IGTRCN Peer-to-Peer Training Fellowship Report

Project title: CRISPR/cas9 in the butterfly *Bicyclus anynana* for functional analysis of the evolution of adaptive traits

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Host lab: Dr. Christine Merlin, Texas A&M University, College Station, Texas

Period of visit: 4th July 2016 – 11th August 2016

Objectives of the training

The overarching goal of the proposed training was to establish efficient strategies for genome targeted mutagenesis in the butterfly *Bicyclus anynana* using CRISPR/Cas9 to ultimately genetically dissect the molecular basis of pheromonal communication in butterflies.

Bicyclus anynana is one of the few butterfly species in which the chemical composition of the male sex-pheromone (MSP) blend, which contributes to mate choice, has been characterized (Nieberding *et al.* 2008). The molecular pathways involved in butterfly pheromone production remains however unknown. Evidence suggests that Pheromone Biosynthesis Activating Neuropeptide (PBAN) could control like in moths pheromone biosynthesis in *B.anynana*, in spite of the fact that butterflies diverged from moths over 100 million years ago. Developing CRISPR-Cas9-mediated targeted mutagenesis in *B. anynana* would allow us to functionally test the role of PBAN in MSP production for the first time in a butterfly, and establish *B. anynana* as a viable model in which to functionally study the genetic basis of morphological novelties and their evolution.

The specific objectives of my visit to the Merlin lab were: 1) to learn butterfly rearing and egg injection techniques to ultimately apply them to *B. anynana*, and 2) to learn to assess their targeting efficiency *in vivo* in somatic cells through mutations screening strategies developed in the Merlin lab (Markert *et al*, 2016) and construct plasmids containing sgRNAs targeting a gene encoding *B. anynana* PBAN.

Outcome of the training

Objective 1: Butterfly rearing and egg microinjection technique

During my stay in the Merlin lab, I learned butterfly-rearing techniques from egg to adults that minimize bacterial and viral contaminations by participating in the maintenance of a large colony of monarch butterflies (*Danaus plexippus*). I also learned how to prepare injection needles and how to microinject freshly laid eggs of *D. plexippus*. To this end and to learn to assess sgRNA/Cas9 targeting efficiency *in vivo* in somatic cells, I injected ~300 eggs with a mix containing Cas9 mRNA, and either of two individual sgRNAs targeting a gene of interest to the Merlin lab.

Specifically, I collected eggs as they were laid on milkweed plant and injected them within 20 min under a dissecting microscope with a pulled borosilicate glass needle (World Precision Instruments, Inc.) attached to an IM 300 microinjector (Narishige) and filled with a solution containing Cas9 mRNA, sgRNAs, and blue food coloring dye to visualize the injection. After injection, embryos were placed in an incubator at 25° and 70% relative humidity, and developing embryos were transferred into individual small petri dishes until larvae hatched. I learned to screen for the

presence of mutations at the targeted site in somatic cells of surviving fourth instar larvae by performing noninvasive genotyping from clipped larval sensors. Genomic DNA from larval sensors was extracted using 0.01 × proteinase K in lysis buffer (100 mM Tris pH 8.0, 25 mM NaCl, 1 mM EDTA) and subjected to PCR using primers flanking the targeted regions. PCR products were purified using 1.5 × modified Sera-Mag Magnetic Speed-beads as described by Rohland and Reich (2012) and resuspended in 10 μl of water. I screened mutations using a Cas9-based cleavage assay in which purified PCR products (150–200 ng) were incubated for 3 hr at 37° with a purified Cas9 protein (100 ng), the sgRNA used for *in vivo* targeting (100–300 ng), BSA (1 μg/μl), and NEB Buffer 3 (1X), as described in Markert *et al*, 2016. By resolving the digested products on agarose gel electrophoresis, it was possible to determine the presence of mutated alleles, which appeared to be resistant to digestion by Cas9.

Objective 2: Construction of expression vectors containing BaPBAN sgRNA, *in vitro* transcription and quality control of the produced sgRNA

I learned that two major criteria need to be considered when designing sgRNAs for gene targeting *in vivo*: 1) the target site needs to be readily accessible *in vivo* to induce double-stranded breaks with high frequency and 2) one should be able to specifically amplify the genomic region flanking the target site for screening for mutations. Therefore, I selected three individual sgRNA target sites to be tested individually. The *B. anynana* PBAN (*Ba*PBAN) gene is composed of five small exons and four larger introns. Due to the fact that the sequence of the introns is not yet firmly established, I designed three sgRNAs targeting sites respectively on exon 1 (sgRNA_1), 2 (sgRNA_2) and 3 (sgRNA_3) using an online gRNA finder tool (http://crispr.mit.edu). In parallel, I tested primers to amplify the genomic regions flanking each target site from genomic DNA extracted from ethanol-preserved *B.anynana* bodies. Only two of the three primer pairs tested yielded specific amplicons. This potential target site was therefore excluded from further experiments. Synthetic oligomers specific for the remaining two sgRNAs (sgRNA_1 and sgRNA_3) were ordered, annealed and subcloned into a DR274 plasmid at the *Bsa*I cleavage site to construct two BaPBAN sgRNAs expression vectors, as previously described in Markert *et al*, 2016. These vectors were purified and sanger-sequenced to confirm proper integration.

I then learned how to perform *in vitro* transcription to obtain the corresponding sgRNAs. Briefly, each sgRNA-containing DR274 vector was linearized with *Dra*I, purified by phenol-chloroform extraction, and subjected to a T7 RNA polymerase. The resulting sgRNAs were further purified with acid phenol-chloroform and quantitated. I finally checked the quality of these sgRNAs by performing the Cas9 *in vitro* cleavage assay I described above, and found that both are of good quality as each can cleave *in vitro* the PCR fragments flanking their corresponding target sites of *BaPBAN*. I obtained several micrograms for each sgRNA that I will now be able to use for microinjections of *B.anynana* eggs.

Conclusions

The IGTRCN Peer-to-peer training program offered me a unique opportunity to gain the necessary hands-on experience to carry out an efficient CRISPR/Cas9-mediated gene knockout assay from egg injection to mutation screening. The BaPBAN sgRNA is now ready to be injected in *B. anynana* eggs. I am awaiting approval of the government agencies to raise *B. anynana* in my lab in Puerto Rico and

start generating *B. anynana* PBAN knockouts. Meanwhile, using Merlin's lab training, I have raised the first colony of Puerto Rican *D. plexippus* in my lab, and will use it for local outreach activities. I am also organizing a one-day workshop on the Basic Concepts of CRISPR/cas9 at the Inter American University of Puerto Rico in February 2017 to disseminate the knowledge I gained from the IGTRCN training program.

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References

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