Equipped with a Cre-Dependent CRISPR-CAS9 System to Investigate Amyotrophic Lateral Sclerosis in Bengalese Finch Models

B. Colquitt, A. Meduri, C. Ortiz [1,2,3]

[1] *University of California, Santa Cruz. Santa Cruz, 95064 California, United States*

The Peroxisome Proliferator-Activated Receptor gamma (PPARγ) transcription factor is a major contributor to antioxidant defense, inducing expression of neuroprotective factors that suppress proinflammatory genes and stimulate antibiotic enzymes. A mutation in this gene could disrupt antioxidant defense mechanisms, causing amplification of Reactive Oxygen Species (ROS) and consequential cell death. If this genetic manipulation were to occur in neurons, or more specifically, motor neuron pathways, the occurrence of cell death would have the capacity to lead to the progression of Amyotrophic Lateral Sclerosis (ALS) – a motor neuron degenerative disease.

With ALS causal effects still being investigated, we intend to contribute to this research focus through a genetic engineering research project. This project aims to induce the disease through genetic manipulation, revealing subsequent effects on endogenous molecular pathways and unraveling distinct mechanisms driving this incurable Neurological disease. Hidden in the specificities of these mechanisms could be a promising approach to target these diseases therapeutically. To fulfill this research goal, the Colquitt Lab at the University of California, Santa Cruz has constructed a Cre-Dependent CRISPR-CAS9 system targeting the first and second exons on the PPARγ gene. This plasmid vector is intended to be made readily available for AAV production at any time, allowing for cerebral injection in Bengalese Finch songbirds.

Keywords: PPARγ, Antioxidant Defense, Reactive Oxygen Species (ROS), Cell Death, Motor Neuron, Amyotrophic Lateral Sclerosis, Cre-Dependent, CRISPR-CAS9, Genetic Manipulation, Exons

Introduction

In order to allow for the high motor activity driving birdsong, high neuron firing is essential, which causes high stimulation of calcium influx into the cell. Amplified production of this intracellular calcium leads to enhanced free radical production, as it augments stimulation of Calcium-dependent enzymes, including NADPH Oxidases, which produce Molecular Oxygen as a by-product. The accumulation of Reactive Oxygen Species, or free radicals, could lead to cell death of motor neurons, as the buildup of free radicals could oxidize mitochondrial proteins, cause DNA defects, or damage the lipid membrane, eventually leading to mitochondrial dysfunction and activation of apoptotic signals. In order to prevent this from occurring, the PPARγ transcription factor activates expression of neuroprotective factors that mediate free radical production and allow for oxidative stress regulation, promoting cell health despite high neuron firing during birdsong.

The PPARγ transcription factor, bound by PGC1a transcriptional co-activator, is required to activate expression of genes that allow for mitochondrial biogenesis and antioxidant defense. In the context of neuronal activity, PPARg regulates oxidative stress regulation, or antioxidant defense, which

allows for healthy mitochondrial function, and thereby the regular production of ATP for cell health, which drives connections between neurons. The ATP produced by the mitochondria is co-released with Acetylcholine at the synaptic terminal of the neuromuscular junction – the connection between the motor neuron and muscle fiber. This allows for the opening of active sites on the actin filaments of the muscle fibers, recruiting myosin for muscle contraction. The myosin then hydrolyzes this ATP, the released energy allowing for temporary disengagement of myosin from the actin-myosin cross bridge, resulting in the muscle contraction mechanism. PPARγ dysfunction would lead to a disruption in oxidative stress regulation, and the subsequent amplification of Reactive Oxygen Species would cause ATP Synthase malfunction and unavoidable motor neuron death, or neurodegeneration. Without neurotransmitter release from the neuromuscular junction to the muscle fiber, muscle contraction would not occur, which would lead to Amyotrophic Lateral Sclerosis. Additionally, even if the actin filament active sites were exposed and myosin did bind, the lack of ATP production would prevent effective myosin-actin interaction, and prevent proper muscle contraction. Therefore, the downregulation of the PPARγ transcription factor or any component associated with PPARγ, could lead to progressive degeneration of motor pathways.

In order to understand the molecular effects resulting from PPARγ mutations, it is essential that the endogenous pathways undertaken by PPARγ are understood. The production of PGC1a, the transcriptional coactivator that allows for PPARγ function, relies on the MAP-Kinase pathway. In skeletal muscle cells, the MAP-Kinase pathway leads to the expression of the CREB and MEF2C/MEF2D transcription factors, which then bind to the promoter region of the PGC1a gene and allow for PGC1a transcription. The produced PGC1a would then bind to and activate PPARγ. The produced PPARγ transcription factor binds to PPRE (a DNA-binding response element) in neuronal DNA that stimulates expression of genes involved in mitochondrial biogenesis and antioxidant defense. In order to bind to PPRE, the PPARγ transcription factor heterodimerically binds to the nuclear retinoic X receptor (RXR) – a receptor localized at the nucleus of cells, playing a crucial role in gene regulation. The PPARγ-RXR complex then binds to PPRE and influences expression of mitochondrial genes, including the SOD1 gene. The SOD1 gene encodes an antibiotic enzyme that interacts with copper or zinc to break down the free radicals, further reiterating the importance of PPARγ in antioxidant defense, as free radicals are Reactive Oxygen Species, so a build-up of free radicals would lead to mitochondrial dysfunction. PPARγ gene mutations also influence glucose metabolism -- a process that is essential for the preservation of antioxidants, and the production of ATP. Glucose is metabolized through the pentose phosphate pathway to produce NADPH, which is needed to keep glutathione in its antioxidant reduced state and prevent oxidative stress.

Identifying the main mechanisms that are impacted by PPARγ mutations could reveal the causal effects driving the progression of Amyotrophic Lateral Sclerosis, and could also suggest approaches on how to cure this disease. In order to make this possible, we constructed a Cre-Dependent CRISPR-CAS9 system targeting the first and second exons in the PPARγ gene – a plasmid vector that is readily available for cerebral insertion.

Methods/Data

The Cre-Dependent CRISPR-CAS9 system is regulated by the Cre-Lox flip-excision (FLEx) vector switch. During Cre-Lox Recombination, there are two components involved: Cre recombinase (site-specific) and LoxP DNA sequences. The LoxP DNA sequence is a

thirty-four base-pair (bp) sequence with two palindromic thirteen bp sequences on either side of an eight bp spacer. The gene resides in between two LoxP sites, which can either be in cis, or surrounding the gene, or can be in trans – on opposite ends of the genome. Cre-Recombinase recognizes these sites, and binds to each site to induce recombination between the sites. To clarify, recombination occurs between two DNA strands, not the same DNA strand, so with two LoxP sites on each strand, there are four sites involved. If in cis, and the LoxP sites are in the same orientation, then the gene will be excised after recombination, which is an irreversible process. If LoxP sites are in opposite orientation to each other, the gene will be inverted, which is a reversible process. FLEx switch constructs are only successful if the two LoxP sites have the same spacer sequence. The fulfillment of this criteria allows for Cre to recombine the LoxP sites.

We made use of this criteria when constructing our plasmid vector, in order to make the CAS9 expression Cre-Dependent. We placed an inverted mCherry (Red Fluorescent Protein) in between two different LoxP sequences, and an inverted Cre-Dependent SaCAS9 between the other pair of the LoxP sequences. Then, ensured that the LoxP sites are in opposite orientation to each other. This way, once Cre-Recombinase recombines the LoxP sites, both sequences will be inverted in the right-side-up direction, and the CAS9 will be expressed along with the other CRISPR components.

The CRISPR-CAS9 system allows for excision at any area in the gene of interest in order to induce genetic mutations and observe the resulting effects on the model organism. This system involves two components – the expression of gRNA and CAS9 in the target cell. The first component is the guide-RNA, which is made up of the scaffold sequence and the spacer sequence. The scaffold sequence of 80 bp adds functionality to the gRNA, allowing it to bind to the target sequence, or in this case, the target PPARg exon. The spacer sequence is a 20 bp stretch that is complementary to the target sequence followed by the Protospacer Adjacent Motif (PAM) sequence, which is the anchor for the gRNA and allows for site-specific cleavage of the target gene. Once the gRNA binds to the PAM sequence, which is encoded by NGG, with N being any nucleotide, CAS9 then recognizes and binds to the gRNA. CAS9 is an endonuclease, and excises the target sequence by inducing a double-stranded break at three nucleotides upstream of the PAM site on the non-target strand and on the target strand. In this process, the sequence in between is cleaved out.

As for the process of building the final plasmid construct, containing the Cre-Dependent CRISPR-CAS9 system for the purpose of mutating PPARγ, the techniques applied include: nucleic acid amplification, microbial manipulation, and cell culture. This section covers a thorough description of the methodology that has led to the construction of the final plasmid vector.

To begin the research project, we used the CRISPOR database to search for gRNA primer sequences that target the first and second PPARγ exons. Using these primer sequences, we would be able to generate the corresponding gRNA sequences through the Polymerase Chain Reaction (PCR) afterwards. After checking the Basic Local Alignment Search Tool (BLAST) in order to confirm whether these sequences are found in the Bengalese Finch genome, the following primer sequences were ordered: gRNA forward and reverse primers encoding a gRNA sequence that targets the 82nd base-pair on the first exon (82-exon 1), gRNA forward and reverse primers encoding a gRNA sequence that targets the 97th base-pair on the first exon (97), and gRNA forward and reverse primers encoding a gRNA sequence that targets the 82nd base-pair on the second exon (82-exon 2).

These sequences came in as single-stranded

oligonucleotides in solution. After annealing these sequences to the plasmid vector PMB1052, a plasmid that we had separately ordered to contain the Cre-Recombinase gene and the gRNA promoter, U6, the plasmid vectors were sent for PCR, where DNA Polymerase used the forward and reverse primers to generate the gRNA sequences – fill the space in between the primers. Through PCR, we would also obtain a large enough concentration of plasmids to move forward with ligation of the gRNA sequences into the main backbone plasmid vector.

After the primer oligonucleotide annealing procedure, we then moved forward with the Golden Gate Ligations, which was intended to remove each gRNA sequence from its respective vector containing their primers, and ligate each of them into a separate PMB1052 backbone plasmid vector. This method was executed using the following reagents, increased by five times the original concentration in order to have enough concentration for E. Coli Transformations.

Reagents for Golden Gate Ligations (5x reactions = 5x original concentrations):

- quick ligase buffer - 62.5 ul
- BSA (recombinant) -=> recombinant albumin - 0.625 ul
- BSA1 HF - 5 ul (high fidelity enzyme used instead of standard BSA1 NEB enzyme)
- quick ligase - 0.625 ul
- backbone vector PBM 1052- 5 ul
- H2O - 46.25 ul
- diluted oligo anneal - 1 ul --> add after creating PCR tubes with each above mix => 120 ul total
- **doing x5 reactions, so for example: instead of 12.5, doing 62.5

The BSA1 restriction enzyme was used to digest the original vectors containing each gRNA sequence and their primers, in order to splice out each gRNA sequence. The oligonucleotide indicates the corresponding gRNA sequence and its vector. The quick ligase enzyme and buffer were used to ligate the gRNA sequences into their new backbone plasmid vector, which is also included as a part of the reagents.

At this point in the methodology, we have three plasmid vectors, each containing its corresponding gRNA sequence, Cre-Recombinase gene, and U6 promoter gene. We now move forward with E. Coli Transformations in order to obtain large enough plasmid concentrations for cerebral injection, or more specifically, pallial injection.

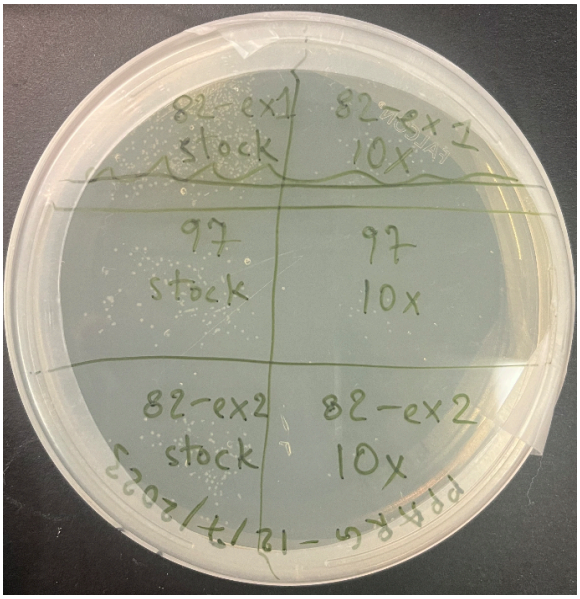
The plasmid vectors were integrated into the E. Coli competent cell genome, and grown on agar plates with ampicillin in order to grow transformed bacterial colonies that are ampicillin-resistant. The following procedure is as follows:

NEB Stable Heat Shock Transformation - Modified SOP
Ask to schedule use of incubator beforehand so someone else isn't already using it when you intend to use it during your experiment
When labeling => initials/date/gene name/way to differentiate between the tubes - A, B, C/etc
Get a bucket of ice and the 50ul sample of E.Coli competent cells from -80 degrees Celsius (freezer)
working with NEB C3040H Stable (competent E.Coli High Efficiency)
invert them, flick them, spin them down using centrifuge => NO VORTEX => throughout this protocol
Pre-warm water bath to 42 degrees Celsius (**do beforehand**, check thermometer to accurately confirm water bath temperature)
Get SOM (Stable Outgrowth Media) from Deli Fridge and warm to room temperature (**do beforehand**)
Pre-make aliquots of 450ul SOM media for future 10x dilution of stock concentration

(competent cells + plasmid mixture) ==> USING LATER
For 10 minutes, thaw the tubes of 50ul competent cells (1 tube competent cells for each PCR sample of [plasmid + primers]) --> working with 3 PCR samples, so 3 tubes of E.Coli
set timer/write down time on paper => throughout this protocol
Add 2ul PCR sample (plasmid) into the 50ul tube of corresponding competent cells
flick tube, invert, spin them down => DON'T VORTEX
****At this point, you have 52ul of cells****
Place the three mixtures on ice for 30 minutes => Don't mix
Heat shock the mixtures in the 42 degree water bath for 30 seconds//Don't mix
Place mixtures immediately on ice for 5 minutes//Don't mix
Check both shaking and non-shaking incubator to see if both are set at 30 degrees Celsius => preparing for next steps
Pipette 950ul SOM into each of the mixtures => Place at 37 degrees Celsius in shaking incubator (shaker) for one hour
while waiting, warm the selection plates to 37 degrees celsius in the non-shaking incubator
After 1 hour incubation, mix cells thoroughly by flicking/inverting/spinning them down
****Now you have 1000ul of each stock solution****
Prepare 10x dilution using **STEP 4 ALIQUOTS** => Pipet 50ul of each of the three stock solutions into each of the three 450ul SOM aliquots respectively
****Now you should have 950 ul of each stock solution, and 500ul of each 10x dilution****
PLATING: => plates have agar gel and ampicillin antibiotic so the colonies that grow are ampicillin resistant cells => Takes 1 hour
take the three warmed plates => label them half 10x, half stock
get three glass sticks to use to streak the plates
get two petri dishes to pour the ethanol (one to rest the glass sticks before and after use, one to actually dip into when plating)/make sure to keep using different glass sticks so no double dipping
turn on bunsen burner

place the stick into the middle/bottom part of the bunsen burner to melt half the stick, then melt half of the top half => place in ethanol
add 50ul of stock => dip glass stick in ethanol, dry => wait 10 seconds => place stick on a dry part of agar gel to cool it off => streak the 50ul across the stock-half of the plate
repeat step f with 10x dilution
clean petri dish/throw away glass stick after completing protocol with one plate

However, the first trial for Transformations produced satellite colonies, for which we believed was due to the use of an older version of Ampicillin, but after a second trial, we had received no colonies. We traced back to the reagents used during Golden Gate Ligations, and saw that we had been using a version of BSA1 that is of low fidelity, so we ordered a BSA1-High Fidelity. Using the BSA1-HF, we tried both a second trial of Golden Gate Ligations and a third trial of Transformations, and colonies were produced!



The resulting colonies were then grown even further through cell inoculations in order to further grow out each plasmid. This was to make its extraction through MiniPrep much smoother. Glycerol stocks of each plasmid were also made in

order to begin MidiPrep preparation if successful Sanger Sequencing results were obtained. The cell inoculation protocol is as follows:

Bacterial cell cultures grown even further to grow the plasmid out into large enough concentrations for a MiniPrep => to extract and purify the plasmid to send for Sanger Sequencing

Followed Protocol:

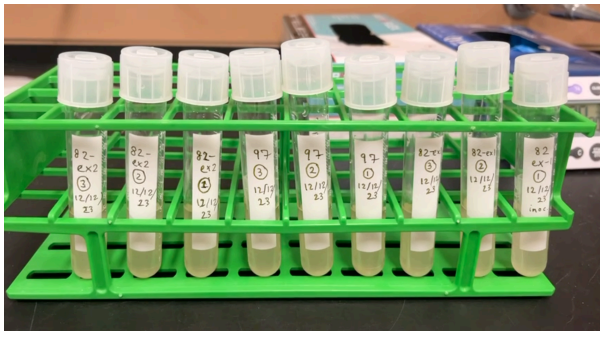
Made mix of [9 tubes x 3ml in each = 27ml =>] 30ml LB liquid and 30ul of ampicillin (9 tubes x 3ul ampicillin in each)

Took nine culture tubes, and filled each with 3 ml mix

Picked three colonies (from each of the three plasmids) and added to the 3ml mix in each respective tube

Glycerol stocks made in order to prepare for MidiPrep after Sanger Sequencing (if sequencing results are successful): Added 0.5ml bacteria suspension to 0.5ml 50% glycerol – store at -80°C

Used ~2 ml of bacteria suspension for DNA miniprep (ZymoPURE plasmid miniprep)



The ZymoPure Plasmid MiniPrep Kit was used to purify and extract the plasmids from these bacterial cell culture concentrations. The results are as follows:

A - Plasmid with sgRNA targeting 82nd base pair in exon 1

A1 (first colony picked):

- 167.2 ng/ul
- A260/280 = 1.92
- A260/A230 = 2.23

A2 (second colony picked):

- 280.0 ng/ul
- A260/280 = 1.92
- A260/A230 = 2.19

A3:

- 323.8 ng/ul
- A260/280 = 1.92
- A260/A230 = 2.20

B - Plasmid with sgRNA targeting 97th base pair in PPARG gene (either in exon 1 or 2)

B1:

- 359.1 ng/ul
- A260/280 = 1.93
- A260/A230 = 2.24

B2:

- 520.6 ng/ul
- A260/280 = 1.93
- A260/A230 = 2.28

B3:

- 578.5 ng/ul
- A260/280 = 1.94
- A260/A230 = 2.25

C - Plasmid with sgRNA targeting 82nd base pair in exon 2

C1:

- 530.9 ng/ul
- A260/280 = 1.95
- A260/A230 = 2.23

C2:

- 301.2 ng/ul
- A260/280 = 1.92
- A260/A230 = 2.21

C3:

- 420.9 ng/ul
- A260/280 = 1.93
- A260/A230 = 2.26

Successful results are indicated by three factors. The first factor is the ng/ul concentration, which is considered sufficient depending on the experiment conducted. For a Plasmid MiniPrep, results that are 200 ng/ul or above are considered more than satisfactory amounts of plasmid concentration. The second factor is the A260/280 ratio, which indicates the level of contamination of the sample. A ratio that is around 1.80 is considered the least concerning level of contamination. If the ratio is considerably less than 1.8, the sample has been contaminated. The third factor is the A260/230 ratio, which indicates the level of contamination by phenols or carbohydrates. The ideal ratio would be above 1.8, which would indicate little to no contamination.

After obtaining successful MiniPrep results, the plasmids were then prepared for Sanger Sequencing by diluting 10 ul of each plasmid from [its respective plasmid concentration ng/ul] to 80 ng/ul. After preparing each sample, they were then sent for Sanger Sequencing in order to determine whether the gRNA sequences aligned with the template gRNA sequences. The following results were obtained, with two of the plasmids swapped for different gRNA sequences:

3-3 —> A - guide RNA targeting 82 Exon 1
1-2 —> A
1-1 —> A
1-3 —> C - 82 Exon 2
2-3 —> B
2-2 —> B
3-1 —> C
3-2 —> C
2-1 —> B

If indicated by a-b (ex: 3-3), if a = 1, then the sequence is 82-exon1, if the a = 2, then the sequence is 97-exon 1, and if a=3, then the sequence is 82-exon 2. ‘b’ stands for the three samples of bacterial colonies picked from the same plasmid group (three bacterial colonies were selected from each plasmid group to move forward with cell inoculations). 3-3 and 1-3 were both switched. 3-3 was the gRNA for 82 Exon 1, and 1-3 was the gRNA for 82-exon 2.

After obtaining successful Sanger Sequencing results, the previously stored Glycerol stocks were then taken out for MidiPrep – a Plasmid Purification protocol for larger obtaining of plasmid concentration. This level of concentration would be used for pallial injection in the Bengalese Finch.

Due to the switch observed in the Sanger Sequencing results, the glycerol stocks were chosen carefully. Specifically, the glycerol stocks chosen were: 1-2, 2-2, and 3-2. These glycerol stocks are E. Coli competent cells containing each plasmid variation, so in order to extract enough plasmid concentration for long-term storage, and for immediate use of pallial injection, these stocks first had to be streaked out on plates to grow out their respective plasmids, and be further grown out through cell inoculations. The last step was the ZymoPure Plasmid MidiPrep, for which the results are the following:

Considering the requirement for a large plasmid concentration extracted through the MidiPrep, these plasmid concentrations are considered to be sufficient, allowing for the successful completion of the project – the construction of a Cre-Dependent CRISPR-CAS9 plasmid vector targeting PPARγ, that is readily available for

future use of cerebral injection.

Results

Our research project was to construct a plasmid vector containing a Cre-Dependent CRISPR-CAS9 system for the successful mutation of PPARγ – a vector made for long-term storage, to be readily available for a cerebral injection at any time.

We have successfully accomplished this goal, which is indicated by the Plasmid MiniPrep results, Sanger Sequencing results, and MidiPrep results. The Plasmid Extraction results from both the MiniPrep and the MidiPrep display sufficient amounts of plasmid concentrations indicated by >200 ng/ul and >350 ng/ul respectively, low contamination of foreign particles in each plasmid sample, indicated by the A260/280 ratio averaging 1.9, and low contamination of phenols and carbohydrates in each sample as well, indicated by the A260/230 ratio averaging 1.97. The Sanger Sequencing results demonstrated close to 100% alignment between the gRNA sequences produced theoretically and experimentally.

If this plasmid vector were to immediately be sent for AAV production, then given the results obtained through this research project, there is a high likelihood of PPARγ mutations occurring in neuronal cells, and a high possibility of observing drastic molecular and macroscopic effects on motor neuron function, from which we would gain deeper insights on Amyotrophic Lateral Sclerosis.

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