Generation of genomic editing resources for NBRP Medaka: Experiments with RXFP4, PERIOD2, and Cas12a

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Abstract

Oryzias *latipes*, the Japanese rice fish commonly known as medaka, is a model research organism used worldwide for the biomedical, evolutionary, and ecotoxicology studies. As a part of NSF Project #1952513, we assisted the Japanese National Bioresource Project Medaka in three studies: 1) We designed primers for the gene Relaxin family peptide receptor 4 (RXFP4) and generated G0 RXFP4 KO medaka, 2) We participated in the synthesis of donor DNA for knockin experiments in an attempt to create the fusion protein PERIOD2::LUCIFERASE and alterative proteins PERIOD2::mNeonGreen and PERIOD2::nLucP, and 3) Tested the genome editing efficiency of LbCpf1 (Cas12a) in medaka by performing experiments testing various concentrations of Cas12a RNPs, measuring the proportion of survival and mutated phenotypes in injected embryos, and prepared donor DNA for an mNeonGreen knock-in into the middle of the slc45a2 gene.

Introduction

The phenotype of gene knockout (KO) and gene knock-in (KI) individuals can be used to infer the normal function of a gene or study the gene expression of a gene of interest in real time (Hoshijima et al., 2019). Gene editing technology has allowed researchers to create transgenic lines of organisms for study. One such organism is *Oryzias latipes*, the Japanese rice fish, commonly known as medaka. Medaka are used as a model organism used worldwide for studies including but not limited to human disease, ecotoxicology, and evolution of sexual systems (Murata et al., 2020). Medaka have a number of characteristics that make them an ideal animal model. The species has a relatively short generation time (6-10 weeks), is capable of laying approximately 10-30 eggs daily, and is easy to raise in the lab. Extensive research on medaka have also provided today's researchers with an abundance of genomic resources, inbred lines, and established protocols (Sasado et al., 2010; Wittbrodt et al., 2002). The Naruse lab at the National Institute for Basic Biology provides genetic strains of medaka for research purposes via the National Bioresource Project (NBRP) for Medaka (Sasado et al., 2010). Through NBRP, researchers can submit orders for medaka of their chosen strain, mutant, or transgenic line and access the Medaka Genome Project website which allows for users to search for genes of interest and BLAST against the medaka genome.

Relaxin family peptide receptor 4

Relaxin family peptide receptor 4 (RXFP4) has been showed to be involved in appetite and metabolism regulation and was recently hypothesized along with insulin-like peptide 5 (INSL5) to be a protective energy sensor; a regulatory element linking metabolism, inflammation, and homeostasis (Ang et al., 2017; Hechter et al., 2022). Still, the exact function of RXFP4 remains unknown. The creation of an RXFP4 KO medaka line will help to discover the gene's function.

PERIOD2

PERIOD2 (PER2) is a key gene in the regulation of circadian rhythm and is involved in modulating a number of processes including food and drug anticipation and sleep cycles; disruption of PER2 gene expression in mice has been associated with cardiovascular disease (Albrecht et al., 2007). Studying expression of PER2 and understanding the regulation of the circadian clock could assist in treatment of diseases like sleep disorders and diabetes (Iida et al., 2022; Itokawa et al., 2013). To assist in the monitoring of PER2 expression, previous studies in mice have shown that using a PERIOD2::LUCIFERASE fusion protein allows for real-time reporting of PER2 transcription (Yoo et al., 2004). While PER2 has recently been shown to have disrupted expression in medaka showing behavior patterns akin to seasonal depression (Nakayama et al., 2020), creation of a PERIOD2::LUCIFERASE line in medaka would further allow researchers to explore the expression of PER2 under various conditions in real-time. Although PERIOD2::LUCIFERASE transgenic mice have been used in research for nearly a decade, no ideal Cas9 PAM recognition site exists in the medaka PER2 gene, preventing the easy establishment of a PERIOD2::LUCIFERASE line in medaka. In addition, the large size of the luciferase gene may reduce genome-editing efficiency. Nowadays, a wide variety of luciferases both natural and synthetic are available for purchase; alternatively, creating a PERIOD::mNeonGreen fusion gene may also help to monitor PER2 transcription, although other studies have shown that using florescent proteins like GFP and mNeonGreen are less sensitive and produce more background signals than bioluminescent tags such as luciferase (Choy et al., 2003).

CAS12a

While the medaka PER2 gene lacks an ideal CRISPR-Cas9 PAM recognition site, an alternative Cas protein, LbCpf1 hereafter called Cas12a, is a promising alternative for the PERIOD2::LUCIFERASE knockin. Cas12a's PAM recognition site is 5'-TTTV-3', creating a double strand break approximately

18bp away from the PAM site (Moreno-Mateos et al., 2017). This extended distance between the PAM site and the cutting site has the potential to increase genome editing efficiency by reducing physical congestion near the cutting site. Although Cas12a has previously been used in zebrafish and mice, it has yet to be tested in medaka (Fernandez et al., 2018; Kim et al., 2016; Moreno-Mateos et al., 2017).

NSF-IRES Japan

In the summer of 2022, we performed the following three projects with support from an NSF IRES research grant (NSF Project #1952513) for NBRP Medaka: 1) Generated RXFP4 knockout G0 embryos for a collaboration project with Dr. Drake Hechter at the University of Winnipeg, 2) Attempted to synthesize donor DNA fragments for PERIOD2::LUCIFERASE, PERIOD2::mNeonGreen and PERIOD2::nLuc to generate a fusion protein to allow for real time reporting of PER2 3) Tested the Cas12a system in medaka by knocking out the slc45a2 phenotype with different concentrations of Cas12a ribonucleoprotein complexes and prepared donor DNA fragments to knockin mNeonGreen to the middle of the slc45a2 gene.

Methods

RXFP4 primer design

We designed primers to amplify our region of interest in RXFP4 using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012)(Table 1). To confirm the accuracy of our primers, we amplified crude DNA from *cab* strain medaka to confirm that our primers amplified a fragment of the expected amplicon size. DNA extraction was performed according to established protocols from NIBB (NIBB, 2018). Embryos were fixed in 100% ethanol for 15 minutes; residual ethanol was removed by vaporization at 70°C on a thermocycler. Proteinase K digestion was performed using 1mg/mL of Proteinase K in TE

buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) at 55°C. After 15 minutes, embryos were homogenized using tweezers. Proteinase K digestion continued for 2-3 hours before denaturing Proteinase K at 95°C for 10 minutes. Digested product was centrifuged at 4000 rpm for 3 minutes and crude DNA was taken from the resulting supernatant.

Each 11µL PCR reaction contained 2.55µL of Milli Q water, 5µL of 2X PCR buffer (Toyobo, Osaka, Japan), 2µL of 2mM dNTPs, 0.2µL each of 10µM forward and reverse primers, 0.05µL KOD-FX Neo polymerase (Toyobo, Osaka, Japan), and 1µL of template DNA. PCR was performed using the following program: An initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 20 seconds, and extension at 68°C for 30 seconds. PCR products were evaluated using a 4% Agarose E-Gel with SYBR Safe (Invitrogen, Massachusetts, USA) and E-GelTM 1 kb Plus Express DNA Ladder (Invitrogen, Massachusetts, USA).

Preparation of microinjection solution for RXFP4 targeted medaka

crRNAs were designed for two separate sites in *RXFP4* to test the efficiency of two separate DSB formation locations – one site near the C-terminal and one site by the N-terminal of one exon. To synthesize sgRNA, 1.2 μ L 100 μ M crRNA was combined with 1.2 μ L 100 μ M tracrRNA and incubated at 95°C for 5 minutes before cooling down to 24°C at a cooling rate of 0.1°C/second. 2.4 μ L sgRNA was held at 24°C for 5 minutes and subsequently mixed with 2.4 μ L Nuclease-free duplex buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate, Integrated DNA Technologies) to create 4.8 μ L of 25 μ M sgRNA which was stored at -80°C. The following day we combined 2 μ L of 25 μ M sgRNA, 0.8 μ L of 10mg/mL Cas9 protein, and 7.2 μ L of Milli Q water to create a microinjection solution that incubated at 37°C for 5 minutes. Prior to microinjection, the solution was centrifuged at 13000 rpm for 1 minute. To create large deletions, microinjection solutions using the two different sgRNAs were mixed in equal proportions.

Microinjection of RXFP4 targeted medaka

On June 3, 2022, we collected (n=70) fertilized embryos from *cab* strain medaka. Attachment filaments were removed from embryos using tweezers and clean embryos were prepared for injection under a dissecting microscope. Embryos were injected with microinjection solutions using a PLI-90A pico-liter injector (Warner Instruments, Massachusetts, USA). Injected embryos were raised in methylene blue and checked daily for death and mold growth. Methylene blue solution was replaced at least every other day and when dead embryos were found.

Heteroduplex mobility assay on RXFP4 targeted embryos

Embryos in which the exon's C-terminal were targeted for indels (n=15), whose N-terminal were targeted for indels (n=15), and where both C and N-terminals were targeted for indels (n=8) were randomly selected for a heteroduplex mobility assay (HMA) to test the efficiency of both sgRNAs in creating double strand breaks (DSBs). Crude DNA extraction and PCR amplification was performed using the same protocols as the RXFP4 primer test. Three combinations of custom primers were used to evaluate DSB activity and the formation of large deletions: 1) *RXFP4_F1* and *RXFP4_R1* to amplify the region near the N-terminal, 2) *RXFP4_F2* and *RXFP4_R2* to amplify the region near the C-terminal and 3) *RXFP4_F1* and *RXFP4_R1* to seek the potential for the creation of a large deletion between the N and C-terminals (Table 1). PCR products were evaluated using microchip gel electrophoresis on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using a DNA 500 Reagent Kit and pUC19DNA/MspI ladder.

PER2 crRNA test

To test our design of the crRNA for the C-terminal region of the PER2 gene, we microinjected medaka embryos with CRISPR-Cas9 RNPs at a concentration of 5uM; RNP solution was prepared using the same protocol as in the RXFP4 knockout experiment. Crude DNA was extracted, and PCR was performed using the primers *PER2_F* and *PER2_R* using the same protocols as in the RXFP4 primer test (Table 1). PCR products were visualized on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using 500bp reagents and a pUC19DNA/MspI ladder.

Luciferase Donor plasmid synthesis

We transformed *E. coli* DH5α Competent Cells (Takara Bio, Kusatsu, Japan) with pLV6-Bmal-luc plasmid (Addgene plasmid # 68833); pLV6-Bmal-luc was a gift from Steven Brown (Addgene plasmid # 68833; http://n2t.net/addgene:68833; RRID:Addgene_68833). Transformed cells were grown overnight on an LB plate treated with ampicillin at a concentration of 50mg/µl. A single colony was randomly selected to be cultured overnight in CircleGrow medium treated with an equal concentration of ampicillin. The following morning we removed the plasmid from the *E. coli* using a QIAPrep Spin Mini Kit (QIAGEN, Hilden, Germany).

An HA epitope flag, 3xFLAG epitope tag, and linker sequences were added using two rounds of PCR using KOD -plus- DNA polymerase (Toyobo, Osaka, Japan). The first round of PCR used the primers *FLAG-luc_F* and *linker-HA-luc* with the extracted plasmid as the template (Table 1). The second round of PCR used the product from the first PCR as a template and the primers *Linker+FLAG+F* and *linker-HA-luc* (Table 1). PCR solutions consisted of 2.5µL of 10µM of each primer, 5µL Buffer for KOD -Plus- (Toyobo, Osaka, Japan), 5µL of 2mM dNTPs, 1µL of KOD -plus-, 2µL of 25mM MgSO₄, 32µL Milli Q water and 0.5µL of template DNA. The PCR program for the first round of PCR was as follows: An initial denaturation step holding at 98°C for 2 minutes followed by 30 cycles of 98°C at 30 seconds,

annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds followed by 10 minutes of extension time at 72°C for 10 minutes. The second PCR program was identical to the first except with an annealing temperature of 45°C.

To confirm the addition of epitope tags and linker sequences to the plasmid, we performed a 1% agarose E-Gel with SYBR Safe (Invitrogen, Massachusetts, USA) and using a 1 Kb Plus Express DNA Ladder (Invitrogen, Massachusetts, USA) to view the presence of our amplicon around our expected size of 1.8kb. To discriminate the 43 bp difference in the products of the first and second rounds of PCR, PCR products were digested with XbaI restriction enzyme in M-Buffer for 1 hour at 37°C (New England Biolabs, Massachusetts, USA) and analyzed with a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using a DNA 500 Reagent Kit and pUC19DNA/MspI ladder. The second PCR product was cleaned with a NucleoSpin Gel and PCR clean up kit (Takara Bio, Kusatsu, Japan) and through gel purification.

Plasmids were ligated to PCR-blunt II TOPO plasmids with a Zero Blunt PCR Cloning Kit (Invitrogen, Massachusetts, USA) and inserted into One Shot *E. coli* cells following kit protocol. Transformed *E. coli* were plated on an LB agar plate containing Kanamycin (50ug/mL) and allowed to grow colonies overnight at 37°C. 184 colonies were randomly selected for Sanger Sequencing to confirm that donor DNA would be without mutations and contain linker sequences and epitope tags. Selected colonies were inoculated in CircleGrow with Kanamycin (50ug/mL) overnight and plasmids were extracted using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany).

Using the plasmid as a template, we prepared samples for Sanger sequencing using M13 Forward (-20) and M13 Reverse primers to amplify our sequence of interest within the plasmid and confirm the presence of epitope tags and linker sequences. In addition, we used Primer3 to design a custom primer, *luc_seq_internal_lee*, to sequence the internal luciferase region to confirm that no mutations had occurred during plasmid synthesis (Koressaar and Remm, 2007; Untergasser et al., 2012)(Table 1).

To prepare samples for sequencing we created a solution containing 0.5uL of 5µM plasmid template, 6.5uL Milli Q water, 1uL 10X Sequencing Buffer (ThermoFisher, Massachusetts, MA), 1uL BigDyeTM (ThermoFisher, Massachusetts, MA), and 1ul of 5µM primer. Sequencing reaction occurred in a thermocycler using the following program: 96°C for 1 minute followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 1 minute. Sequencing reaction samples underwent ethanol precipitation and were heated at 96°C for 2 minutes to ensure DNA denaturation before being sequenced on a 3130x1 Genetic Analyzer (Applied Biosystems, Massachusetts, USA and Hitachi, Tokyo, Japan). Sequences were aligned with the expected plasmid in SnapGene (GSL Biotech LLC, Chicago, USA).

PER2::mNeonGreen

We created a donor fragment to attach mNeonGreen to the C-terminal of the PER2 gene in order to create a PERIOD2::mNeonGreen fusion protein by using PCR to add PER2 homology matching regions and biotin-ends. We used the aforementioned KOD -plus- protocol to do so with an annealing temperature of 56C and an extension time of 2 minutes and 15 seconds using the primers *btn_per2_mNeonGreen-R* and *btn-per2-linker-F* (Table 1). PCR product was cleaned with a Nucleospin gel and PCR Clean-up kit (Takara Bio, Kusatsu, Japan) Amplicon size was confirmed using a 1% agarose E-gel with SYBR Safe using a 1kb plus express DNA ladder (Invitrogen, Massachusetts, USA).

sgRNA was synthesized by mixing equal parts 100uM crRNA and 100uM tracrRNA and incubating at 95C for 5 minutes before slowly cooling down at a rate of 0.1C per second to room temperature (24C). sgRNA was held at 24C for 5 minutes before 1.2uL of sgRNA was diluted with 18.8uL of nuclease-free duplex buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate). 3ul of the diluted sgRNA is added to 3ul Cas9 protein (1000ng/uL) and incubated at 37C for 10 minutes before adding 2.4uL of donor DNA fragment (50ng/uL) and 3.6uL HEPES buffer (200mM HEPES-KOH, 150mM KCl, pH 7.5). Microinjection solution was centrifuged at 13,000rpm for 1 minute prior to use.

Embryos (n=126) were injected using a PLI-90A pico-liter injector (Warner Instruments, Massachusetts, USA) and kept in methylene blue. Dead and moldy embryos were removed, and methylene blue solution was replaced at least every other day.

To test the efficiency of the knockin experiment, we genotyped embryos (n=45) approximately 4 days post-fertilization. Crude DNA extraction and the PCR program were the same as in the RXFP4 primer test. Three sets of primers were used. 1) *PER2_F and PER2_R*, 2) *PER2_F and MNG_R*, and 3) *MNG_F and MNG_R* (Table 1). The PCR for the first primer set used an extension time of 1 minute; the other primer sets used an extension time of 30 seconds. PCR products were visualized using PCR products were visualized on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using 2500bp reagents or 500bp reagents and a pUC19DNA/MspI ladder as appropriate.

Individuals with all 3 expected amplicon sizes were scored as having a correct knock-in, and efficiency percentage was calculated as the number of individuals with correct-knockins divided by individuals genotyped.

nLucP Methods

We created an alternative donor fragment using NanoLuc (NLucP). NLucP is a smaller insertion (~600bp) compared to luciferase (~1650bp); using a smaller insertion may increase the chance of KI success. We performed reverse PCR on the vector pUC19 (Addgene plasmid # 50005) - pUC19 was a gift from Joachim Messing (Addgene plasmid # 50005; http://n2t.net/addgene:50005; RRID:Addgene_50005; (Norrander et al., 1983)) - and PCR was used to add on homology matching regions to the pNL2.2 NLucP Hyglo vector(Promega, Wisconsin, USA). A 1% agarose E-gel was used to confirm the approximate size of the reverse PCR product (~3kbp) and the PCR product (~630bp) using a 1kb plus DNA ladder (Invitrogen, Massachusetts, USA).

Following the In-FusionHD Cloning Kit (Takara Bio, USA), we annealed NLucP to our modified pUC19 plasmid. We transformed *E. coli* with this plasmid and allowed them to grow overnight on an LB plate treated with ampicillin at a concentration of 50mg/µl. 16 colonies were randomly selected to check for the expected plasmid insertion size using *E. coli* colonies as our DNA template for PCR using M13 (20) F and M13 primers. Crude DNA was extracted and PCR was performed using the same protocols as in the *RXFP4* primer test. We selected 4 colonies with the expected amplicon size in the PCR test for overnight colony growth in 3mL CircleGrow and 3uL Ampicillin (50ng/uL). 5uL of colony saved in LB broth was allowed to incubate in the CircleGrow solution overnight at 37C with maximum shaking.

The next day, plasmids were removed from *E. coli* following the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). Insertion size of plasmids was confirmed on a 1% Agarose E-Gel with SYBR Safe (Invitrogen, Massachusetts, USA) and 1 Kb Plus Express DNA Ladder (Invitrogen, Massachusetts, USA). Colonies with the expected amplicon size were prepared for Sanger sequencing following the same protocol as when creating the luciferase donor DNA fragment.

slc45a2 knockouts

To test the genome editing efficiency of Cas12a in medaka, we performed a knockout experiment of the gene slc45a2 to easily identify individuals with altered phenotypes. We prepared three different concentrations (20uM, 10uM and 5uM) of RNP microinjection solution to test. To prepare these solutions we diluted our 100uM of stock solution crRNA by mixing with equal parts nuclease-free duplex buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate). 50uM crRNA was incubated at 70°C for 5 minutes and dropped to room temperature (24°C) at a rate of 0.1°C per second. RNP mixtures were prepared by mixing 50uM crRNA, 65uM Cas12 protein, and Milli Q water (Table 2). RNPs were incubated at 37°C for 5 minutes and held at room temperature prior to microinjection. A total of 341 embryos were injected over 3 days beginning on June 21st. Embryos were kept in methylene blue solution and monitored at least every other day for mold or death. Methylene blue solution was refreshed at least every other day. On June 27th, embryos were sorted into white-eye, mosaic-eye, and black-eye phenotypes. A heteroduplex mobility assay was performed to genotype individuals. Crude DNA was extracted and PCR was performed using the same protocols as in the RXFP4 primer test using the primers $45a2_F$ and $45a2_R$ (Table 1). Results were visualized on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using 500bp reagents and a pUC19DNA/MspI ladder. Lethality was calculated by taking the number of surviving embryos on phenotype scoring day and dividing by the total number of embryos microinjected. Efficiency of mutation phenotype generation was calculated by taking the number of non-black eyed (white-eyed or mosaic-eyed) individuals and dividing by the number of individuals that were scored.

mNeonGreen Cas12a KI into slc45a2

We synthesized an mNeonGreen DNA fragment to be inserted into the middle of the slc45a2 gene by performing PCR to add homology regions and biotin-ated ends to the mNeonGreen from the PAP27 plasmid. PCR was performed using the protocol used to add epitope tags and linker sequences to the luciferase plasmid but with an annealing temperature of 55°C and an extension time of 45 seconds. PCR product was cleaned with a Nucleospin gel PCR cleanup kit (Takara Bio, Kusatsu, Japan) and amplicon size was checked on a 1% agarose E-Gel with SYBR Safe (Invitrogen, Massachusetts, USA) and 1 Kb Plus Express DNA Ladder (Invitrogen, Massachusetts, USA).

Results

RXFP4 primer test and heteroduplex mobility assay

Gel electrophoresis showed the expected amplicon size for our *RXFP4* primer sets (Fig 1). Our heteroduplex mobility assay showed high efficiency in creating double strand breaks at both N and C-terminals of the RXFP4 exon, although the area near the N-terminal showed more multiple banding patterns than those at the C-terminal (Fig 2). We similarly report high amounts of DSB creation when both sgRNAs were used in combination.

PERIOD2 Fusion proteins

Embryos that were microinjected with our crRNA targeting the C-terminal of the PER2 gene showed strong banding patterns, suggesting that the crRNA was efficient at creating double strand breaks in our desired location (Fig 3). Wildtype individuals showed the expected amplicon size. It was observed that a number of amplicons larger than 500bp were also amplified.

Amplification of XbaI digested DNA showed amplicons of the expected size; 95bp for the first PCR product and 138bp for the second PCR product (Fig 4). After epitope tag and linker sequences were added to the plasmid, visualization of transformed *E. coli* colonies was possible via chip electrophoresis (Fig 5.) Sanger sequencing confirmed 2 plasmids that appeared to have the full, non-mutated luciferase sequence, correctly added epitope tags, and linker sequences.

We successfully synthesized a donor DNA fragment to insert mNeonGreen into the C-terminal of the PER2 gene. 22% of 45 microinjected individuals genotyped appeared to have a successful insertion (Fig. 6). However, we found that the non-specific amplification by *PER2_F* and *PER2_R* caused bands around the expected amplicon size of the luciferase insert.

While the initial M13 PCR results suggested the successful fusion of nLucP into the puc19 plasmid (Fig. 7), the subsequent gel and Sanger sequencing results showed that transformed colonies contained mNeonGreen instead of nLucP (Fig. 8).

Cas12a Results

A total of 116, 103, and 122 embryos were injected with RNPs at concentrations of 5µM, 10µM, and 20µM respectively. We generated mutated phenotypes successfully with all three concentrations (Table 3)(Fig. 9). Embryos injected with the highest concentration of RNPs showed the highest mortality rate with a survival rate of only 40.2%, followed by embryos injected with the lowest concentration of RNPs with a survival rate of 50%. Embryos injected with 10µM of RNPs had the highest survival rate of 68% and the highest proportion of embryos that had a mosaic or white eye phenotype with 59 mosaic embryos (84.3%) (Table 4). The highest concentration of RNPs had only 31 embryos with an altered phenotype (63.3%) and the lowest concentration of RNPs had 43 mosaic or white embryos (74.1%). Heteroduplex mobility assay showed strong banding patterns in embryos with altered phenotypes and primarily single bands in those with unaltered phenotypes. (Fig. 10).

Discussion and Conclusions

RXFP Project

Given the high proportion of multiple-band patterns shown in the heteroduplex mobility assay when each sgRNA is used separately and the implication of a large deletion of the RXFP4 gene region when both sgRNAs are used in tandem, we have high confidence that RXFP4 gene function will be disturbed in mutated cells. Embryos could be used to establish a G0 population for RXFP4-KO medaka. Future work will require the raising of G0 population and the breeding, selection, and maintenance of F1 and F2 generations to create individuals with loss of gene function in all cells following previously established protocols (NIBB, 2018).

PERIOD2 Fusion Proteins Project

We created a luciferase plasmid with epitope tags and linker sequences attached that could be used for the creation of donor DNA fragments for insertion into the C-terminal of the PER2 gene or any other locus. While our mNeonGreen insertion appeared successful, the non-specific amplification by *PER2_F* and *PER2_R* may be over-representing the number of successfully mutated individuals. *PER2_R* may also be prone to hair-pinning, reducing amplification efficiency. We suggest designing new primers for the continuation of this study to increase amplification efficiency and reduce non-desired amplicon byproducts. Our nLucP results suggested that the initial reverse PCR done to prepare for the In-Fusion HD cloning kit was a failure. We suggest that future studies troubleshoot the failure of the reverse PCR if necessary.

Cas12a Project

Our study showed that injecting medaka embryos with a CRISPR-Cas12a RNP concentration of 10 μ M is the most effective. Embryos injected with RNPs at a concentration of 10 μ M showed higher survival rates and higher rates of altered phenotypes than embryos injected with 5 μ M or 20 μ M of RNPs. This concentration is identical to the concentration of CRISPR-Cas12a RNPs previously found to be optimal in zebrafish (Fernandez et al., 2018). Heteroduplex mobility assay showed the expected appearance of indels in individuals with altered phenotypes and a lack of indels in individuals with unaltered phenotypes. The genome editing efficiency of Cas12a is comparable to that of Cas9, at least in this case. We suggest testing knockin efficiency in other genes to confirm these results. Should Cas12a have comparable efficiency to Cas9, the number of genome editing sites could dramatically increase, allowing researchers a much broader range of genome editing targets.

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Figures and Tables



Figure 1) RXFP4 primer test. 4% Agarose E-gel containing crude DNA from *Cab* strain medaka amplifying A) *RXFP4_F1* and *RXFP4_R1* and B) *RXFP4_F2* and *RXFP4_R2*. Expected amplicon sizes are 130bp and 144bp respectively. Ladder shown on the left is E-GelTM 1 Kb Plus Express DNA Ladder (Invitrogen, Massachusetts, USA).



Figure 2) **Heteroduplex mobility assay on RXFP4 KO embryos.** Samples run on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using 500bp reagents and a pUC19DNA/MspI ladder. *RXFP4_F1* and *RXFP4_R1* primers target an area near the N-terminal of one of the RXFP4 exons, whereas *RXFP4_F1* and *RXFP4_R1* primers target an area near the C-terminal of the same exon. *RXFP4_F1* and *RXFP4_R2* amplify an area spanning the N-terminal to C-terminal of the exon and signifies the creation of large deletions. The expected amplicon size of *RXFP4_F1* and *RXFP4_R2* for wildtype cab medaka is 1614bp. Numbers below the lanes represent individuals. Wt represents the wildtype – un-injected cab strain medaka.



Figure 3) **Chip electrophoresis for crRNA test for PER2 C-terminal region.** Chip electrophoresis performed on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using 500bp reagents and a pUC19DNA/MspI ladder. PCR was performed using *PER2_F* and *PER2_R* primers on crude DNA from medaka embryos injected with CRISPR-Cas9 using crRNA targeting the C-terminal region of the PER2 gene. Wildtype is represented by cab strain medaka. The expected size of the wildtype amplicon is 151 bp.



Figure 4) PCR Product of XbaI digest during the creation of the luciferase donor DNA plasmid. A)

XbaI digest of the first PCR product, B-E) XbaI digest of the second PCR product. Expected amplicon sizes were 95bp and 138bp respectively. Size difference between the products suggests the successful addition of the full linker and 3XFLAG sequence to the luciferase plasmid.



Figure 5) Example of colonies showing correct luciferase plasmid insertion sizes. Using PCR and the *M13 (-20) Forward* and *M13 Reverse* primers, epitope tags and linker sequences were added to the luciferase sequence from pLV6-Bmal-luc plasmid. Transformed colonies (n=184) were randomly selected to check plasmid insertion size. In this example, A) shows 4 colonies with the expected insertion size (~1.8kb), and B shows colonies that have an incorrect plasmid insertion with a smaller size. Chip electrophoresis was performed using a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using a DNA 2500 Reagent Kit.



pUC19DNA/MspI ladder. B) Individuals genotyped with PER2_F and PER2_R. Expected amplicon size is 949 bp. Individuals with a band within the green box are scored as having the correct amplicon size. Genotyping performed on a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, size is 237 bp. Individuals with amplicon sizes within the red box are considered as having the correct amplicon size. Individuals genotyped with the example, Individual 1 was genotyped using three primer sets. A) Individuals genotyped with the primers PER2_F and MNG_R - expected amplicon amplicon size. Samples were genotyped using a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using 500bp reagents and a primers MNG_F and PER2_R. The expected amplicon size is 112. Individuals with bands within the blue box are considered as having the correct Figure 6) Heteroduplex mobility results of PERIOD2::mNeonGreen experiment. Numbers underneath the lanes represent individuals – for Japan) using 2500bp reagents.



Figure 7) **Gel showing the plasmid size of colonies transformed with "pUC19-nLucP".** Expected PCR product size using M13 (-20) F and M13 R for both nLucP and mNeonGreen is around 800bp. Sanger sequencing later revealed that these colonies contain mNeonGreen.



Figure 8) Gel showing plasmid size pUC19-NLucP after removal of plasmid from transformed *E*.

coli colonies. Samples were run on a 1% agarose E-Gel with SYBR Safe (Invitrogen, Massachusetts, USA) alongside a 1kb Plus Express DNA Ladder (Invitrogen, Massachusetts, USA). Yellow box shows 4 samples of plasmids removed from *E. coli*. Plasmid was created by synthesizing the pUC19 plasmid and the nLuc sequence from the vector pNL2.2 NLucP Hyglo. B, C, D, and E colonies indicate plasmid size is too large.



Figure 9) **Representative medaka phenotypes from each concentration of RNPs for Cas12a slc45a2 knockout experiments.** Embryos injected with 5μM and 20 μM RNP microinjection solution are approximately 4 days post-fertilization. Embryos injected with 10 μM RNP microinjection solution are approximately 6 days post-fertilization, and the wildtype cab strain medaka is approximately 5 days postfertilization. Missing phenotypes lacked representative individuals or photographs.



Figure 10) **Heteroduplex mobility assay on Cas12a slc45a2 knockout embryos.** Heteroduplex mobility assay was performed through a PCR using the primers *45a2_F* and *45a2_R*. Chip electrophoresis was performed with a MultiNA MCE-202 instrument (Shimadzu Corporation, Kyoto, Japan) using a 500bp reagent kit and a pUC19DNA/MspI ladder.

Table 1) Primers used in this study. For primers that were used in this study that do not have sequences,

please contact Dr. Kiyoshi Naruse (<u>naruse@nibb.ac.jp</u>).

Primer name	Sequence
RXFP4_F1	CTTGACCTGGAAAGAATGGAGTT
RXFP4_R1	ATCCATCATAAGTCTCCAAGCC
RXFP4_F2	CTCAGACATACTTCTTTCCTCTGGC
RXFP4_R2	TGGACATAACTGCCAGTCTAAGT
FLAG-luc_F	ACGGGGATTATAAAGATCATGATATAGATTATAAAGATGATGATGATAAAGAAGACG CCAAAAACATAAAGAAAG
linker-HA-luc	CGAGCTTGATCCACTTGGACCAGAGCTAGCGTAATCCGGGACATCATATGGGTACAAT TTGGACTTTCCGCCCTTCTTG
Linker+FLAG+F	TCTAGCGGACCTTCAGGTAGTTCGAGCGACTACAAGGACCACGACGGGGATTATAAA GA
luc_seq_internal_ lee	TCGTGACAAAACAATTGCACTGATA
btn_per2_mNeon Green-R	CTCCGCCCGGGTGAGTGGGTCCGCCTGCTGGTTCACTTGTAGAGTTCGTCCATTCCCAT
btn-per2-linker-F	CAGCAGAGACTCCACCCCAGCCGGACTCTGGCTCCTCTAGCGGACCTTCAGGTAGTTC GA

btn_per2_luc-R	CTCCGCCCGGGTGAGTGGGTCCGCCTGCTGGTTCACAATTTGGACTTTCCGCCCTTCTT G
PER2_F	AGCAGAGAGGGTCGCAGCTC
PER2_R	CTTTCTGTGGGGATGACACAC
45a2_F	GCGTCTTTCTCATCGTGCACC
45a2_R	CTTCCCCAGTGCTCCGTAGCCA
MNG_F	GTAATGGGAATGGACGAACTCTAC
MNG_R	ACCGTTTATGGACCCAAAGATATG

Table 2) Recipe for various concentrations of RNP mixtures for use in slc45a2 knockout experimentwith Cas12a.

	crRNA	Cas12a protein	Milli Q
	(50µM)	(67µM)	
5μΜ	1µl	0.74µl	8.26µl
10μΜ	2.4µl	1.5µl	6.1µl
20μΜ	4.8µl	3.0µl	2.2µl

Table 3) Phenotype scoring of surviving embryos under different Cas12a slc45a2 knockout RNP concentrations. Embryos were injected over three days beginning on June 21, 2022. Phenotypes were scored on June 27, 2022.

	White	Mosaic	Black
5μΜ	10	33	15
10μΜ	0	59	11
20μΜ	18	13	18

Table 4) Results of Cas12a slc45a2 knockout trials with varying levels of RNP concentrations.

Embryos were injected over three days beginning on June 21, 2022, with phenotype scoring performed on

June 27, 2022.

Concentration of	Total	Survived	Survival %	Phenotype white	% Phenotype white
Cas12a RNP	injected			or mosaic	or mosaic
$5\mu M$	116	58	0.5	43	74.13793103
10μΜ	103	70	0.67961165	59	84.28571429
20μΜ	122	49	0.401639344	31	63.26530612

References

Albrecht, U., Bordon, A., Schmutz, I., and Ripperger, J. (2007). The Multiple Facets of Per2. Cold Spring Harbor Symposia on Quantitative Biology *LXXII*.

Ang, S.Y., Hutchinson, D.S., Evans, B.A., Hossain, M.A., Patil, N., Bathgate, R.A., Kocan, M., and Summers, R.J. (2017). The actions of relaxin family peptides on signal transduction pathways activated by the relaxin family peptide receptor RXFP4. Naunyn Schmiedebergs Arch Pharmacol *390*, 105-111.

Choy, G., O'Connor, S., Diehn, F., Costouros, N., Alexander, H.R., Choyke, P., and Libutti, S.K. (2003). Comparision of noninvasive fluorescent and bioluminescent small animal optical imaging. BioTechniques *35*, 1022-1030.

Fernandez, J.P., Vejnar, C.E., Giraldez, A.J., Rouet, R., and Moreno-Mateos, M.A. (2018). Optimized CRISPR-Cpf1 system for genome editing in zebrafish. Methods *150*, 11-18.

Hechter, D., Vahkal, B., Tiede, T., and Good, S.V. (2022). Reviewing the physiological roles of the novel hormone-receptor pair INSL5-RXFP4: a protective energy sensor? J Mol Endocrinol *69*, R45-R62.

Hoshijima, K., Jurynec, M.J., Klatt Shaw, D., Jacobi, A.M., Behlke, M.A., and Grunwald, D.J. (2019). Highly Efficient CRISPR-Cas9-Based Methods for Generating Deletion Mutations and F0 Embryos that Lack Gene Function in Zebrafish. Dev Cell *51*, 645-657 e644.

Iida, M., Nakane, Y., Yoshimura, T., and Hirota, T. (2022). Effects of cryptochrome-modulating compounds on circadian behavioural rhythms in zebrafish. J Biochem *171*, 501-507.
Itokawa, M., Hirao, A., Nagahama, H., Otsuka, M., Ohtsu, T., Furutani, N., Hirao, K., Hatta, T., and

Shibata, S. (2013). Time-restricted feeding of rapidly digested starches causes stronger entrainment of the liver clock in PER2::LUCIFERASE knock-in mice. Nutr Res *33*, 109-119.

Kim, Y., Cheong, S.A., Lee, J.G., Lee, S.W., Lee, M.S., Baek, I.J., and Sung, Y.H. (2016). Generation of knockout mice by Cpf1-mediated gene targeting. Nat Biotechnol *34*, 808-810.

Koressaar, T., and Remm, M. (2007). Enhancements and modifications of primer design program Primer3. Bioinformatics *23*, 1289-1291.

Moreno-Mateos, M.A., Fernandez, J.P., Rouet, R., Vejnar, C.E., Lane, M.A., Mis, E., Khokha, M.K., Doudna, J.A., and Giraldez, A.J. (2017). CRISPR-Cpf1 mediates efficient homology-directed repair and temperature-controlled genome editing. Nat Commun 8, 2024.

Murata, K., Kinoshita, M., and Naruse, K. (2020). Medaka: Biology, Management, and Experimental Protocols, Vol 2.

Nakayama, T., Okimura, K., Shen, J., Guh, Y.J., Tamai, T.K., Shimada, A., Minou, S., Okushi, Y., Shimmura, T., Furukawa, Y., *et al.* (2020). Seasonal changes in NRF2 antioxidant pathway regulates winter depression-like behavior. Proc Natl Acad Sci U S A *117*, 9594-9603.

NIBB, N.I.o.B.B. (2018). Genome Edititing and Imaging of Fish and Amphibians. In The 10th NIBB International Practical Course.

Norrander, J., Kempe, T., and Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene *26*, 101-106.

Sasado, T., Tanaka, M., Kobayashi, K., Sato, T., Sakaizumi, M., and Naruse, K. (2010). The National BioResource Project Medaka (NBRP Medaka): An Integrated Bioresource for Biological and Biomedical Sciences. Experimental Animals *59*, 13-23.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., and Rozen, S.G. (2012). Primer3--new capabilities and interfaces. Nucleic Acids Res *40*, e115.

Wittbrodt, J., Shima, A., and Schartl, M. (2002). Medaka--a model organism from the far East. Nat Rev Genet *3*, 53-64.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., *et al.* (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A *101*, 5339-5346.