To the rescue: Testing the functional complementation of microRNA family members in the nematode *Caenorhabditis elegans*

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Abstract

MicroRNAs are grouped into families based on shared sequence homology of the first 2-7 nucleotides. This region of the miRNA is called the “miRNA seed” and is considered to be essential for miRNA function. Early studies suggest that the seed dictates miRNA target recognition. This implies that miRNAs in the same family can have the same targets. In this study we test if *lin-4* family members, *lin-4* and *miR-237*, are functionally equivalent in living animals. We synthetically express miR-237 with the lin-4 promoter to rescue loss-of-function *lin-4* phenotypes in *C. elegans*. Results show that even though both microRNAs share the same functional region (seed), they are functionally distinct. This finding challenges the current theory that microRNAs with the same seed are functionally equivalent.

1. Introduction

1.1 Review of the Literature

MicroRNAs (miRNAs) are a huge class of small RNAs of approximately 23 nucleotides (nt) in length that are found in plants and animals (Bartel, 2009). MiRNAs do not encode for proteins and instead negatively regulate gene expression in their RNA form. Animal miRNAs function by binding semi-complementarily to the 3' untranslated region (3'UTR) of its target messenger RNA (mRNA) (**Fig. 1**). This interaction inhibits the mRNA's ability to be translated into protein and/or leads to the degradation of the mRNA. Thousands of these tiny miRNAs have been discovered in animals, however only a fraction have been assigned biological functions.

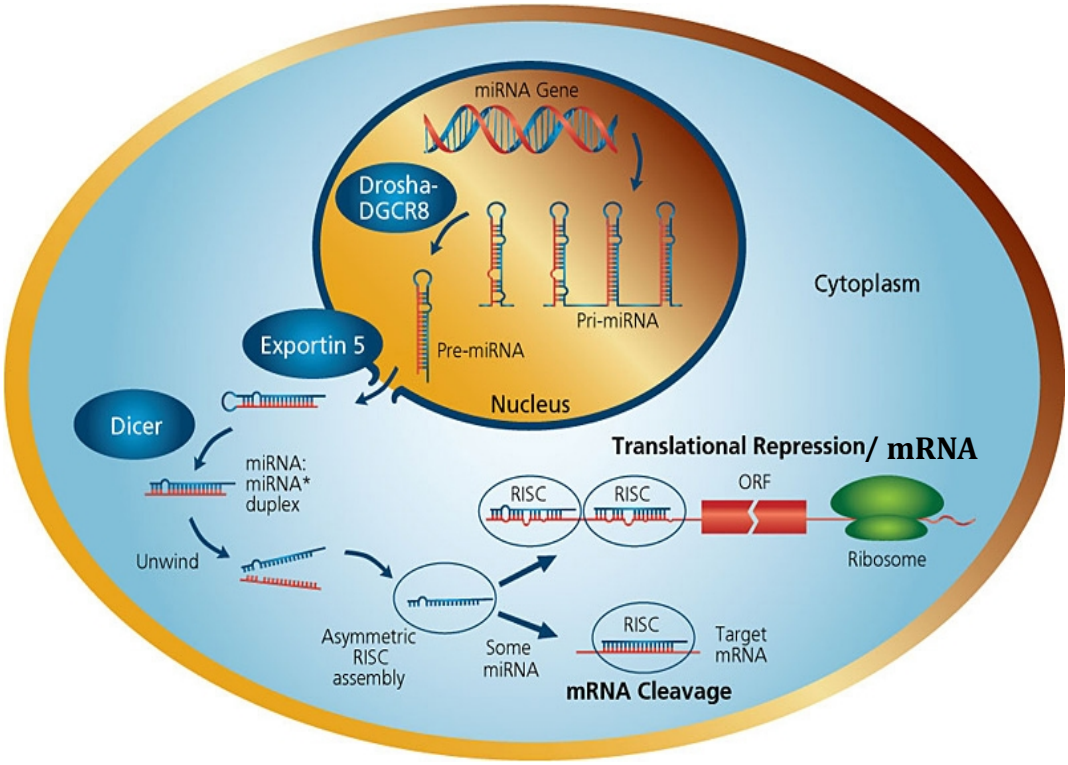

Figure 1. MiRNA biogenesis. MiRNA genes are transcribed in the nucleus. The resulting pri-miRNA transcript is processed by the Drosha-DGCR8 complex to generate a ~70-nt pre-miRNA hairpin precursor. The pre-miRNA is exported into the cytoplasm and cleaved by Dicer into a ~23 nt miRNA duplex. One side of the miRNA duplex is loaded into the RNA-induced silencing complex (RISC). The mature miRNA binds to mRNA targets within the 3'UTR region with incomplete complementarity and leads to a block in protein translation and/or mRNA degradation.

The characterized miRNAs play essential roles in cellular processes related to growth, differentiation, apoptosis, metabolism, and the immune response (Kato and Slack, 2008). MiRNA misexpression has been closely associated with human cancers and recent work reveals that miRNAs can directly influence tumor growth and metastasis (Esquela-Kerscher & Slack, 2006). Therefore, careful analysis of the biological activities of conserved miRNAs especially using *in vivo* animal models will be important when utilizing miRNAs' unique properties in the future as therapeutic tools to treat diseases such as cancer.

MiRNAs are organized into families based on their sequence homology, which is shared primarily in the 5' portion of the mature miRNA sequence. This region includes the "miRNA seed", defined as the first 2-8 nts of the mature miRNA sequence (Bartel, 2009). The miRNA seed has been shown using *in vitro* tissue culture assays to be important for miRNA-target recognition and controlling down regulation of target expression (Doench and Sharp, 2004; Kloosterman et al., 2004; Brennecke et al., 2005; Lai et al., 2005). Therefore, the miRNA seed is considered the essential element of a miRNA to dictate biological function. However, the fact that the *let-7* miRNA is 100% conserved from worms to humans implies that the entire length of the miRNA is important for biological activity (Pasquinelli, et al 2000). This study will determine if a highly conserved group of miRNAs belonging to the *lin-4* family can functionally compensate for one another using the simple model *Caenorhabditis elegans* (*C. elegans*) and directly test the biological significance of the miRNA seed in the intact animal.

The lineage-defective-4 (*lin-4*) miRNA was the first miRNA ever discovered in 1993 by Victor Ambros's laboratory through genetic analysis in *C. elegans*. This miRNA was found to be an important regulator during development by dictating the timing of cell fate specification and cell cycle exit of certain lineages such as skin cells during early larval stages in *C. elegans* (Lee et al., 1993). This miRNA was characterized as a major player in the heterochronic pathway in the nematode that controls the correct timing of the larval transitions during the nematode life cycle. In *C. elegans, lin-4* has only one close homologue, called *miR-237,* whose biological function remains unknown (**Fig. 2A**).

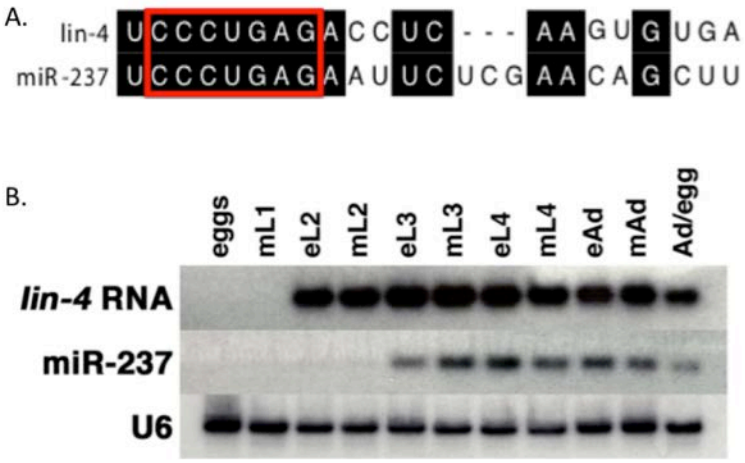

Figure 2. The *lin-4* miRNA family in *C. elegans*. A. Sequence alignment of *lin-4* and *mir-237*. The "miRNA seed" region is boxed in red. B. Northern blot analysis showing temporal expression of *lin-4* and miR-237 throughout the *C. elegans* life cycle (larval stages L1-L4 and adult). U6 is used as an RNA loading control. Abbreviations: e-early, m-mid-larval stage, Ad-adult. (Esquela-Kerscher et al, 2005).

In *C. elegans*, the *lin-4* family members *lin-4* and miR-237 are uniquely expressed both temporally and spatially during development. We believe this difference in expression indicates distinct biological roles for *lin-4* and miR-237 in nematodes. For example, by Northern blot analysis, *lin-4* expression is first noted after the mid-larval 1 stage (mL1) and continues to be expressed at high levels throughout development and into adulthood (Lee, et al. 1993; Esquela-Kerscher et al, 2005) (**Fig. 2B**). In contrast, miR-237 is shown by Northern blot analysis to be expressed at very low levels at larval stage 2 (L2), elevated at the larval stage 3 (L3), and expression continues into adulthood (Esquela-Kerscher et al, 2005). *lin-4* and miR-237 also exhibit unique expression patterns in different tissues and cell types throughout *C. elegans* development. This was noted when the miRNA promoter of either *lin-4* or miR-237 was fused to the reporter green fluorescent protein (GFP) in order to visually detect the time and places that these miRNAs are normally expressed in the nematode (Esquela-Kerscher et al, 2005). For instance, miR-237 is expressed in the gonad of the worm at early larval stages but *lin-4* is devoid of expression in this tissue at these time periods. The *lin-4* family members also showed distinct cellular expression patterns in the egg-laying structures (the vulva) of the worm using

miRNA promoter:GFP fusion studies. These results indicate that *lin-4* and miR-237 direct unique biological roles in tissues such as the gonad and vulva due to their different temporal and spatial expression patterns during *C. elegans* development.

The present study will test the dogma long held in the miRNA field - that closely related miRNA family members sharing a common "miRNA seed" also share identical biological activities. Although past studies appear to support this notion in cell culture experiments, the validity of the "miRNA seed" has never been tested using a functional assay in living animals. Focusing on the *lin-4* miRNA family in *C. elegans*, we asked if the *lin-4* homologue miR-237 could functionally rescue the loss-of-function *lin-4* phenotypes associated with nematode fertility.

1.2 Hypothesis

The hypothesis to be tested is that only the first few residues of the ~23 nucleotide long miRNA - designated the "microRNA seed" - is essential for biological activity and therefore miRNA families sharing a common seed sequence will be functionally equivalent. This study will challenge the hypothesis and ask if the *lin-4* miRNA family in *C. elegans*, *lin-4* and miR-237, are functionally distinct due to their unique temporal and spatial expression patterns (via promoter level control) or if they are really biological different despite possessing identical "miRNA seeds". We will address this by answering the following questions:

a. Do the loss-of-function phenotypes for *lin-4* and miR-237 in *C. elegans* resemble one another?

b. Can miR-237 functionally compensate for *lin-4* when miR-237 expression is driven under the control of the *lin-4* promoter and rescue the fertility defects observed in *lin-4(e912)* loss-of-function animals?

2. Methods

2.1 *C. elegans* brood size analysis

Brood size assays were conducted in order to measure the fertility of loss-of-function *C. elegans* strains *lin-4(e912)* or *mir-237(tm2238)* compared to wild type worms (N2). Larval stage 4 (L4) animals were placed on individual NGM plates seeded with OP50 bacteria. Parent worms were grown at 20˚C and adult staged worms were transferred to new plates every day for five days. The total number of progeny for each parent worm was counted.

2.2 Generation of the microRNA fusion expression construct using Gateway cloning

The *lin-4* promoter::*mir-237* hairpin fusion construct (depicted in **Fig. 6A**) was designed to include the *lin-4* promoter (Lee et al, 1993) upstream of the miR-237 hairpin precursor sequence followed the 3' UTR derived from the *unc-54* gene using the Multisite Gateway Cloning System (Invitrogen). The *lin-4* promoter, *mir-237* precursor and *unc-54* 3' UTR sequences were cloned into the appropriate 5'(pDONRP4P1R), middle (pDONR201) and 3' (pDONRP2RP3) Entry vectors, respectively, by a BP Clonase reaction (PCR fragment + Donor vector = Entry Clone). These Entry Clones were subsequently recombined with Destination vector (pDESTR4R3) via an LR Clonase reaction (3 Entry clones + Destination vector = Expression Clone) to create the final *lin-4* promoter:*mir-237* precursor:*unc-54* 3'UTR fusion expression construct shown in **Fig. 3**.

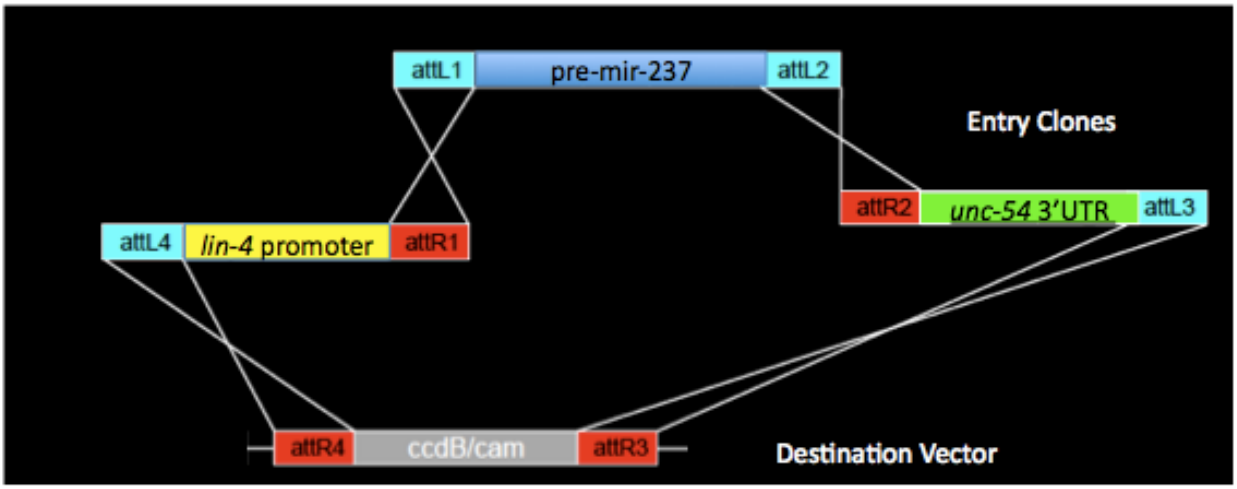

Figure 3. Gateway cloning was used to create the *lin-4* promoter:*mir-237* precursor:*unc-54* 3'UTR expression construct.

Gateway cloning utilizes site-specific attachment (att) recombination events based on the mechanisms used by lamda phage for DNA integration. This technique allows for the efficient cloning of three different PCR fragments (promoter, miRNA precursor, 3'UTR) by DNA recombination at unique att sites into the appropriate "Entry" and "Destination" vectors and occurs in the correct orientation without the need for time consuming and laborious restriction digests or ligase reactions. The BP and LR cloning reactions lasted only 1.5 hours at room temperature. The cloned vectors were then transformed into TOP10 bacterial cells and grown on appropriate LB plates with certain antibiotics (kanamycin for Entry clones and ampicillin for

Destination clones) to select for cells that contain the plasmid. Moreover, successful integration of all three PCR fragments into the Destination vector resulted in the elimination of the ccdB toxic “death” gene from the Destination clone. This selection allowed for the survival of bacteria transformed with the expression construct versus empty Destination vector. Plasmid DNA was isolated from bacterial cells using the QIAprep Spin miniprep kit (Qiagen). All of the experimental constructs were sequenced and verified.

2.2.1 The *lin-4* miRNA promoter:

The *lin-4* promoter consists of the regulatory elements shown by Victor Ambros group to be the minimal sequences needed to drive *lin-4* miRNA expression and rescue the *lin-4* deletion phenotypes in *C. elegans* (Lee et al, 1993). The 517 nucleotide (nt) fragment was amplified using the forward (AEK 35) and reverse (AEK36) primers by PCR from genomic wild type *C. elegans* DNA. The underlined sequences included in the PCR primers denote the recombination sequences required for insertion into the 5’ (pDONRP4P1R) Gateway Entry vector.

AEK 35 – forward
GGGGACAACTTTGTATAGAAAAGTTGGTCGACGAGACGCCGAGTCTCCC

AEK 36 – reverse
GGGGACTGCTTTTTTGTACAAACTTGCAGGCCGGAAGCATAAACTCATAAACC

2.2.2 The *mir-237* precursor:

The *mir-237* precursor consists of a 407 nt fragment that contains the 98 nt *mir-237* pre-miRNA hairpin sequence (shown below, mature *mir-237* sequence indicated in red) as well as ~150 base pairs of flanking sequence on either side of the hairpin to allow for proper recognition and processing by Drosha and Dicer to generate the biologically active mature miRNA.

***mir-237* hairpin precursor sequence**

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--------------     ca   cg      c   g  uu     a      c  a
              uucua  uug  uggucc uga aa  cucga cagcuu aa g
              |||||  |||  |||||| ||| ||  ||||| |||||| ||
              aagau  aac  accagg acu uu  gagcu gucgaa uu u
caaggguucacuag     --   aa      a   g  uu     -      c  g
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The fragment was amplified by PCR using the forward (AEK 37) and reverse (AEK 38) primers from genomic wild type *C. elegans* DNA. The underlined sequences included in the PCR primers denote the recombination sequences required for insertion into the middle (pDONR201) Gateway Entry vector.

AEK 37 - forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAAAAGGTCGTGCCAGCTTGTATCAC

AEK 38 – reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTCATTTTACGGCCGTCGACGATTATC

2.2.3 unc-53 3'UTR:

The *unc-54* 3'UTR fragment was amplified by PCR from the plasmid pPD95.75 (gift from Dr. Andrew Fire, Stanford University) using the forward (AEK 5) and reverse (AEK 8) primers. This 3'UTR sequence does not contain any regulatory elements or miRNA binding sites to affect gene expression of the fusion construct and was included to ensure proper expression and processing of the miRNA precursor. The underlined sequences included in the PCR primers denote the recombination sequences required for insertion into the 3' (pDONRP2RP3) Gateway Entry vector.

AEK 5 - forward
GGGGACAGCTTTCTTGTACAAAGTGGGATAAGTAGAATTCCAACTGAGCGCCGGTC

AEK 8 - reverse
GGGGACAACTTTGTATAATAAAGTTGAAGGGCCCGTACGGCCGACTAGTAGG

2.2.4 The *lin-4* rescue construct:

The wild type *lin-4* rescue construct (pHZ018) was provided by Dr. Andrew Fire at Stanford University (Zhang et al, 2010) and consists of a 695 nt genomic fragment that can rescue the *lin-4(e912)* loss-of-function vulva phenotypes (Lee et al, 1993), consisting of the 94 nt *lin-4* precursor sequence, flanking genomic 5' (498 nt) and 3' (103 nt) sequences. This construct was used as a positive control for scoring the rescue of the *lin-4* loss-of-function phenotypes in *C. elegans*.

2.3 Worm Microinjection and Rescue Assay

MicroRNA expression constructs were mixed with the *pha-1*(+) injection marker plasmid (pC1) (Granato et al. 1994) and microinjected into the gonads of early adult (eAd) stage worms carrying the *lin-4* null mutation (*lin-4e912*). Since animals deleted for the *lin-4* miRNA lack egg-laying structures (vulvaless) and die at the early adult stage when fertilized eggs hatch within the animal due to egg-laying defects (Egl), direct injection into these animals did not yield viable transgenic progeny. Therefore, microinjection of the *lin*-4 promoter:*mir-237* precursor:*unc-54 3'UTR* expression construct (75 ng/uL) or the *lin-4* rescue construct (30 ng/uL) was performed in the strain PD7143: *lin-4(e912)/mIn1[dpy-10(e128)mIs14(gfp)]* II*; pha-1(e2123ts)* III (gift from Andrew Fire, Stanford University). These animals carry a GFP-tagged genetic balancer (*mln 1*) that includes the wild type *lin-4* miRNA and allows for the microinjection of constructs that are essentially wild type in phenotype. Once these injected hermaphrodite worms spontaneously lost the (*mln1*) genetic balancer, we were able to analyze rescue in the null *lin-4(e912)* genetic background.

To select for transgenic worms carrying the miRNA expression constructs upon injection, the PD7143 strain also was deficient for the *pha-1* gene (pha-1(e2123ts)). *pha-1(e2123ts)* animals are healthy at 16˚C. However, at 23˚C, all animals lacking *pha-1* arrest at early stages of development and die. Therefore, the *pha-1* expression construct (pC1) was co-injected with our *lin*-4 promoter:*mir-237* precursor:*unc-54 3'UTR* expression construct or the *lin-4* rescue constructs described above, and only transgenic worms were able to grow at 23˚C.

Multiple independent transgenic lines were obtained and assayed for rescue of the *lin-4*-null mutation *e912 vulva* phenotype. Worms scored as "rescued" included presentation of both partial and complete *lin-4* rescue phenotypes (egg-laying, pvul (protruding vulva), and vulva bursting).

2.4 Microscopy for *C. elegans* imaging

Worms were placed on agarose pads mounted on glass slides after immobilization by levamisole and viewed on an Axioplan2imaging microscope (Zeiss) using Normarski optics and Kohler illumination. All pictures were taken with an AxioCam using AxioVision version 2.0.5 (Zeiss).

3. Results

MiRNAs that share a common “seed” sequence are hypothesized to bind to the same targets and direct identical biological activities. Our past data showed that members within the *lin-4* family exhibit distinct expression patterns during development due to unique promoter/transcriptional elements. Therefore, we wanted to test if miRNAs sharing a common “seed” are functionally distinct because they are expressed in different tissues and at different times during development and thus have access to distinct targets – or if they are really functionally distinct. We focused our studies on the *lin-4* family, composed of *lin-4* and its closest homologue, miR-237.

We first wanted to compare the loss-of-function phenotypes for *lin-4* and its closest homologue, miR-237, in *C. elegans.* As shown in Figure 4, when *C. elegans* do not produce *lin-4* miRNAs, they are unable to develop the egg laying structure (the vulva). This causes the fertilized eggs to remain inside the uterus and develop within the parent. Hatching of eggs within the hermaphrodite result in early adult lethality. Only a low number of viability progeny are noted in *lin-4* loss-of-function animals (*lin-4(e912)*) compared to up ~250 progeny noted in wild type hermaprhodites (Fig. 5). Animals mutant for *lin-4* also exhibit other developmental abnormalities such defects in the differentiation of specialized skin cells (seam cells) and possess an elongated body structure (not shown). In contrast, we found that loss-of-function animals for the *lin-4* homologue, miR-237, do not show any obvious developmental abnormalities and exhibited normal fertility and brood sizes (Figs. 4 & 5). These results indicate that the deletion phenotypes for these two miRNAs are distinct and miR-237 likely possesses functional overlap with other miRNAs.

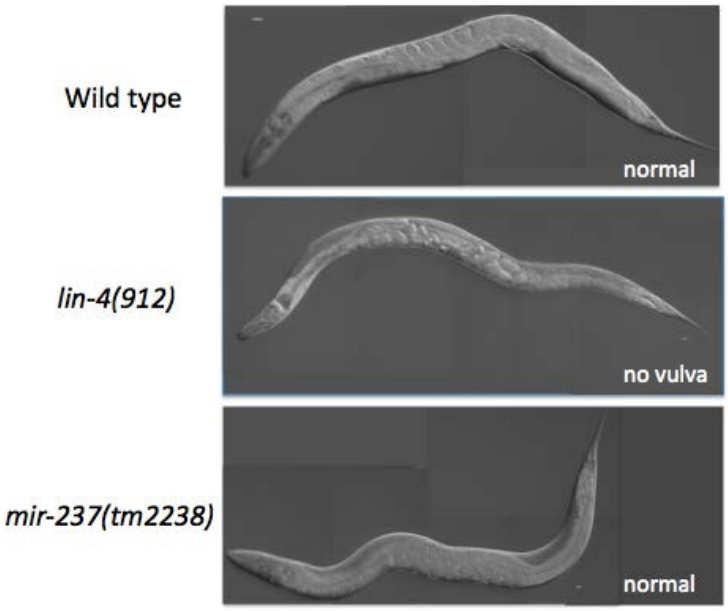

Figure 4. The miRNA deletion mutant animals for *lin-4* and miR-237. The *lin-4(e912)* null animals fail to form egg-laying structures (the vulva) and die when fertilized eggs hatch within the adult hermaphrodite. The *mir-237(tm2238)* null animals do not exhibit any abnormal phenotypes and possess normal vulval morphology compared to wild type worms.

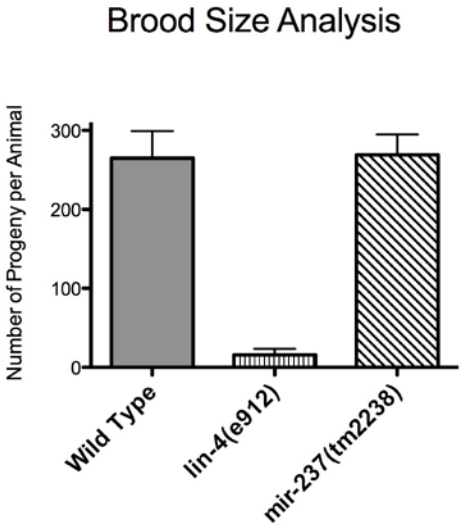

Figure 5. Brood size analysis of *C. elegans* carrying deletions for the *lin-4* miRNA or the *lin-4* homologue, miR-237. The *lin4(e912)* animals have significantly decreased fertility compared to wild type and *mir-237(tm2388)* nematodes.

To test the theory that microRNAs sharing a common miRNA seed are functionally equivalent, we attempted to rescue the *lin-4* loss-of-function vulvaless phenotype in *C. elegans* by synthetically expressing miR-237 under the control of the *lin-4* promoter. We generated the *lin-4* promoter:*mir-237* precursor:3'UTR expression construct using PCR and Gateway cloning (Fig. 6A).

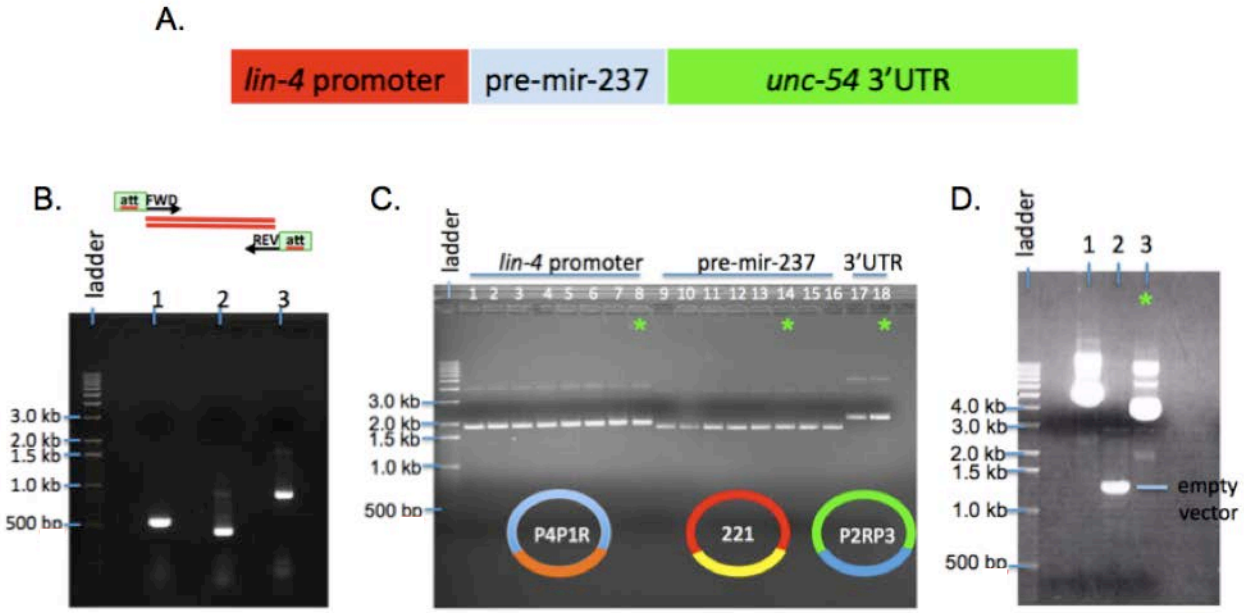

Figure 6. Generation of the *lin-4* promoter:*mir-237* precursor:3'UTR expression construct using Gateway cloning. A. Diagram of the synthetic miRNA expression construct. B. Gel analysis of the PCR fragments for (1) the *lin-4* promoter, (2) the *mir-237* precursor, and (3) the *unc-54* 3'UTR. C. Gel analysis of the promoter, miRNA precursor and 3'UTR fragments cloned into the Gateway Entry vectors pDONORP4P1R, pDONOR221, and pDONORP2RP3 respectively. D. Gel analysis of the *lin-4* promoter, *mir-237* precursor and *unc-54* 3'UTR Entry clones integrated into the Gateway Destination vector pDESTR4R3. Green asterisks indicate verified clones by DNA sequencing.

The synthetic miR-237 expression construct was first verified by DNA sequencing and then we proceeded to perform a *lin-4* rescue assay in living worms (Fig. 7). All animals lacking the *lin-4* miRNA fail to form egg-laying structures (the vulva). We wanted to assay if animals null for *lin-4* could be rescued for this vulvaless phenotype if they were engineered to expressed the *lin-4* homologue, miR-237, in all the cells and at all the times during development that would normally express *lin-4.* We microinjected the *lin-4* promoter:*mir-237* precursor:3'UTR expression construct into the distal arm of the gonad of early adult hermaphrodite worms carrying the *lin-4* loss-of-function mutation (*lin-4e912*). The distal arm of the gonad is the area where germ cells reside and are devoid of cell membranes (the syncitial germline). Therefore, injected DNA is taken up into multiple germ cells once membranes form and these germ cells develop into oocytes.

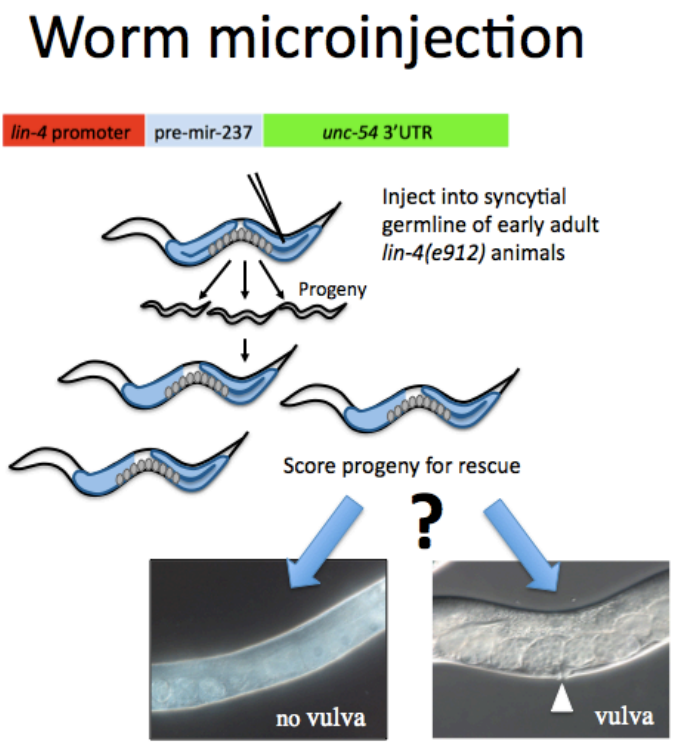

Figure 7. Schematic of the *in vivo* rescue assay. The *lin-4* promoter:*mir-237* precursor:3'UTR expression construct was injected into the germline of *lin-4(e912)* null animals and their progeny were assayed for rescue of the vulvaless phenotype.

This technique resulted in the progeny of the injected adult animals to carry extra-chromosomal arrays containing multiple DNA copies of the *lin-4* promoter:*mir-237* precursor:3'UTR expression construct in their cells. Therefore, the miR-237 synthetic construct was expressed in the *C. elegans* progeny during all stages of development. As a positive control, we injected *lin-4(e912)* animals with a *lin-4* rescue fragment that was previously shown to rescue the *lin-4(e912)* loss-of-function vulva phenotypes (Zhang et al, 2010). This 695 nt genomic fragment contained the 94 nt *lin-4* precursor sequence and flanking genomic 5' (498 nt) and 3' (103 nt) sequences required to drive wild type *lin-4* expression (gift from Andy Fire, Stanford University). The transgenic progeny were scored for rescue of the *lin-4* loss-of-function phenotype - their ability to develop egg laying structures (vulva). We scored worms as "rescued" if they presented partial or complete rescue of the vulva phenotypes (egg-laying, protruding vulva structures (pvul), and vulva bursting). We obtained four independent transgenic lines that expressed the *lin-4* promoter:*mir-237* precursor:3'UTR expression construct. However, these lines were sick and yielded low numbers of viable progeny so only a few worms from each line were scored. Only one independent transgenic line carrying the *lin-4* rescue fragment was obtained and scored for rescue.

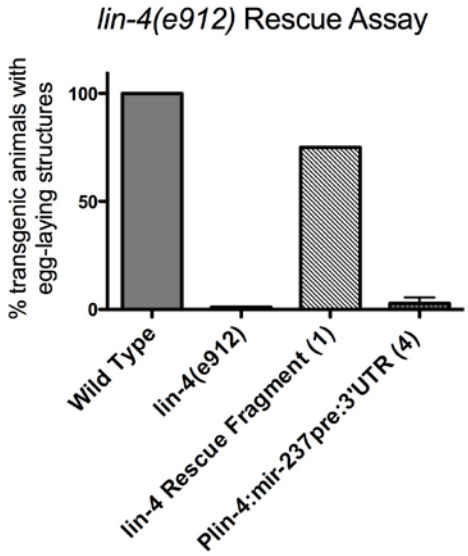

Figure 8. The *lin-4* homologue, miR-237, is not functionally equivalent to *lin-4*. Transgenic worms carrying the *lin-4* promoter:*mir-237* precursor:3'UTR construct failed to rescue the *lin-4(e912)* deletion vulva phenotypes compared to animals transgenic for the *lin-4* rescue fragment. Numbers in parenthesis indicate the number of independent lines that were assayed for the presence or absence of egg-laying structures. Note that the data shown for wild type and *lin-4(e912)* are for un-injected animals.

We found that expression of miR-237 under the control of the *lin-4* promoter was unable to rescue the vulvaless phenotypes in the *lin-4(e912)* animals. These findings indicate that despite sharing a common miRNA "seed", *lin-4* and its closest homologue, miR-237, are not functionally equivalent. In the future, we will need to verify that worms transgenic for the *lin-4* promoter:*mir-237* precursor:3'UTR expression construct are properly expressing processed and mature miR-237 using Northern blot and PCR assays.

4. Conclusions and Discussion

Many studies have shown that when only one microRNA gene has been deleted, the *C. elegan* mutants show a lack of distinct phenotypes due to possible overlapping function of miRNAs family members. For instance, microRNA-237 deletion mutants do not have a detectible phenotype in the egg laying structure (Fig. 4). Following the miRNA "seed" theory, overlapping function is very possible since microRNAs in the same family are theoretically functionally equivalent. However, some studies have shown that the lack of phenotype is *not* due to overlapping function. In one study, observations of deletion mutants of the miR-51 family found that the microRNAs in the family were probably not responsible for the non-detectible phenotypes of single mutants. In the study done by Esquella-Kerscher et al. 2008, they tested the overlapping function between microRNAs in different families. They found that when a C. elegan is missing *miR-48*, *miR-84* (both are from the let-7 family), and *miR-237* serious defects develop. In miR-48 and -84 double mutants, there were only a few minor defects. This shows that there is possible overlapping function between microRNAs of *different* families. Our results prove that *lin-4* and *miR-237* are not functionally equivalent even though they have identical "seeds". These results are very significant for it disproves the longstanding miRNA "seed" theory and our understanding of the functionality of microRNAs. More research needs to be done to find if these results are consistent in other families in addition to research on the interactions between microRNAs of different families.

Our results affect not just research on microRNA functionality in the worm, but in human cancers as well. Human *lin-4* family members are VERY similar to C. elegan members since lin-4 is evolutionarily conserved. As *lin-4* family members are involved in many cellular processes, lin-4 family members have been found to cause as well as prevent problems in multiple human cancers. The three human *lin-4* family members are known as miR-237a, miR-237b-1, and miR-

237b-2. All three are expressed in a large range of tissues during development, however their biological role still unsure. *In vitro* assays indicate that miR-125b can block cellular proliferation in breast, ovarian, hepatocellular and thyroid cancer cell lines and act as a tumor suppressor gene. Recent studies have also shown that miR-125b has converse effects in prostate, pancreas, and myeloid leukemia cancer cell lines and rather induces cellular growth, functioning more like an oncogene. This implicates that the roles microRNAs play in cellular process may not only be affected by their seed, but other interactions such as microRNAs from different families. The results of our study will significantly change the approach towards miRNA family member interaction and miRNA functionality.

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