RNA Interference and Biophysical Modulation Are Two Independent Mechanisms of MicroRNAs

Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in the undergraduate colleges of The Ohio State University

By

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Abstract

Background:

MicroRNAs (miRs) are small non-coding RNA (~22 nucleotides in length) that are well conserved in plants and animals [1,2]. It has been widely known that miRs regulate gene expression via the classical mRNA interference (RNAi) mechanism [3, 4]. In brief, miR is loaded into an Ago (Argonaute) family protein to form an RNA-induced silencing complex and directly binds to the 3' untranslated region (UTR) of mRNA with partially complementary sequences of the seed sequence (the 2nd to 8th nucleotides of miR), resulting in reduced translation and/or stability of the mRNA of targeted genes. Each miR has hundreds of directly-targeted genes and subsequently regulates thousands of genes. The classical RNAi mechanism is a powerful mechanism that miRs regulate all biological activities.

Our lab, in previous studies, revealed a novel action of miRs beyond the RNAi mechanism. miR-1 are a predominant miR in the heart and play a critical role in the regulation of cardiac development and physiology. We found that endogenous miR-1 directly binds to an inward rectifier potassium ion channel Kir2.1 membrane protein and physically suppresses the inward rectifier potassium current (I_{K1}) leading to biophysical modulation of cardiac electrophysiology [5]. Our studies suggest that miRs modulate the homeostasis of tissues/organs via two mechanisms: 1) conventional RNAi to regulate the expression of proteins, including ion channels, which requires hours to days to take effect, and 2) newly-discovered direct binding with ion channels that quickly (seconds to minutes) results in biophysical modulation.

Hypothesis:

From our previous studies we found that a core sequence (AAG AAG, the 10th – 15th nucleotide of miR1) of biophysical action is outside the seed sequence of RNAi mechanism. We identified that some single-nucleotide miR-1 mutants (Mus), such as MU-10U, MU-14G,

or MU-15A, are RNAi-only mutants that specifically disrupt biophysical action of miR1 while maintaining the RNAi mechanism. Therefore, we hypothesize that the RNAi and biophysical modulation are two independent mechanisms. As we have discovered RNAi-only mutants of miR1; here, we specifically hypothesize that there are also biophysical-only mutants of miRs that specifically disrupt the RNAi mechanism while maintaining biophysical actions. The separation of two mechanisms in miR1 mutants will demonstrate that biophysical modulation and RNAi are completely independent two mechanisms.

Methods:

The seed sequences (2-8 nucleotides) of miRNA are critical to miR-mRNA pairing for the RNAi mechanism [6, 7] . To identify biophysical-only mutants, therefore, we mutated one or two nucleotides of miR1's seed sequence and studied the biophysical modulation of Kir2.1 channel. To characterize the RNAi function of miR-1 mutants, we adopted two mechanisms to measure the relative expression of the target gene (e.g., *Kcnj2*, *Gja1*, and *Ptk9*). Dual luciferase assay and Western Blot were used to quantify the amount of target protein that was produced under varying conditions (different RNAi- and biophysical-defect miR mutants, different amount of RNA, single stranded miR versus double stranded miR mimics, etc).

Results:

Preliminary results from Luciferase Assay and Western blot establish Mu2C and Mu2C3U as a good candidate for RNAi mutants only. Computational analysis indicates a downregulation of the target gene in presence of Mu2C and Mu2C3U (RNAi-defect mutants). As hypothesized, mutations in the seed sequence region of the miRNA disrupted the RNAi mechanism and consequently changed its regulation of the expression of downstream target genes, such as *Kcnj2*, *Cx43*, *Ptk9*.

Conclusions:

Our study will demonstrate that miRs regulate the function of targeted genes by two independent RNAi and biophysical actions mechanisms and will significantly and innovatively expand the biological significance of RNA-protein interaction and will provide a better understanding of how RNA and proteins effectively respond to environmental and genetic perturbations. Considering a broad range of cellular activities regulated by miRs [8] and ion channels [9]. Our studies have broad implications and great scientific significance for RNA biology and ion channel biology. A mechanistic understanding of two independent mechanisms of miRs could be applied to develop RNA-associated medicine for various diseases.

Introduction

Cardiac arrhythmias result in irregular heartbeats due to abnormal electrical impulse [10]. Electrical impulse is tightly regulated in cardiomyocytes (cells responsible for the contraction of heart). Ion channels and transporters play a critical role in preserving normal electrical impulse and maintaining the rhythm of the heart. In the cardiomyocytes, microRNA (miR) play a significant role in the regulation of target regulatory proteins, ion channels and transporters [11, 12].

MicroRNA (miRNA) are small non-coding RNA (~22 nucleotides in length). Presently, couple thousands of miRNAs have been discovered in mice and humans respectively [13]. The relative abundance of different types of miRNA is dependent on the function of the cell. The miRNA pulldown result below indicates the relative abundance (percentage of miRNA-specific reads) in male and female mice heart tissues [13]. Since miR-1 is one of the most prevalent forms of miRNA in cardiomyocytes, our study will primarily concentrate on it.

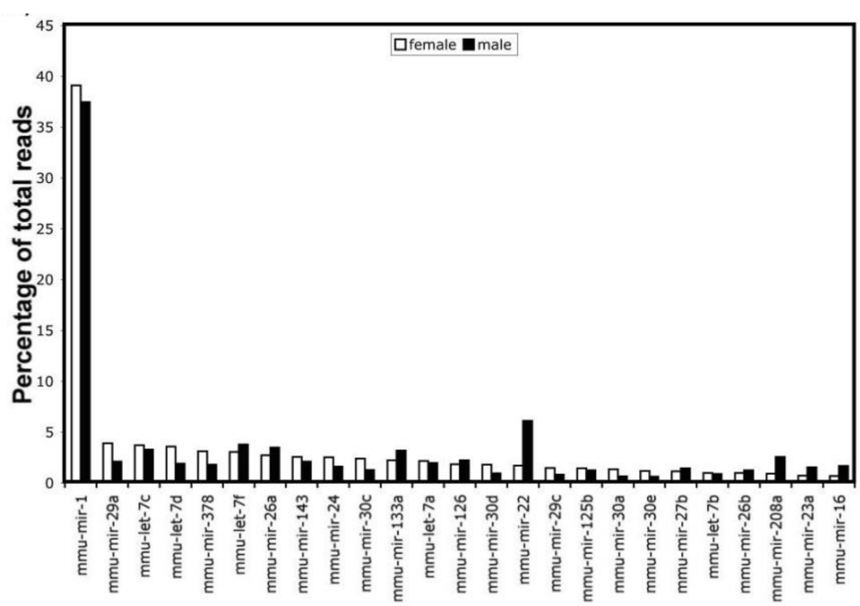


Figure 1: miR-1 is the predominant miRs in the heart. The relative abundance of miRNA (normalized read number) in male and female mice heart tissues (Circulation research, 2009)

Biogenesis of MicroRNA :

Similar to mRNA, miRNA is synthesized from DNA sequences. The genome has individual genes that encode certain miRNAs. DNA transcription is carried out by a complex of assembled transcription factors and RNA polymerase. Primary miRNA transcript is produced in the nucleus. Drosha complexes in the nucleus process the primary miRNA before transporting it to the cytoplasm. In the cytoplasm, Ago2 (Argonaute protein) processes the primary miRNA to produce the mature miRNA. Mature miRNA-Ago complexes can bind to their target mRNA to cause mRNA degradation or translational repression (in both cases, the expression level of target gene will decrease).

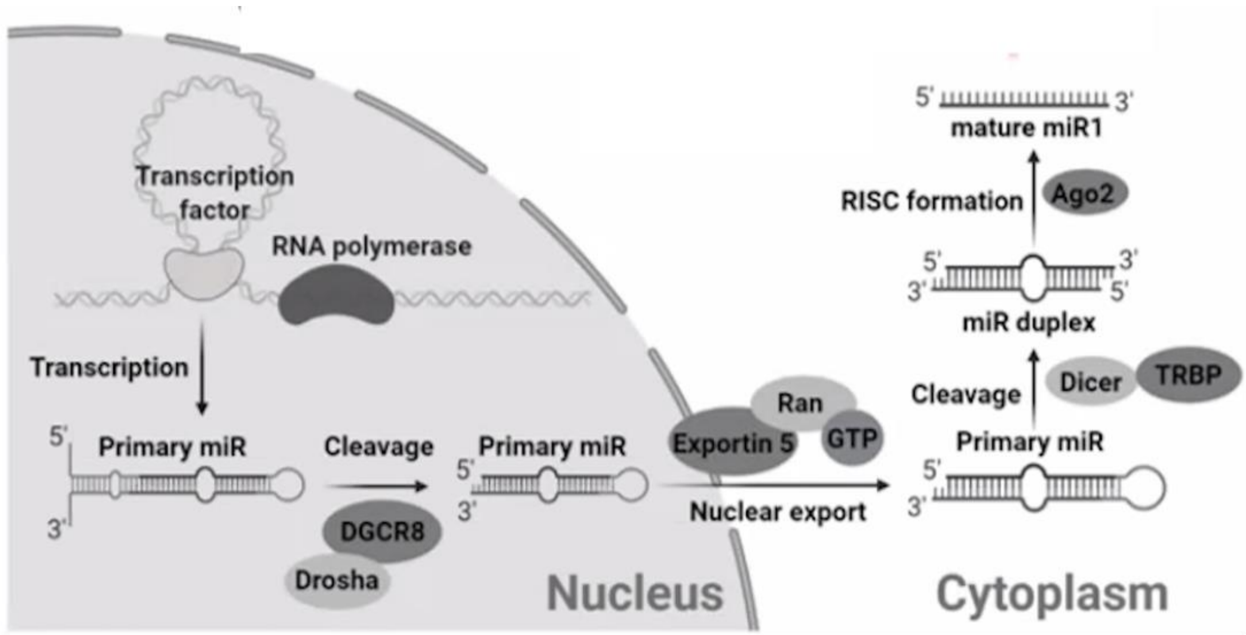


Figure 2: A schematic of miRNA-1 biogenesis in cardiomyocytes.

Gene Expression Regulation via RNAi Mechanism:

The fundamental tenet of a cell is that DNA is transcribed into mRNA (a single-stranded RNA sequence), which is then translated into a functional protein. RNA interference is the technique most frequently used in cells to regulate gene expression. In this process, the target mRNAs downstream translation is impacted by partial or full complementation between miRNA and mRNA. miRNA binds to Arg protein and guide the formed RISC complex to bind to the 3' UTR of the mRNA. The RISC complex, which consists of multiple types of binding proteins, Dicer, and RNA activator, binds to miRNA and prevents mRNA from binding to cytoplasmic translational factors and prohibits translation (translational repression). In addition, the RISC complex can also facilitate the degradation of target mRNA. As a result, the target gene's expression is either fully suppressed or downregulated. Due to the complexity of gene expression regulation, it takes hours to days to observe changes in the expression level of the target gene.

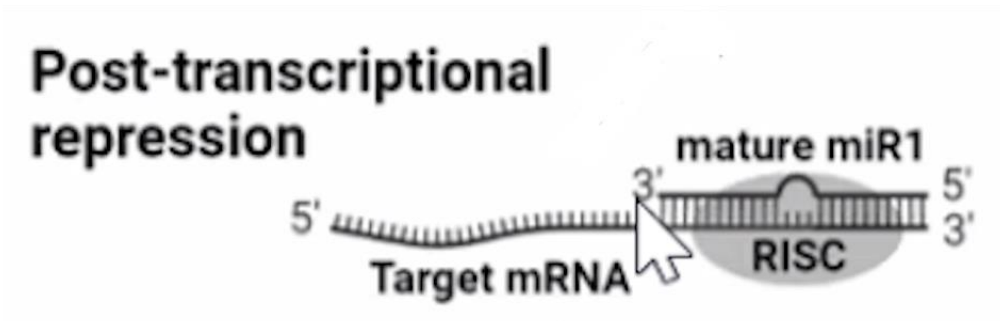
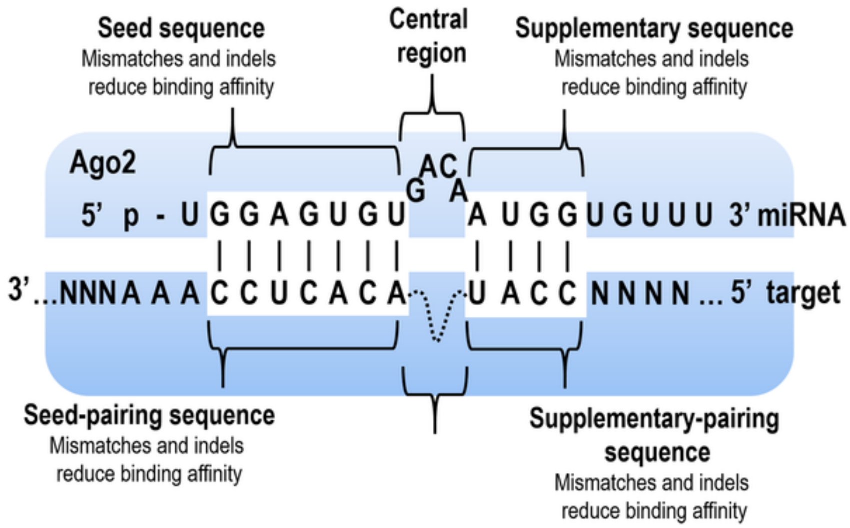


Figure 3: Target mRNA binding with miRNA (via partial or complete complementation) facilitated by RISC complex.

miRNA-mRNA binding is dependent on the seed sequence of the miRNA. The seed sequence contains the 2nd to 8th nucleotide of the miRNA. The partial or complete complementarity between the seed sequence and mRNA dictates the stability and the strength of binding. Therefore, mutations in the seed sequence will impact the stabilization of miRNA-mRNA complex and consequently the regulation of gene expression via RNAi mechanism.

Figure 4: Overview of miRNA-mRNA interaction in human Ago2 protein.



Target Genes for miR-1:

miR-1 has a large number of target genes. Several ion channels and transporter genes, which are involved in the generation of electrical impulses, are directly targeted by miR-1. One of miRNA1's direct targets is the prevalent inward rectifying potassium ion channel Kir2.1 [5], which controls the final repolarization of arrhythmogenic potential, managing cardiac excitability, and regulating the resting membrane potential [15,16]. It is known that a number of genes influence the expression of the Kir2.1 protein. The expression of the membrane-bound ion channel in cardiomyocytes has been documented to decrease in response to downregulation of *Ptk9*, *Kcnj2* (Kir2.1), and *Gja1* (Cx43) [14].

Prior research has demonstrated that miR-1 silences *PTK9, Kcnj2*, and *Gja1* through RNAi-mediated translation suppression [14]. For this study, the expression of these three genes will be studied to test the RNAi function of Biophysical-only mutants (miR-1 with mutations in the seed sequence).

Gene Expression Regulation via Novel Biophysical Modulation:

Previously, we thought that miRNA could only control gene expression through the traditional RNAi mechanism. Recently, our lab discovered a novel action of gene regulation via biophysical modulation. Unlike the RNAi mechanism, miRNA can directly bind to functional protein to regulate their expression. According to our publication of "MicroRNA Biophysically Modulates Cardiac Action Potential Binding to Ion Channels," miR-1's direct binding to its target protein (Kir2.1) decreased its expression level (decreased action of ion channel). The regulation of target gene can be characterized by: 1) conventional RNAi to regulate the expression of proteins, including ion channels, which requires hours to days to take effect, and 2) newly-discovered direct binding with ion channels that quickly (seconds to minutes) results in biophysical modulation.

In the study, miR-1 mutants were mutated at different nucleotides to observe the changes in relative gene expression. Findings indicated that biophysical control of the target protein was impaired by alterations in the miRNA's core region (nucleotides 10-15). These were termed RNAi-only mutants, because they specifically disrupted the biophysical function while maintaining the RNAi function. The findings suggested that the nucleotides in the core sequence may be crucial for miR-1's interaction with the target protein (Kir2.1).

Based on the conclusions of our previous study, we hypothesized that biophysical-only miR-1 mutants would specifically disrupt the RNAi function while maintaining biophysical function. As mentioned earlier, seed sequence plays a critical role in the RNAi mechanism of gene expression regulation. Therefore, we hypothesize that mutations at the seed sequence of miR1 would make good candidates for biophysical-only mutants. A biophysical-only mutant would be able to biophysically modulate Kir2.1

(observe decrease in ion channel function) but would not be able to translationally repress the mRNA expression of *Ptk9*, *Kcnj2*, and *Gja1* genes .

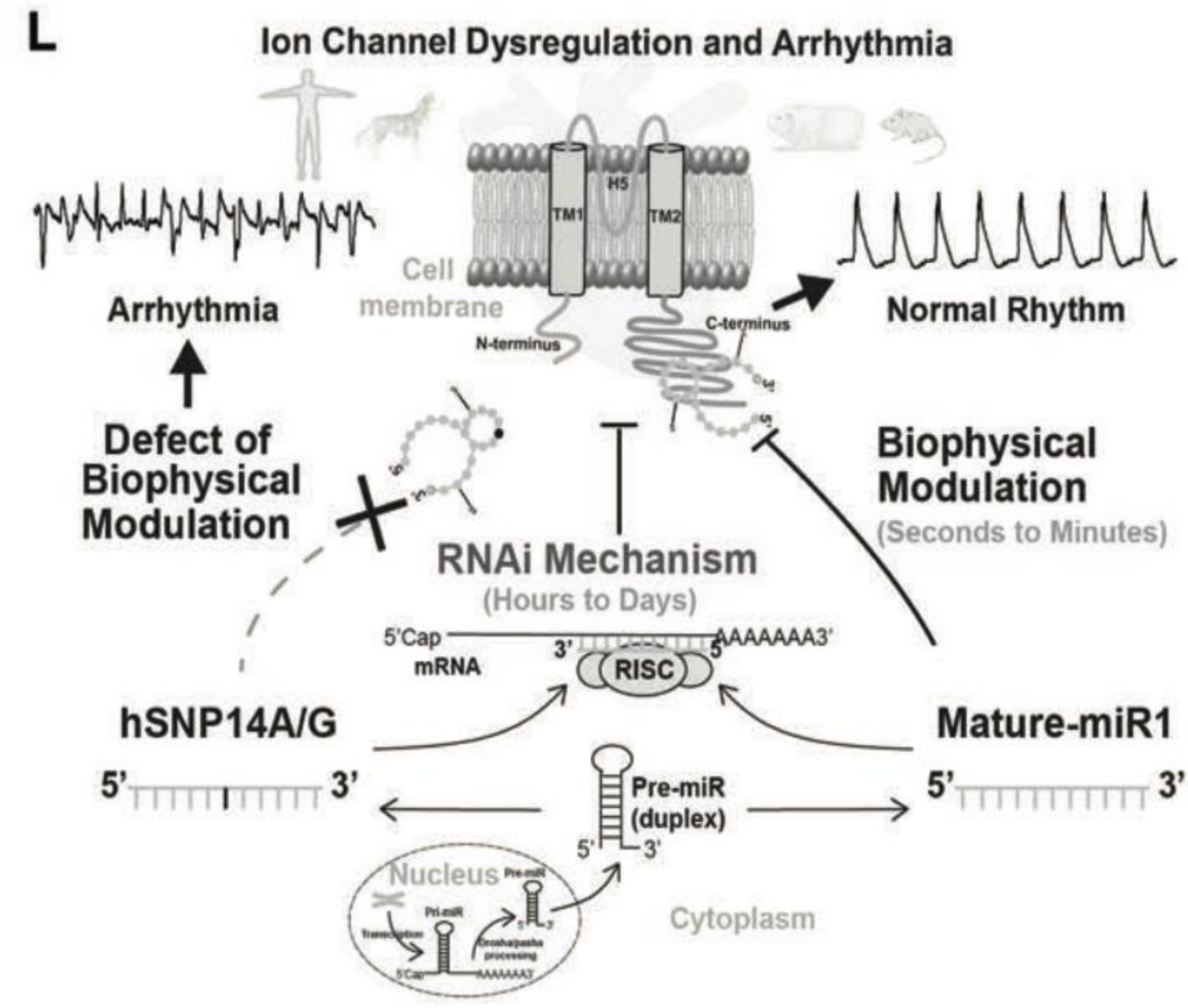


Figure 5: Role of the traditional post transcriptional RNA interference (RNAi) mechanism of ion channel expression and the recently discovered biophysical modulation, in cardiac ion channel dysregulation and arrhythmia.

Materials and Methods

Molecular Cloning

Basic molecular cloning techniques were used to construct a recombinant DNA molecule of interest. For the experiment, the recombinant DNA sequence pSin-EF1a-Kir2.1GFP with mKCNJ2 3'UTR region was created using pSin-EF1a-GFP as the construct and pSin-Kir2.1-3'UTR as an insert. I optimized the digestion of the construct and insert, DNA extraction (using miniprep, gel electrophoresis, and gel extraction), ligation, and transformation. The creation of pSin-EF1a-Kir2.1GFP-mKcnj23'UTR and pSin-EF1a-Kir2.1GFP-hKCNJ23'UTR was confirmed using diagnostic PCR. In the list of protocols is a full protocol used for the production of recombinant DNA of interest.

The recombinant DNA pSin-EF1a-Kir2.1GFP-mKcnj23'UTR expressed the target gene of miR-1 (kir2.1GFP fusion gene) followed by the 3'UTR region of mouse or human KCNJ2. The 3'UTR region of Kcnj2 contains a sequence targeted by miR1, which is critical for the dual expression of both genes.

Lentiviral Production

To successfully transduce the viral genome into the host cell (HEK293 or HEK293T), lentivirus LV-EF1a-Kir2.1GFP-mKcnjg23'UTR was created . In order to create lentivirus, HEK293T cells were transfected with the target DNA (pSin-EF1a-Kir2.1GFP-mKcnj23'UTR), pMD-G (vector envelope plasmid), and pSPAX-7 (vector packing plasmid). The lentiviral vector was harvested 24-48 hours later and stored at -80C. Please look at the protocol list to get the detailed method used for lentiviral production.

The final lentiviras included a viral genome with integrated recombinant DNA produced via molecular cloning (LV-EF1a-Kir2.1GFPmKcnj223'UTR). The harvested lentivirus was used to introduce a stable integration of expressed EF1a-Kir2.1GFPmKCNJ2-3'UTR into host cells.

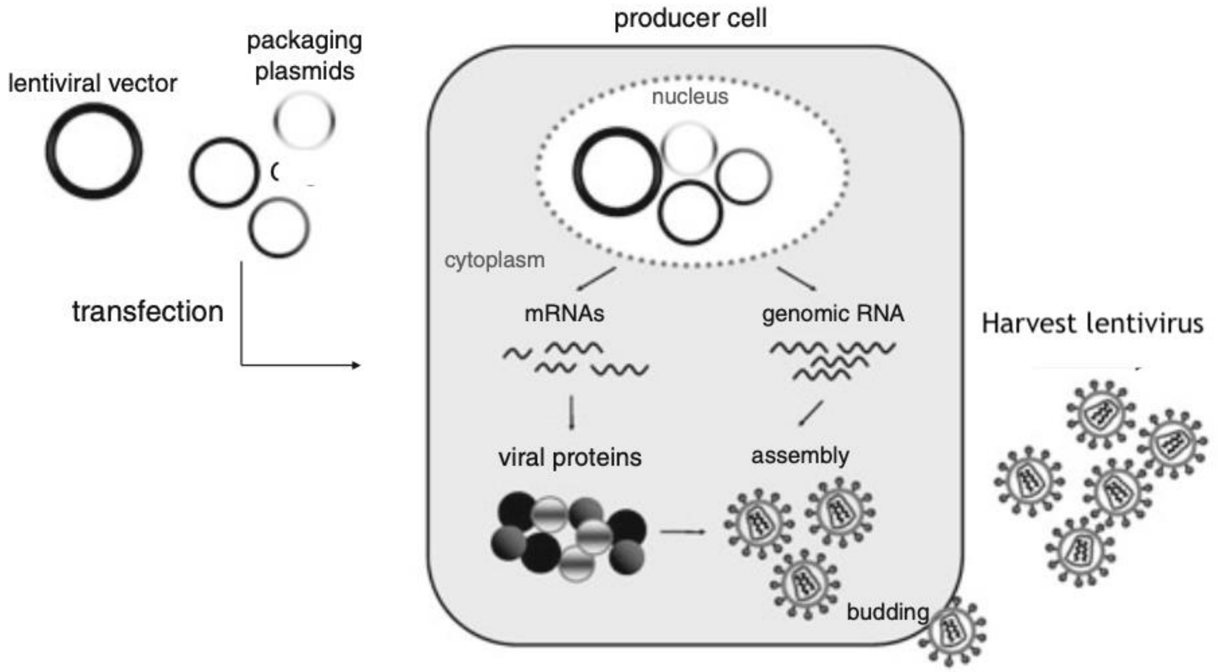


Figure 6: Production of lentivirus vector using target DNA (pSin-EF1a-Kir2.1GFp-mKcnj23'UTR), pMD-G (vector envelope plasmid), and pSPAX-7 (vector packing plasmid).[17]

Stable Cell Line Establishment

Lentiviral vector was transduced into host cells (i.e., HEK293 and HEK293T cell). The EF1a-Kir2.1GFPmKCNJ2-3’UTR DNA was incorporated into the genome of host cells. Therefore, lentivirus-infected cells with successful integration would have green fluoresce because of the expression of GFP gene (fluorescence producing gene in the DNA of interest). GFP-fluorescence was used as indicator of positively-infected host cells with integrated DNA of interest, which were sorted by FACS machines. Purified GFP+ host cells were expanded to established stable cell line and used to study the RNAi action of miR1 and mutants.

Note: The generation times of HEK293 and HEK293T vary. HEK293T are faster growing cells. Either maintained cell line was employed depending on the requirements of the experiment.

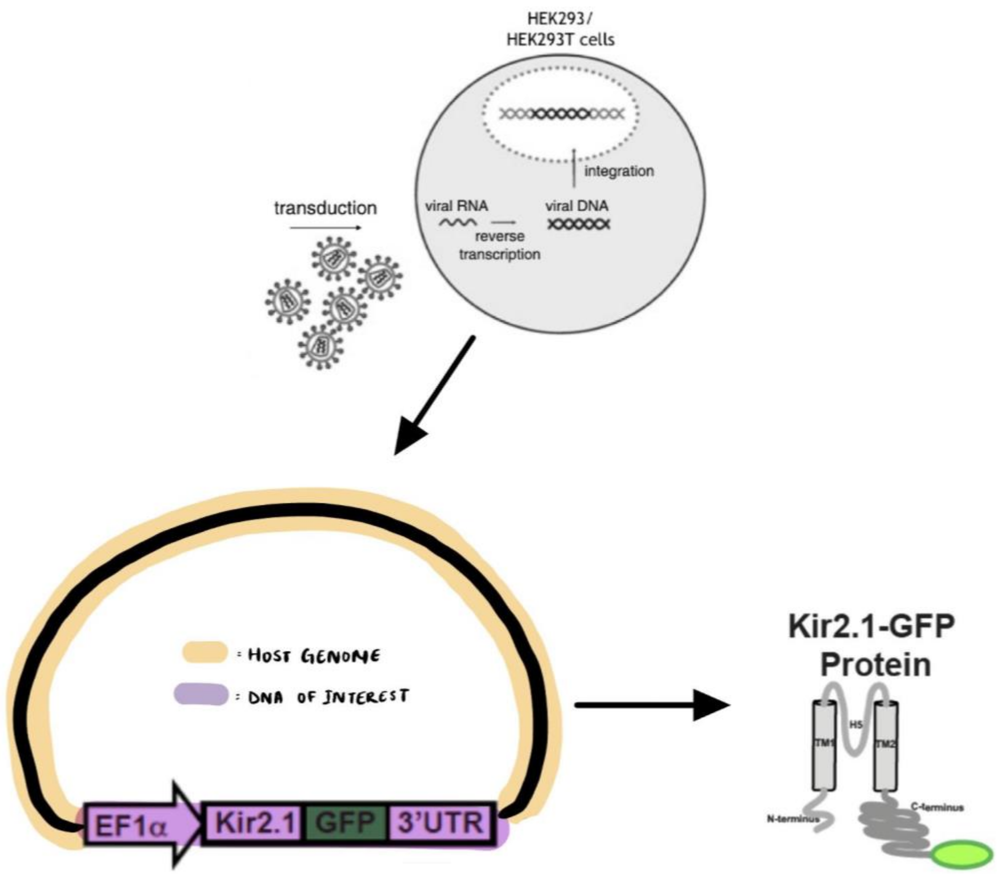


Figure 7: Visualization of host cell with successful integration of DNA of interest. Fluorescein host cells were used to produce and maintain and produce stable cell lines.

Dual Luciferase Assay

Host cells (HEK293 or HEK293T) were transfected using 0.45 uL of PEI. 100 ng of DNA (mPTK9, mKCNJ2 or mCx43) and 1uL of RNA (miR-1, miR-451, MU2C, MU2C3U, MU14G). We had optimized experiment conditions, including quantity of transfection agent, amount of DNA plasmid required to see suppression effect, cell confluency, amount of RNA, volume of luciferase reagents used, type of RNA (single-stranded versus double-stranded RNA) and the amount of incubation time after transfection.

Promega's Dual Luciferase Assay kit was used to quantify the relative gene expression of the target gene. The amount of firefly luciferase protein produced under differing experimental conditions was used to quantify the relative expression of target gene (expression of both firefly luciferase protein and target protein are controlled by the upstream 3'UTR region).

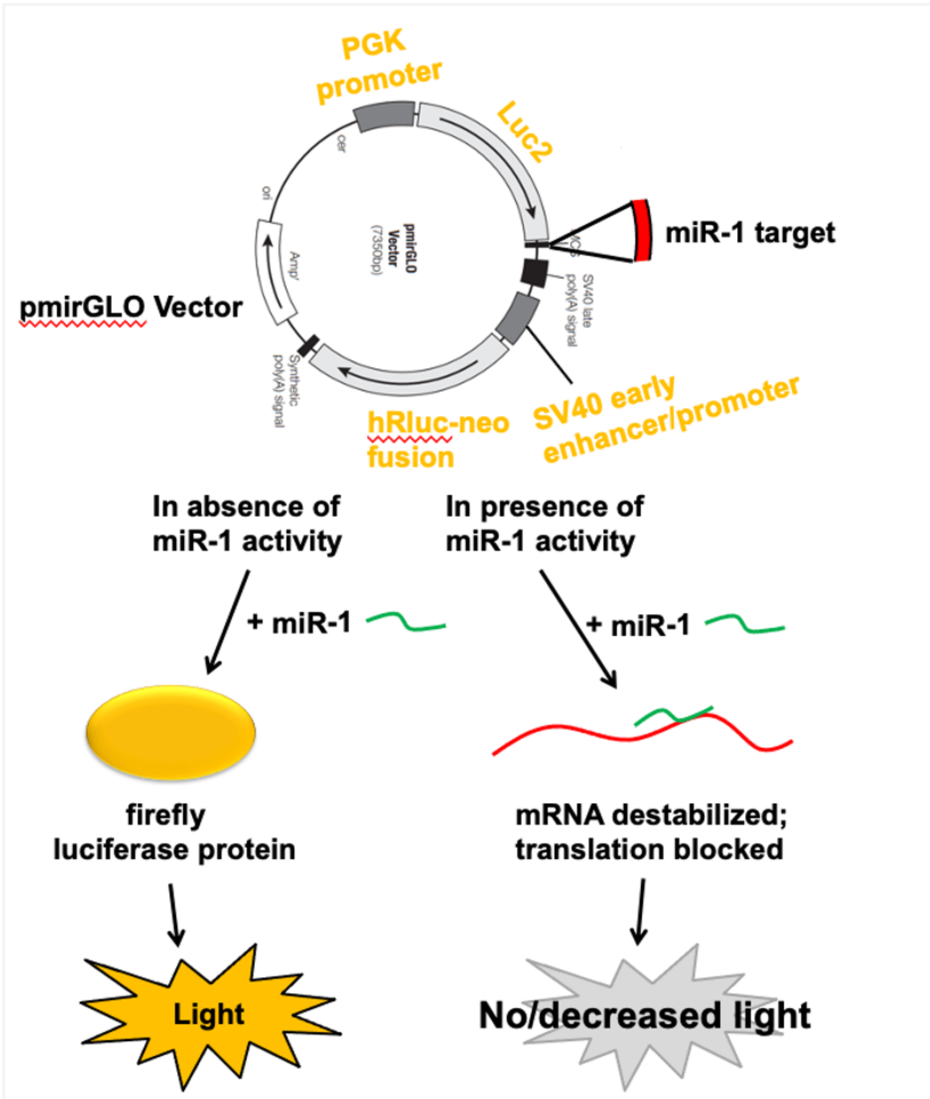


Figure 8: Suppression of Firefly Luciferase and Target Protein (IK1) are controlled by the conjugation of miRNA and 3'UTR (upstream to both Firefly Luciferase gene and Target Gene). Light (produced by Firefly Luciferase Protein) can be quantified to observe the relative suppression effect of miRNA on the target mRNA.

Western Blot

Western blot was used to observe the suppression/ downregulation of the functional target protein produced after the transfection of the host cell with RNA (miR-1, miR-451, MU2C, MU2C3U, MU14G). We had also optimized the experiment conditions of western blot, including quantity of transfection agent, amount of DNA plasmid required to see suppression effect, cell confluency, amount of RNA, and the amount of incubation time after transfection. Cells were harvested 24-48 hours after transfection. Host cells were lysed to release proteins into the sample used for western blot. Please find the detailed western blot protocol used. Data collection and analysis was done by my colleague, Saanvi Ganesh.

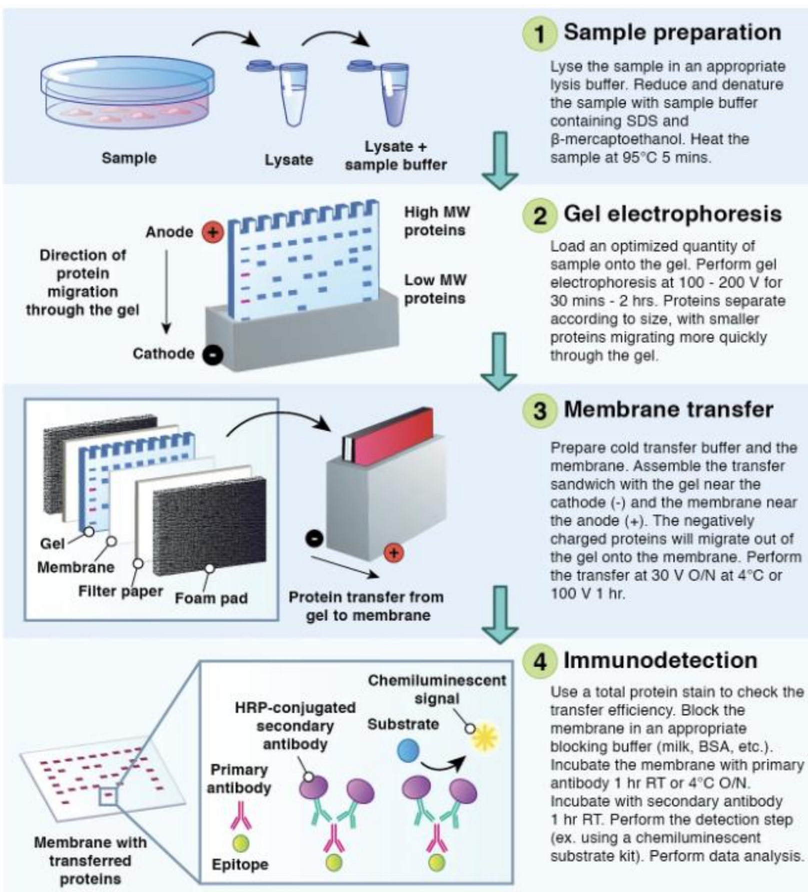


Figure 9: Visual schematic of western blot procedure used for quantification of relative target protein expression.

Results

Recombinant DNA Sequence of Interest: pSin-EF1a-Kir2.1GFP-mKCNJ23'uTR

For the molecular cloning experiments, the construct (pSin-Ef1a-GFP) and the insert (pSin-Kir2.1GFP) were individually extracted. Following PCR amplification of the insert, gel electrophoresis was used to visualize the results. DNA from the gel was cut and extracted to isolate the insert plasmid.

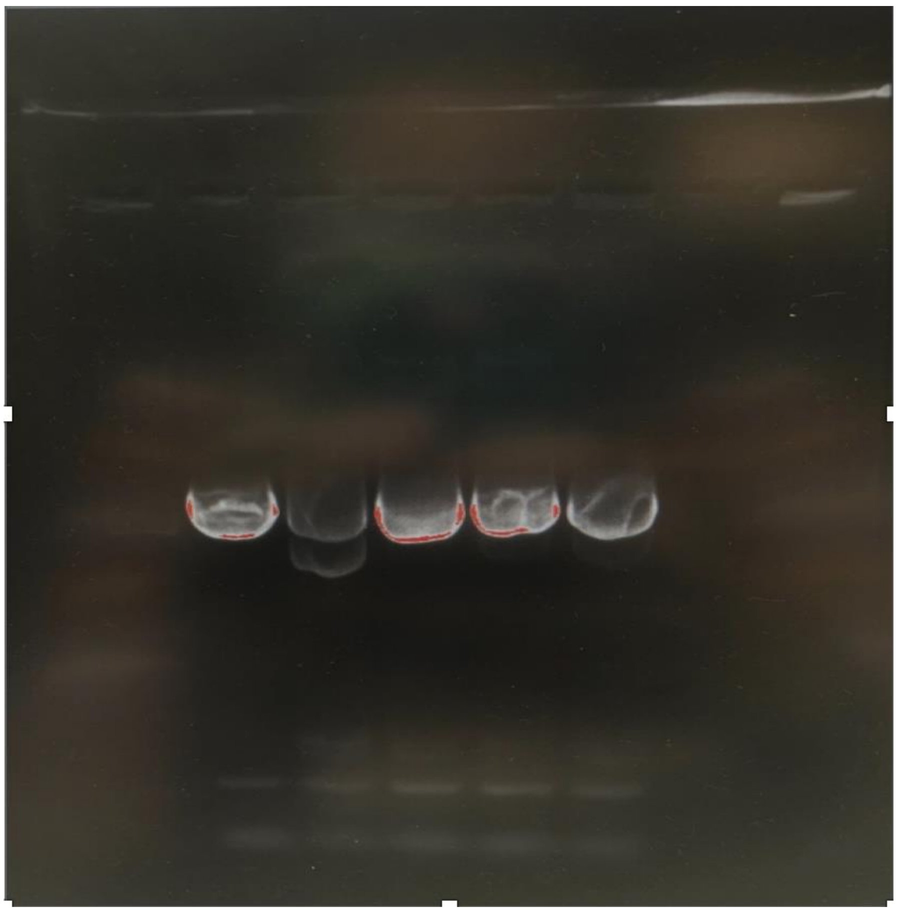


Figure 10: Visualization of amplified insert plasmid compared to the DNA ladder. As expected, the size of the insert plasmid was ~2 Kb, indicated by the DNA ladder.

The construct and the insert plasmid were individually digested with respective restriction enzymes. Information about the restriction enzymes used and the expected band size are attached in the protocol. The correct digested band (examined by the band size) was cut and extracted from the gel.

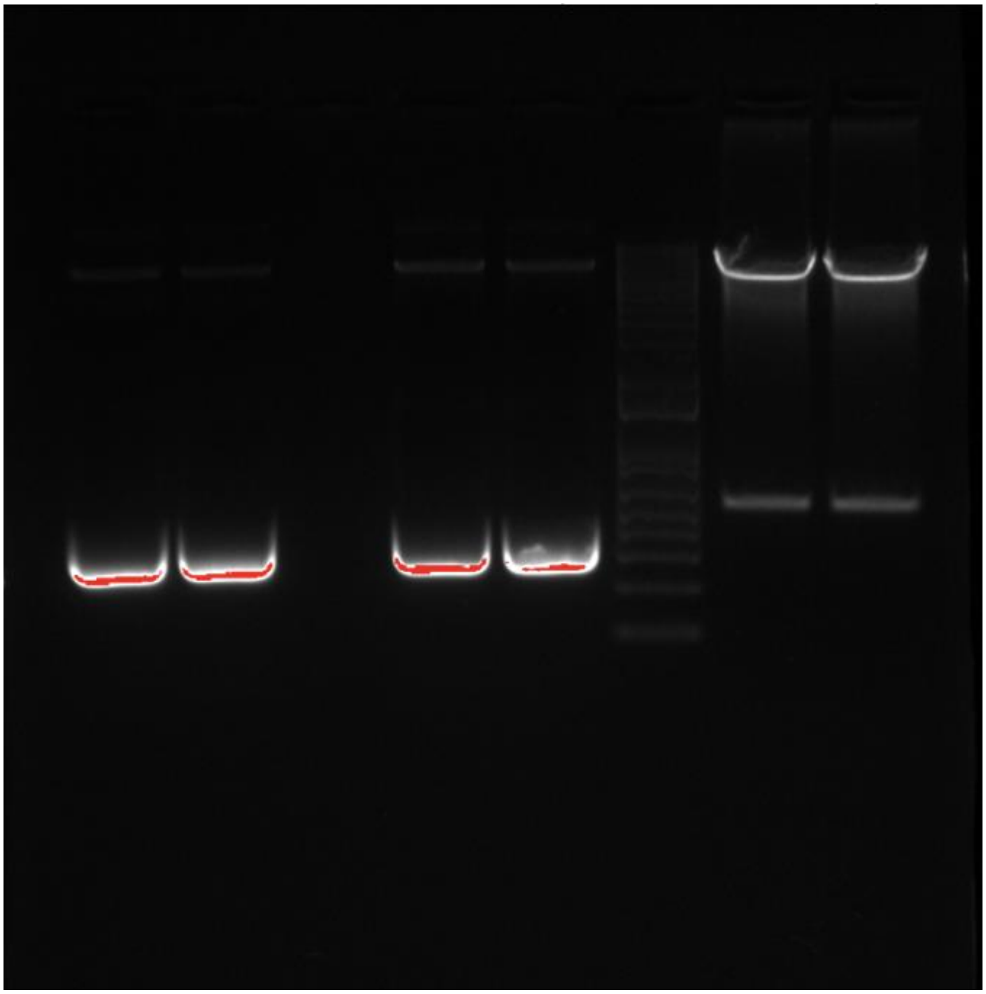


Figure 11: Lane 1 and 2 (from left side) on the gel shows digested mouse Kcnj23'UTR insert plasmid (expected band side ~300 bp). Lane 4 and 5 on the gel shows digested human KCNJ23'UTRinsert plasmid (expected band side ~300 bp). Lane 6 is the D NA ladder. Lane 7 and 8 on the gel shows digested construct plasmid digested with SexAI and BsrGI (expected band side at ~8000 bp and ~400 bp).

Digested construct (from band size of ~8000 bp) and the insert (from band size of ~300 bp) plasmid were extracted and purified. Ligation of the construct and insert plasmid was performed to produce the final DNA recombinant sequence (pSin-EF1a-Kir2.1GFP mKCNJ2-

3’UTR and pSin-EF1a-Kir2.1GFP hKCNJ2-3’UTR). Diagnostic PCR was followed by digestion of DNA of interest and visualization via gel electrophoresis.

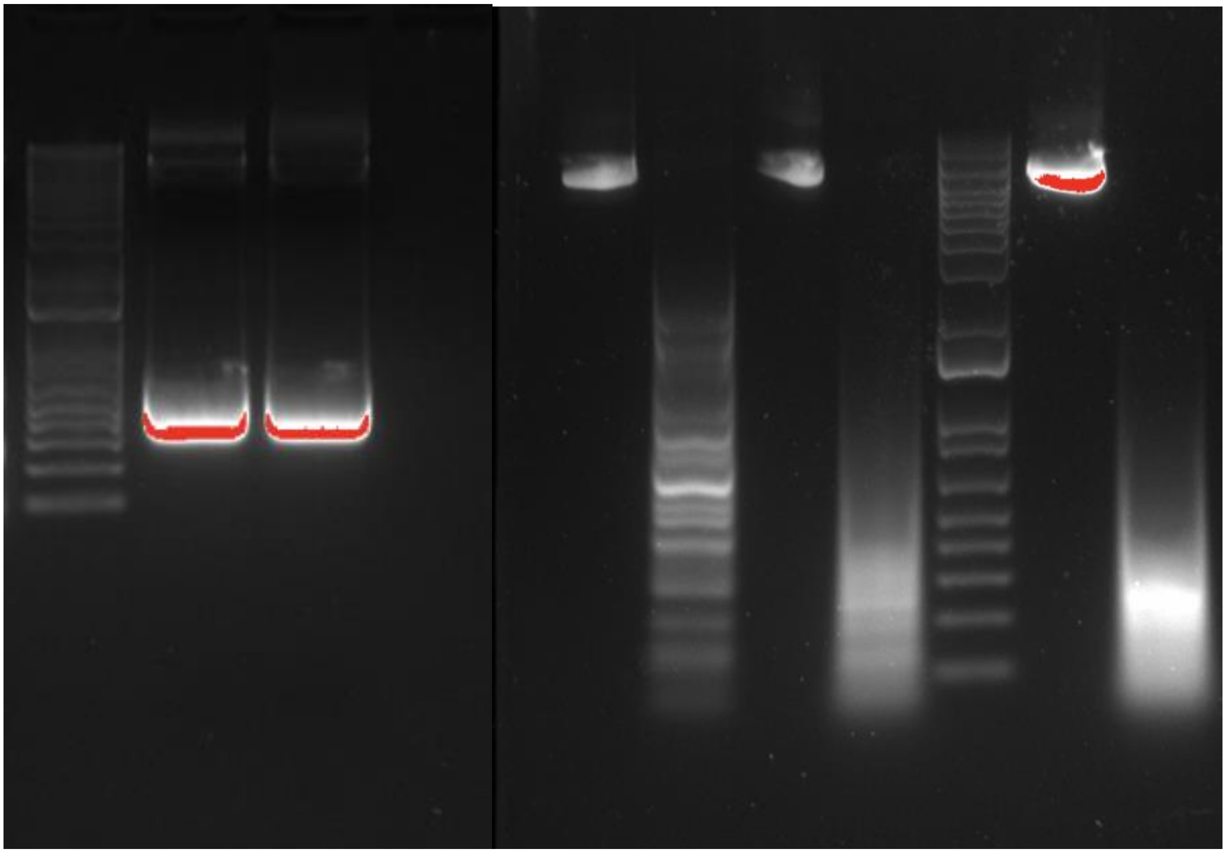


Figure 12: Panel A: Digestion of recombinant DNA sequence with AscI RE (expected band at ~568 bp). Panel B: Lane 1 and 2 (pSin-EF1a-Kir2.1GFp-mKCNJ2 3’UTR plasmid 1 undigested and digested respectively), Lane 3 and 4 (pSin-EF1a-Kir2.1GFp-mKCNJ2 3’UTR plasmid 2 undigested and digested respectively), Lane 5 is the DNA ladder and Lane 6 and 7 ((pSin-EF1a-Kir2.1GFp-hKCNJ2 3’UTR plasmid Pa1 undigested and digested respectively).

HEK293/ HEK293T Stable Cell Line Expressing LV-EF1a-Kir2.1GFP-mKCNJ2-3'UTR

A. B.

Figure 13: Panel A: Top brightly fluorescing colony indicates positively-infected HEK293 cells with expression of target DNA. Panel B: Bottom brightly fluorescing colony indicates a good colony of HEK293 cells with expression of target DNA. (Other brightly fluorescing colonies in the fluorescence image were not used for stable cell line formation due to irregular morphology of HEK293 cells.

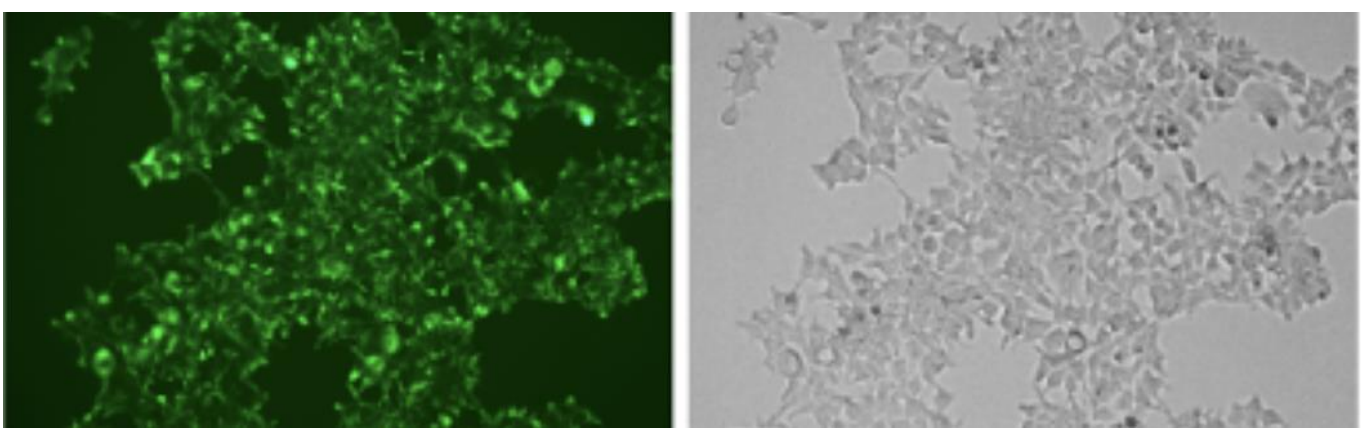


Figure 14: Stable HEK293 cell line expressing LV-EF1a-Kir2.1GFP-mKcnj2-3'UTR vector. Fluorescence is observed due to the expression of the GFP gene from the lentivirus vector.

Relative Target Gene Expression for Biophysical-only Mutants

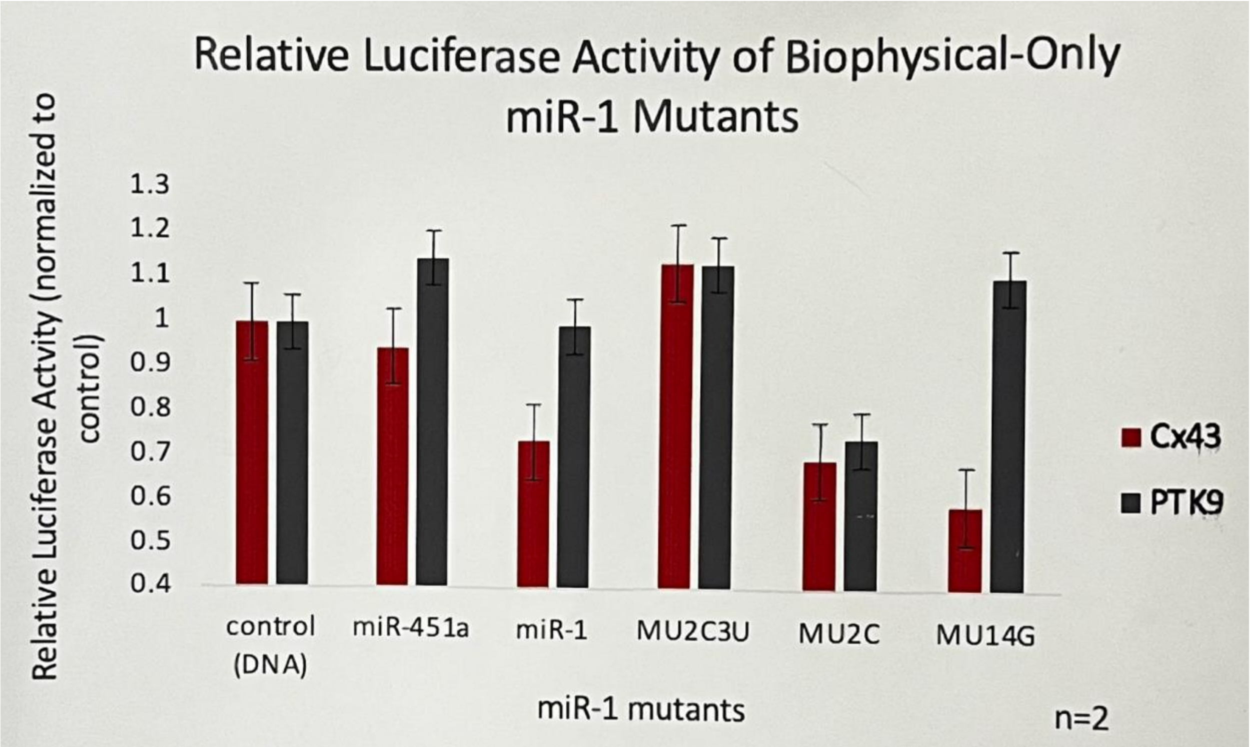


Figure 15: Luciferase activity of miR-1, miR-451a and miR1 mutants showed that miR-1, MU2C and MU14G suppressed the expression of Cx43, while MU2C3U and miR-451a didn't. Further optimization of experiment conditions for PTK9 gene is required, because under current conditions, miR-1 didn't suppress the expression of PTK9.

Relative suppression of target protein was also evaluated via western blot to validate the results from the luciferase assay. Data was gathered and analyzed by my colleague Saanvi Ganesh. This paper focuses on my contribution to further understanding of the subject matter. Therefore, western blot data will not be discussed in this section of my paper.

Discussion

Cardiac arrhythmias are caused by an irregular electrical impulse that leads to an abnormal heart rhythm [10]. Factors contributing to the maintenance of electrical impulse are critical for maintaining the normal rhythm of the heart. A wide range of ion channels in the cardiomyocytes are responsible for maintaining the normal electrical impulse[18]. In the cardiomyocytes, microRNA (miR) play a significant role in the regulation of ion channels and transporters that are responsible to generate cardiac electrical signals [11, 12].

MicroRNA (miR) are small non-coding RNA that play an important role in the regulation of their target gene. miR-1 is a predominant miRNA in cardiomyocytes. *PTK9*, *Gja1*, *Kcnj2*, and other target genes are directly regulated by miR-1. Kcnj2 encodes an inward rectifier potassium ion channel that conducts I_{K1}. Downregulation or partial suppression of I_{K1}, a potassium ion channel in the cardiomyocytes, are correlated with increased risks of cardiovascular diseases such cardiac arrhythmias [19].

In cardiomyocytes, miR-1 can suppress or downregulate the expression of target gene/ protein via two different mechanisms: 1) the RNA interference (RNAi) mechanism, the miR-1 binds to the 3'UTR region of the target gene via partial or complete complementation. The mRNA-miRNA binding either degrades the mRNA or inhibits it from interacting with the ribosome and complete translation. In both cases, the expression of the target gene is suppressed. 2) biophysical modulation of miR-1, miR-1 directly binds to functional target proteins (e.g., Kir2.1) and suppresses the activity of the membrane protein (decreased potassium ion transportation across the ion channel). The mechanism of approach and the amount of time required to observe suppression effect are distinct for RNAi and biophysical modulation of miR-

1. Therefore, we hypothesized that these regulatory mechanisms may be ,in fact, independent of one another.

Our lab's previous research had shown that some special mutatants of miR-1 have effects on the target gene/ protein's suppression via one mechanism while still maintaining the regulation of the other mechanism. Such as RNAi-only mutants (mutated at the core sequence- 10-15th nucleotide) specifically disrupt biophysical action of miR-1 while maintaining the RNAi mechanism[5]. Similarly, biophysical-only mutants (mutated at the seed region- 2-8th nucleotide) should specifically disrupt the RNAi mechanism while maintaining the biophysical action of miR-1.

We hypothesized that biophysical-only mutants can be separated from RNAi-only mutants, which will demonstrate that RNAi and biophysical action of miR-1 are two independent mechanisms. To test this hypothesis, a new recombinant DNA sequence (pSin-EF1a-Kir2.1GFP-3'UTR) was generated that allowed co-expression of miR-1 target gene (Kir2.1) and GFP gene together with miR1-bound 3'UTR sequence. Biophysical-only mutants (e.g., Mu2C3U) were used for the experiment to study the effect of suppression via RNAi mechanism, while RNAi-only mutant (e.g., MU14G) was used as negative control.

Co-transfection of DNA (PTK9, Kir2.1 or Cx43) with RNA (miR-1, miR-451a, MU2C, MU2C3U AND MU14G) into HEK293 stable cell line was established to observe the relative suppression of target gene via the RNAi mechanism. Luciferase assay was used to quantify the relative gene expression. Computational data from dual luciferase assay showed the MU2C3U had weaker suppression effect on the expression of Kir2.1GFP via RNAi mechanism than miR-1 and RNAi-only mutants (Mu14G). The data supports that MU2C3U may be a good candidate for Biophysical-only mutant.

Inside out patch clamp data generated by my colleague Dr. Xiaoping Wan evaluated the suppression effect of I_{K1} membrane protein for different biophysical-only mutants. Data showed no suppression in the biophysical action of miR-1 for biophysical-only mutants (data not shown). The data from inside-out patch clamp (biophysical function assay) and quantification of luciferase assays (RNAi action assay) demonstrate that Mu2C3U is a biophysical-only mutant that specifically disrupts the RNAi action while maintaining the biophysical action of miR-1.

To further support the findings of this study, optimization and repetition of dual luciferase assay would be considered. In addition, western blot analysis and qPCR analysis can be done to validate the conclusions of the study. Other gene expression quantification methods such as: Northern blot, quantitative reverse transcription PCR (qRT-PCR), serial analysis of gene expression (SAGE) and DNA microarrays, can be employed to further validate the results of the study.

In summary, this study attempts to separate the two discovered mechanisms of miRNA, biophysical vs. RNAi actions. We have identified biophysical-only and RNAi-only mutants and different nucleotides are required for each mechanism. A future direction for this study would be identifying how mutation of a given nucleotide affects the binding efficiency to target gene (in RNAi mechanism) and target protein (in biophysical action of miRNA). Understanding the role of a nucleotide in the binding can help us appreciate the complexity of each mechanism. In addition, currently the biophysical modulation of miR-1 is only discovered in cardiomyocytes. It would be interesting to observe if similar biophysical action of miRNA is observed in different types of cell in regards to a wide range of miRNA or if this mechanism is specific to miR-1 in cardiomyocytes.

Acknowledgement:

I want to express my gratitude to Dr. Jidong Fu for his unwavering support and direction during my research career. He has advanced my knowledge, both as a researcher and as a student. I want to express my gratitude to Dr. Dandan Yang and Dr. Lalita Uttarwar for helping me advance my study and teaching me essential research methods. I appreciate my colleague Saanvi Ganesh's help in generating the western blot data I needed for my study. Finally, I want to express my gratitude to the OSU Undergraduate Research committee for its financial assistance. Thank you everyone for your ongoing assistance in making this research feasible.

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Protocols

Molecular Cloning:

pLV-THM-Kir2.1GFP-h3'UTR

1. PCR to amplify Human KCNJ2 3'UTR

   hKCNJ2UTR-AscI-sen: GATggcgcgCCGAGTCTAGTCAGTGTA

   hKCNJ2UTR-NdeI-sen: ATCcatatgCTAAGGTGAAGGTGGGCTG

   Cut out a band of 382bp.

2. Digest PCR hKCNJ2 3'UTR with AscI + NdeI

3. Digest pLV-THM-Kir2.1GFP with AscI + NdeI

4. Ligation and transformation with STBL3

5. Digest with Pme I, Fragments: 10.3 + 2.3 Kb

6. Send two clones for sequence with primer:

   GFP-s-sen: CACTACCTGAGCACCCAGTC. (primer box 4, 2-9) 5378

7. Make a Glycerol stock of pLV-THM-Kir2.1GFP-h3'UTR

pLV-THM-Kir2.1GFP-m3'UTR

1. PCR to amplify Mouse Kcnj2 3'UTR

   mKcnj2UTR-AscI-sen: TGGggcgcgccGGTGTGAAGCAGTCCGTTCA

   mKcnj2UTR-AscI-AntiS: TCTggcgcgccTCTTTCTTAATGTGACACTGAGC

   Cut out a band of 384bp.

2. Digest PCR hKCNJ2 3'UTR with AscI

3. Digest pLV-THM-Kir2.1GFP with AscI. After digestion, de-phosphorylation of CIP.

4. Ligation and transformation with STBL3

5. Diagnosis by PCR

mKcnj2UTR-AscI-sen: TGGggcgcgGGTGTGAAGCAGTCCGTTCA

WPRE-S-anti: CCACAACTCCTCATAAAGAG (primer box-8, 2-5) (prepared in first round cloning)

PCR band: 568 bp

6. Send two clones for sequence with primer:

   GFP-s-sen: CACTACCTGAGCACCCAGTC. (primer box 4, 2-9) 5378

7. Make a Glycerol stock of pLV-THM-Kir2.1GFP-m3'UTR

Lentiviral Production

1. Use lipofectamine 2000 (use according to manufacturer's protocol).
2. Mix lipofectamine with OPTI-MEM®I medium (Mixture 1).
3. Mix all three DNA with OPTI-MEM medium (Mixture 2).
4. Let mixture 1 and mixture 2 sit at room temperature for 5 minutes.
5. Mix Mixture 1 and Mixture 2 together and let stand for 20 minutes at room temperature.
6. Change the medium of HEK T-cell to OPTI-MEM°I medium (do not contain serum).
7. Add the mixture (of M1 and M2) into the HEK T-cell.
8. Put back the cells into the incubator for 6 hours.
9. Change the medium of HEK T-cell back to DMEM.
10. Collect the supernatant at 24 and 48 hours after transfection.
11. Filter the supernatant using 0.45pm filter (Millipore).
12. Place the virus at -80°C before use.
13. Below are the reagents and their quantity used for the production of target lentiviral.

    Please use the given amount of each reagent and optimize depending on your experiment condition.

Keeping and Transfection of 293T cells:

Medium:

DMEM 10%FBS 1% Pen Strep

Use 0.05% Trypsin to split cells

Cells double every 24 hours, and need to change medium frequently.

LF-2000=Lipofectamine 2000

	100mm Plate	150mm Plate
Area	1	2.92
Cell/ Plate (the day before)	6.7*10^6	19.6*10^6

Use the table above to find the ratio and the number of cells to be plated for your experiment.

Next Day:

In 5mL Tube 1

100mm Plate	**150mm Plate**
10ug delta 8.9	30ug delta 8.9
2.5ug pMD.G	7.5ug pMD.G
5.0ug DNA	15ug DNA
1.5 mL opti-MEM	4.3mL opti-MEM

In tube 2+

100mm Plate	**150mm Plate**

1.5mL opti-MEM	4.3mL opti-MEM
50uL LF-2000	150uL LF-2000

14. Incubate 5 mins
15. Mix tube 1 and 2
16. Wait 20 mins and then add the mixture into HEK293T cells for successful transfection.

Dual Luciferase Assay

1. Aspirate the media from the plate. Use 1-2 mL of PBS to wash cells.
2. Make a lysis buffer using 1:5 ratio of PLB lysis buffer and DEPC. Multiply the recommended amount of lysis buffer to be added in each well (variable depending on well size) with the number of wells you need to lyse. Please find the information of the recommended lysis buffer in Promega’s Dual Luciferase Assay Protocol.
3. Place the cell plate on a plate tilter for 5 minutes to allow equal lysis of cells.
4. Pipette the cell lysate into a 1.5mL eppendorf tube.
5. Prepare L and S buffer as recommended by Promega’s Dual Luciferase Assay Protocol.
6. Add the recommended amount of L buffer to a blocked cell plate (to avoid leaking of luminesce from neighboring wells).
7. Add L buffer first with the recommended amount from Promega’s Dual Luciferase Assay Protocol. You can increase the amount of L buffer to get a higher read value.
8. Use a spectrometer to measure the firefly luciferase activity.
9. Add S buffer with the recommended amount from Promega’s Dual Luciferase Assay Protocol. You can increase the amount of L buffer to get a higher read value.
10. Use a spectrometer to measure the renilla activity.

Additional Data

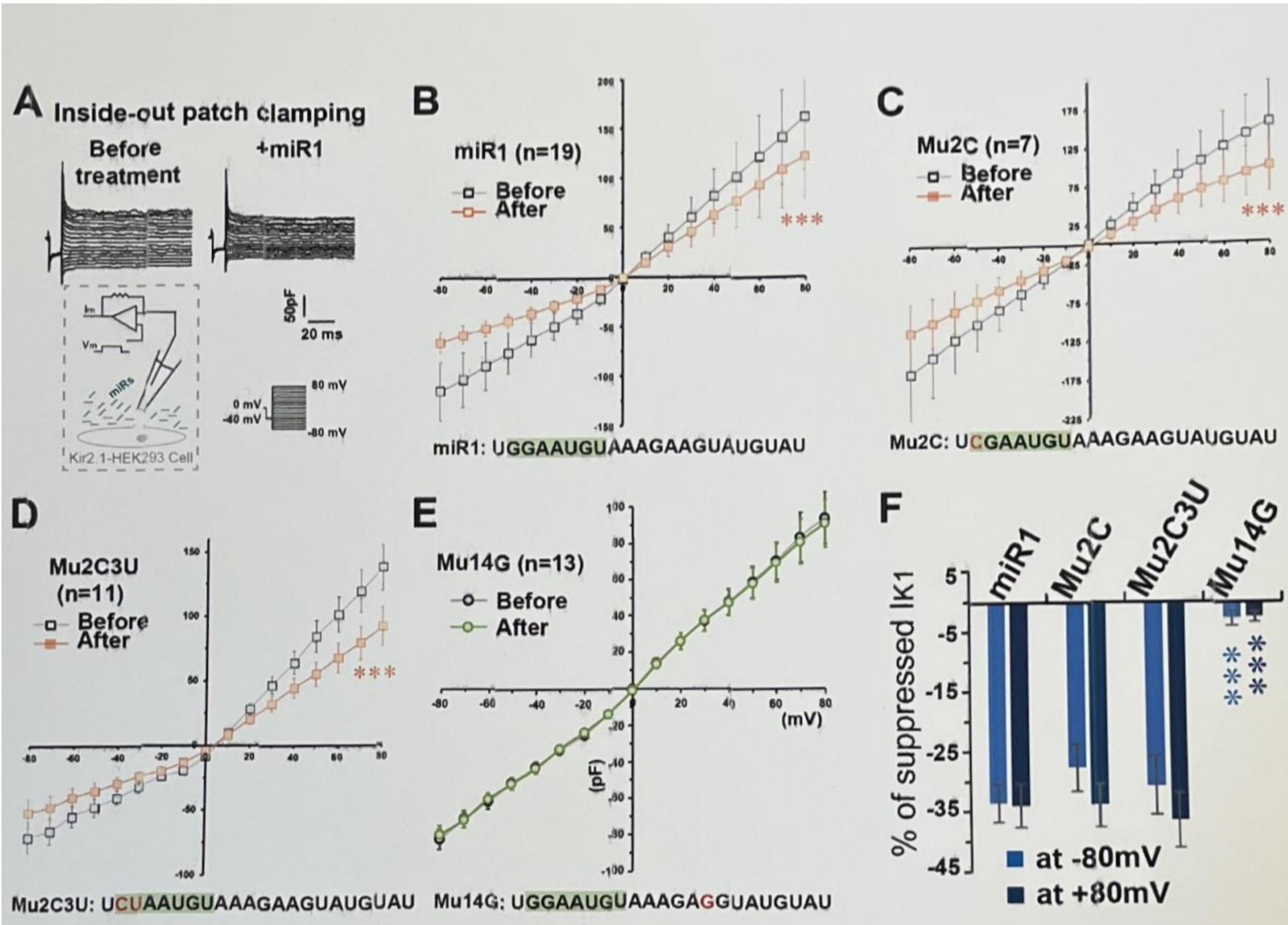

Figure 16: Inside-out patch clamp data produced by Dr. Dandan Yang. Inside-out patch clamp measures the voltage difference across the membrane. Results from the inside out patch clamp shows that the suppression of Ik1 membrane protein is suppressed when the miRNA is mutated in the core sequence (10-15th nucleotide). Whereas, the suppression of Ik1 is relatively constant when the miRNA is mutated in the seed region. Results show the biophysical-only mutants have maintained the biophysical action of miR-1.