Dissecting the piRNA pathway in *Bombyx mori* using the CRISPR/Cas9 System

Senior Thesis

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Chapter 1: Adopting Immunomagnetic Cell Sorting in the *Bombyx mori* System

I. Abstract

Fluorescent activated cell sorting (FACS) is a common cell sorting method, however, there are issues associated with stringency, cost and efficiency. Immunomagnetic cell sorting (iMACS) addresses these concerns by the use of superparamagnetic beads conjugated with antibodies against a group of well-characterized cell surface glycoproteins in the CDx family which are expressed on target cells. Previous studies in *Spodoptera frugiperda* (Sf21) insect cells showed that using iMACS yielded 99% of CD4 expressing cells after sorting. Here we describe attempts to adopt the iMACS system in the *Bombyx mori* ovary cell line BmN4s. *Bombyx mori* has been shown to have homologous proteins to species like *Drosophila melanogaster* and *mus musculus* that are involved in piRNA biogenesis. The power of adopting iMACS in the *Bombyx mori* system will allow the study of piRNA biogenesis in *Bombyx mori* more efficient and cost effective when compared to FACS. We encountered problems after transfection of vectors expressing murine CD4 in *Bombyx mori* cells and subsequent Western immunoblotting experiments. The lack of visualization of any bands either in our samples or the positive control may point to issues either with the antibodies’ specificity or underlying problems with the expression of CD4.

II. Introduction

Fluorescent activated cell sorting (FACS) has long been an established method to select for targeted cell populations (Dittrich and Göhde 1968, Van Dilla et al 1969). FACS offered a faster and wider range of cell types that could be used when compared to traditional sorting techniques at the time like somatic cell fusions in selective media (Wanda et al. 1982). Despite its advantages, FACS does have a number of drawbacks. First, flow cytometers are relatively complex devices that require specialized training (Shapiro, 2003). This training is not only time consuming but the operator requires compensation for their kind contributions. In addition, there are issues with stringency, the output of living cells after sorting, high cost and efficiency (Shapiro, 2003, Chalmers, 1998). However, looking at the bigger picture of modern day cell sorting technology, immunomagnetic cell sorting (iMACS) offers an alternative method to FACS.

iMACS was developed roughly 15 years ago and solves many of FACS’ shortcomings (Chalmers et al., 1998). iMACS is based on targeting cells expressing specific members of the CDx family (under the superfamily immunoglobulin), a group of well characterized cell surface glycoproteins, with antibodies which are adhered to superparamagnetic beads (Chalmers et al., 1998). This method does not require rigorous and specialized training like FACS therefore iMACS can be used by a wider population of researchers. Additionally, due to the two-fold specificity of the antibody targeting the CDx family as well as magnetic pull down of targeted cells; concerns with stringency are alleviated when compared to FACS (Chalmers et al., 1998). Issues with cell survivability after sorting are addressed due to the fact that target cells can be eluted from the antibody by a low pH wash. When compared to FACS, iMACS is a less expensive alternative because of the lack of specialized personnel and the relative low cost of the antibody superparamagnetic beads. Finally, iMACS can sort up to \(10^{10}\) cells per hour as opposed to FACS which can process only \(10^7\) cells in the same time frame (Miltenyi et al. 1990, Chalmers et al. 1998). Many studies have adopted iMACS in order to satisfy their cell sorting needs.

Philipps et al. of Novartis Pharma used the iMACS system in sorting cells from a *Spodoptera frugiperda* ovary cell line, Sf21 (Philipps et al 2004). By infecting their cells
using the baculovirus expression vector system (BEVS) which contained a CD4-EGFP C-terminal fusion in the pFASTBac™ DUAL vector, they utilized the iMACS method and observed 99% of fluorescing (and therefore CD4 expressing) were present in their positive fraction (Philipps et al., 2004). These promising results have inspired us to attempt to adopt a similar system in Bombyx mori ovary cell lines. Bombyx mori ovary cells have been shown to express piRNAs and their associated PIWI-PIWI interacting RNA proteins that have homology to other species (Kawaoka S et al, 2008, Kawaoka S et al 2009, Zhang P et al., 2012). By adopting this method we seek to dissect and clarify the mechanisms of piRNA biogenesis in Bombyx mori as well as other species that share the well conserved piRNA pathway.

Here, we report attempts to adopt an analogous experimental system to of iMACS seen in Philipps et al. in BmN4 (Bombyx mori ovary cell line). We show our efforts of transfecting mouse CD4 in BmN4 cells and probing for CD4 expression via Western immunoblot.

**III. Materials and Methods**

*Isolation of Plasmid Constructs*

The plasmids in question: plBG64, plBG64 ZsGreen, and pCGPO (murine CD4 cloned into the plBG64 vector under the OpIE1 promoter) cultures of *E. coli* (DH5α) were grown up overnight at 37°C and DNA was isolated via standard Midiprep procedures.

*Transfection of BmN4 Cells with plBG64, plBG64 ZsGreen and pCGPO*

~10^6 BmN4 cells (larval ovary cells extracted from Bombyx mori) were plated in tissue culture treated polystyrene 6-well dishes. 24 hours prior to the transfection and incubated at 26°C 0% CO₂ in 2 mL IPL-41 media + 10% fetal bovine serum. 1µg and 3µg of plBG64, plBG64 ZsGreen and pCGPO isolated plasmids were mixed with FuGENE® HD in a 2:1 weight to volume ratio. Next, 100µl of 100 mM sterile NaCl was added to the DNA-FuGENE® mixture and was incubated at 25°C for 15 minutes. Each mixture was added to their respective wells drop-wise and left to incubate at 26°C, 0% CO₂ for 48 hours.

*Preparation of Transfected BmN4 Cells for Western Blot Analysis*

The media of the transfected BmN4 cells was aspirated and then the cells were washed with sterile 1x PBS (phosphate buffer saline). The BmN4 cells were gently lifted from the 6-well dish mechanically with a cell scraper, resuspended in 1mL of sterile 1x PBS and were then spun down at 13,000 rpm for 1 min. The supernatant was removed and resuspended in 150µl of 3x Laemmli Sample Buffer. Samples were then boiled at 95°C for 10 minutes to denature the proteins.

*Jurkat Cell Culture and Preparation for Western Blot Analysis*

The Jurkat cell line used as a positive control for CD4 expression was obtained from Seble Asrat in Dr. Ralph Isberg’s lab at Tufts School of Medicine (150 Harrison Ave, Boston MA 02111, Department of Molecular and Microbiology). Note Jurkats are immortal T-lymphocytes from a male patient with acute T-lymphocyte leukemia. Jurkats were grown in RPMI-1640 +10% FBS H.I in a 75 cm² tissue-culture treated polystyrene cell culture flasks at 37°C at 5% CO₂ for 3 days until they were harvested. ~10^6 cells were harvested, spun down at 1000 RPM for 10 minutes at 4°C. The supernatant was removed and the cells were resuspended in 250µl of 3x Laemml Sample Buffer and boiled at 95°C for 10 minutes.
Western Immunobloting

Samples were run on 10% SDS-polyacrylamide gels and transfer reactions were run using activated PVDF (polyvinylidene difluoride) membranes. After blocking in 5% milk, monoclonal mouse αFLAG antibody (1:500) was used as a primary antibody and added to the membranes suspended in 5% milk which were left to incubate and shake at 4°C overnight. The next day goat α-mouse Horse Radish Polymerase (HRP) (1:5,000) was used as a secondary antibody and the immunoblots were developed.

Transfected CD4 Expressing Cells

Apply Magnetic Field

Target Cells-Bound by αCD4 and are pulled down

Legend:

Low pH wash to elute

CD4

αCD4-superparamagnetic Beads

Transfected Cell

Figure 1- Model of immunomagnetic cell sorting—CD4 expressing cells (green circles with red hexagons) are targeted with superparamagnetic beads conjugated with αCD4 antibodies (black circle with blue Y-shape). After application of a magnetic field CD4 expressing cells bound by the superparamagnetic beads are pulled down while those that do not express CD4 are discarded. Finally, target cells are eluted from the
IV. Results

Testing the Expression of CD4 in Bombyx mori

Figure 1 refers to the overall methodology behind immunomagnetic cell sorting. The expression of mouse CD4 from the pIBGP64 plasmid was tested by first transfecting pIBGP64, pIBGP64 ZsGreen and pCGPO (murine CD4 expresser) in BmN4 cells. Transfection efficiency and overall success was based on analyzing the expression of pIBGP64 ZsGreen transfected BmN4 cells. Cells were then harvested and subject to Western immunoblot analysis in order to observe CD4 protein expression by pCGPO in BmN4 cells. Figure 3A shows a clear band ~40 kDa in lane 2, containing the Jurkat lysate. Other lanes either show bands at very high molecular weights or

![Image of figure 3A showing a clear band ~40 kDa in lane 2](image)

nothing at all. Figure 3B shows a high background signal and bands at high molecular weights but there is not any evidence of clear bands in any other lanes.

Figure 2-Transfection of BmN4 cells - Panel A shows from left to right: the brightfield, pIBGP64 Zs Green expression, and the merged image of BmN4 cells transfected with 1 µg of pIBGP64 ZsGreen. Panel B shows from left to right: the brightfield, pIBGP64 Zs Green expression, and the merged image of BmN4 cells transfected with 3 µg of pIBGP64 ZsGreen. Panels C and D show the same information conveyed in A and D however the samples were harvested on 4/9/13 after a 48 hour transfection. Panels E and F show similar information to the panels A-D however it shows 48 hour transfections of 3 µg (E) and 5 µg(F).
Here, we describe our efforts to express murine CD4 in *Bombyx mori* ovaries (BmN4 cell line) in the pIBGP64 vector as the first step to adopting the powerful, efficient and cost effective cell sorting iMACS system in *Bombyx mori*. As seen in Figure 3A-D, although transfection did occur for samples from 3/29/13 and 4/9/13, the transfection efficiency was very low, especially when compared to transfections done on 5/5/13 (Figure 2E-F). The low transfection efficiency may have occurred due to the fact the sample and FuGENE® were mixed directly as opposed to the 5/5/13 technique where both were diluted in 150 mM sterile NaCl beforehand. When compared, Figures 3A-D fluorescence is brighter however less dispersed to in Figures 3E-F. Direct mixing may have been detrimental due to the high concentration of DNA could have ended up in larger micelles formed by the FuGENE® and therefore more DNA went into the cells that phagocytosed the lipids but less cells were exposed to the DNA. However, transfection efficiency did not improve the results observed by Western immunoblotting.

As Figures 3A and B show the only bands seen are faint, ubiquitous in most samples and at high molecular weights. Concerns recognizing the positive control Jurkats cells with the αCD4-αmouse were alleviated by purchasing αCD4-αhuman which also recognizes mouse and rat CD4. This suggests both antibodies; αCD4-αmouse (Figure3A) αCD4-αhuman (Figures3B) are binding nonspecifically, more so with the αCD4-αhuman antibody due to the high background. The band observed in lane 2 of Figure3A at ~40 kDa is an aberration not observed in any other blots and not in the vicinity of the expected 51 kDa of CD4.

These data presents us with a major question: why are the positive control and our samples not being recognized by the previously mentioned antibodies? One possibility is that the Jurkat cell line that we received does not express CD4 and instead another CDx family member. Another is that the antibodies that we used don’t bind specifically enough or at all to any CD4 that is expressed in our system. It should be noted that recombinant CD4 peptide was to be delivered but the commercial vendor had our group waiting an exorbitant amount of time and therefore the order was canceled.
In order to address these concerns, we will seek out a new positive control and a more specific antibody. If the current antibodies work with the new positive control, this would point to issues with the expression system. In order to address concerns with expression, CD4 could be moved to be under the GP64 promoter which has been previously shown to work in our lab (data not shown), instead of the OIEP2 promoter. Also, human CD4 will be cloned into the pIBGP64 vector to address concerns over antibody specificity. When the concerns with CD4 expression and a positive control are addressed we can fully implement iMACS and subsequently increase the efficiency and scale of cell sorting in our lab.

**VI. References**


Chapter 2: Bm Yu/Spoonbill and its Potential Role in Regulating piRNAs

I. Abstract

PIWI-interacting RNAs (piRNAs) and their associated PIWI proteins are essential for germ line development. This highly conserved biological “vanguard” protects host genomes by silencing transposable elements. Numerous studies have shown that TUDOR domains are important in piRNA biogenesis and have been shown to recognize PIWI proteins based on symmetrical dimethylated arginines (sDMAs). This study will focus on Bm Yu/spoonbill, an AKAP protein, which has been previously shown to contain the TUDOR domain and be essential in oogenesis in Drosophila. Here we describe the molecular cloning of FLAG-tagged Bm Yu into our expression vector as well as protein expression of cloned Bm Yu in the Bombyx mori ovary cell line BmN4, visualized using Western immunoblotting. We showed that 2xFLAG-BmYu was expressed in BmN4 cells. Further studies in our lab will study if Bm Yu interacts with proteins related to piRNA biogenesis through co-immunoprecipitation.

II. Introduction

The study of small RNAs (sRNAs) specifically PIWI-interacting RNAs (piRNAs) has grown exponentially since their official discovery in 2003 (Aravin et al 2003). piRNAs are highly conserved and an integral part of germ line development and maintenance among both vertebrates such as Mus musculus and Rattus norvegicus and invertebrates such as Drosophila melanogaster and Bombyx mori (Aravin et al 2006, NC Lau et al, 2006, Aravin et al, 2003, Kawaoka S et al 2008). piRNAs defend the germ line by silencing transposable elements (TEs) which by hopping around the genome, can insert in the middle of genes causing them to lose function and overall alter the entire genome (Siomi et al 2011, Siomi et al 2010). An important regulatory mechanism of piRNA biogenesis has been shown to be proteins containing TUDOR domains.

TUDOR domains originate from a series of repeated motifs observed in the Drosophila melanogaster gene tud, which was shown to be necessary for the formation of the germ plasm (Boswell and Mahowald 1985). Additional research in multiple model organisms has shown that proteins other than tud can have TUDOR domains (Ponting, 1997, Talbot et al., 1998, Maurer-Stroh et al., 2003). TUDOR domains recognize PIWI proteins via symmetrical dimethylated arginines (sDMAs) and the interaction between TUDOR domains and PIWI proteins has been shown to be integral in localization and function (Kirino et al., 2009, Nishida et al, 2009, Vagin et al., 2009, Chen et al, 2009, Reuter et al, 2009, Shoji et al., 2009, Vasileva et al., 2009., Wang et al., 2009, Kirino et al., 2010). This particular study focuses on the Bombyx mori gene, bm yu or its homolog in Drosophila, spoonbill.

Bm yu was first discovered by Lu et al. in 2007 in a long term memory study in Drosophila. They found that yu is an A-kinase anchoring protein (AKAP) which were previously shown to be critical in localizing cAMP-protein kinase A which has been implicated in long term memory formation (Lu et al., 2007, Kandel et al., 2001). They also found that yu localized in the Drosophila mushroom body (Lu et al. 2007). Additional studies have shown that yu contains a TUDOR domain, is essential for oogenesis in Drosophila and has a localization pattern to the mitochondria and Golgi apparatus (Handler et al., 2011, Hadad et al. 2011). It should be noted that analysis of the localization patterns of yu and the Drosophila zucchini which has been implicated in piRNA genesis are very similar and exhibit the same female sterility phenotype.
We are interested in studying if Bm Yu interacts with other PIWI proteins in the *Bombyx mori* ovary cell line BmN4 and how that fits into the bigger picture of piRNA biogenesis using the iMACS methodology mentioned previously. Here, we report the successful cloning of 2xFLAG-bm yu into our insect vector expression system and subsequent Western Blot analysis of transfected BmN4 cells.

III. Materials and Methods

**PCR Amplification of Bm yu from Bm yu in the pCRII-TOPO Vector**

Previous work in the lab by Dr. Nelson Lau and Christina Post cloned Bm Yu from BmN4 total RNA into the pCRII vector. The following primers were used to amplify Bm Yu from the pCRII vector and were produced by Integrated DNA Technologies (IDT):

- **Forward Primer**: 5’AACTCCGAGGAATGGACTACAAGACGATGACGACGACAAGCACCTTGTCGTCAACTACTC-3’
- **Reverse Primer**: AATTCTAGATCAGGAGTCTGGTGGAACATCCCACTC-3’.

**Gel Extraction and Purification**

After PCR products were subject to gel electrophoresis, gels were observed under ultra violet light and the target bands were excised and massed. Qiagen QG was then added in a 3:1 w/v ratio and the gel was dissolved at 65 C for 10 minutes. Isopropanol was added in a 1:1 w/v ratio to the dissolved gel fragments and then applied to silica membrane spin columns and spun for 1 min at 13,000 RPM. The columns were then washed to remove any unwanted cellular debris with 500 µl of Buffer PB and spun as noted previously. Columns were subject to an additional wash with 750 µl Buffer PE and spun twice as mentioned previously. The DNA was eluted from the column with ddH2O with a spin as mentioned previously.

**Restriction Enzyme Digest of Bm Yu and pIBGP64**

2.5 µg of the pIBGP64 vector and 1.5 µg of the gel purified Bm Yu product were digested by the restriction enzymes *XbaI* and *XhoI* at sites +2664 and +881 of the vector overnight at 37 C in the presence of 1% BSA. Calf intestine phosphatase (CIP) was added to the pIBGP64 reaction to prevent self-ligation for downstream procedures. After the overnight digest both products were subject to PCR clean-up to remove the restriction enzymes and unwanted buffer components from the target DNA. 5:1 (v/v) of Buffer PA was added to the digestion product, mixed well and applied to silica membrane spin columns and spun for 1 min at 13,000 RPM. 750 µl Buffer PE was then used to wash the column and the column was spun twice as mentioned above. The DNA was eluted with ddH2O with a spin as mentioned previously.

**Ligation of Clean Digested Bm Yu and pIBGP64**

The clean digested Bm Yu and pIBGP64 vector were mixed in a 10:1 (insert to vector v/v) ratio with T4 ligase and its corresponding buffer from NEB and incubated at 16 C for 16 hours.

**Transformation of pIBGP64-Bm Yu Ligation into Chemically Competent DH5α E. coli**

The pIBGP64-Bm Yu ligation product was added to chemically competent DH5α E. coli and incubated on ice for 30 minutes. The DH5α was then subject to a heatshock at 42 C for 1 minute. 500 µl of SOC was added to the bacteria and left to incubate and shake at 37 C in order to recover for 1 hour. After 1 hour, 50 µl of the cultures were plated on LB+Amp (100mg/mL) and then incubated at 37 C overnight.
The next day, single colonies were picked and liquid cultures were grown up overnight at 37 C.

**Isolation of pIBGP64-BmYu Product from DH5α via Miniprep**

Overnight liquid cultures were spun down and the supernatant was removed and the cell pellet was resuspended in P1 buffer. Next P2 lysis buffer was added, mixed well and incubated at room temperature. After the incubation, N3 neutralization and binding buffer was added and then the mixture was at 4 C. The supernatant was removed and applied to silica membrane spin columns and spun for 1 min at 13,000 RPM. Buffer PE was then used to wash the column and the column was spun two times as mentioned above. The DNA was eluted with ddH2O with a spin as mentioned previously.

**Colony PCR Screen**

Colony PCR was used to verify the insert was present before sending the samples off for sequencing. Single colonies from successful ligations were picked, mixed into the reaction mixture and plated onto a new LB+Ampicillin plate. Two sets of primers were used to screen for the presence of the insert, one targeting the N-terminus (1) of Bm Yu and the other targeting the C-terminus (2) : 1- Forward Primer 5'- AGCTATCTGTGCTGCGCCTACTGAA-3' and reverse 5'CTGAATAATCTTAGTTTGTATTGTC-3'(expected size: 441 bp) 2- Forward 5'- ACTGTGCTCTTCACAGGAAC-3' and reverse 5' CAGACCTTAGGACACCATT-3'(expected size: 335 bp). Taq DNA polymerase was used for the PCR reactions. Colonies were plated on LB+Amp plates were incubated overnight at 37 C. Positive based on gel electrophoresis were grown in liquid culture in LB media +Amp (100mg/mL) overnight and DNA was isolated via miniprep (see above protocol).

**DNA Sequencing**

Positive colonies based on the colony PCR screen were sent to an offsite DNA sequencing company, Genewiz. Samples were sent with the following primers: Forward 5'- ACTGTGCTCTTCACAGGAAC-3' and reverse 5'CTGAATAATCTTAGTTTGTATTGTC-3'. Sequencing data yielded 95% coverage of the Bm yu gene inside the pIBGP64 vector.

**Transfection of BmN4 Cells with pIBGP64, pIBGP64 ZsGreen and pIBGP64-BmYu**

~10^6 BmN4 cells (larval ovary cells extracted from *Bombyx mori*) were plated in tissue culture treated polystyrene 6-well dishes. 24 hours prior to the transfection and incubated at 26 C 0% CO2 in 2 mL IPL-41 media + 10% fetal bovine serum. 1µg and 3µg of pIBGP64, pIBGP64 ZsGreen and pCGPO isolated plasmids were mixed with FuGENE® HD in a 2:1 weight to volume ratio. Next, 100µl of 100 mM sterile NaCl was added to the DNA-FuGENE® mixture and was incubated at 25 C for 15 minutes.

An alternative procedure was used for experiments conducted after 5/5/13 in which both the FuGENE® and DNA were mixed in 100 µl of 150 mM sterile NaCl, then mixed together and incubated for 15 minutes at 25 C. Each mixture was added to their respective wells drop-wise and left to incubate at 26 C, 0% CO2 for 48 hours.

**Preparation of Transfected BmN4 Cells for Western Blot Analysis**

The media of the transfected BmN4 cells was aspirated and then the cells were washed with sterile 1x PBS (phosphate buffer saline). The BmN4 cells were gently lifted from the 6-well dish mechanically with a cell scraper, resuspended in 1mL of sterile 1x PBS and were then spun down. The supernatant was removed and resuspended in 3x
Laemmli Sample Buffer. Samples were then boiled at 95 C for 10 minutes to denature the proteins.

**Western Immunoblotting**

Samples were run on 10% SDS-polyacrylamide gels and transfer reactions were run using activated PVDF (polyvinylidene difluoride) membranes. After blocking in 5% milk, monoclonal mouse αFLAG antibody (1:500) was used as a primary antibody and added to the membranes suspended in 5% milk which were left to incubate and shake at 4 C overnight. The next day goat α-mouse Horse Radish Polymerase (HRP) (1:5,000) was used as a secondary antibody and the immunoblots were developed.

**IV. Results**

**Molecular Cloning of Bm Yu into pIBGP64**

Molecular cloning has long been an established method in order to selectively express a target gene. PCR, gel electrophoresis, the use of restriction enzyme digests, ligation, colony PCR and DNA sequencing was used to clone Bm yu into pIBGP64 under the GP64 promoter as seen in Figure 1. Bm yu was first amplified from *Bomyx mori* mRNA (cDNA) by Dr. Nelson Lau and cloned into the pCRII-TOPO vector. Bm yu was then amplified from the pCRII-TOPO vector using PCR (as described previously) and run on a 1% agarose-ethidium bromide gel a band was observed at the expected size of ~1.8 kb in addition to a band around 5.0 kb (Figure 2). Gel purification was used to isolate the 1.8 kb band and both the Bm yu fragment and the pIBGP64 vector were digested with *XbaI* and *XhoI* while pIBGP64 was also treated with calf intestine phosphatase in order to prevent self-ligation (see Figure 1). Both digested products were PCR purified and then were mixed together in a ligation reaction.

The ligation mixture was then transformed into chemically competent DH5α and then subject to a colony PCR screen using two sets of different primers. Analysis of the colony PCR reaction on a 1% agarose-ethidium bromide gel shows bands at ~340 bp for primers targeted to the N-terminus of Bm yu in lanes 3, 5 and 7 and another targeting the C-terminus of Bm yu with bands around 510 bp (Figure 2). Sequence data...
confirmed that Bm yu successfully was cloned into \textit{pIBGP64}. With the sequence confirmed the next step was to test the expression of Bm yu in BmN4 cells.

\textbf{Testing \textit{pIBGP64-Bm yu} in \textit{Bombyx mori} cells}

The \textit{pIBGP64-Bm yu} plasmid was transfected for 48 hours in BmN4 cells with two different amounts of DNA: 3 μg and 50 μg. In order to assess the success of the transfection and its efficiency a positive control, \textit{pIBGP64 ZsGreen} was also transfected under the same conditions as \textit{pIBGP64-Bm yu} and observed ZsGreen expression under fluorescent conditions (Figure 3). The transfection efficiency was calculated to be 27.2% for the 3 μg transfection while 5 μg yielded an efficiency of 27.5%. Efficiency was assessed by dividing the number of ZsGreen positive cells by the total number of cells within the field of view. Next, FLAG tagged Bm yu protein production was tested via Western Blot. Samples were run on 10% SDS polyacrylamide gels, transferred for 1 hour onto a PVDF membrane, blocked for 1 hour and probed with αFLAG propagated in mice. 2R TALEN expressed in HEK 293T cells was used as a positive control from FLAG expression. Figure 4 shows a band in the positive control lane (Lane 2) at roughly 100 kDa while in lanes 3 and 4 (3 μg and 5 μg of \textit{pIBGP64-Bmyu} respectively) there is a band at ~76 kDa. It should be noted the band observed in lane 3 is fainter when compared to that of the band in lane 4.
V. Discussion

Here, we describe the molecular cloning of 2xFLAG-Bm yu and the subsequent expression of the pIBGP64-Bm yu vector in BmN4 cells. Previous work has shown that Bm yu may be related to piRNA biogenesis due to its TUDOR domain and its knockdown phenotype which leads to female sterility in flies. Figure 2A shows evidence that the fragment that was amplified from the pCRII-TOPO-Bm yu vector is the correct size, 1.8kb. Figure 2B’s bands at above 500 bp seen in lanes 2, 4 and 8 when the expected size was 444 bp may have been due to the DNA forming supercoils in the agarose gel and therefore retarded the movement. The bands observed in lanes 3 and 7 of Figure 2B were of the expected size of 335 bp. These data suggest that previous steps such as amplification, digestion, ligation and transformation were successful. Sequencing data however confirmed that Bm yu was successfully cloned into pIBGP64. Although there were some “mutations” (G→C @ position 1051, A→T1183, A→G @1565, T→C @1651) this may have been due to the fact the Bm yu sequence originated from REFseq and therefore may not be the true sequence. Once we obtained pIBGP64-Bm yu as seen in Figure 1, we went on to see if this construct would be produced in BmN4 via transfection and Western immunoblotting.

BmN4 cells were transfected with varying amounts of sample (3µg and 5µg) for 48 hours as seen in Figure 3. The transfection efficiency based on pIBGP64ZsGreen fluorescence, was average but could have at least been 1.5 fold

![Image](image-url)

**Figure 3- Transfection of BmN4 Cells with pIBGP64-Bm yu** - Panel A shows from left to right: the brightfield, pIBGP64 Zs Green expression, and the merged image of BmN4 cells transfected with 3 µg of pIBGP64 ZsGreen. Panel B shows from left to right: the brightfield, pIBGP64 Zs Green expression, and the merged image of BmN4 cells transfected with 5 µg of pIBGP64 ZsGreen. Any cells partially in view were not considered in the calculation of transfection efficiency.
higher based on previous transfections in our lab (data not shown). Transfection is not exactly well understood and efficiency may vary from time to time. Transfected cells were harvested, lysed and subject to Western immunoblotting. As Figure 4 shows, pIBGP64-Bm yu was expressed in the transfected cells as seen by the ~75 kDa bands in lanes 3 and 4. The validity of the αFLAG antibody is shown in lane 2 of Figure 4 which is the 2R TALEN HEK 293T lysate which contains FLAG peptide. The cloning of FLAG tagged Bm yu is an important step in dissecting the piRNA pathway not only in Bombyx mori but other organisms as well.

Further studies surrounding Bm Yu will involved co-immunoprecipitations in BmN4 cells using FLAG-conjugated beads in order to see if PIWI proteins like SIWI, interact with Bm yu. If indeed Bm Yu interacts with PIWI proteins, a ΔTUDORBm yu could be made to test if Bm Yu interacts with PIWI proteins via its TUDOR domain. In addition, a cell line expressing FLAG-tagged Bm yu will be established via drug selection and perhaps iMACS allowing the piRNA pathway to be more readily studied in Bombyx mori. Other studies on the AKAP family also provided impetus to further study Bm yu.

Interestingly enough, a mouse study of D-AKAP1 showed intracellular localization at the mitochondria and the endoplasmic reticulum as well as very strong expression in the testes (Huang et al., 1999). These data are consistent with the intracellular and overall body localization pattern of Bm yu. Bioinformatic analysis by our group shows that D-AKAP1 and spoonbill show homology to one another and both contain a KH domain (which binds DNA and RNA) and TUDOR domains. An additional study in humans showed that hAKAP220 was found to be localized during spermatogenesis (Reinton et al., 2000). These data suggests that Bm yu and D-AKAP1 may be homologues or that the AKAP family has a greater role in piRNA biogenesis. Once data is collected on the interacting partners of Bm yu it would be interesting to see if D-AKAP-1 could rescue a Bm yu knockdown in BmN4 cells. In addition, the same co-immunoprecipitation studies related to Bm Yu could be applied to exogenously expressed D-AKAP1 in order to further elucidate not only their relationship to one another but to AKAPs and piRNA biogenesis.
VI. References


Chapter 3: Adopting the CRISPR System in *Bombyx mori*

I. Abstract

PIWI-interacting RNAs (piRNAs) and their associated PIWI proteins are essential for germ line development and have been found in *Bombyx mori*. In the past decade genomic editing tools have been developed, one of which, called CRISPR has arisen as an attractive option due to its ease of use and cost efficient methods. This study will focus on adopting the CRISPR system in the *Bombyx mori* cell culture line BmN4s in the first step to using CRISPR to understand piRNA biogenesis in *Bombyx mori*. We demonstrate that sgRNAs in conjunction with Cas9, targeting ZsGreen can reduce but not eliminate the levels of ZsGreen fluorescence. However, targeting proteins involved in piRNA biogenesis using both normal and modified sgRNA designs such as Siwi has yet to be observed via protein and genetic analysis. Future studies will focus on optimization of the CRISPR/Cas9 system before studying factors involved in the piRNA pathway in *Bombyx mori*.

II. Introduction

The manipulation of an organism’s genome has long been a useful tool to dissect biological mechanisms by observing the phenotypic effects of genetic alterations. Recent advances over the past decade have allowed for more efficient site specific genome editing technologies such as zinc finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs) system. Both these technologies rely on the activity of site specific DNA binding proteins bound to an endonuclease, *FokI*, to induce double strand breaks (DSBs) at the targeted region (Kim et al. 1996, Daimon et al. 2013, Gaj et al 2013). The DSBs in turn induce insertion or deletion mutations via non-homologous end joining resulting in the knockout of gene function due to frameshift mutations. (Gaj et al. 2013). Although ZFNs and TALEN’s have been shown to effectively edit the genomes wide range of organisms from *Drosophila* to mice, they are limited by their high cost and can be time consuming to engineer (Beumer et al 2006, Lui et al. 2012, Mani et al. 2005). A time and cost efficient alternative known as CRISPR has recently emerged as powerful tool in the field for genome engineering.

The CRISPR/Cas9 system is derived from a bacterial adaptive immune system against invading viral DNA (Sorek et al 2013). Bacteria acquire foreign DNA and incorporate it within specific regions of their genome at clustered regularly interspaced short palindromic repeats (CRISPR) loci. The incorporated DNA is then transcribed and processed into CRISPR RNAs (crRNAs). crRNAs then anneal to a trans-activating CRISPR RNA (tracrRNA) forming the guide strand. The guide strand subsequently directs gene specific silencing via Cas (CRISPR-associated) proteins (Brouns et al. 2008, Hwang et al. 2013). An important feature of crRNAs are the protospacer adjacent motifs (PAM) having the sequence NGG, which are recognized by Cas9 and are required for inducing double strand breaks at the sites specified by the crRNAs (Gaj et al. 2013). A fusion of crRNA and tracrRNA into a synthetic guide RNA (sgRNA) along with Cas9 expressed from a plasmid in *Drosophila* was sufficient for gene knockout (Gratz et al. 2013). The power of the CRISPR system lies in the ease and cost effective manner in which target sequences can be designed within the constant regions of the sgRNA. We seek to take advantage of
the CRISPR system as a tool to study piRNA (piwi-interacting RNA) biogenesis in *Bombyx mori*.

PiRNAs are a group of highly conserved small RNAs integral to germ line development due to their ability to silence transposable elements (Siomi et al 2011). Homologs of PIWI and AGO3, proteins essential for primary and secondary piRNA biogenesis in a variety of organisms, including *Drosophila melanogaster*, have been identified in the *Bombyx mori* ovary cell culture line BmN4 (Kawaoka 2008). Previous studies by Daimon et al. have demonstrated the CRISPR/Cas9 system in *Bombyx mori* worms resulted in mosaic loss of function that they argue may be due to improper expression of Cas9 and the need for further optimization for the concentrations of the sgRNAs and Cas9 (Daimon et al 2013). Adopting the CRISPR/Cas9 system in cell culture may provide an easier and more efficient alternative studying piRNA biogenesis to whole organism *in vivo* studies. Despite the CRISPR/Cas9 system is efficient, previous efforts by Chen et al. have shown that modifications to sgRNA design can improve stability of the Cas9-sgRNA complex by extending the stem loop and increase efficacy by preventing premature Pol-III termination by flipping an adenine for a uracil at the termination site. These modifications are referred throughout the manuscript as the FE modification (Chen et al 2013). In addition to the basic goal of knocking out the key Piwi protein in *Bombyx mori*, Siwi, we aim to assess whether or not the FE modification is an improvement over conventional sgRNA design. Also, we demonstrate that the CRISPR/Cas9 system can effectively and specifically decrease ZsGreen expressing BmN4 cells. However, western immunoblotting and DNA digestion assays of *Bombyx* cells transfected with either conventional sgRNAs or FE modified sgRNAs targeting Siwi, failed to demonstrate clear editing of the genome and therefore knockdown.

III. Materials and Methods

*Cas9 Expression Vector and sgRNA Design*

In order to express Cas9 in Bmn4 cells, Cas9 with a C-terminal 3XFLAG tag originally from the pJDS246 plasmid, was placed under the OPIE2 promoter in the pIBGP64 plasmid backbone. The *Bombyx mori* Cas9 expression plasmid was named pJDSIB-Cas9.

sgRNAs targeting ZsGreen were identified using the ZiFiT program ([http://zifit.partners.org/ZiFiT/](http://zifit.partners.org/ZiFiT/)) and candidates closer to the 5' end of the gene and included restriction enzyme recognition sites were preferentially selected. The final sgRNA construct consisted of the target sequence inserted between two flanking constant regions. The final 60 bp sgRNA was constructed through ligating the oligonucleotides of the target sequence and the constant regions as described by Hwang et al 2013. The complete target sgRNA was subsequently cloned downstream of the 453 bp U6 promoter in the pCRII plasmid. Additionally, as described in Hwang et al. 2013, sg RNAs were cloned under the T7 promoter for direct transfection of the sgRNAs into BmN4s. FE modified sgRNAs were also engineered using a similar stitching together method, gel purified and cloned into the pCRII TOPO TA vector. DNA sequencing was used to confirm the identity of the sgRNA PCR and plasmid products. It should be noted that Jenny Klein constructed the pJDSIB-Cas9-3xFLAG, U6 sg RNA
backbone and the U6 ZsGreen and SIWI sg RNA plasmids. Also, the hCD4-mCherry and hCD4-ZsGreen fusion plasmid was constructed by Sally Demirdijan.

**Transfection of BmN4s**

~10^6 naïve or BmN4 cells stably expressing ZsGreen (larval ovary cells extracted from *Bombyx mori*) were plated in tissue culture treated polystyrene 6-well dishes 24 hours prior to the transfection and incubated at 26 °C 0% CO2 in 2 mL IPL-41 media. Desired plasmids were diluted in 150 mM sterile NaCl. FuGENE® HD in a 2:1 volume: Total DNA (or RNA) ratio was also diluted in 150 mM sterile NaCl. Next, both the FuGENE® HD and DNA NaCl mixtures were combined, mixed and incubated at 25 °C for 15 minutes. After incubation samples were applied drop-wise to each well and incubated at 26 °C 0% CO2 for 14 days. To test for Cas9 expression BmN4s were transfected with 5 μg of pIBGP64, of 8 μg of U6 ZsGreen sg1 and sg2 with pJDSIB-Cas9-3xFLAG 5 μg of pJDSIB-Cas9-3xFLAG alone. Experiments attempting to optimize the method of sgRNA delivery in comparing plasmid based and PCR based products transfected BmN4s with 2 μg pJDSIB-Cas9, 2 μg Siwi sg1 and sg2 plasmid based products, 200 ng of Siwi sg1 and sg2 FE modified PCR products and 1 μg of hCD4-ZsGreen. The transfection from 4/12/14 used similar conditions as mentioned previously but instead used 2 μg of Siwi sg1 and sg2 FE plasmid based products. Additionally, the transfection from 4/13/14 only differed in that 2 μg of hCD4-ZsGreen was used. It should be noted in the control wells containing only hCD4-ZsGreen and Cas9, a benign vector, pIBGP64 was added to ensure equal amounts of DNA were transfected.

**Preparation of Transfected BmN4 Cells for Western Blot Analysis**

The media of the transfected BmN4 cells was aspirated and then the cells were washed with sterile 1x PBS (phosphate buffer saline). The BmN4 cells were gently lifted from the 6-well dish, resuspended in sterile 1x PBS and were then spun down. The supernatant was removed and resuspended in of 3x Laemmli Sample Buffer. Samples were then boiled at 95 °C.

**Western Immunoblotting for Cas9 Expression in BmN4s**

Samples were run on 10% SDS-polyacrylamide gels and transfer reactions were run using activated PVDF (polyvinylidene difluoride) membranes. After blocking in 5% milk, monoclonal mouse αFLAG antibody (1:500) was used as a primary antibody and added to the membranes suspended in 5% milk which were left to incubate and shake at 4 °C overnight. The next day goat α-mouse Horse Radish Polymerase (HRP) (1:5000) was used as a secondary and the immunoblots were developed.

**Western Immunoblotting for Siwi Expression in BmN4s**

Samples were run on 10% SDS-polyacrylamide gels and transfer reactions were run using activated PVDF (polyvinylidene difluoride) membranes. After blocking in 5% milk, monoclonal rabbit αSiwi antibody (1:2,000) and mouse αEa7 Tubulin (1:10,000) were used as primary antibodies and added to the membranes suspended in 5% milk which were left to incubate and shake at 4 °C overnight. The next day goat α-mouse Horse Radish Polymerase (HRP) (1:10,000) as well as goat α-rabbit Horse Radish Polymerase was used as a secondary in 5% milk, wash and finally the developed.
Siwi loci Digestion Assay
After 7-9 days of transfection, the genomic DNA of BmN4s was phenol-chloroform extracted and the loci of interest, Siwi sg1 and Siwi sg2, were PCR amplified. Amplicons were then ethanol precipitated to purify the products and subject to overnight digests with either XhoI or EcoRV. Before being loaded on a 2% agarose gel, samples were incubated at 65°C to heat inactivate any restriction enzyme in the mixture.

IV. Results
To test whether Cas9 could be expressed in BmN4 cells a construct, pJDSIB-Cas9-3xFLAG along with ZsGreen sg1 and 2 target sequences (Figure 1A and 1B) was transfected into BmN4s and cellular extracts were harvested two days post transfection as described in the Materials and Methods section. As seen in Figure 1C, bands were present at around the expected size of Cas9 of 153 kDa, when probed with antibodies against the FLAG peptide in lanes 4 and 5, containing 8 μg U6 ZsGreen sg RNA 2 and pJDSIB-Cas9-3xFLAG and 5 μg pJDSIB-Cas9-3xFLAG alone respectively. Lane 4 also had a band at roughly 140 kDa. There were not any visible bands in lane 2 which contained 5 μg of pIBGP64 which lacks the FLAG peptide. Curiously, there were not bands in lane 3 which contained containing 8 μg U6 ZsGreen sg RNA 1 and pJDSIB-Cas9-3xFLAG. With the presence of Cas9 confirmed we next sought to test whether the

Figure 1-Cas9 and sgRNA Design- Panel A shows Cas9 and GP64 under the regulation of the insect promoter OPIE2. The plasmid map was generated with Vector NTI v10. Panel B shows the generalized format of the complete sgRNA which consists of the 20 bp target sequence (green rectangles ZsGreen in this study’s experiments) and 19 bp sgRNA scaffold regions (blue rectangles) flanking the target sequence. The top sgRNA is under the U6 promoter (yellow arrow) was incorporated into the PCRII vector. Panel C shows a Western immunoblot of BmN4s stably expressing ZsGreen with the pJDSIB-Cas9-3xFLAG expression vector probing with monoclonal mouse αFLAG antibody (1:500) and goat α-mouse Horse Radish Polymerase (HRP) (1:5000). Lane 1 shows the protein standard, while lane 2 shows BmN4s transfected with 5 μg of pIBGP64, lanes 3 and 4 show transfections of 8 ug of U6 ZsGreen sg1 and sg2 with pJDSIB-Cas9-3xFLAG while lane 5 shows a transfection 5ug of pJDSIB-Cas9-3xFLAG alone.
sgRNAs against ZsGreen would affect its expression in BmN4s stably expressing ZsGreen.

**Figure 2** - Target sgRNA mRNA was not effective at knocking out ZsGreen expression. **Panel A** shows the PCR amplification of the ligated oligonucleotides of the sgRNA scaffold (blue), the ZsGreen target sequence (green) and the T7 promoter (grey arrow) of both ZsGreen sg1 (Lanes 2 and 3) and ZsGreen sg2 (Lanes 4 and 5) on a 2% agarose gel. Lane 1 shows the DNA standard with the green arrow denoting the expected size of the product, 162 bp. **Panel B** shows a 14 day transfection of BmN4s stably expressing ZsGreen that were transfected with 8 μg (4 μg for the U6 Backbone) pJDSIB-Cas9-3xFLAG with either 4 μg of the U6 sgRNA backbone (top row) or 1 μg of T7 ZsGreen sg1 (middle row) or T7 ZsGreen sg2 (last row).

sgRNAs against ZsGreen would affect its expression in BmN4s stably expressing ZsGreen. **Figure 2A** shows the PCR amplification of the T7-sgRNA target sequence.
after isolating the mRNA after a T7 transcription reaction, 1 μg of each individual ZsGreen sg RNA was transfected in conjunction with 8 μg of pJDSIB-Cas9-3xFLAG and observed over the course of 14 days. Comparing the ZsGreen sg RNAs to the U6 RNA backbone there does not appear to be any loss of ZsGreen expression over time. It should be noted that ZsGreen expression is variable in the BmN4s stably expressing ZsGreen. These data suggest that transfection of mRNA derived sgRNAs at low concentrations are not sufficient enough for proper Cas9 mediated knockout in BmN4s.

To control for transfection efficiency and the specificity of the ZsGreen sg RNAs, tdTomato under the OPIE2 promoter was used in a plasmid deemed pIBGP64-tdTomato (5084 bp). As Figure 3 shows, tdTomato fluorescence is observable under the excitation conditions for both ZsGreen and tdTomato and widely dispersed in the cytoplasm of many of the BmN4s in the field of view. The intensity and subsequent bleed through of tdTomato fluorescence has led us to seek an alternative option for a transfection control, hCD4-mCherry under the OPIE2 promoter.

To determine whether or not the CRISPR/Cas9 system was fully operational in BmN4 cells, naïve BmN4’s were all transfected with 2 μg hCD4-mCherry fusion under the OPIE2 promoter, 1 μg of ZsGreen under the OPIE2 promoter and 2 μg of pJDSIB-Cas9-3xFLAG. 2 μg of the U6 sgRNA Backbone and both ZsGreen sg RNAs under the U6 promoter in the pCRII vector were transfected into their own respective wells. It is expected that at early time points ZsGreen and mCherry expression would be in the same cells and they would appear to fluoresce as yellow. In time if the CRISPR system was working, a loss of ZsGreen expression should be observed in wells transfected with ZsGreen sg RNAs under the U6 promoter, expressed along with Cas9 in theory leaving only the mCherry expressing cells. We should not observe any change in ZsGreen expression in the U6 sgRNA backbone due to its lack of a target sgRNA.
Figure 4- sg RNAs Targeting ZsGreen Confer Lower ZsGreen Expression: Naïve BmN4’s were all transfected with 2 μg hCD4-mCherry fusion under the OPIE2 promoter, 1 μg of ZsGreen under the OPIE2 promoter and 2 μg of pJDSIB-Cas9-3xFLAG. 2 μg of the U6 sgRNA Backbone and both ZsGreen sg RNAs under the U6 promoter were transfected into their own respective wells. The above images were captured at 2 (top) and 7 (bottom) days post transfection.

Figure 4 shows 2 days post transfection ZsGreen expression in the U6 backbone seems to be bright but in a small number of cells. Both U6 ZsGreen sgRNAs had dimmer ZsGreen expression at day 2. However, it should be noted that there is a lack of clear hCD4-mCherry expression. After 7 days, clear colocalization of both ZsGreen and hCD4-mCherry was present as seen in the merged panel of the U6 sgRNA backbone.
Both ZsGreen sgRNAs exhibited considerably fainter ZsGreen expression while hCD4-mCherry expression appeared brighter than the ZsGreen expression in the same wells. The merged images of the ZsGreen sgRNAs shows BmN4s with solely hCD4-mCherry expression, albeit it is faint due to its membranous localization. Data from Figure 4 indicates that the CRISPR/Cas9 system targeting ZsGreen may be functional to a certain extent. Based on these results we wanted to test alternative strategies to construct the U6 target sgRNAs.

**Figure 5-U6 and ZsGreen sgRNA Oligonucleotide Annealing**- Panel A shows the overall scheme of overlapping primers between the U6 promoter (green, 453 bp) and the sgRNA constant region plus the target sg RNA (blue and green, 120 bp) to give a final 584 bp fragment. Panel B shows a 1.5% agarose gel stained with ethidium bromide with 1 kb ladder plus DNA ladder in lane 1, the sgRNA scaffold +Target sequence annealed products for ZsGreen sg1, 2 and SIWI sg1 and 2 in lanes 2, 3, 4 and 5 respectively. Lane 6 shows the isolated U6 promoter while lanes 7-10 show the final product of the U6 promoter and the target sg RNA sequence along with the sg RNA scaffold for ZsGreen sg1, 2 and SIWI sg1 and 2 in lanes 7, 8, 9 10 respectively.

A similar technique to constructing the T7- target sgRNAs was adopted to engineer the U6-target sgRNAs as described in the Materials and Methods. Figure 5A shows the stepwise progression of annealing the 453 bp U6 promoter to the amplified product of the target sgRNA associated with the sgRNA scaffold (120 bp) via complementary regions to yield a final 584 bp product. As seen in Figure 5B, both the target sgRNA +the sgRNA scaffold (lanes 2-5) and the U6 promoter (lane 6) appear to be the correct size though the U6 promoter has an unspecific band around 3.5 kb. As
we look at lanes 7-10, the expected final products at 584 bp are present (faintly in U6 SIWI sg2, lane 10) but there is an unknown lower molecular weight band and 220 bp. The unknown band is not the same size of the target sgRNAs + sgRNA scaffold which would rule out the possibility of an excess of that species in the reaction and would suggest it is due to one of the primer binding non-specifically to a region on either the U6 promoter or the target sgRNA complex. To circumvent problems that would arise from including the whole PCR product in the cloning process, the target band was excised and gel purified and subsequently cloned into the pCRII-TOPO-TA vector.

**Figure 6 Siwi Protein Levels Unaffected by Siwi sgRNAs** - shows a 10% SDS PAGE gel comparing plasmid based conventional Siwi sg RNAs and FE modified Siwi sgRNA PCR products of transfected BmN4 cells. All cells were transfected with 2 μg of Cas9, 1 μg of hCD4-ZsGreen. Plasmid based Siwi sgRNAs received 2 μg total while the FE modified Siwi sgRNA PCR products received 200 ng. The blots were probed with rabbit 521 α Siwi (1:2,000), Mouse α Ea7 Tubulin (Lab Isolated Stock) (1: 10,000) and HRP goat α mouse/rabbit (1:10,000). The expected size for Siwi is 100 kDa while tublin is 50 kDa. The blot was exposed for 2 seconds using ECL Prime.

After engineering the sgRNAs, plasmid based and FE modified sgRNA PCR products were transfected in BmN4s. **Figure 6** shows Siwi bands at the expected size of 100 kDa and of similar intensity despite having sgRNAs targeting Siwi or not. Upon observing a lack of knockdown in the PCR product, the effectiveness of the FE modified sgRNAs in plasmid form were tested.
Figure 7 shows the general transfection efficiency and brightness of the hCD4-ZsGreen is variable evident especially in comparing the Siwi sg1 and Siwi sg2 FE to the ZsGreen alone in 7A. It would seem the increase in hCD4-ZsGreen confers increased brightness and the number of cells that are transfected. After incubating for 8 and 9 days, Siwi loci were amplified from the genomic DNA of the transfected cells and subject to restriction digests to monitor whether or not these specific Siwi loci were edited. In Figure 8, we see the correct sizes for uncut Siwi loci 1 and 2 (164 and 131 bp respectively) however there are deviations from the expected sizes of the digested products. In the XhoI digestions of the Siwi sg1 locus there are 3 observable bands: one around 200 bp, 120 bp and 75 bp. The EcoRv digestes of Siwi sg2 locus shows 4 bands: one at around 175 bp, 130 bp, 120 and 50 bp. When comparing the Bmn4s that lack sgRNAs targeting Siwi and those who have them, it appears that there is a decrease in
intensity in the EcoRV digested product of the Siwi sg2 locus targeted by the Siwi sg2 FE RNA in 8B.

Figure 8 Siwi Loci Digestions Do Not Show Clear Editing- Panel A shows the general schematic of the DNA digestion protocol. The Siwi sg1 locus (amplified from Siwi6 Fwd and Rev primers) is shown in blue with a red XhoI restriction site. It is expected that with editing the 164 bp fragment will not be cut while without editing the XhoI site is retained and will yield products that are 46 and 188 base pairs. Siwi sg2 (amplified from Siwi7 Fwd and Rev primers shown in green with a yellow EcoRV site follows a similar logic in that if editing occurs a 131 bp band will be present while if there is not editing the site is cut and 94 and 37 bp bands. Panel B shows the digestions of samples from the 4/12/14 transfection. Panel C shows the digestions of samples from the 4/13/14 transfection.
I. Discussion

The CRISPR/Cas9 system is a popular and effective tool for genome editing. In this work we demonstrate the partial loss of ZsGreen mediated by the CRISPR system in BmN4s. However, the data presented in this report raises questions on optimization of the CRISPR/Cas9 system in *Bombyx mori*. First, the variable intensity between Cas9 +ZsGreen sg RNA 2 and Cas9 alone must be addressed. The difference may either be due to the variable amounts of Cas9 that were transfected or it may suggest that the sgRNAs somehow stabilize the Cas9 structure. The lower band at ~140 kDa may be a destabilized Cas9 without sgRNA and is not observed in the Cas9 alone sample due to the lower concentration. The absence of a band in the U6 ZsGreen sg RNA 1 was most likely due to experimental error based on downstream experiments (Figure 1). Next, the lack of a change in ZsGreen expression with regards to the T7 target sgRNA may suggest that the amount of mRNA may need to be increased to yield a visible effect on ZsGreen expression. Using mRNA is also difficult due to the issues with stability (Figure 2).

The knockdown of ZsGreen expression suggests a few areas where improvement can be implemented (Figure 4). First, hCD4-mCherry expression is too dim to clearly see colocalization which may be attributed to the localization on the plasma membrane or to dampening effects of hCD4. Future experiments should address this concern by comparing mCherry alone and by placing fusing tdTomato to hCD4 to observe a possible dampening effect. Also, the loss of ZsGreen expression should be better characterized by either a genome type assay based on the loss of restriction sites found in the targets of the sgRNAs or by probing against ZsGreen expression on a Western immunoblot. If we focus on targeting an endogenous locus, Siwi the results are slightly different than those seen in the sgRNAs targeting ZsGreen.

The lack of a clear difference in intensity of Siwi protein as seen in Figure 6 comparing conventional Siwi sgRNAs on a plasmid and modified Siwi sgRNA PCR products suggests that editing did not occur. This may be due to poor transfection efficiency of BmN4s or the concentration of sgRNAs is not high enough and they are getting degraded too quickly to assemble with Cas9 and initiate editing. Although the overall transfection efficiency was satisfactory as seen in Figure 7, it was still very variable and it appears in the context of the DNA digestion assay did not make a large difference.

Despite having being digested the products do not appear to the correct size which may suggest either off target cutting or the remnants of the restriction enzymes is effecting the shift of the bands. To address this concern samples should be heated inactivated for longer. In every amplicon subject to a restriction digest, cutting occurs which suggests that there is little to no observable genome editing. However, the change in intensity in the 120 bp band between the EcoRV digested ZsGreen transfected BmN4s and those transfected with the Siwi sg2 FE RNA may suggest some protection occurred. Although it is fainter in Figure 8B a similar trend is seen. It would be helpful to repeat this experiment with perhaps more sgRNAs. Overall, the data suggests that editing via CRISPR using conventional or FE modified sgRNAs of the endogenous Siwi locus is not occurring. This may be due to technical issue that require optimization or the *Bombyx mori* is especially resistant to NHEJ (non-homologous end joining).
mutations due to its slow growth and its ability to repair these mutations before they present an observable phenotype.

VI. References


