song, 2015).

#### 3.2.3 Site-directed mutagenesis

Upon successful cloning of a 3′UTR of the potential miRNA target gene downstream of *rLuc*, perform site directed mutagenesis to specifically mutate the bioinformatically predicted miRNA binding sites. Mutation of the third and fifth base pair on

the seed sequence of the target mRNA is sufficient to abolish miRNA to mRNA target recognition (Staton & Giraldez, 2011). We use the QuikChange Lightning kit by Agilent (catalog #210515) and design mutagenesis primer sets using the Agilent website (https://www.genomics.agilent.com/primerDesignProgram.jsp). If the 3′UTR of the gene of interest has more than three miRNA target sites, one can use the QuikChange Multi Site-directed Mutagenesis kit (Agilent, catalog # 200514) to efficiently mutate multiple sites in one reaction. Transform cells into chemically-competent XL-10 Gold cells (Agilent, catalog #200315) in 14 mL polypropylene round-bottom tube (BD Falcon catalog #14-959-10B), as specified by the manufacturer. Validate all mutated seed sites by DNA sequencing.

### 3.2.4 Dual luciferase assay

This assay tests the direct regulation of miRNA at a specific mutated seed site.

**a.** Linearize *rLuc* containing desired 3′UTR with a unique restriction enzyme that cuts 3′ to the 3′UTR insert and gel purify. Using mMessage machine kit (ThermoFisher, catalog #AM1340), *in vitro* transcribe with Sp6 RNA polymerase (or T7 RNA polymerase, depending on the *rLuc* backbone).

**b.** As a control for the dual luciferase assay, use Firefly luciferase (*FF*) with the SV40 polyadenylation site. This is subcloned from psiCheck into pSP6 plasmid flanked by *Xenopus β-globin* UTRs (Gustafson & Wessel, 2010).

**c.** Linearize *FF* with a unique enzyme that cuts after the polyadenylation site. *In vitro* transcribe with Sp6 RNA polymerase or other RNA polymerase, depending on the cloning vector (Stepicheva et al., 2015; Stepicheva & Song, 2015).

**d.** Coinject 50–150 ng of *FF* and 100–250 ng of *rLuc* with 3′UTR with wild type seeds or *rLuc* with 3′UTR with mutated seeds. The amount of *FF* and *rLuc* you inject must be the same between the *rLuc* with wild type and mutated 3′UTRs.

**e.** Perform dual luciferase quantitation using the Promega™ Dual-Luciferase™ Reporter (DLR™) Assay Systems with the Promega™ GloMax™ 20/20 Luminometry System (Promega, Madison, WI). Collect 20–50 embryos at the desired developmental stage in 22 µL 1 × lysis buffer and vortex in maximum speed until the cells have lysed open (∼20 min of vortexing). The number of embryos collected must be the same between the embryos injected with *rLuc* constructs with the wild type and mutated seeds. Embryonic lysates can be stored at −80 °C or processed immediately.

**f.** Prior to luciferase readings, add 100 µL of the Luciferase Activating Reagent II (LAR-II) to each well of the MicroAmp™ Endura Plate™ Optical 96-well clear plate (ThermoFisher Scientific, catalog #4483348). Add 20 µL of the embryonic lysates to the LAR-II (thaw to room temperature) immediately prior to luciferase readings.

**g.** Obtain the FF luciferase reading first. Then, add 100 µL of the Stop and Glow solution to quench the FF luciferase signal and obtain the rLuc reading next.

**h.** Normalize the rLuc reading to the FF luciferase reading. Once the normalized readings are obtained, they may be compared between the wild type and the mutated 3′UTR constructs. The expectation is that the *rLuc* with the 3′UTR with mutated miRNA seeds would abrogate miRNA binding and relieve translational repression. Thus, if the miRNA predicted binding site is a *bona fide* regulatory miRNA target site, one would expect to observe approximately a 1.2- to 3-fold increase in normalized luciferase read out in the embryos injected with the *rLuc* with the 3′UTR with mutated miRNA seeds compared to the embryos injected with the *rLuc* with the 3′UTR with wild type miRNA seeds. It is important to perform at least three biological replicates for statistical analysis, and each replicate should contain at least 20–50 embryos.

## 3.3 Assaying the impact of miRNA inhibition *in vivo*

### 3.3.1 Target protector morpholinos (TP MASOs)

Once the miRNA regulation of a target gene is identified using dual luciferase reporter assays, one can assess the impact of blocking specific miRNA regulation of a target gene in the embryo. To disrupt the binding of a miRNA to a specific site on a target mRNA, a miRNA TP MASO is designed to be complementary to the validated miRNA seed and specific flanking sequences (Fig. 1). This is a powerful

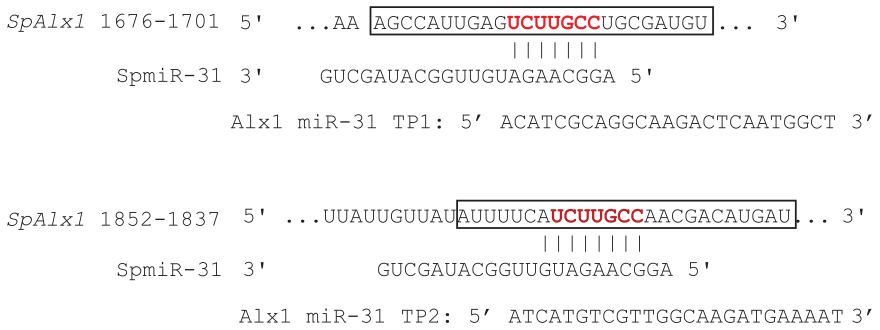


**FIG. 1**

Design of miRNA target protector. In the sea urchin, one can bioinformatically identify a miRNA's targets by searching for perfect complementary sequence to the particular miRNA within the target genes. For example, the exact match for the reverse complement of the miR-31 seed is 5′ UCUUGCC 3′. After validation of miRNA regulation of a specific gene, one can design a miRNA TP MASO target protector to assess the impact of miRNA suppression of that gene. To design the miR-31 TP against *SpAlx1*, we designed the 25 base pair TP MASO sequence against the miR-31 seed and the flanking sequences (shown in the boxed area with seed sequence in bold) (Stepicheva & Song, 2015). The sequence complementary to the miR-31 seed is in red. Notice that *Sp*miR-31 has two target sites within the *SpAlx1* gene. Each TP is unique to the specific region of *SpAlx1*.

approach to address the impact of a specific miRNA's regulation of a specific target gene. Each miRNA TP MASO is uniquely designed to bind to one specific miRNA binding site within the target gene. By binding to the mRNA, the miRNA TP MASO prevents the endogenous miRNA/RISC complex from binding to the target mRNA to mediate translational silencing (Staton & Giraldez, 2011). It is essential to include a control MASO This can be scrambled or the standard control from GeneTools that is not found in the sea urchin genome to ensure that any phenotypes observed are specifically due to inhibition of miRNA binding.

**a.** To determine the amount of miRNA TP MASO to inject, one can estimate the number of transcripts and miRNA binding sites of the target mRNA. However, this must be validated by testing a range of miRNA TP MASO concentrations (i.e. 3 to 300 μM). The injected negative control MASO should contain the same molar concentration as the corresponding miRNA TP MASO.

**b.** The injection solution consists of 20% glycerol, a final concentration of 2 mg/mL Texas Red (Thermo Fisher Scientific, catalog # D1863), and miRNA TP MASO or negative control at the appropriate concentration. Texas Red is used to mark injected embryos and can be replaced with a different fluorescent dextran. It is important to centrifuge the solution for at least 15 min at 21,000 × *g* prior to injections to prevent clogging of the needle.

**c.** Typically, one would inject 100–200 zygotes per bioreplicate. At least three bioreplicates would be necessary for these assays: RT-qPCR, whole mount *in situ* hybridization, immunolabeling, western blotting and phenotyping experiments to define the function of the miRNA or miRNA regulation of a developmental pathway.

## 3.4 Evaluation of translational inhibition mediated by miRNAs of target genes

### *3.4.1 Western blotting*

If an antibody against the target protein tested is available, conduct western blotting (Mahmood & Yang, 2012) to determine the level of target protein in the presence or absence of a miRNA inhibitor or miRNA TP MASOs (Stepicheva et al., 2015). Of note is that a single miRNA targets many genes, so the observed effect may be direct or indirect (Sood, Krek, Zavolan, Macino, & Rajewsky, 2006). On the other hand, a miRNA TP MASO is designed to block a single miRNA's binding to a specific site within the target gene. The western blot using an antibody against the miRNA target protein will provide the estimated level of translational regulation mediated by miRNAs. Determine empirically the number of embryos necessary for antibody detection first with 50–500 uninjected embryos. Then inject this minimum number of embryos with the miRNA inhibitor or miRNA TP MASO with appropriate controls and run their lysates on the sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel.

Quantify signals of protein bands in control MASO and miRNA TP MASO-injected embryos by using image analysis software such as ImageJ (Schneider, Rasband, & Eliceiri, 2012). At least three biological replicates should be performed for statistical analysis.

### 3.4.2 Quantification and spatio-temporal analysis by immunolabeling

The level of miRNA target proteins can also be assessed semi-quantitatively with immunolabeling. Control TP MASO and miRNA TP MASO-injected embryos are immunolabeled with the specific antibody. An equal number of scanning confocal *Z*-stack images at specific developmental time points are collected. The maximum projections of these *Z*-stacks of each embryo are collected and the amount of fluorescent pixels are quantified with ImageJ (Schneider et al., 2012) or Metamorph (Molecular Devices, LLC, San Jose, CA). The differential protein levels between the control and miRNA TP MASO-injected embryos are expected to be relatively small, approximately 1.5- to 2-fold change. We have found that blocking miRNA suppression of target gene translation is sufficient to impact early development (Sampilo et al., 2018; Stepicheva et al., 2015; Stepicheva & Song, 2015).

## 4 Conclusion

miRNAs play an important role in many cellular processes and have been found to be vital in regulating developmental processes (Baek et al., 2008; Campo-Paysaa et al., 2011; Song et al., 2012; Yang et al., 2016). Using the described techniques and strategies, the sea urchin researcher will be able to study regulatory roles of miRNAs during development.

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