5-2020

Evaluating biogenesis of 5’-tailed mirtrons

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by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2020

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Abstract

MicroRNAs are 22 nucleotide, non-coding RNAs that serve as substrates for Argonaute proteins to induce RNA interference pathways. Intron-derived miRNA precursors called “mirtrons” have been identified and classified primarily through deep sequencing methods. Unlike most miRNAs, mirtrons are derived from splicing events and also exhibit high levels of post-transcriptional nucleotide addition to hairpin precursors. Most relevant among these modifications is 3’ uridylation as it inhibits mirtron biogenesis in multiple model systems. Mirtrons may also possess additional nucleotides adjacent to the pre-miRNA hairpin at the 3’ and/or 5’ ends. These nucleotide “tails” are removed prior to Dicer cleaving the hairpin. In 2010, Flynt et al. reported that tail trimming activity in the highly conserved 3’-tailed mirtron dme-miR-1017 was performed by the RNA exosome. The insertion of a tract of guanines within the tail would prevent trimming if the activity is performed by an exoribonuclease. To determine if tail removal in 5’-tailed mirtrons is also exosome-mediated, three mutant constructs including a 12 G insert, 20-bp splice site insert and a splice site mutant (negative control) were derived from hsa-miR-5010 and used for comparing target repression levels and base modification. We employed cell culture, transfection, luciferase assay and sequence analysis to determine the effect of differential tail elements on miRNA processing and activity. Our findings confirm selective uridylation at the 3’ end for all miR-5010 variants. The Poly G mutant had greater activity and stability compared to both the wild-type and the splice site mutant. These data imply that 5’-tailed mirtron tail removal is mediated by an endoribonuclease unlike the 3’-tailed variants.

Keywords**:** RNA interference, microRNA, mirtron, transfection, uridylation, RNase.

Acknowledgements

I would like to thank my thesis advisor, Alex Flynt, for teaching me all that I know, inspiring me to pursue a career in biotechnology research and lighting a fire beneath me when I need it. I would also like to also express my gratitude to my wonderful mentor and friend, Farid Zia, for his encouragement, patience and empathy throughout the research process.

I would also like to thank Mississippi INBRE, the National Institutes of Health and the Drapeau Center for Undergraduate Research for funding and providing the required facilities to make this project possible.

I would also like to thank my associates within the Honors College and my USM family for their role in pushing me to succeed as an undergraduate student and beyond. Finally, all the love and appreciation in the world for my partner, Emily Welch, for her role in supporting me in all of my academic endeavors.

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List of Abbreviations

AGO	Argonaute
DMEM	Dulbecco's Modified Eagles Medium
dsRNA	double stranded RNA
FBS	fetal bovine serum
GM	genetically modified
miRNA	microRNA
mRNA	messenger RNA
PAP	poly A polymerase
RISC	RNA induced silencing complex
RNAi	ribonucleic acid interference
RNase	ribonuclease
siRNA	small interfering RNA
shRNA	short hairpin RNA
ssMut	splice site mutant
TUT	Terminal Uridyltransferase, Tailor

Chapter 1: Introduction

A brief overview of genetic manipulation

During the 20th century, humanity experienced incredible advances in molecular biology and biotechnology. Since the structure of DNA was elucidated in the 1950s, progress has been rapid in understanding and manipulation of genetic material. Many modern technologies have been developed to understand and influence genetic variability in bacteria, plants and, more recently, animals.

Some notable innovations in the industry include the development of recombinant DNA and transgenic methods, the refined production of high-quality gene products from non-native organisms, and the development and popularization of genetically modified (GM) plants and animals for human use. Recent advances in the field, including the discovery and use of RNA interference and related technologies to selectively control gene expression in model systems, depend on understanding the genetic basis of these tools (Hammond, 2006). In order to produce the next breakthrough in genetic engineering, research efforts must be focused on understanding biological systems with potential to inspire the next developments in biotechnology.

RNA interference

RNA interference (RNAi) is a biological process that uses double-stranded (ds) RNA to regulate gene expression via the interactions between protein-RNA complexes and messenger RNAs with complementary regions to the dsRNA (Lee et al., 1993). RNA interference can be initiated by both native and exogenous dsRNA (Fire et al., 1998; Timmons et al., 2001). Exogenous dsRNA enters the RNAi pathway by interacting with

the RNase III Dicer (Bernstein et al., 2001). Dicer cleaves the dsRNA into double-stranded 20-25 bp fragments called small interfering (si) RNAs. siRNAs associate with Argonaute (Ago) proteins, but only one strand of the siRNA guides the complex to target mRNA. The passenger strand is ejected from the Ago-RNA complex to form the RNA-induced silencing complex (RISC) (Parker, 2010). The guide RNA leads the RISC to complementary mRNA to regulate gene expression (Gregory et al., 2005).

Discovery of microRNAs

MicroRNAs (miRNAs) were discovered in 1993 in *Caenorhabditis elegans* and function as native substrates to initiate RNAi (Lee et al., 1993). These novel transcripts made by RNA polymerase II had a previously undescribed activity in regulating gene expression during larval development (Abbott et al., 2006; Bhaskaran and Mohan, 2014). Since their discovery in *C. elegans*, most research in the area has focused on the model organisms *C. elegans* and *Drosophila melanogaster*, but more recent efforts have described multiple variations of the original miRNA pathways in plants, animals and viruses (Xie and Steitz, 2014). In 2003, the Tuschl lab discovered 31 miRNAs in humans and mice, which were classified into 15 families conserved between both vertebrates and invertebrates (Lagos-Quintana et al., 2003).

MicroRNA biogenesis pathways

Canonical microRNAs (miRNAs) are approximately 22 nucleotide (nt) hairpin RNAs transcribed by RNA polymerase II (Lee et al., 2004). Generally, miRNAs are transcribed within the nucleus and are excised from the primary miRNA (pri-miRNA) transcript by the RNase III Drosha (Lee et al., 2003). Exportin-5 transports these pre-miRNA hairpins out of the nucleus (Murchison & Hannon, 2004). Once in the cytoplasm,

the pre-miRNA hairpins serve as substrates for another RNase III, Dicer, which cleaves the hairpin at the loop (Lund & Dahlberg, 2006). The annealed strands separate, one strand is selectively degraded, and mature miRNA proceeds through the RNAi pathway (Siomi & Siomi, 2010).

In addition to the canonical miRNAs described previously, atypical miRNA precursors have been discovered using high throughput sequencing data (Ladewig et al., 2012). Intron-derived microRNAs, dubbed "mirtrons", are derived from splicing events that liberate short introns competent to fold into miRNA-precursors (Ruby et al., 2007). Detection of mirtrons requires genomic analysis using algorithms to recognize elements that are common to a majority of mirtrons, but mirtrons have heterogenous elements that add further variability to miRNA precursors and make detection difficult (Valen et al., 2011). The most relevant of these heterogenous elements are nucleotide "tails" extending from the miRNA hairpin. These elements vary in both composition and location. Tails may be present on the 3' and/or 5' end of the harpin and must be removed before the pre-miRNA can proceed into the RNAi pathway. Interestingly, 5'-tailed mirtrons account for the majority of all mirtrons, but their complete biogenesis pathway has yet to be described (Fig. 1) (Wen et al., 2015).

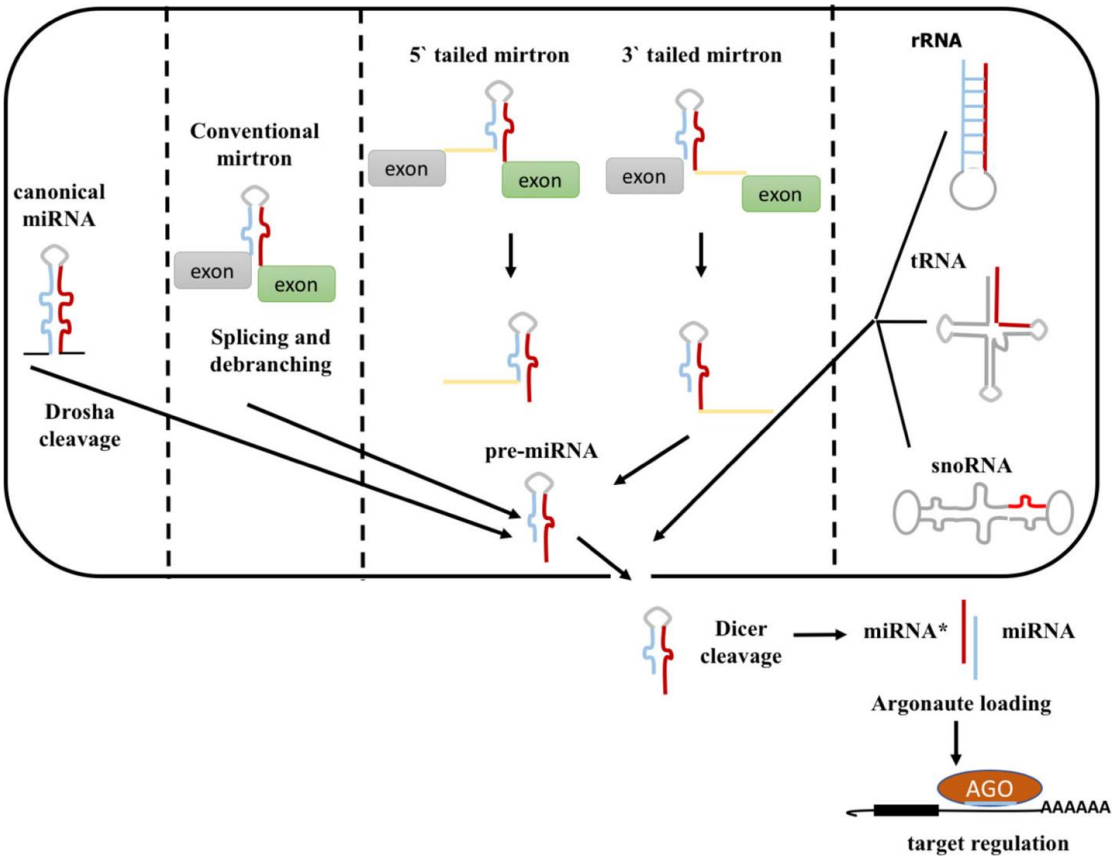

Figure 1. Classes of miRNAs separated by biogenesis pathway. *The core RNAi pathways are shown as a Dicer substrate (pre-miRNA) is cleaved, and one strand preferentially associates with an Ago protein and guides the RISC to target mRNA. Canonical miRNAs are processed by the nuclear RNase Drosha prior to export into the cytoplasm. Mirtrons are formed through a different pathway involving a splicing event before joining the RNAi machinery. Tailed mirtrons require 3' and/or 5' tail removal by RNase prior to Dicer cleavage (Zia and Flynt, 2018).*

In addition to tail removal, post-transcriptional addition of component nucleotides is commonly seen in miRNAs (Westholm et al., 2012). Post-transcriptional uridylation is performed by a noncanonical Poly(A) Polymerase (PAP) which later was discovered to be the Terminal Uridyltransferase, Tailor (TUT). Among recognized nucleotidyl transferases in mammals, TUT1, TUT4 and TUT7 enzymes preferentially perform uridylation of human RNA (Bortolamiol-Becet et al., 2015; Yashiro & Tomita, 2018). Previous studies have reported that the untemplated monouridylation at the 3' end has various effects on miRNA stability and gene expression, and it is likely that effects differ between plant and

animal systems. This uridylation causes miRNAs to be less active and more prone to degradation; however, the level of suppression is more pronounced in plants than in animals (Gutiérrez-Vázquez et al., 2017). The utility of this activity is still debated, but one possibility is that newly evolved regulatory RNAs may affect gene expression in a deleterious way. Limiting small RNA activity using post-transcriptional modification would allow for evolutionarily conserved Dicer substrates access to the RNAi machinery with less competition.

MiR-1017: Conservation and processing in Drosophila melanogaster

The evolutionary history of mirtrons and their variable elements is an ongoing area of research, and computational methods using model systems with large genomic databases are often combined with laboratory methods such as knockdowns and Northern Blotting to provide insights into the conservation of various elements. In 2010, Flynt et al. combined computational and laboratory methods to describe the conservation and biogenesis pathway of dme-miR-1017. In *D. melanogaster*, a few mirtrons, such as the 3'-tailed mirtron miR-1017, are highly conserved within the genus, but many others are not. Like many mirtrons, miR-1017 contains additional sequence elements in addition to the functional miRNA sequence. Within the 5' arm the 5' splice donor is present and highly conserved, and the 3' arm contains the "seed" sequence (short segment of nucleotides that must be a perfect complement to target mRNA) that is also highly conserved. However, the loop between the arms is much less consistent and is not conserved. If the mirtron contains a tail on either or both ends, the tail's sequence is generally much less conserved than the previously described elements.

Work with mir-1017 in the Flynt lab has supported the claim that disorganized nucleotide modifications in the mirtron tail do not likely impact its ability to function as a Dicer substrate, but the addition of a 12 G tract inhibits exosome-mediated tail removal due to interference by the resultant secondary structure.

5'-tailed mirtron biogenesis: endonuclease or exonuclease?

Hsa-miR-5010 is a 5'-tailed mirtron located on chromosome 17 and annotated on the basis of ~ 2200 small RNA reads present in the miRbase.org database. This mirtron is found within an intron of one of the vacuolar ATPase (V-ATPase) domains. V-ATPase is a multi-subunit enzyme and mediates acidification in eukaryotes for zymogen activation, protein sorting and other intracellular processes. The Hsa-miR-5010 mirtron served a model to determine the effects of tail modification on 5'-tailed mirtron maturation.

The presence of a 12-mer of guanine on the mirtron tail would prevent tail removal if the activity is exosome-mediated. Tracts of guanines tend to form stable secondary structures called a G quadruplex, and the presence of these structures prevents the activity of the RNA exosome (Capra et al., 2010; Liu et al., 2007). If tail removal is mediated by the RNA exosome, the maturation process would be inhibited by the Poly G tract and no pre-miRNA would form. However, if the tail removal of miR-5010 is performed by an endoribonuclease, the Poly G tract would not have any negative effect on the maturation process. The presence of this element in the 5' tail that inhibits exonuclease activity may improve the miRNA's ability to suppress target mRNA as it would be protected from cytoplasmic exoribonuclease activity.

To test if adding a 12-mer of guanine to the tail of miR-5010 will impact its maturation, vectors altered by adding a 12-polymer of guanine and a 20 bp insert into the

mir-5010wt (wild type) vector within the 5’ tail were transfected into HEK 293 cells for expression. These changes may result in altered mirtron expression, degradation, or target suppression as well as possible variation in 3’ uridylation. Luciferase assay was conducted to determine the effect of the insertions on the level of target miRNA suppression. Next generation sequencing will be employed to determine the exact relationship between untemplated nucleotide addition and these changes in the mirtron tail sequence (Behjati & Tarpey, 2013).

Chapter 2: Materials and Methods

Construction of vectors

Previous studies have shown that 3'-tailed mirtron dme-miR-1017 may possess many nucleotide changes in the tail, suggesting that flexibility in the tail is compatible with access to the miRNA pathway (Flynt et al., 2010). To confirm this with hsa-miR-5010, we cloned wild-type miR-5010 into pcDNA 3.1 (Addgene) expression vector (Fig. 2) using a Cold Fusion Cloning Kit (SBI). The vector containing miR-5010 was subjected to additional cloning to generate the miR-5010 Poly G and Insert variations by adding a 12 G tract and a 20 bp insert within the 5' tail, respectively. miR-5010 splice site mutant (ssMut) variation included a mutation that prevents splicing activity. This mutated construct serves as a negative control for the luciferase assay as no mature hairpins should be detected. All vectors included restriction sites for Renilla luciferase for use in downstream luciferase assay and next generation sequencing. The completed vectors were transformed into competent cells for amplification and extracted using Miniprep Plasmid Extraction Kit (Thermo Fisher). All extracted vectors were confirmed by sequence analysis.

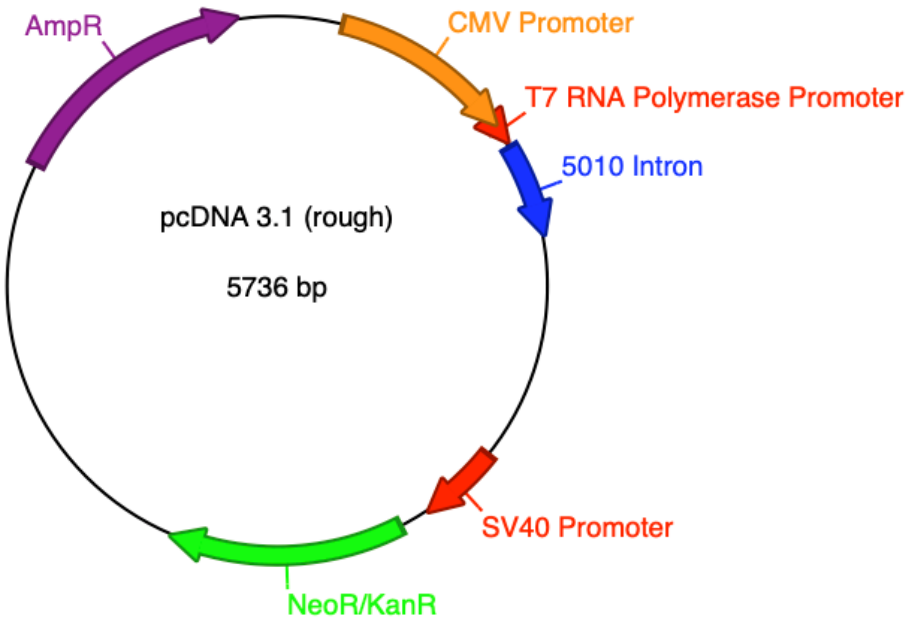

Figure 2. pcDNA 3.1 miR-5010 wild type. *This vector serves as the primary transfectant to express miR-5010 in HEK 293 cells. Poly G, Insert, and ssMut variants were derived from this construct.*

Cell culture and transfection

DMEM (Dulbecco's Modified Eagles Medium) with 10% FBS, 1% Pen/Strep (CORNING cellgro) media was used to culture HEK-293 cells (American Type Culture Collection) in standard methodology of cell culture (Tom et al., 2008). HEK cells were seeded in 6-well plates for plasmid transfections and FUGENE 6 transfection reagent (Promega) was used to transfect the cells (4 μg of mirtron plasmids for 6-well plate). 24 to 48 h after transfection, the transfection efficiencies were checked, and RNAs were extracted using Tri Reagent (Molecular Research Center, USA) according to manufacturer instructions.

Luciferase assay

A Luciferase assay was used to determine the pre-miRNA viability as a Dicer substrate. Dual-luciferase assay is a method that uses cotransfected constructs containing firefly luciferase upstream of a target gene that is used to report the translation of that gene (Brasier & Ron, 1992). The firefly luciferase vector contained a synthetic miR-5010 target sequence to report the target suppression. The cotransfectant includes a weaker *Renilla* luciferase gene as a background control. We cloned mir-5010 mutants and wild type downstream of the *Renilla* luciferase gene to determine if the mutant mirtrons could suppress the target mRNA.

The assays were done in 96-well plates, and 24 h before the transfection HEK 293 cells were seeded in wells. When the confluency of cells was about 90%, 250 ng of mirtron constructs were co-transfected with the psiCHECK2 plasmid. The 5010 target was cloned adjacent to the *Renilla* luciferase gene in the psiCHECK2 vector. Then the luciferase activity was measured by micro-plate reader for 72 h after transfections were completed using Dual-Luciferase Reporter Assay System (Promega). The ratios of *Renilla* luciferase to firefly luciferase were normalized. Tukey ANOVA was used for statistical analysis.

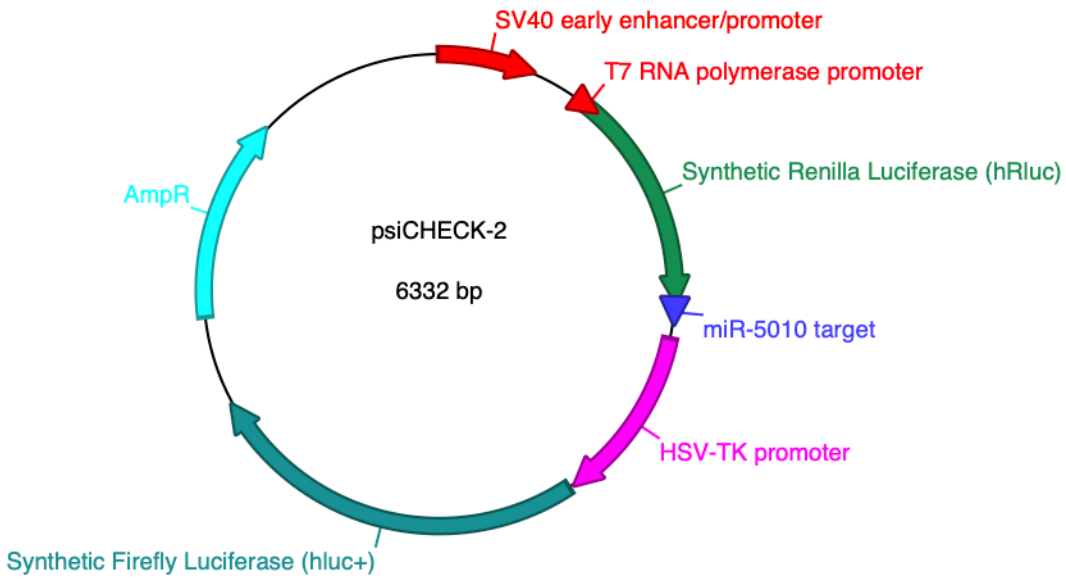

Figure 3. psiCHECK-2. *This vector map details the components of the psiCHECK-2 vector used in the luciferase assay. A synthetic miR-5010 target was cloned directly downstream of the Renilla luciferase gene. Firefly luciferase functions as a reporter, and Renilla luciferase controls for differences in transfection efficiencies.*

RNA extraction, sequencing and analysis

After transfected cells were incubated at 37° C for 48 hours, total RNA extraction was performed using chloroform and TRI Reagent (Molecular Research Center, USA) according to manufacturer's guidelines. RNAs were stored at -80 °C and held until all samples were collected and prepared for sequencing. Illumina sequencing data was evaluated using bioinformatic methods (see below) to determine the effects miRNA addition and ribonuclease knockdown.

After collecting the RNAs from all required transfections, samples were sent for sequencing on Illumina platform. FastQC was used to check the quality of the sequenced library. The FastQ files were trimmed to remove the 3' adapter sequences. Each read should have an adapter if it is a mature hairpin. Bowtie 2 index files of the intron that

contains miR-5010 were generated using human (Dec. 2013 GRCh 38/hg38) sequences available on UCSC website. Then, the clipped reads were aligned to the Bowtie 2 index created in the previous step. This alignment generates .sam (sequence alignment Mao) files. The .sam files are compressed into a binary form (as .bam files) to make downstream manipulation more efficient and order the reads. Bedtools was used to count the number of 3p and 5p reads that align with the 5010 intron. Bedgraph was used to convert the alignment into a histogram with genome position on the x-axis and the number of reads on the y-axis (Fig. 4)

Chapter 3: Results

Cloned vectors were verified by sequencing, and HEK 293 cells were successfully transfected with all constructs. The splice site mutant (ssMut) did not produce any mature miRNAs; 5010 wt, Poly G and Insert yielded mature miRNAs. The compatibility of different tail sequences with the mirtron biogenesis pathway is verified. Additionally, all mature miRNAs included the post-transcriptional 3' uridylation (shown in red) conserved in the majority of native mirtron biogenesis pathways (Fig. 4)

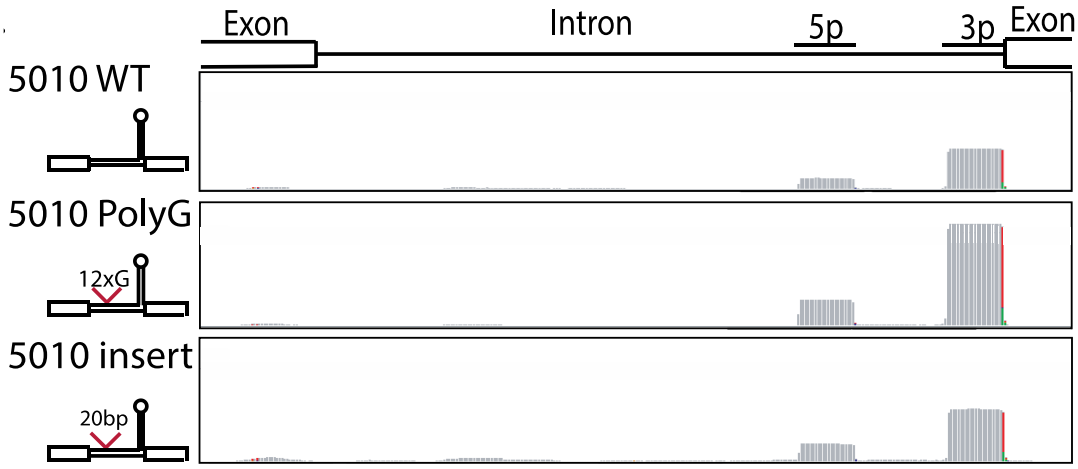

Figure 4. Ectopic expression of hsa-miR-5010. *Three constructs were transfected into HEK cells: Wild Type, a 12-guanine version (Poly G) and a 20 bp inserted version (insert). Mapping of reads to the miR-5010 intron following transfection of each, and sequencing of small RNAs. All three illustrating high frequency of untemplated "T" nucleotide reads at the 3' end, shown in red.*

To better observe the post-transcriptional modifications, all 3' end nucleotide additions were determined, and each is shown as a percentage (Fig. 5). We expected to see a preferential addition of a Uracil at the 3' end of the hairpin. In the transfected samples U is added to the 3' end in almost 80% of the reads and 70% in unresected samples (Fig 4). It is widely accepted that uridylation is a conventional modification which happens at the 3' end of 5'-tailed mirtrons, therefore the retention of this hallmark activity supports the

inference that tail sequence changes do not inhibit 5'-tailed mirtrons from normal maturation.

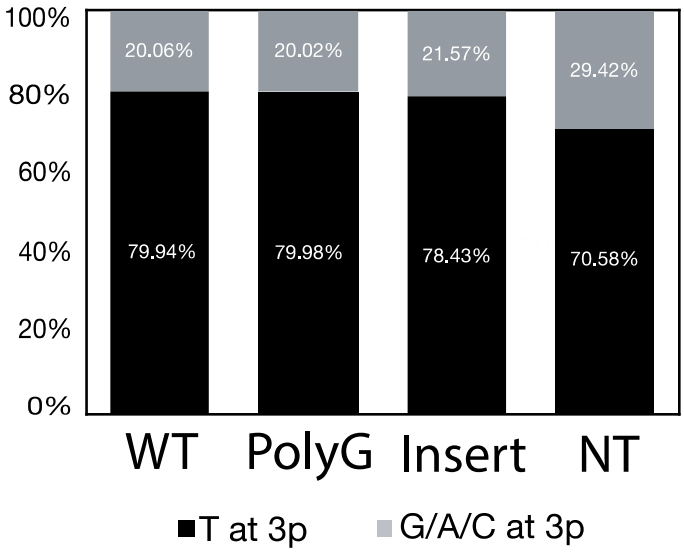

Figure 5. Untemplated nucleotides. *Bar graph showing the percentage of 3' untemplated "U" nucleotides on 3' small RNA in Wild Type, Poly G, Insert and not transfected (NT) HEK cells.*

Analysis of the sequencing data was used to determine that the Poly G tract did not inhibit the mirtron maturation or post-transcriptional modification. If the maturation of hsa-miR-5010 was mediated by the RNA exosome, the Poly G tract would prevent the RNase activity and miRNA biogenesis. The detection of pre-miRNA hairpins was used to verify if tail removal is occurring, because trimming is required for progression to the hairpin form (Fig. 1).

Enumerating hairpin reads for each mutant and the wild type further described the impact of tail sequence modifications on mirtron biogenesis. We expected to see similar numbers of reads in wild type and insert and fewer in Poly G if the hypothesis that tailed mirtron biogenesis is exosome-mediated is valid. However, we found that Poly G had the most hairpin reads at almost 2.5 times the number in wild type and insert constructs (Fig. 6).

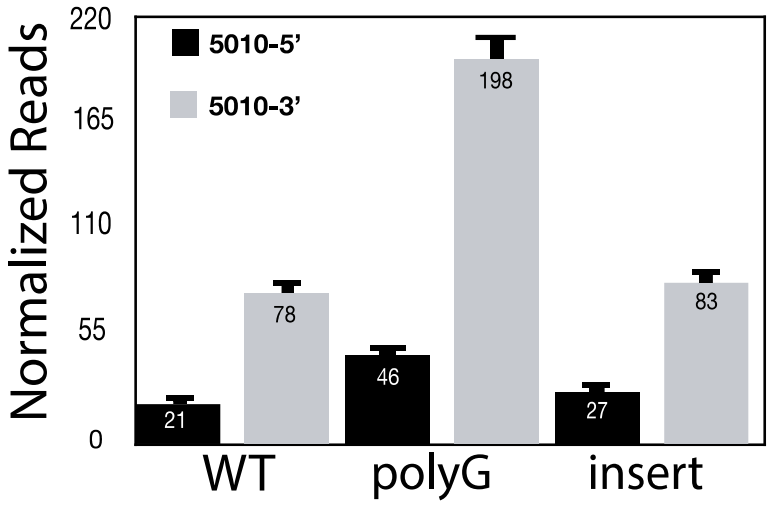

Figure 6. Hairpin mapping reads. *Normalized reads from transfected libraries arising from both 5' and 3' arms of the mirtron hairpin.*

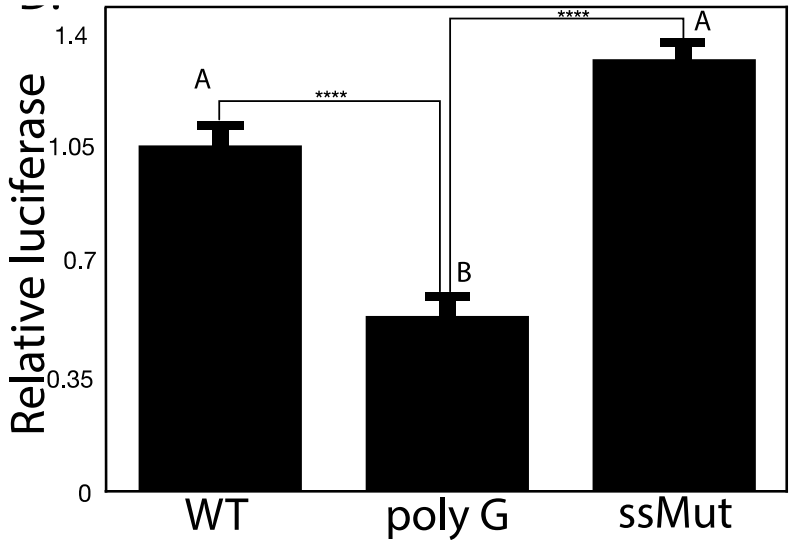

Figure 7. Regulation of sensor. *Relative luciferase activity from 5010 luciferase sensor when cotransfected with miR-5010 WT, Poly G, and Splice Site Mutant expression constructs. Tukey ANOVA was done for statistical Analysis, P < 0.0001.*

If the tail removal activity in miR-5010 is mediated by the RNA exosome, the luciferase assay should show less effective target suppression for the Poly G variant compared to the wild type. Results from luciferase assays showed that the Poly G mutant was able to repress the target more than effectively than either the wild type or Insert mutant (Fig. 7). Considering the increased number of reads and greater target suppression by miR-5010 Poly G compared to the wild type and the 20 bp insert, an exosome-mediated maturation process in 5'-tailed mirtrons is unlikely.

Chapter 4: Discussion and Conclusion

Since the discovery of RNAi in the 1990s, many dsRNA variants have been described and cataloged. Canonical miRNAs comprise the majority of possible Dicer substrates; the various types of mirtrons make up a significant minority. The 5'-tailed mirtron biogenesis pathway provides the greatest number of possible substrates of any mirtron variant (Wen et al., 2015). However, the enzymatic components of this biogenesis pathway have not all been identified. The RNase responsible for the tail removal is still undiscovered. Previous findings by Flynt et al. in 2010 determined that the conserved 3'-tailed mirtron in *Drosophila* miR-1017 relied on an exosome-mediated tail removal process before functioning as a Dicer substrate. To identify the RNase responsible for this activity in 5'-tailed mirtrons, methods to validate the exosome-mediated processing hypothesis were employed. Since 5'-tailed mirtrons are the most abundant variant in mammalian genomes, insight into this pathway will provide a nearly complete picture of the overall miRNA biogenesis system.

The exosome-mediated degradation of mirtron tails can be inhibited by the presence of a secondary structure that blocks exoribonuclease processing (Anderson, 1998). Our data show that introducing a secondary structure to the tail of miR-5010 did not inhibit its maturation. Also, the retention of 3' uridylation adds validity to the assertion that the mirtron tail sequence is a flexible element, and modifications likely do not inhibit the functionality of mature miRNAs. Overall, this data does not support the hypothesis that tail removal in 5'-tailed mirtrons is dependent on the RNA exosome. Therefore, it is most

likely that the tail removal activity is mediated by an endoribonuclease. This finding will require additional methods using other 5'-tailed mirtrons to determine its validity.

I also see increased target suppression and total reads for the Poly G mutant compared to both the Insert mutant and the wild type. The presence of a guanine tract likely protects the 5'-tailed mirtron from degradation by cytoplasmic exoribonucleases the same way it prevents 3'-tailed mirtron maturation (Flynt et al., 2010). This increased stability is a likely cause for the increase in target suppression seen in Poly G mutant.

RNAi has become a powerful, modern tool for manipulating gene expression in vivo and in vitro, but some efforts using transfected dsRNAs have had unexpected results. Instances of dsRNA transfection disrupting global gene expression have been reported (Khan et al., 2009). Developmental issues in model systems of human disease have been reported in cases when exogenous dsRNA was used to knock down a relevant gene (Stewart et al., 2008). The most likely cause of this disruption is the saturation of the nuclear export protein Exportin-5 because the increased traffic caused by expression of non-native short hairpin RNAs (shRNAs) would compete with native pre-miRNAs and inhibit their function (Colledge, 2006). Our results have shown that modifying tail elements of mirtrons does not significantly affect their maturation process and functionality as Dicer substrates. Therefore, a possible way to avoid the Exportin-5 saturation problem is to modify dsRNA elements to avoid the need for nuclear processing by delivering the modified RNA to an Argonaute protein directly. If this strategy proves viable, the use of exogenous siRNAs would be more effective at fine-tuning gene expression without competing with native miRNA activity.

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