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piRNA expression in the tube feet of *Lytechinus variegatus*

by

Reagan Milliet

A Thesis
Submitted to the Honors College of
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ABSTRACT

Tissue regeneration is an area of research with implications for medicine and animal health. While nearly all living multi-cellular organisms are capable of regeneration, there are major differences in the animal kingdom. Some organisms are capable of regenerating virtually every cell in their bodies. In hopes of engineering tissue regeneration for medical applications, the mechanisms by which organisms regenerate are being widely investigated. To better understand regeneration, the role of P-element Induced WImpy testis (PIWI) proteins are being evaluated. In collaboration with PIWI-interacting RNAs (piRNAs), PIWI proteins have been proven instrumental to transposon silencing and maintenance of the genome in germline cells. In recent years, PIWI-piRNA functions have also been discovered in somatic cells. As research continues, the link between piRNA and tissue regeneration is expanding. To further understand this connection, this study aims to investigate the relationship between somatic piRNAs and regeneration in the non-model organism *Lytechinus variegatus*. A member of the echinoderm phylum, green sea urchins can regenerate their spines and tube feet. While some studies have been done, piRNA expression has yet to be observed in the somatic tissues of this organism. In this study, piRNA expression was examined in regenerating and control tube feet samples through high throughput small RNA sequencing. While differential expression (DE) did not occur between these two conditions, piRNA expression was observed in somatic cells. Through Gene ontology (GO) and RepeatMasker analyses, it was determined that these piRNAs target transposons and gene segments involved in cell division, DNA Replication, transcription, and translation.

Keywords: piRNA, PIWI, Transposons, *Lytechinus variegatus*

DEDICATION

I would like to dedicate this thesis to my soon-to-be wife Hannah for always providing me with unconditional love and encouragement, to my family for supporting me as I strive to further my education, and to my best friends Kyle and Logan for always being there for me.

ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Alex Flynt, for taking me under his wing and allowing me to conduct research in his lab. His guidance regarding the research process and synthesis of this paper was invaluable. I would also like to thank the graduate student, Beatriz Zancanela. I am extremely grateful for her kindness and the many hours she spent helping me every step of the way with this project. Finally, I would like to thank the University of Southern Mississippi, the School of Biological, Environmental, and Earth Sciences, and the Honors College for allowing me the opportunity to participate in research of this magnitude.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

Ago	Argonaute
Ago3	Argonaute3
Alu	Arthrobacter luteus
Aub	Aubergine
cDNA	Complementary DNA
DAG	Directed acrylic graph
DE	Differential expression
DGE	Differential gene expression
DNA	Deoxyribonucleic Acid
eIF3	Eukaryotic translation initiation factor 3
ERVs	Endogenous retroviruses
GO	Gene Ontology
GOA	Gene Ontology Analysis
H3K9	Lysine 9 residue in histone 3
L	Linker
LINEs	Long interspersed nuclear elements
LINE-1	Long interspaced nuclear element 1
LTRs	Long terminal repeats
MID	Middle
MITEs	Miniature inverted-repeats
mRNA	Messenger RNA
N	Amino-terminal

NFW	Nuclease free water
Nxf2	Nuclear export factor 2
Nxt1	Nuclear transport factor 2 like export factor 1
PAZ	PIWI–ARGONAUTE– ZWILLE
piRNA	PIWI-interacting RNAs
PIWI	P-element Induced WImpy testis
pi6	Locus on chromosome 6
RNA	Ribonucleic Acid
RNA Seq	RNA sequencing
SINEs	Short interspersed nuclear elements
siRNA	Small-interfering RNA
TEs	Transposable elements
UMMC	University of Mississippi Medical Center
Zuc	Zucchini

CHAPTER I: INTRODUCTION

One of the frontiers of modern medical research is regenerative medicine. Due to the prevalence of cancer, diabetes, and other diseases, amputations are a very common medical procedure. Additionally, over 104,000 Americans are currently waiting for an organ transplant (HRSA, 2023). Due to these factors, the ability to regenerate entire limbs and organs is currently the focus of intense research. Studies have shown that stem cells may play a vital role in advancing regenerative medicine. To better understand the role of stem cells in regeneration, scientists look to model organisms that are capable of regeneration.

Planarians, also known as flatworms, are a group of emerging model organisms. Planarians are capable of extensive regeneration through the function of neoblast cells (Wenemoser & Reddien, 2010). Upon injury, neoblasts migrate to the site of infection and proliferate. This proliferation leads to the formation of a structure called a blastema. A blastema is a group of cells that form at the site of infection and allow organisms such as planarians to regenerate tissues and even entire organs (Wenemoser & Reddien, 2010).

While the mechanism by which blastema structures lead to tissue regeneration is poorly understood, research has shown that PIWI proteins are key players in the process (Yamaguchi et al., 2020). PIWI proteins are members of the Argonaute (Ago) protein family that interact with piRNA. PIWI-piRNA complexes are most well-known for their ability to silence transposons and regulate the germline genome (Teefy et al., 2020). While it was originally thought that piRNAs are only present in germline cells, recent studies have revelated that they are also expressed in somatic cells (Théron et al., 2014). Moreover, many new functions have been discovered for the PIWI-piRNA complex. Of

these functions, their expression in regenerating tissues is of particular interest to medicine.

PIWI expression has been observed in the regenerating tissues of many organisms. One such organism is the planarian *Schmidtea mediterranea*. In this flatworm, three different PIWI proteins are expressed nearly exclusively in neoblast cells. Further research has shown that when the genes that code for these proteins are silenced, regeneration cannot occur (Li et al, 2021). PIWI proteins ensure proper cell differentiation in *S. mediterranea* by silencing transposons activated during cell division (Li et al., 2021).

The echinoderm *Lytechinus variegatus*, commonly known as the green sea urchin, is of current interest to the scientific community. Green sea urchins and other urchins can regenerate their spine structures and tube feet tissues. The mechanism by which this regeneration occurs is currently being researched. Prior studies have shown that PIWI proteins are expressed in the spines and tube feet of sea urchins (Amir et al., 2020). The presence of these proteins in regenerative tissues indicates that they might play a role in regeneration, possibly through coordination with stem cells (Amir et al., 2020). Using RNA sequencing, this study aims to determine if piRNAs are present in the tube feet of *L. variegatus*. Furthermore, this study aims to determine if piRNAs are differentially expressed in control and regenerating tube feet of *L. variegatus*. The findings of this study will hopefully open up a new avenue of research into the role of piRNAs in regeneration.

CHAPTER II: LITERATURE REVIEW

Tissue Regeneration

As stem cell research related to regenerative medicine has advanced, organisms capable of regenerating complex structures have become increasingly important. One well studied group of emerging model organisms is planarian flatworms. In these organisms, research has shown that PIWI proteins and piRNAs play an important role in their regenerative properties (Kim et al., 2020). One example of this is seen in neoblast cells. Neoblasts are all of the cells present in planarians that are capable of differentiation. These cells are the key to the regenerative properties of planarians, allowing them to regenerate lost body parts (Reddien, 2013). Planarians are capable of anterior and posterior regeneration, a process known as polar regeneration. Polar regeneration is made possible through the formation of the blastema (Umesono et al., 2013). Blastema are masses of cells that allow some adult organisms to regrow organs, a process known as epimorphogenesis. These cell masses form as cells migrate to the site of injury and begin to proliferate (Seifert & Muneoka, 2018). A head blastema is formed when an injury occurs on the anterior side in planarians. A tail blastema is formed when an injury occurs on the posterior side. (Umesono et al., 2013).

Regeneration has also been studied in organisms such as annelid worms. In the same manner as planarian worms, annelid worm regeneration occurs through the formation of the blastema. Unlike planarians, annelids have differing regenerative capabilities. While most annelid worms are capable of posterior regeneration, anterior regeneration varies widely across the phylum. Some annelids have little to no capability for anterior regeneration. Other annelids show decreasing anterior regeneration as the

injury or amputation moves closer to the posterior side (Kostyuchenko & Kozin, 2021). The variation observed among the phylum in anterior regeneration capability is attributed to the process of evolution. Phylogenetic research has shown that the capacity for anterior regeneration has been lost and regained throughout the evolutionary history of the annelid phylum. Annelids such as leeches, dinophilids, and other members of the phylum that lack anterior regeneration did not regain this ability (Kostyuchenko & Kozin, 2021). The regenerative properties of other organisms, such as echinoderms, are also being studied.

Regeneration in Sea Urchins

Regenerative capabilities vary within the phylum Echinodermata. Starfish can regenerate full-body, while sea urchins are capable of less-extensive regeneration. The organism used in our experiment was *Lytechinus variegatus*, commonly known as the green sea urchin. Green sea urchins prefer warm waters and can commonly be found in the western Atlantic Ocean (Moore et al., 1963). Like other echinoderms, sea urchins can regenerate damaged skeletal structures, tissues, and severed limbs. Spine regeneration in sea urchins occurs via biomineralization (Reinardy et al., 2015). While there is little research on how sea urchins regenerate their tube feet, stem cell-associated genes (such as PIWI) and Notch target genes are expressed during sea urchin regeneration. Due to the presence of the Vasa gene in tube feet, research suggests that multipotent stem cells could be the driving mechanism behind regeneration (Bodnar & Coffman, 2016). Sea urchin tube feet have the unique property of plasticity, allowing the overall size and disk area to change depending on the environmental conditions (Narvaez et al., 2020).

PIWI Proteins

PIWI proteins are members of the Ago protein family. These proteins are part of the clade that interacts with piwi-interacting RNAs, also known as piRNAs (Doyle et al., 2012). Piwi is made up of four domains: the amino-terminal (N), PIWI–ARGONAUTE–ZWILLE (PAZ), middle (MID), and PIWI. Piwi is also comprised of three linker (L) regions: L0, L1, and L2. These domains and linker regions are arranged into the N-PAZ and MID-PIWI lobes, which are connected by a hinge region. The N-PAZ lobe contains the L0, L1, and L2 linker regions. The MID-PIWI lobe contains L0 and L2 linker regions. PIWI proteins are structurally rearranged to bind to small RNAs. The 3' end of the small RNA is bent into a binding pocket by the PAZ domain, where it is anchored. The 5' terminal base of the small RNA binds to a conserved tyrosine in another binding pocket, provided by the MID domain. Small RNA loading and duplex unwinding are accomplished via interaction with the N domain.

Recent research has shown that the PIWI domain of Piwi has Asp at position 614, Val at postion 653, Asp, and Lys at postion 818. This is known as the DVDK tetrad. This differs from other Argonaute proteins such as Siwi. Siwi is a PIWI-like protein that has been observed in silkworms (Matsumoto et al., 2016). This protein has a PIWI domain with mutations that form a DEDX (X is D or H) tetrad. Proteins containing DEDX tetrads are capable of exhibiting slicer activity while proteins with DVDK tetrads are not. Piwi mutants with amino acid changes in the sequence of the protein at positions 617 (L617H), 625 (A625S) and 818 (K818H), have a PIWI domain compromised of a DEDH tetrad motif, making them slicer proteins (Yamaguchi et al., 2020).

The PIWI protein family was first discovered in the model organism *Drosophila melanogaster*, commonly known as the common fruit fly. Research on this organism has shown that PIWI proteins have important functions in male germline mobility control, spermatogenesis, retrotransposon silencing, and germline stem-cell maintenance. Due to these functions, they are of great interest to scientists studying regeneration and self-renewal (Han et al., 2017). While PIWI proteins are expressed in both germline and somatic cells, they are mainly expressed in the germline (Palakodeti et al., 2008).

PIWI Expression in Germline Cells

The predominant expression of PIWI proteins in germline cells explains why their function in this context is more comprehensively understood. Prior research has shown that PIWI proteins are responsible for maintaining the integrity of genomic materials by suppressing transposon activity and ensuring fertility in germline cells. The transcription and post-transcriptional silencing function of PIWI has been observed in *Drosophila*. In this organism, transcriptional silencing is carried out by the Nuclear PIWI protein, PIWI, while post-transcriptional silencing is carried out by the cytoplasmic PIWI proteins Aub and Ago3. This mechanism occurs as the PIWI-piRNA complex induces Lysine 9 residue in histone 3 (H3K9) methylation of the target transposon, forcing the transposable element into the repressive chromatin state (Wang & Lin, 2021). Alternatively, transposon silencing has been observed through the functions of Heterochromatin protein 1a and linker histone H1. Heterochromatin protein 1a is recruited by Piwi, resulting in the heterochromatinization of the target transcript. Also recruited by Piwi, linker histone H1 can also force the target transcript into the repressive chromatin state (Wang & Lin,

2021). Ensuring fertility is also a function of PIWI in germ cells. Research on the fertility of male mice has shown that the expression of the piRNA locus on chromosome 6 (pi6) is necessary to produce functional sperm cells. When this locus is not expressed, sperm production and fertilization capacity are negatively affected (Wu et al., 2020). Novel research has led to the observation of a new function of PIWI proteins in the germline. It has recently been discovered that transposons are present in the untranslated region of many mRNAs. Silencing these transposons often leads to mRNA degradation, indicating that PIWI proteins play a role in regulating gene expression (Wang & Lin, 2021).

PIWI Expression in Somatic Cells

Novel research has shed light on the role of PIWI proteins in somatic cells. Expression of PIWI proteins has been observed in the somatic stem cells of organisms possessing regenerative capabilities. This includes sponges, acoels, cnidarians, and planarians. Research has also shown that regeneration cannot occur in these organisms without expressing PIWIs and piRNAs in their somatic stem cells (Li et al., 2021). PIWI expression has been studied in the emerging model planarian organism *Schmidtea mediterranea*. This organism produces three PIWI proteins: SMEDWI-1, SMEDWI-2, and SMEDWI-3. The genes that encode these three proteins are expressed almost exclusively in the neoblast cells of this organism (Li et al., 2021). Prior research has shown that the silencing of smedwi-2 and smedwi-3 genes leads to neoblast population deterioration, ultimately causing the planarian organism to expire (Palakodeti et al., 2008). Further study of these three proteins has shown that they have several key functions in somatic cells. SMEDWI-2 is necessary for tissue-specific gene expression

and proper cell division. Moreover, SMEDWI-2 is responsible for transposon silencing. SMEDWI-2 targets repetitive elements and silences them at the transcriptional level. Research has shown that many transposable elements become active during cell division due to changes in chromatin. If these transposons are not silenced, incomplete or improper cell division occurs. SMEDWI-2 silences these transposons in neoblast cells by inducing H3K9 methylation. SMEDWI-1 and SMEDWI-3 are responsible for the post-transcriptional silencing of any transposons that escape transcriptional silencing by SMEDWI-2 (Li et al., 2021). Due to the pivotal role PIWI proteins have shown in tissue regeneration in planarians, further research is being conducted in less-studied organisms capable of regeneration.

PIWI Expression in **Lytechinus variegatus**

Prior research has shown that PIWI proteins are expressed in *L. variegatus* germline cells and some somatic tissues (Amir et al, 2020). The protein SEAWI is expressed in sea urchins. This protein is a member of the PIWI family and has been observed in the germline and somatic cells of *L. variegatus*. This protein and its associated piRNAs are responsible for silencing transposons in the germline to protect the genetic material. The PIWI-piRNA complex also takes part in gene regulation through post-transcriptional silencing. PIWI expression was noted in the spines and tube feet of sea urchins. While the function of these proteins in the somatic tissues of sea urchins is unknown, the presence of these proteins in regenerative tissues indicates that they may have a role in spine and tissue regrowth, possibly in conjunction with stem cells (Amir et al., 2020).

piRNAs

piRNAs are a class of small non-coding RNAs that have functions in germline and somatic cells related to interactions with PIWI proteins. These small RNAs were first discovered in the *D. melanogaster*, where expression has been observed in germline and somatic cells. These small RNAs are created through a process called piRNA biogenesis (Figure 1).

piRNA biogenesis can be divided into primary piRNA biogenesis (Phasing) and secondary piRNA biogenesis (Ping-Pong cycle). Primary piRNAs are first transcribed from DNA to form a piRNA precursor in primary biogenesis. This precursor is exported to the cytoplasm for further processing. In the cytoplasm, the mitochondrial-membrane-bound endonuclease Zucchini (Zuc) cleaves the precursor to form the 5' end. This precursor is loaded onto a Piwi/Aubergine (Aub) complex where the 3' end is trimmed. This complete piRNA then takes part in the Ping-Pong cycle, producing secondary piRNAs.

In the Ping-Pong cycle, antisense Aub-bound piRNAs cleave transposable element transcripts. This produces a new sense-piRNA transcript that is bound by Argonaute3 (Ago3). This complex targets piRNA cluster transcripts, producing antisense piRNA transcripts. These transcripts are bound by Aub and the cycle repeats. This cycle is dual functioning, as piRNA biogenesis is combined with target silencing. In each step, a 10nt overlap is observed between reads and transcripts. This is a distinguishing feature of the Ping-Pong cycle (Czech & Hannon, 2016).

piRNAs are incredibly diverse, with the number of unique sequences ranging in the hundreds of thousands. These small RNAs are synthesized from two locations in the genome, piRNA clusters and protein-coding genes. piRNA clusters are the main location of piRNA synthesis. These clusters are comprised of repetitive, non-coding sequences. They are divided into two types depending on the direction in which transcription occurs. In the first type, unidirectional clusters, transcription only occurs in one direction. In bidirectional clusters, transcription occurs in both directions, working inward starting from the ends. In *Drosophila,* unilateral cluster expression has been observed in somatic cells while bilateral cluster expression has been observed in germline cells (Le Thomas et al., 2014).

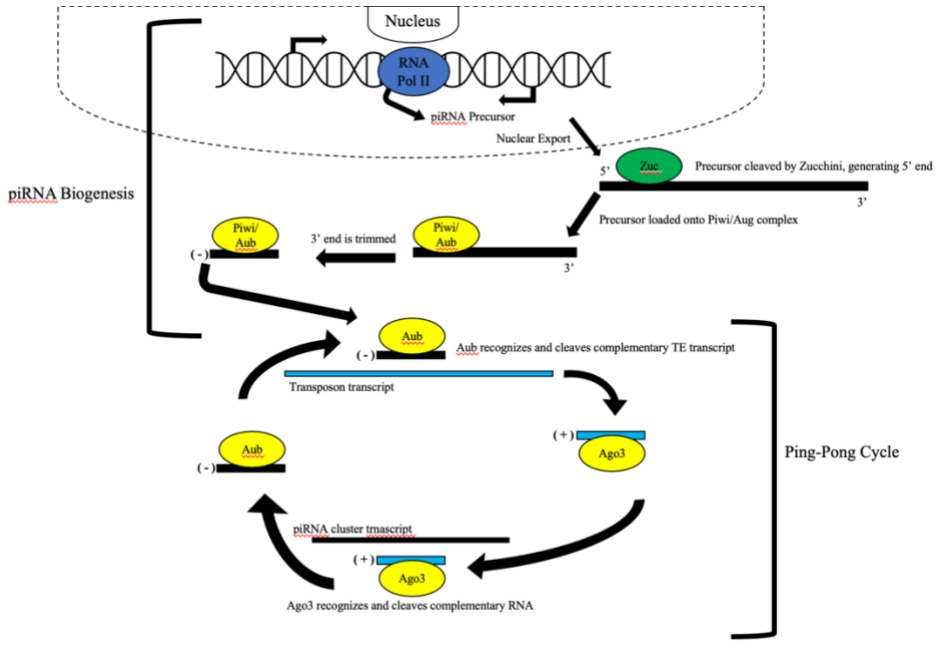

Figure 1: piRNA Biogenesis and the Ping-Pong Cycle. This figure shows the steps that take place in the biogenesis of a new piRNA, as well as the steps of the ping-pong cycle. RNA pol II transcribes a piRNA precursor from DNA which is exported to the cytoplasm. Endonuclease Zucchini, located on the mitochondrial membrane, cleaves the piRNA precursor to generate the 5' end. This precursor is loaded into a Piwi/Aub complex where the 3' end is trimmed. Bound to an antisense piRNA strand, Aub cleaves a complementary TE transcript. This produces a complementary piRNA, now sense, which is bound by Ago3. Ago3 cleaves a complementary piRNA cluster transcript, producing an antisense piRNA transcript. This transcript is bound by Aub, starting the process over.

Transposable Elements

Transposable elements (TE), commonly referred to as transposons, are sometimes described as "parasitic DNA". Transposons are segments of DNA that can replicate independently and relocate themselves throughout the genome. TEs can encode

regulatory sequences and proteins that function to increase their replication. This replication can occur in the germline, causing TEs to be passed on to subsequent generations in a process called vertical transfer. TEs are also capable of being passed between species, a process known as horizontal transfer. The main types of TEs are short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs), miniature inverted-repeat TEs (MITEs), and endogenous retroviruses (ERVs) (Wells & Feschotte, 2020).

TEs are extremely prevalent and have been observed in nearly all eukaryotic genomes. TEs are largely the reason that most genomes are comprised mainly of noncoding regions. TEs far outnumber protein-coding regions in the genomes of most organisms, determining the size of the genomes of these organisms (Wells & Feschotte, 2020). In extreme cases, genomes have been observed in which TEs comprise 90% of the genetic material. TEs account for approximately 45% of the genetic material in the human genome (Ayarpadikannan & Kim, 2014).

TEs can be divided into DNA transposons and retrotransposons. DNA transposons can insert themselves into new locations in the genome. Currently, there are no active DNA transposons in the human genome. Retrotransposons can insert themselves into new genome locations by producing RNA intermediates. These intermediates are converted back into DNA through reverse transcription (Ayarpadikannan & Kim, 2014).

Recent studies have shed light on the impact that TEs have on the human genome. TEs have been shown to create genomic instability, driving evolution as genetic variation is increased. Genomic instability is caused by the creation of inversions, deletions, and

duplications. TEs have also been shown to play a role in gene regulation. The main mechanisms for gene regulation are cis-acting control elements and transcriptome modulation via epigenetic control. Research has shown that certain active retrotransposons in the human genome, such as Arthrobacter luteus (Alu) and Long Interspaced Nuclear Element 1 (LINE-1), are directly linked to the formation of tumors (Bhat et al., 2022). The silencing of these genetic parasites is one of the most well-studied functions of PIWI proteins and piRNAs. To do this, retrotransposon silencing is employed at transcriptional and post-transcriptional locations.

In *Drosophila*, the Aub and Ago3 PIWI proteins take part in post-transcriptional silencing. Also in *Drosophil*a, incipient transposon transcripts are bound by the PIWI-piRNA complex to silence transcription. This complex functions to coerce TEs into the repressive chromatin state, preventing transcription. This is achieved via interaction between the PIWI-piRNA complex and two mediator proteins known as Asterix and Panoramix. Nuclear Export Factor 2 (Nxf2) and Nuclear Transport Factor 2 Like Export Factor 1 (Nxt1) are activated by interaction with Panoramix. Once activated, these factors go on to join dSetDB1 (Eggless) and methylate H3K9. This process induces the repressive chromatin state (Wang & Lin, 2021).

CHAPTER III: MATERIALS AND METHODS

Maintenance of *Lytechinus variegatus* Specimens

Our green sea urchin specimens were obtained from Oceans Reefs and Aquariums. They were kept in an aquarium tank which the Laboratory Technician Beatriz Zancanela was responsible for maintaining. The water was tested weekly to monitor the salinity and pH. The water salinity was sustained at 25ppt. The water was also tested weekly for ammonia, nitrate, and nitrite pollution. The water temperature was maintained at 23℃ using a chiller that was connected to a water pump. The urchins were fed sushi nori strips every other day, which were purchased from the grocery store.

Amputations

Three sea urchins were placed under a dissecting microscope in a saltwater container for the amputation process. From each urchin, both regenerating and control tube feet samples were taken. To do this, any spines that were in the way were removed using nail clippers. The majority of the tube feet were amputated from one side of each urchin using a pair of fine scissors, and a pipette was used to collect the samples. The contents of the pipette were run through a 40 μm cell strainer to remove the seawater. The filtered tissues were placed in TRIzol. The urchins were allowed one week to regenerate their tube feet. After regeneration, the majority of the tube feet (regenerated and control) from the entire organism were removed in the same manner (Figure 2). As sea urchins can retract their tube feet, this process was quite tedious. RNA extraction was performed after collecting the control and regenerated tube feet.

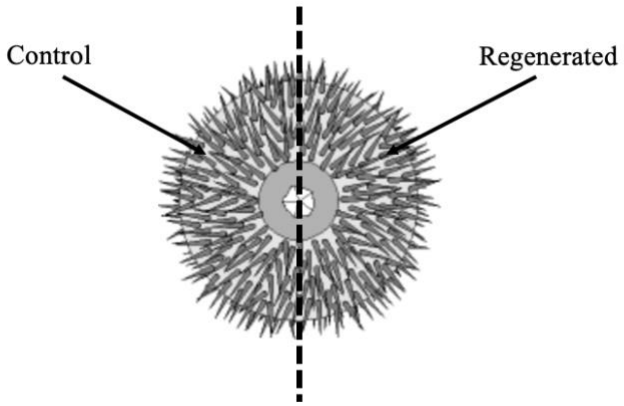

Figure 2: Tube feet amputation of one of the two bilateral halves of Lytechinus variegatus. Tube feet were amputated using fine scissors. Tube feet were not amputated from the control side.

RNA Extraction

RNA extraction was performed using the ThermoFisher TRIzolTM LS Reagent kit following the manufacturer's protocol. Six samples were extracted in total, one control and one regenerating from each of the three urchins. In addition to the manufacturer's protocol, 300µL of nuclease-free water (NFW) was added to the Eppendorf tubes to balance the salinity of the tissues. The RNA samples were washed using a 30% NFW/70% ethanol solution. NanoDrop was used to assess the quality of RNA. Following extraction, samples were kept at -80°C until they were sent off for small RNA sequencing.

RNA Sequencing

RNA sequencing (RNA seq) is a laboratory tool used to study gene expression that was developed in the mid-2000s. This tool has allowed the entire transcriptome of

organisms to be observed and analyzed. (Stark et al., 2019). RNA seq is mainly used to observe differential gene expression (DGE), which is when a statistical difference in gene expression is observed between two different experimental conditions (Anjum et al., 2016). The process of RNA sequencing begins with the extraction of total RNA from sample tissues. The samples can then be enriched or purified for specific RNA types, such as small RNAs or mRNAs, depending on the desired outcome. Reverse transcriptase is then used to convert the RNA sequences into complementary DNA (cDNA). A high-throughput platform, such as Illumina, is then utilized to deep-sequence a library of adaptor-ligated sequences, yielding 10-30 million reads per sample. After that, the final steps involve data analysis. The sequences must be aligned with the genome, read-transcript overlaps must be quantified, insignificant data must be filtered out of the data set, and statistical analysis must be done to determine if there is a significant difference between gene expression in the control and experimental tissues (Stark et al., 2019).

Illumina is the most prevalently used high-throughput platform in RNA seq research. Illumina has been used in 95% of the published research related to RNA seq (Stark et al., 2019). The Illumina Nextseq2000 is capable of RNA seq related to single-cell gene expression, whole-exome sequencing, spatial transcriptomics, shotgun metagenomics, and total RNA sequencing. In our experiment, total RNA extracted from our samples was shipped to the genomics core at the University of Mississippi Medical Center (UMMC) for small RNA sequencing using Illumina NextSeq2000. Library preparations were performed by UMMC as well.

Computational Analysis

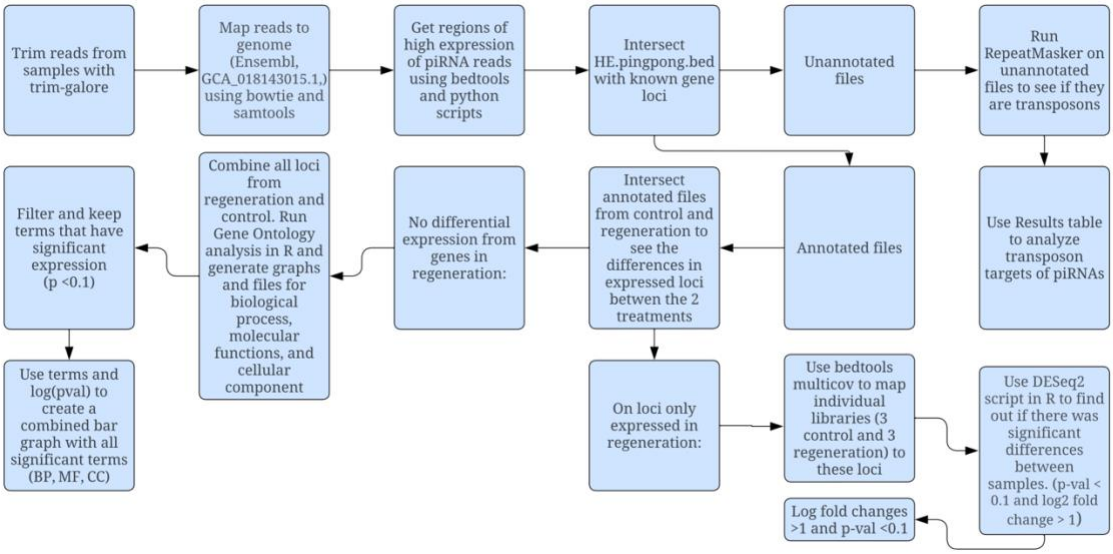

Figure 3: Flow Chart of the computational analysis of piRNA expression in control and regenerating tissue of Lytechinus variegatus. The chart shows all of the steps that were taken to analyze piRNA expression in the control and regenerating tissues and determine if statistically significant differences in expression are observed.

Gene Ontology Analysis

RNA sequencing with high-throughput technologies such as Illumina yields large data sets regarding gene expression. Managing these vast data sets has presented a unique problem to the field of biology. To organize sequencing data into a usable form, The Gene Ontology Consortium has been created. This database organizes genes utilizing unique vocabulary to annotate them with known biological knowledge. There are three main categories by which genes can be organized: molecular function, biological process, and cellular component (Beissbarth, 2006). The GO terms are organized into a directed acrylic graph (DAG), which organizes terms into levels based on specificity. Each

subsequent level increases in specificity, with one or more “parent” terms being linked to a more specific “child” term. Gene Ontology Annotation (GOA) databases exist for specific organisms, allowing the genes of entire transcriptomes of specific organisms to be organized following high-throughput sequencing (Grossmann et al., 2007). GOAs make it possible for the extraordinary number of genes that have been and are still being discovered to be organized using vocabulary that allows genes present in multiple species, or even in all eukaryotes, to be identified (Ashburner et al., 2000).

CHAPTER IV: RESULTS

piRNA Gene Targets in Control and Regenerating Tissue

After the RNA samples were sequenced, pipelines were used to find loci expressing high piRNA counts based on ping-pong signature (Figure 3). DESeq2 was used to generate a volcano plot (Figure 4) displaying values of statistical importance (Love et al., 2014). It was expected that differential expression (DE) would be observed, as piRNA gene targets involved in regeneration would be upregulated. Observation of this plot shows that DE of piRNA gene targets between control and regenerating tissues did not occur at a statistically significant level. This was not the expected result.

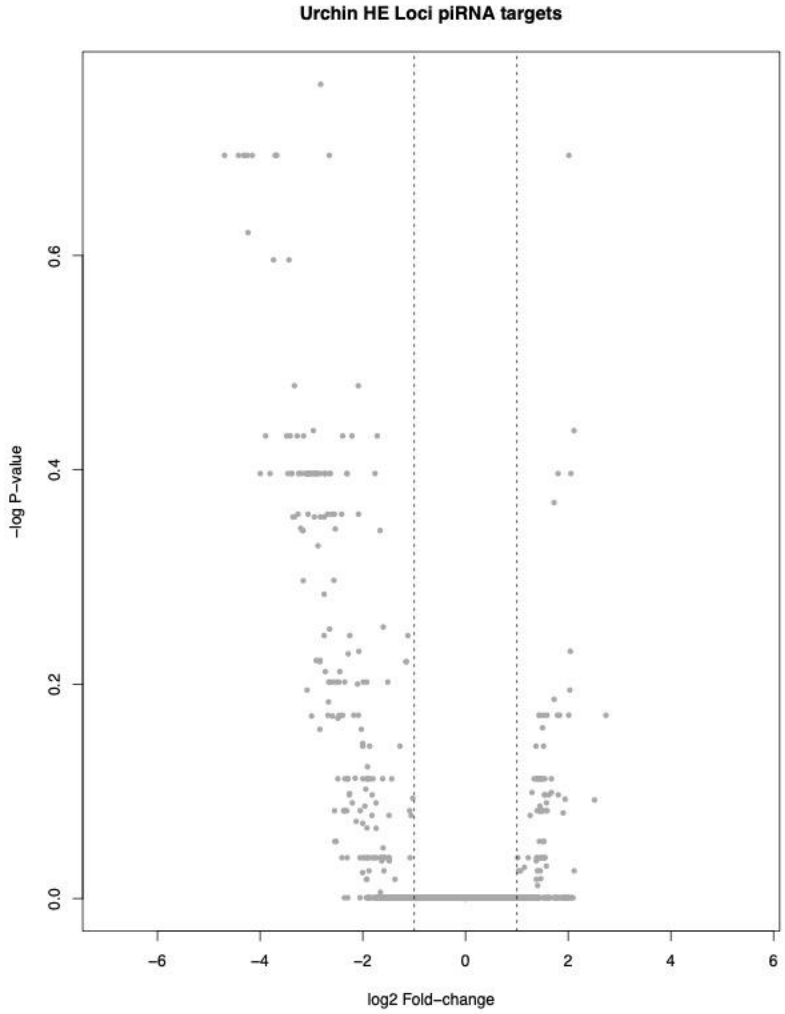

Figure 4: piRNA Gene Targets in Control and Regenerating Tube Feet in Lytechinus variegatus. The volcano plot shows the piRNA gene targets of statistical importance (p-val < 0.1, log2fold change > 1).

GO Analysis of piRNA Gene Targets

While differential expression did not occur, piRNA expression was observed in the tube feet samples. This is the first time piRNA expression has been recorded in sea urchin somatic cells. To shed light on the role of these piRNAs, a GO analysis was

performed to determine the functions of the targeted genes using TopGO in R Studio (Alexa & Rahnenfuhrer, 2022). Several genes of particular interest will be discussed.

Cellular Component

A bar graph was created with all of the genes involved in cellular component (Figure 5). Of these genes, three of particular interest are intracellular membrane-bounded organelle, condensed chromosome kinetochore, and eukaryotic translation initiation factor 3 complex. The intracellular membrane-bound organelle GO term was the most statistically significant. As the name suggests, these genes are related to any membrane-bound organelle, including the nucleus, vacuoles, plastids, vesicles, and mitochondria (OBOF, 2023). One possible explanation for this gene target is endonuclease interaction. The endonuclease Zucchini, which is located on the mitochondrial membrane, cleaves precursor piRNA transcripts, forming their 5' ends. Based on other target genes, it is also possible that piRNAs interact with the nuclear membrane during cell division, allowing nuclear division to be regulated. The condensed chromosome kinetochore GO term defines genes that are related to the kinetochore. The kinetochore is a protein made up of multiple subunits at the central region of the chromosome. This structure is where spindle microtubules attach during cell division, allowing sister chromatids to be separated (OBOF, 2023). The targeting of these genes suggests that piRNAs might play a role in cell division. This has already been observed in the planarian *Schmidtea mediterranea*, as PIWI-piRNA complexes silence transposons that are activated by chromatin rearrangement to ensure proper cell division. The

presence of the eukaryotic translation initiation factor 3 (eIF3) complex GO term is of particular interest. As the name suggests, eIF3 is an important complex that is key to protein synthesis (OBOF, 2023). This complex leads to the formation of the 43S preinitiation complex by binding to the 40S ribosome, allowing the Met-tRNA/eIF2.GTP ternary complex to be loaded. Additionally, eIF3 chaperones messenger RNA (mRNA) to the 43S complex through interaction with eIF4 (Zhou et al., 2005). The targeting of these genes suggests that piRNA could be vital for protein synthesis. piRNA silencing could be a mechanism by which eIF3 expression is regulated.

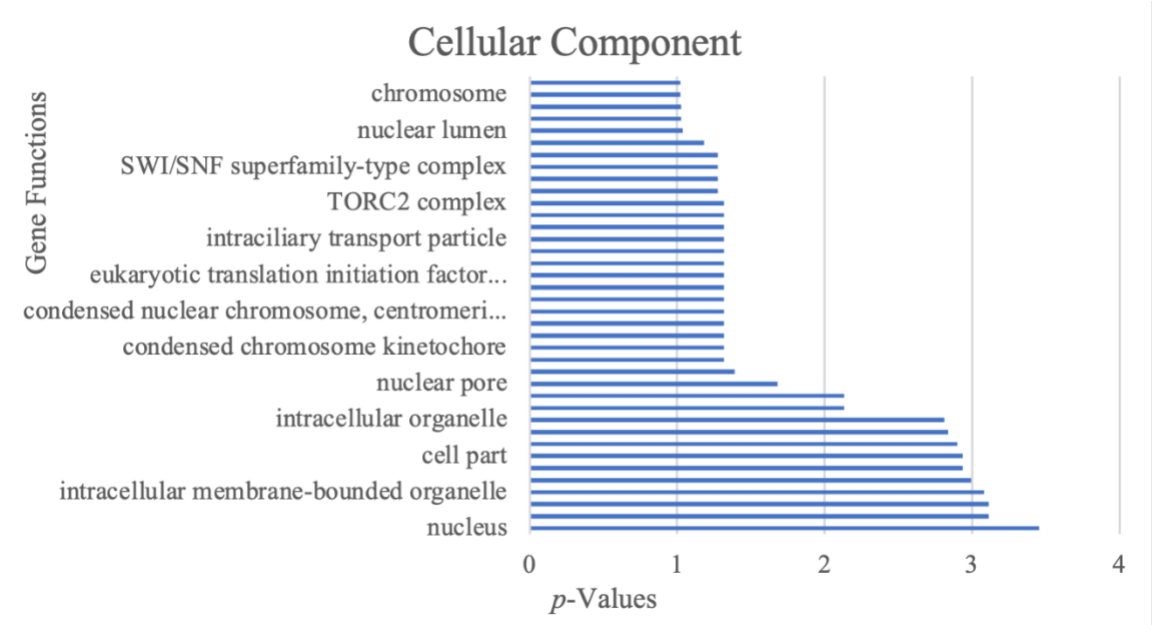

Figure 5: Cellular Component Gene Targeting by piRNAs in the Tube feet of Lytechinus variegatus. This bar graph shows the CP genes that were targeted and their statistical significance. The Log of each p-Value (pval < 0.1) was taken, meaning as the p-Value increases on the graph, genes are of greater statistical significance.

Biological Process

A bar graph was also created with all of the genes involved in biological process (Figure 6). Of these genes, three of particular interest are proteolysis, negative regulation of mitotic cell cycle, and nuclear division. The GO term proteolysis indicates genes that are involved with the process of cleaving protein peptide bonds to form smaller subunits (OBOF, 2023). Research has shown that proteolysis is involved in the interaction between mitotic spindles and chromosomes, making it a necessary component of cell division (King et al., 1996). In addition to targeting genes involved with the kinetochore, it appears that piRNAs could be necessary for cell division. piRNA silencing could be a mechanism by which sister chromatid separation is controlled. The GO term negative regulation of mitotic cell cycle refers to genes that lead to the termination of the mitotic cell cycle (OBOF, 2023). This further links piRNA expression to cell division. piRNA silencing could be the instrument by which the cell cycle is terminated. The GO term nuclear division also connects piRNA to cell division. piRNAs could play a role in regulating nuclear division. Such an interaction was observed in the planarian *Schmidtea mediterranea*. Without piRNA expression, transposons caused mutations during cell division that were often fatal to the organism.

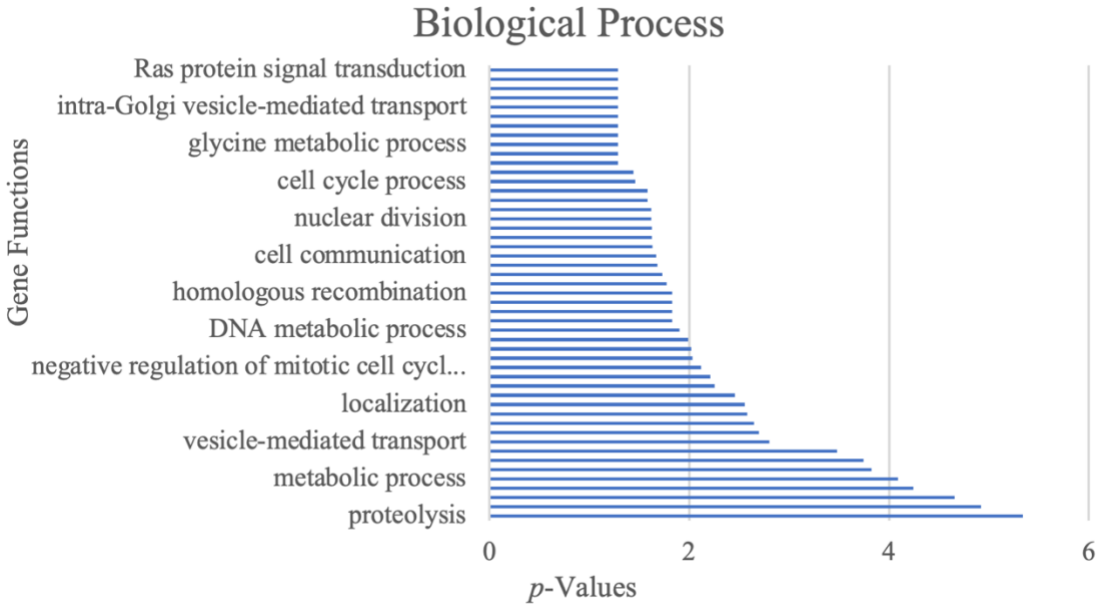

Figure 6: Biological Process Gene Targeting by piRNAs in the Tube feet of Lytechinus variegatus. This bar graph shows the BP genes that were targeted and their statistical significance. The Log of each p-Value (pval < 0.1) was taken, meaning as the p-Value increases on the graph, genes are of greater statistical significance.

Molecular Function

A bar graph was created with all of the genes involved in molecular function (Figure 7). Of these genes, three of particular interest were purine ribonucleoside triphosphate binding, DNA helicase activity, and nucleotide binding. Ribonucleoside triphosphate is a precursor molecule for the synthesis of DNA and RNA (Lam, 1997). The targeting of these genes indicates that piRNAs could be instrumental in the proper synthesis of genetic material. piRNA expression could control when ribonucleoside triphosphate binding occurs. The targeting of genes linked with DNA helicase suggests that piRNA could be necessary for successful DNA replication and transcription. DNA

helicase could be utilized through the function of piRNA silencing. piRNA targeting of genes involved with nucleotide binding may indicate that piRNA is necessary for the formation of the DNA double helix. piRNAs could be the mechanism by which nucleotides bind to their correct counterpart. The targeting of genes involved with these three molecular functions indicates that piRNA could be a key player in the successful formation and utilization of genetic material.

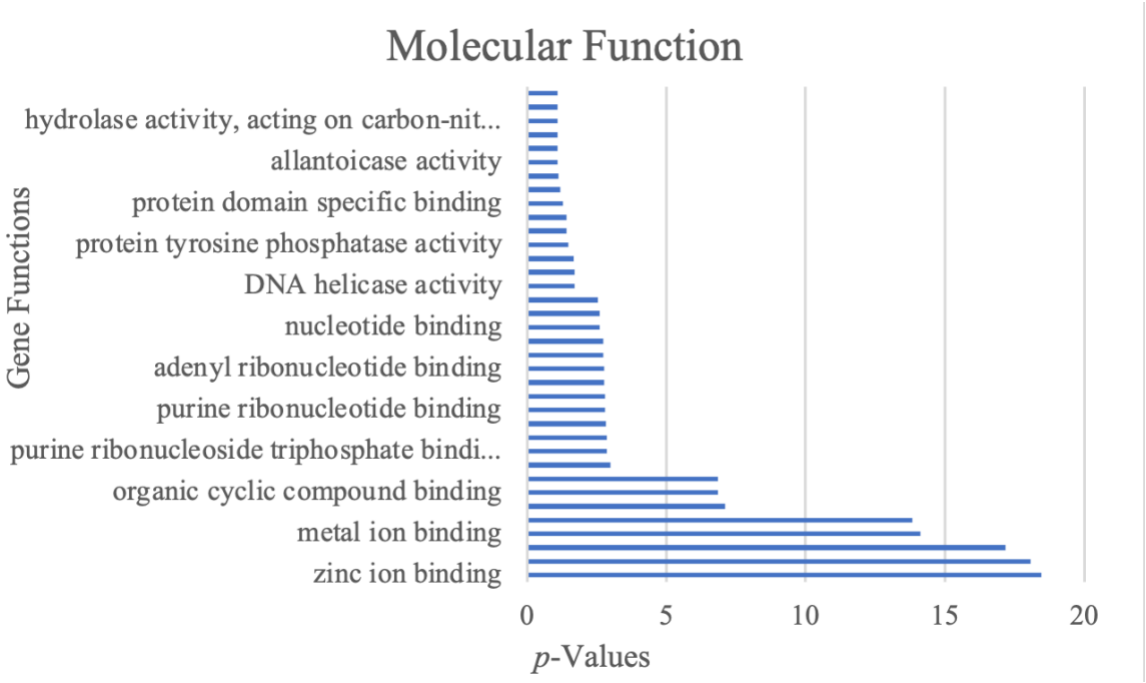

Figure 7: Molecular Function Gene Targeting by piRNAs in the Tube feet of Lytechinus variegatus. This bar graph shows the MF genes that were targeted and their statistical significance. The Log of each p-Value (pval < 0.1) was taken, meaning as the p-Value increases on the graph, genes are of greater statistical significance.

RepeatMasker Analysis

A bar of pie chart (Figure 8) was created with the piRNA transposon targets from RepeatMasker analysis (Smit et al., 2015). This chart shows that the main targets of

piRNAs in the tube feet of *L. variegatus* were retrotransposons and DNA transposons. Retrotransposons, more specifically Long Terminal Repeats (LTRs), were the main targets of piRNAs. This is of no surprise as retrotransposon silencing is one of the most well-known functions of piRNA. LTRs are also the most abundant retrotransposon in the genome. To a lesser extent, DNA transposons were also a main target of piRNA. This is also not surprising as DNA transposons are far outnumbered by retrotransposons.

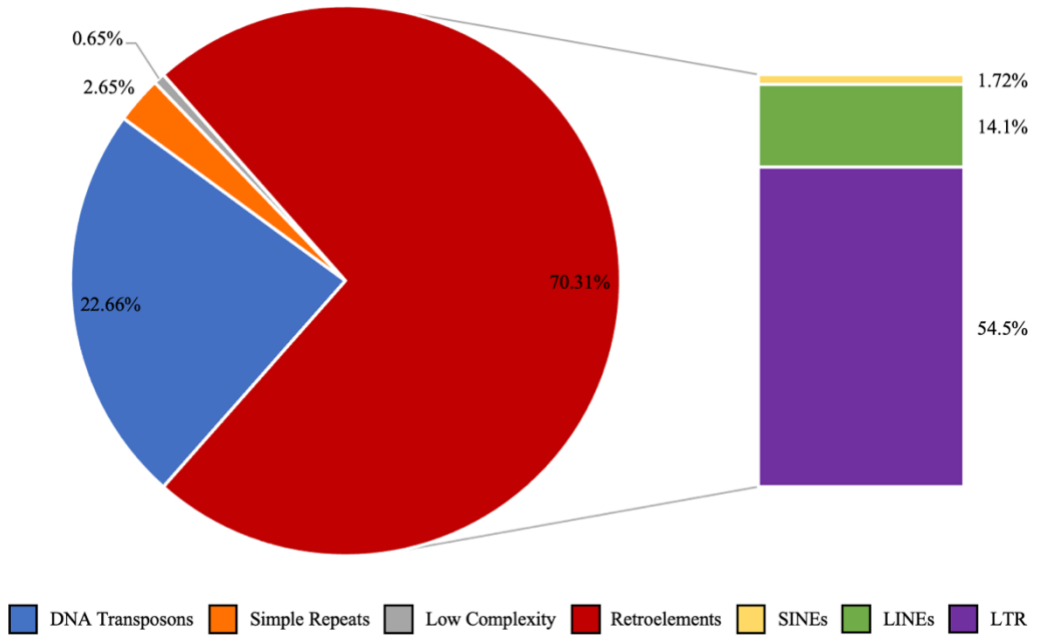

Figure 8: Transposon Targets of piRNA Expressed in the Tube feet of Lytechinus variegatus. This bar of pie chart shows the transposons that were targeted out of the 2850 genes that were run in RepeatMasker.

CHAPTER V: DISCUSSION

To date, piRNA expression in the somatic cells of *L. variegatus* has been poorly studied. Prior research has failed to elucidate the presence and function of piRNAs in sea urchin tube feet. In this study, piRNA expression was characterized in the tube feet of the echinoderm *L. variegatus*.

After RNA seq, statistical analysis using DESeq2 was used to generate a volcano plot that showed a lack of differential expression of piRNA in these two conditions. We expected DE of genes to some level because regeneration is a process that involves stem cell differentiation, proliferation, and cell migration; however, DE was not observed. While the results were not as expected, this is the first time piRNA expression has been recorded in the somatic cells of *L. variegatus.*

Further investigation of piRNA targets using GO Analysis explained the putative function of piRNA in these somatic cells. Thirteen statistically significant Cellular Component gene segments were targeted. Of these segments, membrane-bounded organelle, condensed chromosome kinetochore, and eIF3 complex were of particular interest. These interactions suggest that piRNAs could play a pivotal role in separating sister chromatids, cell division, and protein synthesis (OBOF, 2023). Thirteen Biological Process gene segments were also targeted. The targeting of proteolysis, negative regulation of the mitotic cell cycle, and nuclear division gene segments offer insight into piRNAs' function in these tissues (OBOF, 2023). Proteolysis is required to properly interact with mitotic spindles and kinetochores, suggesting that piRNAs could regulate sister chromatid separation (King et al., 1996). Negative regulation of the mitotic cell cycle would allow piRNA to drive cell division through gene regulation. In conjunction

with the membrane-bounded organelle gene segment, interaction with genes involved in nuclear division further provides evidence that piRNAs may regulate cell division. Twelve statistically significant Molecular Function gene segments were targeted. Of these, purine ribonucleoside triphosphate binding, DNA helicase activity, and nucleotide-binding were noted. These gene targets propose that piRNA could play a pivotal role in synthesizing genetic materials. Purine ribonucleoside is a precursor of both DNA and RNA, meaning that regulation of this precursor could be utilized during DNA synthesis (Lam, 1997). DNA helicase is vital for DNA replication and RNA transcription, meaning that its regulation by piRNA could control these mechanisms. Nucleotide-binding is also required for the formation of the double helix, providing yet another point of regulation for piRNA.

Analysis with RepeatMasker presented the transposon targets of piRNA in *Lytechinus variegatus* tube feet. LTR retrotransposons were the most highly targeted transposable elements. DNA transposons were also targeted to a significant extent. This reinforces the already well-recognized role of piRNA in transposon silencing (Wang & Lin, 2021).

The many transposons targeted by piRNAs in this study add to the foreknown function of piRNA as a silencer of transposable elements. piRNA targets obtained from RepeatMasker show that transposon silencing may occur in these tissues as well. GO analysis elucidated the function of piRNAs in these cells by revealing the targeted genes. Targeting of genes involved in cell division, DNA Replication, transcription, and translation implies that piRNA plays a vital role in many cellular processes.

While the data shows that piRNA could be involved in tissue regeneration in *L. variegatus*, further studies need to be conducted to confirm this role. As in previous studies, the effect that suppressing piRNA expression has on regeneration should be studied in this organism. Somatic piRNAs appear to have a shared role in planarians and sea urchins. An example of this is piRNA expression which coincides with cell division. These piRNAs target transposons, preventing them from damaging the genomes of the daughter cells. This is a fundamental part of animal biology, showing that these two diverse groups of organisms evolved to retain their somatic piRNAs. This proposes another important question, why were somatic piRNAs lost in mammals through evolution? Perhaps the answer to this question will lead to advancements in regenerative medicine.

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