

The Effect of Gene Knockout of CEP290 using CRISPR-Cas 9 Technology on *Xenopus tropicalis*

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Abstract - Modelling human diseases using the *Xenopus* species is an increasingly popular method to study vertebrate embryology and development, basic cell and molecular biology, genomics, neuro-biology, and toxicology. This allows for the elucidation of the regulation mechanisms and interactive networks that affect the direct development of embryos, the adaptation process, and disease and malformation-causing dysregulations. Here we aim to analyze the possible kidney defects during the gene-knockout of CEP290 in *X. tropicalis* species. Our objectives are to produce sgRNA via de-novo synthesis from the constructed DNA template, to microinject synthesized sgRNA into embryos of *Xenopus tropicalis*, and to determine the success of the genome editing via T7-Endonuclease I assay and observation of genetically modified tadpoles for identifying any physical symptoms produced due to gene knockout. The data obtained from the embryos of *X. tropicalis* suggests that complete knock-out of the gene *cep290* results in severe mutation that causes death.

Keywords - CRISPR Cas 9; *Xenopus tropicalis*; Gene knockout; Kidney defects.

1 Introduction

Historically, gene targeting involves homologous recombination of an in vitro engineered exogenous DNA fragment thereby altering the genome of an organism through the process called targeted insertion mutagenesis [1]. This principle is the basis for gene knockout technique, wherein the expression of the target gene is permanently blocked, which may produce undesirable outcomes for the target organisms [1]. The gene of interest for this experiment is *cep290* which encodes a centrosomal protein of 290kD, found on chromosome 3 in *Xenopus tropicalis* [2]. Though the exact function of the gene is undiscovered, it has been found in humans that mutations in *cep290* are responsible for 148 phenotypes even though it only has 102 variants [3]. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) CRISPR-associated proteins (Cas) 9 system is a genome editing technique where a sgRNA is fused to produce synthetic RNA [4]. The target sequence is added to the mixture containing the two RNAs which directs the CAS9 to the target region for cleavage [4]. Non-homologous End Joining (NHEJ) repair mechanisms proceeds to fix the cleavage causing insertions and deletions mutations known as indels [4]. Since Cas 9 protein is used in this research, this is a Class 2, type II system wherein the protein is derived from *Streptococcus pyogenes* [5]. The Cas 9 protein searches for a genomic site containing protospacer adjacent motif (PAM) to the target sequence and the endonuclease cleaves the bonds between the

double strand upstream of the PAM [5]. Modelling human diseases using the *Xenopus* species is an increasingly popular method to study vertebrate embryology and development, basic cell and molecular biology, genomics, neurobiology, and toxicology [6]. This allows for the elucidation of the regulation mechanisms and interactive networks that affect the direct development of embryos, the adaptation process, and disease and malformation-causing dysregulations [6]. Moreover, in addition to the reasons listed in Figure 1, *Xenopus* species are closer to *Homo sapiens* than zebrafish, *Drosophila* or *C. elegans* [6].

Category:	<i>C. elegans</i>	<i>Drosophila</i>	Zebrafish	<i>Xenopus</i>	Chicken	Mouse
Broodsize	250-300	80-100	100-200	500-3000+	1	5-8
Cost per embryo	low	low	low	low	medium	high
High-throughput multiwell-format screening	good	good	good	good	poor	poor
Access to embryos	good	good	good	good	poor	poor
Micro-manipulation of embryos	limited	limited	fair	good	good	poor
Genome	known	known	known	known	known	known
Genetics	good	good	good	fair	none	good
Knockdowns (RNAi, morpholinos)	good	good	good	good	limited	limited
Transgenesis	good	good	good	good	poor	good
Evolutionary distance to human	very distant	very distant	distant	intermediate	intermediate	close

Table 1: Brief comparison between commonly used model organisms to conduct biomedical research and vertebrate embryology with respect to *Homo sapiens* [2].

Besides, administration of hCG (Human Chorionic Gonadotrophin) in *Xenopus* species increases the production of eggs throughout the year, thus ensuring a larger sample size. As a result, two *Xenopus* species are extensively explored in research: *X. tropicalis* and *X. laevis*, however, the former is preferred for this research due to its shorter lifecycle and ploidy (diploid) [6].

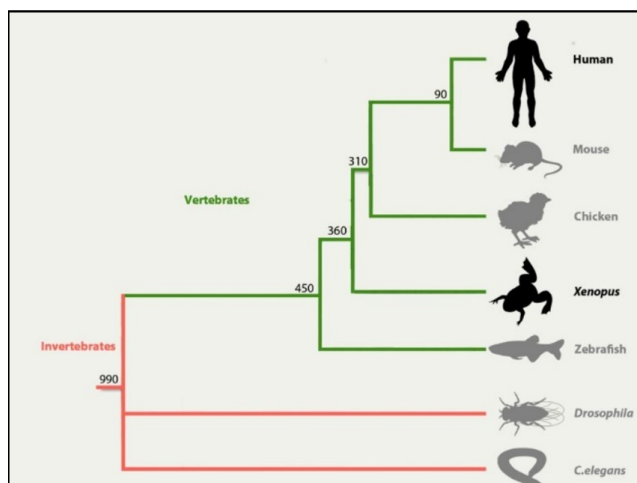


Figure 1: Predicted phylogenetic tree with divergence time in millions of years, the branch length determines the similarity between organisms in a phylogenetic tree, the closer the branch the more similar it is to the target organism (*Homo sapiens*/ Humans) [2].

2 Methods and Methodology

2.1 Designing sgRNA

The gene of interest, *cep290* was searched on the genomic database of *Xenopus* species called Xenbase. The gene structure was observed and the FASTA was downloaded to perform CRISPRScan to identify three sgRNAs from the sequence. All of the RNA were chosen from the seventh exon on chromosome 3, but they had varying CRISPRScan scores, and were chosen from varying strands, while ensuring the absence of off-target regions. sgRNA1 was chosen from the positive strand between positions 44962166-44962189 and was of sequence: taatacgactactataGGATTTGTAATCCTGTCCCCgtttagagctagaa, though sgRNA2 template was of the same strand and CRISPRScan score, its position was between 44962180-44962203 and the sequence was taatacgactactataGaaagcgcaacatattctgcgtagagctagaa. Meanwhile,

sgRNA3 template was selected from the negative strand of chromosome 3 with a CRISPRScan score of 59, between positions 44962207-44962230. The sequence of sgRNA3 template was taatacgactcactataGGGGCAGATTGACTCTCAGAgtttagagctagaa. The specific sgRNA templates were then suspended in nuclease free water and made up to a 100 μ M concentration each for the annealing and extension reaction. The amount of water for the concentration was calculated by multiplying the nanomole quantity of the oligonucleotides by 10.

2.2 Synthesis of dsDNA template for sgRNA production

The synthesized template was further annealed and extended to generate a dsDNA template for coding the sgRNA. The components of the reaction are shown in Table 2 and the thermal cycler conditions were set up as shown in Table 3.

Chemicals	Volume(μ L)
GoTaq G2 Master Mix	50
Nuclease free water	44
100 μ M Specific target Oligo	2
100 μ M Universal CRISPR Oligo	2

Table 2: Components of Annealing and Extension Reaction Mixture.

Step	Cycles	Temperature ($^{\circ}$ C)	Time (Min: Sec)
1	1	95	5:00
2	13	95	00:20
		65	00:20
		68	00:15
3	30	94	00:20
		58	00:20
		68	00:15
4	1	68	5:00
5	1	4	

Table 3: Thermal Cycler Conditions for Annealing and Extension Reaction.

The presence of the template in the product was tested by running 3 μ L of the product against 5 μ L of 100bp ladder on a 1.2% agarose gel, and all gel electrophoresis was performed as described by as shown in Table 4 [7]. The gel was observed under UV light for the presence of DNA template, and was then discarded.

Components	Amount
Agarose	0.6g
1X TBE buffer	5mL
Distilled water	45mL
Ethidium Bromide	5 μ L
Allowed to solidify for 30 minutes	
Loading dye (to be added to sample)	1 μ L
Sample (With loading dye)	3 μ L
100bp Ladder	5 μ L
Run the gel for 30 minutes at 70 Volts.	

Table 4: Procedure for Gel Electrophoresis.

2.3 In vitro Transcription of sgRNA

The DNA template was transcribed into RNA following the MEGAscriptTM T7 Transcription Kit (Table 5). The reaction was mixed and pulsed for a few seconds in a mini

centrifuge for the mixture to collect at the bottom of the Eppendorf tubes. This was followed by overnight incubation at 37°C on a hot block. The incubated mixture was pulsed in the mini centrifuges to remove condensation on the walls of the tubes followed by the addition of 1µL of DNase I to degrade the DNA molecule, and the mixture was further incubated for 15 minutes.

Components	Amount (µL)
T7 10X Reaction Buffer	2
75mM T7 ATP Solution	2
75mM T7 CTP Solution	2
75mM T7 GTP Solution	2
75mM T7 UTP Solution	2
Template DNA	8
T7 Enzyme Mix	2

Table 5: MEGAscript™ T7 Transcription Kit.

2.4 Purification of sgRNA and inclusion of CRISPR Cas9 protein

The sgRNAs were column purified using SigmaSpin™ Sequencing Reaction Clean-Up Kit and following the manufacturer's (SIGMA-ALDRICH) protocol and then visualized on a 2% agarose gel electrophoresis. The column is lined with size exclusion matrix suspended in water and 25ppm of Kathon, which was discarded before pipetting the incubated sample into the center, as the gel shrank due to previous spinning, of the column by centrifugation of the column at 0.6RCF for 2 minutes. The pockets present in the matrix allows the smaller molecules to sit in them while the areas of gaps between the molecules, allows larger molecules to be "excluded" and collected at the bottom of the column when centrifuged at 0.6RCF for 4 minutes. The column was then discarded and the quantity and purity of the resulting RNA samples was assessed using spectrophotometer, the Nanodrop N.D-1000 v3.8.1. The each sgRNA was stored as aliquots at -80°C.

2.5 Microinjection of CRISPR Cas9 construct in *Xenopus tropicalis*

Female *Xenopus tropicalis* were injected with hCG to induce artificial ovulation. The eggs released were collected and fertilised with frozen sperm after which, the embryos formed were de-jelled by cysteine treatment in order to facilitate better injection. The embryos were washed to remove the remnants of the treatment before injection of 4µL CRISPR Cas9 constructs (Table 6).

Components	Concentration (µL/mol)	Dilution	Amount (µL)
sgRNA1	2142	1 in 10	0.8
sgRNA2	794.8	1 in 5	0.8
sgRNA3	27.3		1.6
Cas 9			0.8

Table 6: Components of the Cas 9 mixture.

It should be noted that a typical embryo can intake only 4nL after which it bursts. Since different sgRNAs have different concentration, sgRNA with higher concentrations are diluted to ensure balance.

2.6 Extraction of genomic data for genotyping and observation of phenotypes

Tissue samples of the embryos were collected via digestion using a reaction mixture containing lysis buffer (Table 7) and incubated at 56°C for 2 hours. The samples were then incubated at 95°C for 15 minutes to inactivate proteinase K and then stored at -20°C.

Component	Concentration
Tris(pH 8.5)	50mM
EDTA	1mM
Tween-20	0.5% v/v
Proteinase K	100µg/ml

Table 7: Components of Lysis Buffer.

The samples were thawed and centrifuged at 1 RCF for 1 minute before further use. The EDTA acts as a chelating agent that binds to the metal ions in the enzyme in addition to inhibiting the nuclease activity of the enzyme during DNA extraction.

2.7 Amplification of Target Region

The tissue samples contained gDNA which were amplified via PCR reaction using components shown in Table 8 and thermal cycler conditions as shown in Table 9. The presence of DNA was then visualized on a 1.2% agarose gel electrophoresis.

Components	Amount (µL)
GoTaq G2 Green Master Mix	10
10Mm Forward Primer	1
10mM Reverse Primer	1
50ng Sample	2
Nuclease Free Water	6

Table 8: Components of Annealing and Extension Reaction.

Step	Cycles	Temperature (°C)	Time (Min:Sec)
1	1	95	5:00
2	40	95	00:30
		58	00:30
		72	1:00
3	1	72	7:00
4	1	4	

Table 9: Thermal Cycler Conditions.

3 Results

Double stranded DNA templates were synthesized from sgRNA and trasgRNA for the production of single guide RNA (Fig. 2). The DNA was then transcribed into sgRNA using MEGAshortscript™ T7 Transcription Kit and another gel electrophoresis was conducted to ensure its presence (Fig. 3). Moreover, the quantity and quality of the produced sgRNA was observed via spectrophotometry using Nanodrop ND-1000 v3.8.1 software (Fig. 4).

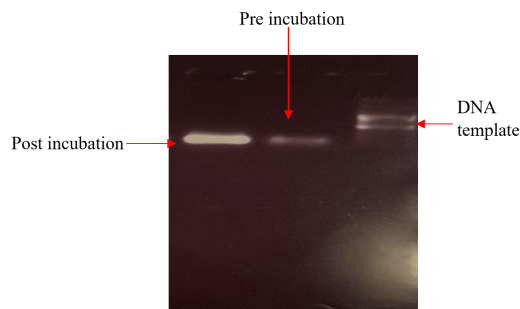


Figure 2: Oligonucleotides containing the target region and Cas9 binding structures were mixed, annealed and extended using Taq polymerase. Equal volumes of the reaction pre- and post- incubation were visualized on a 1.2% agarose gel and made visible by UV.

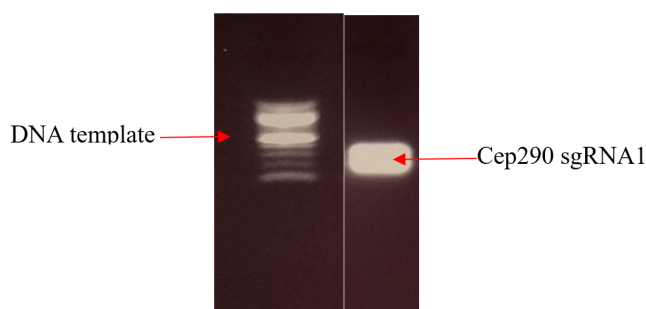


Figure 3: Gel electrophoresis with 2% agarose with bands indicative of presence of sgRNA following the Sigma Aldrich procedure for size exclusion chromatography. The columns contained silica gels that had holes for the bigger undigested DNA molecules. sgRNA molecules were thus collected at the bottom of the column and thereby purified.

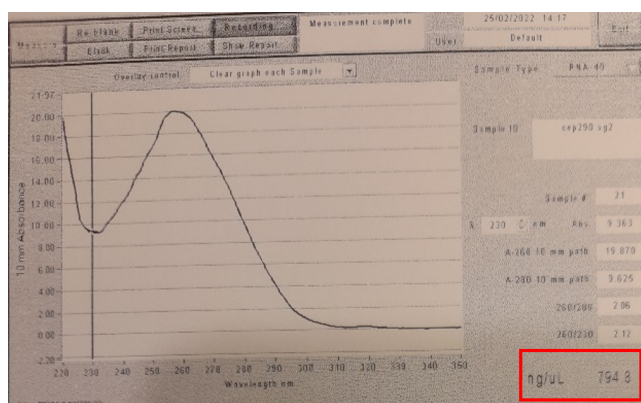


Figure 4: 1 μ L of the purified sgRNA sample was pipetted and the concentration of the sgRNA was tested using Nanodrop ND1000 v3.8.1. The concentration of sgRNA present in the sample was written as 794.8ng/ μ L.

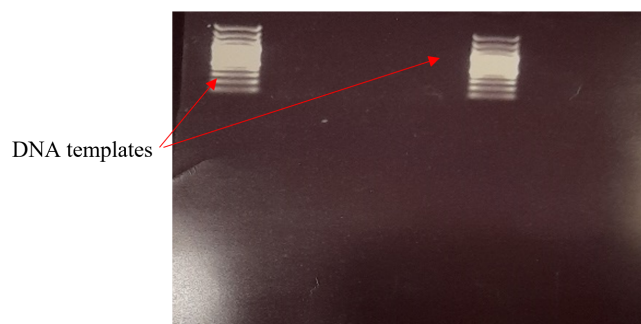


Figure 5: Genomic DNA collected from tissue samples of *Xenopus* embryos underwent PCR reaction for analyzing the expression of gene knockout. The presence of target gene was tested on 1.2% gel electrophoresis. There were no wells that expressed the presence of DNA which implicates the absence of genetic material in the sample obtained. Bands visible on the gel belong to 1000bp ladder.

Xenopus tropicalis embryos were injected with CRISPR Cas9 mixture containing all three sgRNAs targeting the gene *cep290*. Whole or partial tissue samples were collected from

the injected embryos at the neurula stage. The samples underwent purification and PCR reaction, however, during gel electrophoresis DNA bands were not visible (Fig. 5) and it was observed that the tadpoles formed after the gene knockout procedure had stunted development and were weak or dead (Fig. 6).

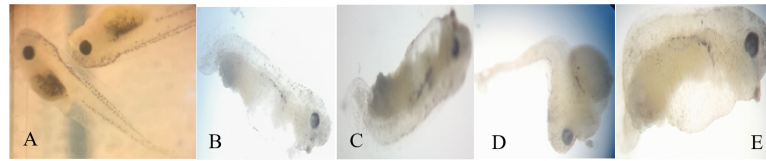


Figure 6: A. Control tadpoles that did not have any gene knock-out done to them; B-E. *Xenopus* tadpoles with gene *cep290* knocked out. Observations showcase tadpoles with stunted growth and development.

4 Discussions

The *Xenopus* species are most commonly used for the purpose of studying for kidney malfunctions as kidneys start developing as early as day 2 [8]. The data obtained from the embryos of *X. tropicalis* suggests that complete knock-out of the gene *cep290* results in severe mutation that causes death. Consecutive presence of the dsDNA template and sgRNA during gel electrophoresis prior to and post- injection suggests that the modified genomic data was successfully transferred into *Xenopus* embryos. Further analysis of the RNA-Seq data of the gene clarified that *cep290* plays a role in the earlier stages of the embryo development prior to its function in the kidney development stage (Fig. 7).

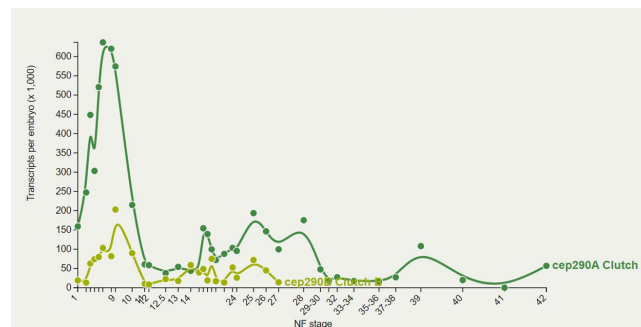


Figure 7: RNA Sequence Data for the *cep290* gene showing that the gene of interest is expressed during the gastrula development stages prior to expression during organogenesis in *Xenopus tropicalis* [2].

As such, the role of *cep290* in renal development could not be observed as DNA could not be purified from samples. Phenotypic analysis via microscopy of some *Xenopus* samples showed no kidney development though some development is visible. This could be indicative of absence of kidneys in individuals in complete gene-knockout. However, since the exact function of *cep290* is not known, further research must be undertaken to determine the lethality of its absence in the genome. Existing studies on *cep290* in humans suggests that the gene is responsible for the functionality of cellular skeletal structures such as cilia and centrosomes. This is due to wide range of ciliopathies and the lack of *cep290* in these individuals. It is hypothesized, however, that microtubules are regions for sequestering mRNA that encode functionally related proteins [9]. If this hypothesis is true, then the results of this experiment could be explained due to the irregularity in the microtubules. Research has shown that motile cilia is pivotal to the central nervous system of mice, zebrafish, humans and *X. tropicalis* with central roles in the brain ventricles during organ development and homeostasis, however, current research in the field is oriented towards epithelial cilia [10]. It is possible that *cep290* might cause kidney disease due to dysfunctional cilia as many mutated genes causing kidney diseases is localized on cilia [11]. In addition to this, cilia is also present in the nephrostomes as well as pronephros [11].

5 Conclusion

Complete gene knockout of *cep290* gene is lethal to *Xenopus tropicalis*, which might be due to its function as an encoding protein for cellular cytoskeleton structures such as cilia [11]. As cilia is found to be key structure for the central nervous system during organogenesis, mutation in the gene might be the reason that the modified embryos were ill. This has prevented the research from progressing further as cilia is present in the excretory system of *Xenopus* [12]. Therefore, partial gene-knockout experiments should be carried out with each sgRNA to investigate the more lethal mutation and to examine if severe oedema is present during the renal development stages of *Xenopus tropicalis*. Congenital anomalies of the kidney and urinary tract remain of the leading causes of pediatric kidney failure due to the long waiting list [8]. Thus, it is imperative that innovative methods be employed aimed at reducing the fatality while ensuring functionality of the kidneys.

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