Genome editing efficiency in cell cultures of honeybees

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Objective of the training

Background

The honey bee, *Apis mellifera* (L.), is the most important managed pollinator for U.S. agriculture and is vital for global and domestic food security. Numerous flowering plants critically depend on insect pollination, and the honey bee is a generalist pollinator that cross-pollinates many plant species. Honey bees pollinate 80% of existing flowering crops, or 33% of human food consumption (Morse & Calderone, 2000, Klein et al., 2007). The value associated with honey bee pollination reached $11.68 billion by 2009 (Calderone, 2012). Honey bee colony populations are in steady decline, especially in the managed honey bee industry (commercial beekeeping). Annual beekeeper losses around 30-40% of their hives in recent years (Seitz et al., 2016). Such losses are unsustainable, although colony losses can be partially compensated by splitting existing hives.

The primary concern of honey bee health is the parasitic mite and pathogens including viruses (Cox-Foster et al., 2007, Evans et al., 2009). One of the most studied viral pathogens is the Israeli Acute Paralysis Virus (IAPV) because it thought to be associated with colony collapse disorder and over-winter losses (Chen et al., 2014, Cox-Foster et al., 2007). IAPV is a dicistrovirus, positive single-stranded RNA (+ssRNA) prevalent in invertebrates including orders of Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera (Bonning & Miller, 2010).

Even though dicistroviruses in bees were the first ones described (Bailey et al., 1963), we still lack critical knowledge on the viral molecular mechanism and signaling pathways to manipulate host genome and physiology. There is an urgent need to develop environmentally benign viral-control measures. One study showed non-target dsRNA may alter or suppress known immune pathways, or may be

predominantly post-transcriptionally regulated and contribute to the antiviral response in adult bees (Flenniken & Andino, 2013).

Integrating recombinant DNA or other genetic material into a host genome has proved to be a powerful tool for genetic and evolutionary studies and promoted research progress of genetics field. Transgenic insect methodology or technologies were developed 49 years ago (Caspari and Nawa, 1965, Handler and James 2002). Advanced development has been achieved in the insect models of *Drosophila melanogaster* with direct injection of DNA into fly embryos (Germeraad, 1976) and P transposable element discovery (Rubin GM and Spradling AC, 1982).

Genome editing is developing at a fast pace in recent few years since technologies of CRISPR (clustered regularly interspaced short palindromic repeats) discovered (Jansen et al., 2002, Barrangou et al., 2007, and Garneau et al., 2010, Cho 2014). The technology requires the adding a mixture of guided RNA and Cas9 proteins into the germ cells. Reports showed successful genome editing in flies (Bassett et al., 2013, 2014, Li and Scott, 2016), silkworm (Tan et al., 2014), but nobody has explored the possibility to establish this method in the honey bee system.

In human *in vitro* studies, a recent report showed that a unique form of prokaryotic gene regulation via Cas9 proteins. Cas9 nuclease from *Francisella novicida* (FnCas9) targets a bacterial mRNA, leading to suppression of gene expression. Together with an engineered small RNA (RNA-targeting guide RNA, rgRNA), FnCas9 can bind to positive single-stranded RNA (+ssRNA) virus such as Hepatitis C virus in human cell culture, which gives hope for virus control (Price et al., 2015). Other evidence also showed that efficacy of CRISPR/Cas9 system can be used for viral inference in plants (Ali et al., 2015).

Even though transgenesis approach has been employed to several insect systems (Coates et al., 1998, Lorenzen et al., 2002), it was still a difficult task to

carry in social insect of honey bee (*Apis mellifera*). One of the reasons is because the handling of honey bee embryos and post-injection rearing environment require high specific conditions, which is perhaps the difference of social insect development and biology versus other insects.

Alternatively, i*n vitro* cell line studies in honeybees may serve a primary role to screen and test for efficiency of designed guided RNAs. Up to date, one bee cell line AmE-711 line is available for testing (Goblirsch et al., 2013) . This cell line will be a fundamental system to study bee viral interaction and viral infections (Carrillo-Tripp et al., 2016). The goal of this training is to learn how to start a cell culture, and test new CRISPR/Cas9 system to achieve genome editing *in vitro*, which will promote deep studies on honeybee genetics, development, and viral interaction with host cells.

The hypothesis we are testing in this project is the CRISPR/ Cas9 system can be harnessed to target the highly conserved sequences identified in the genome, and edit the sequence of the targeted genes. The goal at this stage is a proof of concept to test the feasibility of genome editing via CRISPR/Cas9 in honey bee cell lines. The significance of this project is that the programmable Cas9-mediated DNA targeting may represent one of the most important potential applications of Cas9 in social insects, and provide fundamental screening for valid small guided RNA designs.

The aim of this training was to develop a new screen system of small guided RNAs using *in vitro* cell cultures. However, we experienced difficulties in the cloning a new construct of FnCas9 with a *Drosophila* universal promoter during our planned period of time. So instead, we adjusted our plan, and decided to use traditional SpCas9 (*Streptococcus pyogenes*) and designed rgRNA to target a critical development gene called *dnmt3* (DNA methyl-transferase 3) in the AmE-711 cell

line instead. The rationale was because *dnmt3* is a critical gene linked to honey bee queen and worker caste determination and sociality during social bee development (Kucharski et al., 2008, Li-Byarlay et al., 2013). However, the known biological function of this gene is still limited. An intriguing question to ask is whether we can develop a mutation to target *dnmt3* during honeybee development? Will this mutation line give us a robust system to study the genetic and genomic basis of social behavior and sociality? Therefore, our goal for this project is to create the mutation of *dnmt3* in the AmE-711 cell line. The DNA editing sequence after transfection will be evaluated and compare to control groups.

Trip Report

Preparation before the trip:

The targeted gene to edit is a DNA methyl-transferase 3 (*dnmt3*) with known genome sequence in the honey bee genome (Ensembl Metazoa 76 (GCA_000002195.1)). The trainee has used a suitable online tool of CRISPOR (http://tefor.net/crispor/crispor.py) to design, evaluate, and clone guide sequences for the CRISPR/Cas9 system. Three guide sequences (Sg4, 5, and 6) were selected based on their specificity scores, predicted efficiency, out-of-frame scores, and how many off-targets for mismatches next to PAM sites. The sequence information is listed in table 1 below.

Name	Guide sequence + PAM
Dnmt3-sg1	TCCCTTCCGTCGAACAGAAA*TGG*

Dnmt3-sg2	GCCGTTGAAAGAGCAGAAAC*CGG*
Dnmt3-sg3	GGTAAAGATGCAACAAAGAT*TGG*

Table 1: Sequence information of the guide RNA designs for this project.

Guide RNA oligos were ordered from Eton Bioscience (www.etonbio.com). Mix the final oligomers to a final concentration of 1 μM in NEB#2 buffer (www.neb.com). The mix was heated at 95°C for 5 min, and then cool down to room temperature slowly to form the double strand oligomers. Next, digestion of plasmid pDCC6 was performed by 1ul of FastDigest BpiI (FisherSci), 1ul of pDCC6 plasmid (1ug/ul), 2ul of buffer for BpiI (10x), and 16ul H2O at 37 degree for one hour, then 65 degree for 20 minutes. Then, the ligation the oligomers into pDCC6 was carried using 1ul of BpiI digested plasmid, 1ul of annealed oligomers, 0.5ul of T4 ligase, 0.5 ul T4 buffer (10x), 2ul of H2O at room temperature for 1 hour. After cloning, blue colonies were picked and tested for PCR using Phusion HF polymerase with forward and reverse oligo primers (98˚C for 5 min, 35 cycles of 98 ˚C for 5min/54 ˚C for 30 sec/72 ˚C for 20 sec, then 72 ˚C for 5 min, stay at 4 ˚C). Confirmed DNA gels were cleaned by a PCR purification kit (BS363-50, Bio Basic) and then sent for DNA sequencing to Eton Bioscience Inc. The null pDCC6 plasmid (10ng) was also purified and cloned to E. coli competent cells (50ul), then colonies were purified. SpCas9 plasmid is ordered from MLM3613 plasmid (Addgene, Plasmid #42251), which expresses Cas9 nuclease (*Streptococcus pyogenes*) from CMV and T7 promoters. All verified plasmids by sequencing were freeze-dried at NCSU and then mailed to University of Minnesota before the trainee's arrival.

After comparing different options in transfection reagents and our budget, the Lipofectamine™ 3000 Transfection Reagent was purchased from Invitrogen (L3000001) and shipped to University of Minnesota before December 2016.

Experimental procedures at University of Minnesota

The trainee flew from Raleigh-Durham International airport to the Minneapolis–Saint Paul International Airport and started the lab work started on Dec. 12th. Five tested different groups were 1) blank pDCC6 plasmid and MLM3613 (Cas9 donor), 2) Sg1 and MLM3613, 3) Sg2 and MLM3613, 4) Sg3 and MLM3613, 5) normal cells. Each group had 3 replicates. Reagent tubes were prepared with 17ul of LIPO and 425 ul of cell medium for each well of 17 wells. Tubes for group 1 were prepared with 0.5ug of blank pDCC6 plasmid, 0.5ug of MLM3613, and 25ul of medium. Tubes for group 2 were prepared with 0.5ug of Sg1 plasmid, 0.5ug of MLM3613, and 25ul of medium. Tubes for group 3 were prepared with 0.5ug of Sg2 plasmid, 0.5ug of MLM3613, and 25ul of medium. Tubes for group 4 were prepared with 0.5ug of Sg3 plasmid, 0.5ug of MLM3613, and 25ul of medium. Tubes for group 5 were prepared with 25ul of medium only.

AmE-711 cells were thawed before experiments started in the morning. First step was to wash the cells along the plate wall, take out old cell medium, and add 0.5ml of SFM. Step 2 was to mix 425ul of serum free medium (SFM) and 17ul of Lipofectamine 3000. In step 3, we took 95.7ul out of the LIPO reagent tube, and add it to the group tubes (group 1, 2, 3, and 4). In step 4, we took out SFM in each well, and then quickly added the 50ul of mixed reagents and 50ul of SFM. We used parafilm to seal the 24-well plate, then kept it in a Percival incubator with 32 degree

and 70% humidity. The incubation was 3 hrs with all treatment groups. On December 13th, we repeated the same procedure on a 2nd 24-well plate.

Seven-two hours after the transfection, cells were harvested from each well, and pictures were taken under the microscopes. One picture is listed in figure 1. DNA extractions were carried for 15 samples from the 24-well plate using the One-4-ALL Genomic DNA Miniprep Kit (BioBasic Inc). Primers were ordered at University of Minnesota for PCRs. All the cell DNA were used to amplify the target region of *dnmt3*.

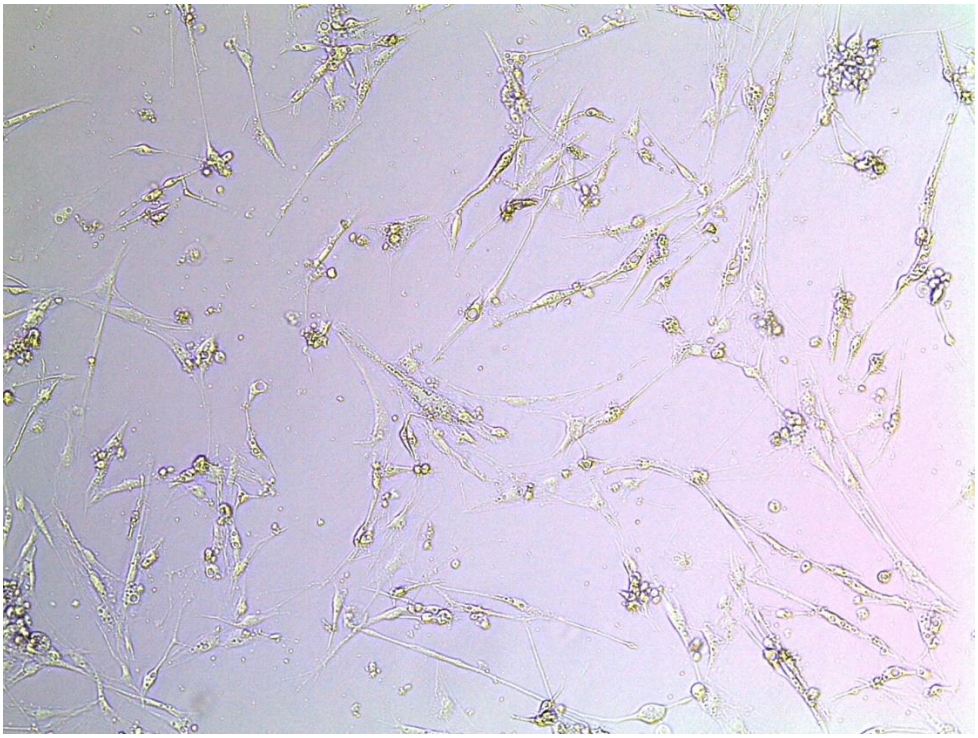

Figure 1: The graph represented one example of AmE-711 cells after 72 hours of transfection.

On December 16th, cell DNA from 2nd 24-well plate was harvested and extracted using the One-4-ALL Genomic DNA Miniprep Kit (BioBasic Inc) and

carried for PCRs. The concentrations were measured by a nanophotometer NP80 (IMPLEN). The PCR products were shipped to NCSU for sequencing.

Post visit experiments

In total 30 DNA samples were tested for PCRs to target dnmt3 region. The polymerase used for this PCR was DreamTaq (Fisher Scientific). The program for the PCRs is 95 Celsius for 2 minutes, 35 cycles of 95 Celsius for 15 seconds, 59 Celsius for 15 seconds, 72 Celsius for 45 seconds, then 72 Celsius for 10minutes, and keep at 4 degree. After gel imaging, 27 purified PCR samples were sent to Eton Bioscience for sanger sequencing. Samples 6, 28, and 29 were excluded due to no PCR products (Figure 2).

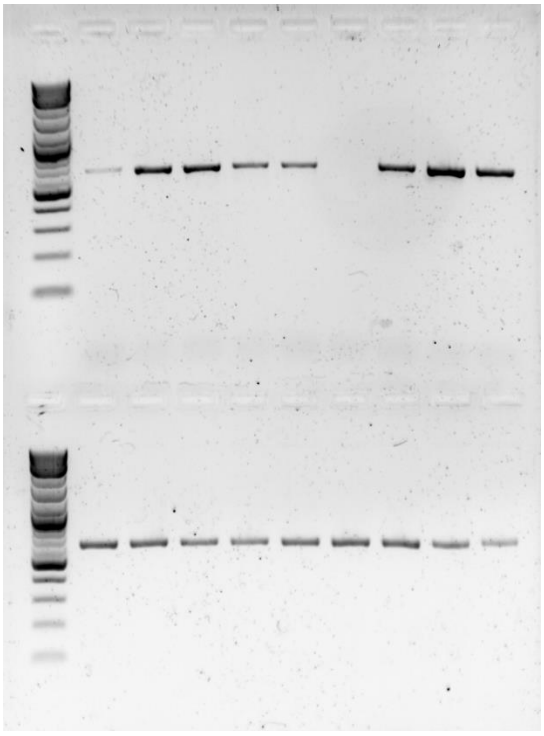

Figure 2: The gel images of PCR products of dnmt3 from cell DNA samples 1-18

The sequencing results had high noise in the background, so it was difficult to detect mutation in terms of inserts of deletions among the samples. Potential reason for high noise was natural polymorphism of the gene *dnmt3* in the cell cultures or

the DNA extracted is not pure. Future considerations are to test alternative methods for DNA extraction such as the phenol:chloroform:isoamylalcohol method.

After discussing with the trainer, we would like to design a new set of SgRNAs (Sg4, 5, and 6) and repeat the experiment. Please see table 2 for the sequence information.

Name	Sequence	Guide sequence + PAM
Dnmt3-sg4-exon 2 fw	gaaat**taatacgactcactata**GGATACGTTGGACCAGTGGGGTTTTAGAGCTAGAAATAGC	GGATACGTTGGACCAGTGGG *CGG*
Dnmt3-sg5-exon 2 fw	gaaat**taatacgactcactata**GGATGGAATTGGGCCTGATATGTTTTAGAGCTAGAAATAGC	GATGGAATTGGGCCTGATAT *CGG*
Dnmt3-sg6-exon 7 fw	gaaat**taatacgactcactataGG**AAATTGCCGGGCACAATTGGGTTTTAGAGCTAGAAATAGC	AAATTGCCGGGCACAATTGG *TGG*
Reverse primer	AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT **GCT ATT TCT AGC TCT AAA AC**	

Table 2: Sequence information of the guide RNA 4, 5, and 6 designs.

All the plasmids were prepared under the same procedures as before. Dried plasmids have been shipping to University of Minnesota for the 2nd attempt. After that, we will obtain the cell DNAs as we carried before, and prepare a DNA library

using the TruSeq DNA Methylation Kit (Illumina, Inc) for next generation sequencing to test the DNA methylation levels in treated cells.

Significance to the applicant and host laboratory

This training has provided a great opportunity for the trainee, Dr. Hongmei Li-Byarlay, to broaden her molecular skills on insect cell biology and promoted collaborations among bee researchers across different institutions between North Carolina State University and University of Minnesota. The host laboratory harvested a good collaboration with the trainee, and gained the potential to publish scientific findings together. The trainee has presented this work at a member symposium on Functional Genomics of Insects via Targeted Genome Editing of the Entomology Society of America 2017 annual meeting at Denver, Colorado.

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