Development of a reactive oxygen species (ROS) biosensor to monitor kidney and liver damage in a zebrafish model of diabetes

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Abstract

Diabetes leads to irregular regulation of blood sugar levels caused by dysfunctional beta cells in the pancreas. The resulting mellitus associated hyperglycemia leads to many devastating complications in the body, including damage to the kidney and liver, via excessive reactive oxygen species (ROS) generation and overwhelming oxidative stress. To better understand the pathophysiology in these organs, we have been developing novel animal models that will permit monitoring of ROS in live animals experiencing hyperglycemia. For this, we have chosen to express the ROS "biosensor" RO-GFP in the zebrafish, Danio rerio. RO-GFP has fluorescent properties that change depending on ROS presence in the expressing cell. Zebrafish are superior models for human disease not only because of their conserved physiology, but also because it is relatively easy to create transgenic zebrafish lines, and they are easy to image. To create biosensor transgenes, we took various molecular cloning approaches. First, we PCR amplified the fabp10 promoter DNA for liver expression and the enpep promoter DNA for kidney expression. Next, we attempted a classic restriction method to insert each promoter into an existing RO-GFP transgene plasmid using restriction enzymes, to build a construct for injection. This was not pursued as we ultimately could not identify a suitable combination of enzymes for specific cuts. As an alternative, we used the Gibson assembly method which stitches multiple DNA components together with DNA polymerase. Although we generated several DNA fragments needed for the transgene, this project is ongoing. In a second overall approach, we used a system of transgenes based on tissue-restricted expression of Cre recombinase that directed liver specific expression of RO-GFP. For this, we injected the transgene: "ubiquitin-loxP-RFP-loxP-RO-GFP" into zebrafish zygotes that already had an fabp10-Cre transgene; using microscopy, we observed fluorescent expression in the resulting embryos. Although it is more complex, this Cre-lox based system has more versatility for examining other tissues that can be engineered to express Cre. In future experiments, the new transgenes described here will be used together with a zebrafish model of diabetes to examine the role of ROS and oxidative stress in the development of diabetic complications. These biosensors can further be used to facilitate the discovery of new therapies to prevent diabetes complications.

Introduction

According to the Center for Disease Control, over 22 million Americans suffer from diabetes mellitus (DM). DM occurs when normal blood glucose homeostasis is disrupted. The disruption is caused by the destruction or dysfunction of beta cells, which produce insulin. The lack of beta cells leads to decreased insulin. Decreased levels of insulin causes hyperglycemia, or high blood sugar, in individuals with diabetes. Diabetes can lead to damage in the kidney and the liver from excessive reactive oxygen species and extreme oxidative stress. The kidney and liver are two major targets of hyperglycemia damage.

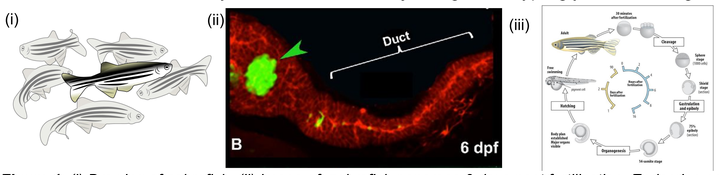


***Figure 1**: (i) Drawing of zebrafish, (ii) Image of zebrafish pancreas 6 days post fertilization. Endocrine cells are marked in green, and the exocrine cells are marked in red. The green arrow points to the pancreatic islet, (iii) zebrafish lifecycle.*

In order to study the diabetes pathways, we used zebrafish models. Zebrafish are great models for studying human diabetes because humans and zebrafish have similar pancreas development and a similar glucose homeostasis system. Zebrafish are also easy to image and it is easy to create transgenic zebrafish lines.[1]

There are various pancreas injury models used to induce diabetes in zebrafish. One example is a hybrid chemical-genetic model of a zebrafish containing a nitroreductase gene, which does not harm the beta cells under normal conditions. When MTZ (metronidazole) is introduced, apoptosis occurs which leads to beta cell injury (Fig. 2). This will induce diabetes into zebrafish embryos.[2]

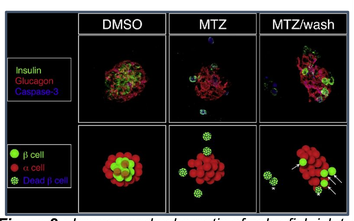


***Figure 2 :** Images and schematic of zebrafish islet with NTR-expressing cells before and after MTZ treatment.*

To study the tissue specific generation of ROS, which may underlie the pathogenesis of complications in diabetes, we used a genetically-encoded biosensor, Grx1-ROGFP. We plan to express Grx1-ROGFP in kidney and liver tissues to study the effects of ROS. Grx1-ROGFP changes fluorescent properties when oxidized. We plan to put this biosensor in to tissues with diabetic complications and measure fluorescence in live tissues using a confocal microscope.

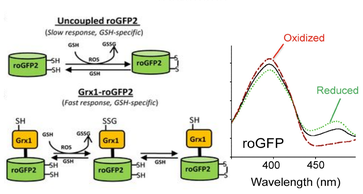


***Figure 3 : S**chematic showing of oxidation or Grx1-ROGFP which causes measurable changes in fluorescence emission.*

Objectives

The purpose of this research project was to build a transgene biosensor to investigate the damaging effects of diabetes on the kidney and liver. Our overall goal was to inject the biosensor into a zebrafish and to induce diabetes. We would then image and analyze the damage using the biosensor.

Methods

Creating Biosensor Transgene

- fabp10 + enpep promoter: We focused on creating transgenes with two promoters: fabp10, which expresses in the liver, and enpep, which expressed in the kidney. We used PCR to amplify these two promoters.
  - Cloning Approaches:
    - Restriction method - (cut and paste) First we attempted using the restriction method to insert the promoter into an existing RO-GFP transgene plasmid using restriction enzymes. We did not further pursue this method as we could not find appropriate enzymes for the specific cuts (Figure 4).
    - Gibson Assembly: Gibson Assembly is another method we used to construct the transgene. This project is ongoing, but starts by amplifying promoter regions from genomic DNA. Then, Gibson Assembly stitches DNA components with DNA polymerase to create the transgene (Figure 5).
- Cre Recombinase
  - We also worked with transgenes based on Cre recombinase that directed liver specific expression of RO-GFP. We injected ubiquitin-loxP-RFP-loxP-RO-GFP into fabp10-Cre transgene (Figure 7).

Injecting the Transgene

- After collecting the embryos, we injected the zebrafish cells with the transgene (Figure 7A).

Image Analysis

- After injecting the embryos with transgenes, we used microscopes to image analyze the transgenic fish. The microscopes used fluorescent light, which is used to image the genes (Figure 7B & 8).

Results

Cloning

- Restriction Method

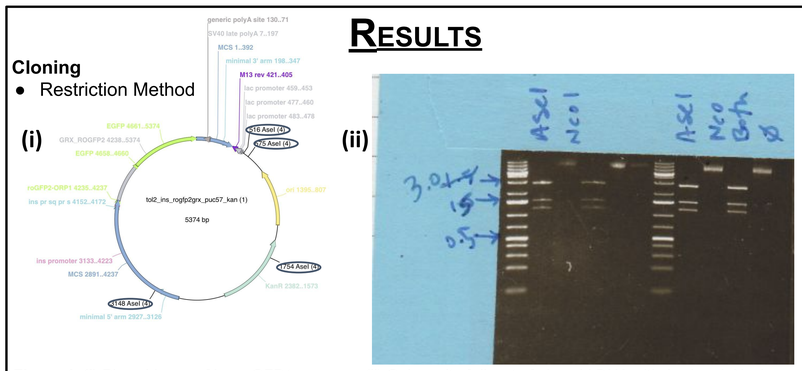


***Figure 4: (i)** Plasmid map of ins:roGFP transgene, (ii) Gel result of digest of plasmid DNA with AseI and NcoI enzymes.*

- PCR and Gibson Assembly

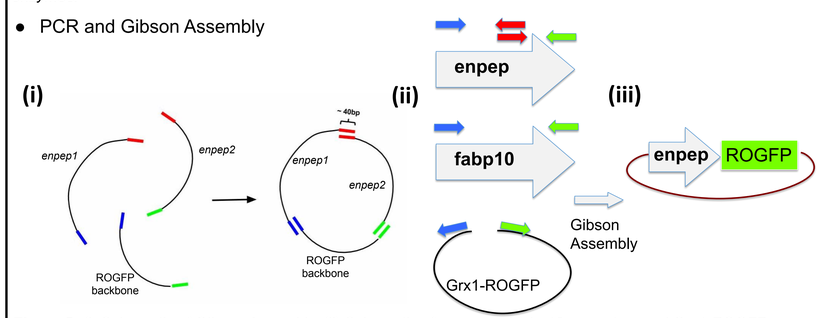


***Figure 5:** (i) Schematic of Gibson Assembly, (ii) Schematic of enpep and fabp10 promoters and Grx1-ROGFP backbone, primers shown as colored arrows (iii) Schematic of enpep:ROGFP plasmid after Gibson Assembly*

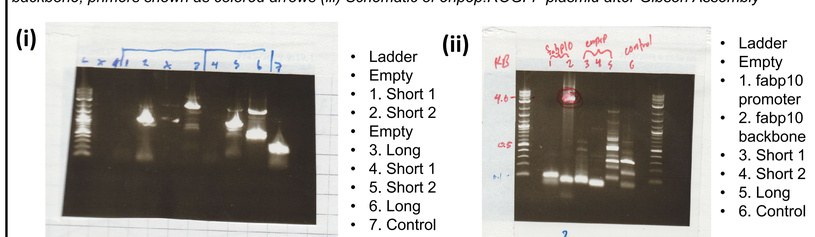
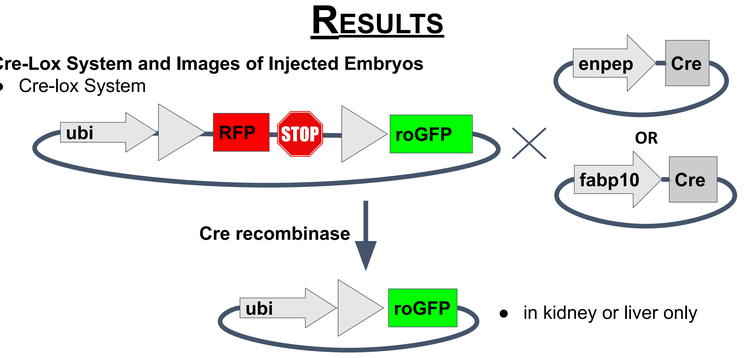


***Figure 6:** (i) PCR of enpep promoter fragments, (ii) PCR of fabp10 promoter, fabp10 backbone, and enpep promoter fragments*

Results

Cre-Lox System and Images of Injected Embryos

- Cre-lox System



- in kidney or liver only
- Figure 7: Images of Cre-lox Injected Embryos

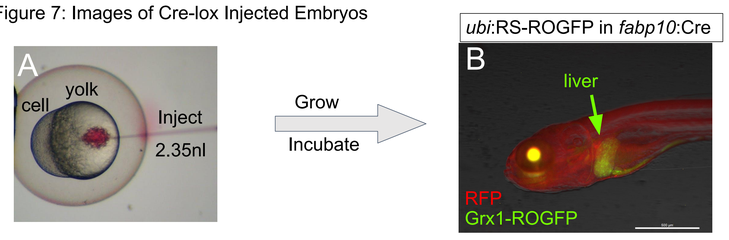
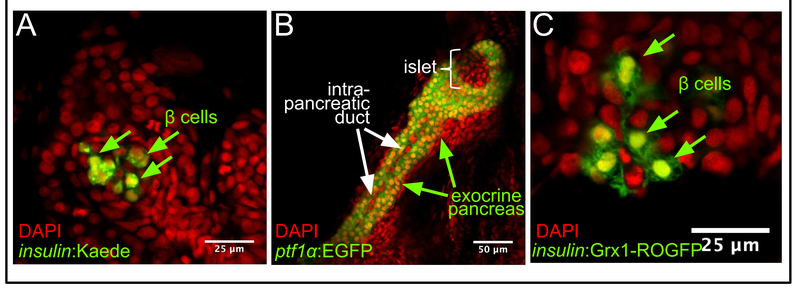


Figure 8: Confocal Images of Zebrafish Pancreas



Conclusion

- Techniques learned: embryo collection, microscopy and confocal imaging, embryo fixing, deyolking, whole mount microscopy, DAPI staining, PCR, Gibson assembly, injections with DNA, morpholinos, and transgenes, and genomic and plasmid DNA extracting.
- We successfully amplified the ROGFP backbone and part of the enpep promoter.
- We validated the ubi-cre-lox-ROGFP construct.

Future Work

- In future experiments, the new transgenes described here will be used together with a zebrafish model of diabetes to examine the role of ROS and oxidative stress in the development of diabetic complications.
- After a biosensor is created in the future, it can be injected into a ins:NTR embryo and be imaged after diabetes is induced. We will study the elevated ROS overtime in the diabetic fish and assess damage in the kidney and liver.
- These biosensors can further be used to facilitate the discovery of new therapies to prevent diabetes complications.

Acknowledgements

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References/Resources

1. Zang L, Maddison LA and Chen W (2018) Zebrafish as a Model for Obesity and Diabetes. Front. Cell Dev. Biol. 6:91. doi: 10.3389/fcell.2018.00091
2. Prince V, Anderson RM, Dalgin G (2017) Zebrafish Pancreas Development and Regeneration: Fishing for Diabetes Therapies. *Current Topics in Dev. Biol. 124. 235-276.*