Generation of a Transgenic Zebrafish Line to Characterize Kidney Injury and Regeneration

Tobechukwu Kenneth Ukah
Indiana University of Pennsylvania

Follow this and additional works at: https://knowledge.library.iup.edu/etd

Recommended Citation

This Thesis is brought to you for free and open access by Knowledge Repository @ IUP. It has been accepted for inclusion in Theses and Dissertations (All) by an authorized administrator of Knowledge Repository @ IUP. For more information, please contact sara.parme@iup.edu.
GENERATION OF A TRANSGENIC ZEBRAFISH LINE TO CHARACTERIZE KIDNEY INJURY AND REGENERATION

A Thesis
Submitted to the School of Graduate Studies and Research
in Partial Fulfillment of the
Requirements for the Degree
Master of Biology

Tobechukwu Kenneth Ukah
Indiana University of Pennsylvania
May 2013
Indiana University of Pennsylvania
School of Graduate Studies and Research
Department of Biology

We hereby approve the thesis of

Tobechukwu Kenneth Ukah

Candidate for the degree of Master of Biology

______________________  Cuong Q. Diep, Ph.D.
Assistant Professor of Biology, Advisor

______________________  Robert D. Hinrichsen, Ph.D.
Associate Professor of Biology

______________________  Robert J. Major, Ph.D.
Assistant Professor of Biology

ACCEPTED

______________________  ________________________
Timothy P. Mack, Ph.D.
Dean
School of Graduate Studies and Research
Humans cannot regenerate their kidneys, whereas zebrafish can regrow new kidney tissues after injury. To study this process, we set out to generate a transgenic zebrafish line in which a specific part of the kidney, the proximal convoluted tubule (PCT) is labeled with \textit{GFP}. A DNA construct carrying the PCT-specific \textit{slc20a1a} gene promoter was chosen for this process. First, we modified it so that it would carry the \textit{Tol2} cassette for transposon-mediated transgenesis. Then, a switchable reporter cassette (\textit{attP1-GFP-2A-CreER-attP2}) was inserted into the DNA construct so that the \textit{slc20a1a} promoter would control expression of the \textit{GFP} reporter. The \textit{attP1/attP2} recombination sites flanking the reporter cassette would allow for future exchange of a different cassette, such as a gene that produces a toxic protein, for PCT-specific ablation. Our data suggest that the final DNA construct was successfully generated and it is ready for injection into zebrafish embryos for transgenesis upon sequencing.
ACKNOWLEDGEMENTS

I am grateful to God Almighty for his continued immeasurable love and sustenance. You have been faithful.

I would like to express my sincere gratitude to my advisor, Dr. Cuong Q. Diep whose unalloyed engagement, guidance and willingness to share from his wealth of research skills made my success story. You will always remain a source of encouragement in my path as a scientist.

To my committee members, Dr. Robert D. Hinrichsen and Dr. Robert J. Major, it was wonderful working with each and every one of you and I greatly appreciate your tremendous support and help.

My greatest appreciation goes to my parents, sister and brothers who remained the financial pillar and motivator of this entire program.

Special thanks to my lab colleagues for all your suggestions and help.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION ................................................................. 1</td>
</tr>
<tr>
<td>2</td>
<td>LITERATURE REVIEW ................................................................ 7</td>
</tr>
<tr>
<td></td>
<td>Vertebrates and Regeneration ............................................... 7</td>
</tr>
<tr>
<td></td>
<td>Vertebrate Kidney regeneration ............................................ 7</td>
</tr>
<tr>
<td></td>
<td>Zebrafish Kidney Regeneration ............................................. 9</td>
</tr>
<tr>
<td></td>
<td>The Zebrafish as a Research and Regeneration Model.................. 10</td>
</tr>
<tr>
<td></td>
<td>Slc20a1a Gene as a Proximal Convoluted Tubule Marker............... 14</td>
</tr>
<tr>
<td></td>
<td>Bacterial Artificial Chromosome (B.A.C.) and E. coli Recombineering</td>
</tr>
<tr>
<td></td>
<td>Strain, SW102 ........................................................................ 16</td>
</tr>
<tr>
<td>3</td>
<td>MATERIALS AND METHODS ........................................................... 19</td>
</tr>
<tr>
<td></td>
<td>Materials ............................................................................. 19</td>
</tr>
<tr>
<td></td>
<td>Methods .............................................................................. 22</td>
</tr>
<tr>
<td>4</td>
<td>RESULTS ............................................................................... 35</td>
</tr>
<tr>
<td></td>
<td>Restriction Digest of slc20a1a-BAC DNA ................................ 35</td>
</tr>
<tr>
<td></td>
<td>Polymerase Chain Reaction (PCR) to Isolate iTol2-amp Cassette ... 35</td>
</tr>
<tr>
<td></td>
<td>iTol2 Cassette Recombination into SW102-BAC and Confirmation</td>
</tr>
<tr>
<td></td>
<td>of Tol2-BAC ........................................................................ 36</td>
</tr>
<tr>
<td></td>
<td>Polymerase Chain Reaction (PCR) to Isolate polyA-KanR Cassette... 38</td>
</tr>
<tr>
<td></td>
<td>KanR Cassette Recombination into #802 (GFP-F2A-CreER) and</td>
</tr>
<tr>
<td></td>
<td>Confirmation ....................................................................... 39</td>
</tr>
<tr>
<td></td>
<td>Isolation of attP1-GFP-F2A-CreER-pA-Kan-attP2 from CQD29 and</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>List of Primers Used in this Study</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>PCR Parameters to Isolate iTol2-amp Cassette</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>PCR Parameters to Isolate FRT-kan-FRT Cassette</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Components of the Injection Mixture</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>PCR Parameters to Isolate CQD29 Cassette</td>
<td>33</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(a) Human kidney showing the internal structures (b) Magnified view of a single nephron</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A stem cell undergoing replication to form another stem cell and a specialized cell</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>The picture depicts a fertilized egg undergoing cleavage to give rise to morula at the early embryonic stage</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>The zebrafish larva (2dpf) showing embryonic kidney with two simple nephrons as indicated by the arrows</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>The nephron depicting reabsorption and solute transport at the proximal convoluted tubule</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>Embryonic kidney expression of known renal genes and solute transporters isolated using functional genomic</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>Agarose gel electrophoresis showing results of the restriction digests of slc20a1a BAC DNA with AgeI</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>Agarose gel electrophoresis showing PCR isolation of Tol2-amp cassette</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>37</td>
</tr>
<tr>
<td>Schematic integration of Tol2-amp cassette into the slc20a1a BAC</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Replica plating of five candidates showing the growth of Tol2-BAC cells on LB-cam-amp plate but no growth on LB-cam-spec and LB-cam-kan</td>
<td></td>
</tr>
</tbody>
</table>
plates .........................................................................................................................37

11 Agarose gel electrophoresis showing the 1153 bp (lane 2) from PCR to
confirm the Tol2 cassette integration into the slc20a1a BAC .........................38

12 Agarose gel electrophoresis showing the PCR isolation of the 1649 bp
(lane 2) KanR cassette ..........................................................................................38

13 Agarose gel electrophoresis to confirm KanR integration into the
intermediate CQD29 .............................................................................................40

14 Confirmation of sequencing result of CQD29 using NCIB BLASTN
program ..................................................................................................................40

15 Mosaic expression of GFP-2A-CreER-KanR in 3 dpi larvae under the green
channel of a fluorescence ......................................................................................41

16 Agarose gel electrophoresis showing in lane 2 the result of the PCR
isolation of the 4663 bp cassette (attP1-GFP-F2A-CreER-pA-Kan-attP2)
from CQD29 ..........................................................................................................41

17 A sketch of our final slc20a1a BAC construct containing the Tol2 cassette
for transposon mediated transgenesis and integrase bound reporter gene
for future genetic switch ........................................................................................42
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>~</td>
<td>Approximate</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Centigrade</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µM</td>
<td>MicroMolar</td>
</tr>
<tr>
<td>amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cam</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>fw</td>
<td>Forward primer</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo bases (1000 bp)</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani agar</td>
</tr>
</tbody>
</table>
M  Molar
MgCl₂  Magnesium Chloride
min  Minute
mL  Milliliter
mM  MilliMolar
mm  Millimeter
N/A  Not Available
NaCl  Sodium chloride
ng  Nanogram
OD  Optical density
PCR  Polymerase chain reaction
pH  Potentia hydrogenii
qPCR  Quantitative PCR
rev  Reverse primer
RNase  Ribonuclease
rpm  Revolutions per minute
sec  Second
TAE  Tris/Glacial acetic acid/EDTA
tet  Tetracycline
Tris  Tris-(hydroxymethyl) aminomethane
UV  Ultra violet
V  Volts
vol/vol  Volume per volume
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>( \lambda )</td>
<td>Lambda</td>
</tr>
<tr>
<td>( \mu l )</td>
<td>Microliter</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Kidneys are essential organs used for blood filtration, reabsorption of water and solutes, and secretion of molecules, resulting in the excretion of waste as urine. This process is carried out by nephrons, the functional units of the kidney (Figure 1). Kidney disease is a serious illness in which a cure has evaded scientists for many decades. In 2010, the prevalence of chronic kidney disease (CKD) in the U.S. was ~20 million (more than 10%) (NIDDK, 2012). CKD ultimately leads to kidney failure (End Stage Renal Disease; ESRD), where ~90% of kidney function has been lost and patients require renal replacement therapy for survival. Currently, renal replacement options for ESRD are dialysis and kidney transplantation (Ludlow, Kelley, & Bertram, 2012). Dialysis treatment results in a poor lifestyle with a high mortality rate (~20%), while kidney transplantation has a chance of immune rejection and an approximate three-year waiting list. ESRD will continue rising to an alarming level because the two major contributors of ESRD (diabetes and hypertension) are also rapidly increasing (Fox et al., 2004). In 2008, ~550,000 people in the U.S. had ESRD and their treatments consumed ~$40 billion that year from public and private spending, causing a huge financial burden on the healthcare system (NIDDK, 2012).

Figure 1. (a) Human kidney showing the internal structures (b) Magnified view of a single nephron.
Due to the limitations of current treatments and their associated costs, there is an urgent need for novel alternative therapies. One possibility that has tremendous potential is a stem cell/regenerative therapy. Stem cells (SCs) are undifferentiated cells with the potential to turn into many different cell types (potency). They can also undergo self-renewal using asymmetric division to replenish the stem cell pool (Figure 2). SCs exist in two forms, adult (tissue-specific) and embryonic stem cells (ESCs). Adult stem cells (ASCs) are multipotent but are restricted to only forming the cell types of that specific tissue (Terskikh, Bryant, & Schwartz, 2006). They are responsible for the replenishment of dead cells resulting from normal cellular turnover in tissues such as the blood, skin, and intestine. A good example is the haematopoietic stem cells which have been applied with great success in treating blood related diseases such as leukemia (Terskikh et al., 2006).

In contrast to ASCs, ESCs are derived from the inner cell mass (ICM) of embryo at the blastocyst stage after fertilization (Figure 3) (Cohen & Melton, 2011; Hopkins, Li, Rae, & Little, 2009). ESCs are pluripotent and can differentiate in vivo into every cell type found in that...
organism. This implies that when the differentiation mechanism is repeated in vitro under controlled conditions, it may be possible to attain the desired cell type for treatment of a specific disease (Daley & Scadden, 2008; Melton, 2011). Recently, a newer “embryonic-like” stem cell called “induced pluripotent stem cell” (iPSC) was discovered. iPSCs are derived from mature adult cells instead of embryos (Takahashi & Yamanaka, 2006). It has been shown that ESCs and iPSCs can be reprogrammed into kidney cells (Cohen & Melton, 2011; Hopkins et al., 2009).

![Figure 3. The picture depicts a fertilized egg undergoing cleavage to give rise to morula at the early embryonic stage. Source: The University of Kansas Medical Centre, http://www.kumc.edu/stem-cell-101/early-stem-cells.html.](image)

Therefore, it may be possible in the future to use a stem cell therapy for treating kidney disease and other types of diseases. One example is type 1 diabetes (T1D), which is caused by autoimmune destruction of the insulin-producing beta cells of the pancreas. In this case, it may be possible to use a patient-derived iPSCs in vitro to generate the beta cells and transplant them into the patient’s pancreas (Melton, 2011). Further studies will be needed to fully understand the mechanism of SC differentiation in order to bring us closer to stem cell/regenerative therapies.
The use of human embryos still remains controversial coupled with limited supplies of human eggs to derive ESCs. Thus, many researchers have directed their efforts toward model organisms such as mice and zebrafish. The zebrafish is a vertebrate which has great capabilities in organ regeneration and an excellent model of human disease because it is evolutionarily conserved to humans (I. A. Drummond, 2005). Zebrafish exhibits many advantages as a model organism, such as a short generation time, optically transparent embryos, and external fertilization, making them accessible for manipulation (I. A. Drummond, 2005; Lieschke & Currie, 2007).

At the embryonic stage, antisense morpholino oligonucleotides can be used to knock down gene function, an important tool to evaluate phenotypic expression of a particular gene of interest (Lieschke & Currie, 2007; Yuan & Sun, 2009). Moreover, forward and reverse genetics enable us to identify the genetic basis of a particular phenotype. Consequently, the identification of genes involved in human diseases and their contribution can be studied (Amsterdam & Hopkins, 2006; I. A. Drummond, 2005; Lieschke & Currie, 2007). It is also easy to make transgenic animals to monitor and visualize the cells of interest using the fluorescent microscopy.

Zebrafish kidney development occurs in two stages. During the first stage, a simplified embryonic kidney (the pronephros, Figure 4) consists of two nephrons (the functional units of the kidney) (Diep et al., 2011; Wingert & Davidson, 2008; W. B. Zhou, Boucher, Bollig, Englert, & Hildebrandt, 2010). The bilateral nephrons of the pronephros have related cellular structures (such as glomerulus and proximal convoluted tubule) and functions similar to the mammalian nephrons by two days post fertilization (dpf) (Davidson, 2011; I. Drummond, 2003; I. A. Drummond, 2000, 2005). In the second stage, development of the adult kidney (the mesonephros) begins at around 10 dpf.
In mammals, a third stage of kidney development occurs to form the adult kidney (the metanephros). Nephrogenesis (the process of forming new nephrons) ceases at around birth. Therefore, mammalian nephrons can undergo hypertrophy to increase in size, but cannot make new nephrons postnatally. Although the mammalian nephrons can mobilize epithelial cells to replace damaged cells in response to injury (Bonventre, 2003; Humphreys et al., 2008), they lack the capability to make new nephrons (neonephrogenesis) during regeneration (Davidson, 2011).

In contrast, the adult zebrafish kidney not only repairs damaged nephrons (Wang et al., 2011), it can form new nephrons through neonephrogenesis, a feature that presents it as a model of choice to study kidney regeneration with respects to repair of damaged nephrons and neonephrogenesis (Davidson, 2011; Diep et al., 2011; Reimschuessel, 2001; W. B. Zhou et al., 2010).

The proximal convoluted tubule (PCT) of the nephron is the major reabsorptive segment and can be analysed to visualize and monitor kidney function. It has been demonstrated that expression of the \( \text{slc20a1a} \) gene (a sodium/phosphate co-transporter) is specific in the PCT and can serve as a marker for nephron function (Diep et al., 2011; Nichane, Van Campenhout, Pendeville, Voz, & Bellefroid, 2006). In this respect, it is our assumption that the promoter sequence controlling \( \text{slc20a1a} \) expression can be used to express a fluorescent protein to visualize and monitor nephron function. The promoter sequence can also be used to drive expression of toxic gene products (such as nitroreductase) to ablate the PCT and induce kidney injury.

There have been reports of injury models for the zebrafish pronephros, such as intravenous microinjection of nephrotoxin (Cianciolo Cosentino, Roman, Drummond, & Hukriede, 2010) and laser ablation (Johnson, Holzemer, & Wingert, 2011). Although these do result in PCT injury, both are time consuming (only one embryo could be treated at a time) and limited by the low consistency of cellular damage (Johnson et al., 2011; Jonker, Woutersen, van
Bladeren, Til, & Feron, 1993; Pfaller & Gstraunthaler, 1998). Therefore, our goal is to generate a genetic model of PCT ablation, which would yield greater consistency and many larvae could be analyzed at one time. To achieve this, the aim of this research will be to generate a slc20a1a BAC DNA construct in which the gene promoter of slc20a1a is positioned to drive expression of an exchangeable GFP.

Figure 4. The zebrafish larva (2dpf) showing embryonic kidney with two simple nephrons as indicated by the arrows.
CHAPTER 2

LITERATURE REVIEW

Vertebrates and Regeneration

Previous investigations have shown that most vertebrates, especially fish and *urodeles* have varying tissue and organ regeneration capabilities compared to mammals (Nakatani, Nishidate, Fujita, Kawakami, & Kudo, 2008). These include regeneration of the retina and spinal cord axons in *Xenopus laevis* tadpole (Gibbs, Chittur, & Szaro, 2011; Martinez-De Luna, Kelly, & El-Hodiri, 2011), limb in salamander (Garza-Garcia, Driscoll, & Brockes, 2010), tail in leopard gecko (DANIELS et al., 2003) and heart in newt (Borchardt et al., 2010). Regeneration in fish include brain (Ilieş, Zupanc, & Zupanc, 2012; Zupanc, 2006), fin (Nakatani et al., 2008), heart (Ausoni & Sartore, 2009) and liver (Zaret & Grompe, 2008).

Though it has been reported that a deer can completely regenerate its antlers, mammals in general lack the capacity to regenerate their damaged tissues and organs but can undergo limited replacement of certain cell types (Ausoni & Sartore, 2009; Osakada et al., 2007). Understanding the behavior of these cells may contribute to studies in mammalian regeneration. Thus, unraveling the regeneration mechanism in fish and other lower vertebrates may provide the required link among species.

**Vertebrate Kidney Regeneration**

The kidney is essential for calcium metabolism, production of renin, which maintains blood pressure, and erythropoietin (EPO), which regulates the production of red blood cells (Agcaoili, Atala, & Yoo, 2010). Its functional units are the nephrons and they could be
compromised in the event of severe damage, leading to tubular atrophy and glomerulosclerosis (Agcaoili et al., 2010). As one of the complex physiological organs in the body, comprising of diverse cell types, regeneration of the kidney remains an intricate task.

Mammals undergo three major phases of kidney development. The paired vestigial pronephric kidney develops from the intermediate mesoderm from where the epithelial cells join and form the pronephric duct (Dressler, 2009). In mammals such as mouse, the pronephric kidney may be unnoticed (Dressler, 2009). Furthermore, the pronephric kidney develops to induce more epithelial cells into mesonephric tubules. This second anterior structure becomes the mesonephric kidney with developed proximal glomerulus and convoluted tubules that empty into the nephric duct (Dressler, 2009; Little, 2011). The final phase as also found in reptiles and birds is the adult metanephric kidney which develops posteriorly and differs from the freshwater mesonephros due to the presence of loop of Henle in the nephrons (Davidson, 2011). The loop of Henle structure aids to concentrate mammalian urine, thereby, conserving body fluid. While the ureteric bud (UB) emerges from the nephric duct to give rise to ureter and collecting duct, the nephrons develop from the metanephric mesenchyme, a process suggested to be initiated by the Wnt signaling pathway (Davidson, 2011; Little, 2011). It is believed that the UB and metanephric mesenchyme work synergistically to regulate UB elongation and branching and any interference could hamper kidney development. Each of the dividing UB bears the cap mesenchyme, which is suggested to function as the transitory stem cells and nephron progenitors (Davidson, 2011; Dressler, 2009).

In humans, nephrogenesis ceases ~35 weeks within gestation due to the loss of the cap mesenchyme which results in the termination of UB branching (Davidson, 2011). Consequently, the nephron population is fixed and can only increase in size and function as mammals grow and
develop postnatally. This implies that congenital and postnatal defects that affect kidney development and functions will definitely lead to diminished number of nephrons. Also, persistent disease states such as diabetes and hypertension, which seriously affect kidney functions, have the tendencies to affect the nephron population as a chronic state. While mammals have lost the capacity to regenerate new nephrons, they only have the potential to repair damaged nephrons due to acute kidney injury (Reimschuessel, 2001). In their demonstration, using genetic fate-mapping techniques and ischemic renal insult, Humphrey et al. (Humphreys et al., 2008) showed that surviving mice kidney cells were able to repopulate damaged nephrons by epithelial cell proliferation and differentiation. Similarly, the human metanephric kidney has such renal regenerative potential in response to minor ischemic or toxic injury or shedding to its proximal tubule but does not include new nephron development (Bonventre, 2003; Davidson, 2011). Mammals can also undergo compensatory renal hypertrophy, a situation where there is increase in cell size of the remaining kidney cells after nephrectomy (loss of nephron) (Reimschuessel, 2001). These are the farthest that mammals can go in terms of kidney regeneration whereas a lower vertebrate like the zebrafish has the capacity to regenerate and also to make new nephrons (neonephrogenesis).

**Zebrafish Kidney Regeneration**

It has been suggested that the mesenchymal cells on the pronephric tubules of the zebrafish aggregate to give rise to the adult mesonephric nephrons (Davidson, 2011; Fedorova et al., 2008). However, there have been suggestions that teleosts develop their mesonephric kidney many days following the formation of the nephric duct and its fusion to the cloaca (Davidson, 2011). The process of epithelial aggregation of the basophilic mesenchyme cells continues to produce new nephrons as growth persists. Besides, the process of nephrogenesis is believed to be
spread throughout the organ, which further suggests that the progenitors may be recruited to sites as the need arises.

Unlike in mammals, where nephrons can only increase in size as body gets bigger, adult fish nephron is reported to increase in number as their body size increases with age (Diep et al., 2011). Upon injury, the zebrafish kidney cells undergo an initial stage of cell death. After some days, the epithelial cells proliferate to repopulate the exposed basement membrane as may be similar to mammals (Bonventre, 2003; Reimschuessel, 2001). However, with nephrotoxins such as gentamycin, the zebrafish not only repairs kidney cells but can completely make new nephrons. This occurs when the basophilic clusters of cells that are adjacent to the collecting ducts proceed to form renal vesicles and S-shaped bodies (Reimschuessel, 2001). The tubular outgrowths eventually fuse with the collecting ducts to give rise to new nephrons. Similarly, Diep et al. (2011) reported the coalescence of kidney stem cells to form pre-nephron aggregates of progenitor cells that eventually differentiate into nephrons. This developmental process can be visualized with the transgenic zebrafish line, Tg(lhx1a:EGFP) where the lhx1a gene promoter drives the GFP expression (Diep et al., 2011; W. B. Zhou et al., 2010).

**The Zebrafish as a Research and Regeneration Model**

There have been tremendous successful investigations that have demonstrated the zebrafish as an excellent model to study tissue and organ regeneration. These are based on their ability to regenerate several of their damaged tissues and organs such as the kidney (Diep et al., 2011), liver (Kan, Junghans, & Belmonte, 2009), heart (Kikuchi et al., 2010; Lien, Harrison, Tuan, & Starnes, 2012), auditory hair cell (Sun, Lin, & Smith, 2011), fins (Sofia Azevedo, Sousa, Jacinto, & Safde, 2012), central nervous system (Becker & Becker, 2008) and pancreas.
(Moss et al., 2009). With basic similarities to humans, it is likely that understanding the molecular basis of regeneration in zebrafish can shed more light into most of the human-associated degenerative diseases.

**Chemical and Genetic Screening**

The zebrafish is a powerful genetic and chemical screening tool. In an investigation that involved the exposure of zebrafish embryos to ten different chemicals in order to understand the biosynthesis of hematopoietic stem cell (HSC), prostaglandin E2 was revealed to enhance HSC homeostasis (North et al., 2007). The 36 hpf *in-situ* hybridization showed that the modulation of the prostaglandin pathway changed the expressions of *runx1* and *cmyb*, which are important genes in HSC development. In fact, *Cox1* and *Cox2*, which are associated with prostaglandin synthesis, were found to be vital in HSC synthesis. Also, the derived prostaglandin E2 improved kidney marrow recovery following irradiation injury in the adult zebrafish. Further research using the zebrafish model, could contribute to the control of diseases such as leukemia.

Similarly, the behavioral profiling in zebrafish larvae using some drugs indicated neurological and pharmacological similarities between zebrafish and mammalian responses to rest and wake states (Rihel et al., 2010). The findings revealed that phenotypic features of the chemicals were related if they belong to the same class, have common targets or have multiple targets but share properties. For instance, the profiling of anti-Parkinson’s drug trihexyphenidyl and the likes corresponded to the observed effects on dopamine reuptake inhibitor and muscarinic acetylcholine receptor antagonist 3α-bis-(4-fluorophenyl) methoxytropane. Also, new rest/wake behavioral pathways were discovered in zebrafish, such as the verapamil class (which are L-type calcium channel inhibitors) promoted rest with reduced effect on wake, increased rest
latency with podocarpatien-3-ones and the role of some anti-inflammatory substances (such as steroidal glucocorticoids and phosphodiesterase (PDE) inhibitors) in daytime activities and the suggested role of Ether-a-go-go-gene (ERG) potassium channels in wakefulness (Rihel et al., 2010).

In another chemical screening, Bergapten (a member of the psoralen family) and asarylaldehyde compounds were suggested to interact with cdx-4 pathway as they restored the expression of cdx-4 in mutant zebrafish embryos (Paik, de Jong, Pugach, Opara, & Zon, 2010). cdx-4 is a transcription factor with regulatory functions over the hox genes and as such is vital in the anterior to posterior patterning during embryogenesis. Bergapten and asarylaldehyde rescued cdx-4 by restoring gata1 expression in the mutant embryos. All these results depict the zebrafish as an important chemical screening tool for in-vivo analyses in drug discovery.

Again, the application of forward and reverse genetics in zebrafish has yielded similar phenotypic consequences as observed in humans (Lieschke & Currie, 2007). For instance, a zebrafish mutant with defective dystrophin gene (homologous to human gene) exhibited similar pathological features as found in humans with Duchenne muscular dystrophy (Bassett et al., 2003). Therefore, we hypothesize that a transgenic zebrafish with a kidney specific expression of a toxic gene has potential to expand investigations into kidney regeneration using chemical and genetic screenings.

**Transgenesis**

Four major molecular methods of generating transgenic zebrafish embryos have been identified. These methods enable the successful insertions of exogenous DNA sequences of interest into the zebrafish genome and have further enhanced its potential as a research model.
The first is the microinjection of linear DNA (with the promoter driving the expression of the reporter gene such as *GFP*) at the one-cell stage of the zebrafish egg. Another is the infection of the egg with pseudotyped viruses for insertional mutagenesis purposes (Gaiano, Allende, Amsterdam, Kawakami, & Hopkins, 1996). Others include the microinjection of I-SceI meganuclease along with the DNA of interest bearing the I-SceI sequences (Thermes et al., 2002) and the use of transposon-mediated transgenesis (Kawakami, 2007; Urasaki, Morvan, & Kawakami, 2006).

Although the above methods have proven to be successful at some rates (linear DNA, ~5%; I-SceI, 50%; retrovirus, 100%; transposon, 50%), the transposon-mediated transgenesis approach has been shown to exhibit high genomic integration in the germline of most vertebrates (Kawakami, Shima, & Kawakami, 2000; M. L. Suster, G. Abe, A. Schouw, & K. Kawakami, 2011; Tsang, 2010; Urasaki et al., 2006). Transposons are genetic elements that can move from one chromosomal locus to another and are found as repetitive sequences in the genome. Investigations have revealed that a transposition method where the nonautonomous *Tol2* plasmid construct and an *in vitro*-generated mRNA from a transposase cDNA template are injected into the zebrafish fertilized eggs, the transposase protein were made from the mRNA and subsequently catalyzed the excision and integration of the *Tol2* construct into the zebrafish genome (Asakawa et al., 2008; Kawakami et al., 2000; Urasaki et al., 2006). The medaka fish *Tol2* element belongs to the *hAT* family of transposons and a construct having inverted minimal ends of 150 bp on the left and 200 bp on the right has been reported as sufficient for excision and transposition by a transposase protein (Urasaki et al., 2006). In contrast, transposon construct with less than 150 bp and 100 bp on the left and right respectively may not be transposable (Urasaki et al., 2006). The *Tol2 cis* sequences are positioned in the left and right ends of each of
the DNA insert to be integrated into fertilized eggs in a transposase-dependent cut-and-paste mechanism (M. L. Suster et al., 2011). Therefore, we expect that our final DNA construct (which will be flanked by the inverted Tol2 sequences) will successfully be excised and integrated into the zebrafish genome by the transposase protein.

**Other features**

It is now a common practice to have transgenic zebrafish lines where the promoter of a target gene has been constructed to drive the expression of a fluorescent protein which can be visualized directly under fluorescent microscopes. For example, using a Tg(lhx1a:EGFP), lhx1a (a LIM homeodomain transcription factor) has been reported as nephron progenitors in transparent zebrafish larvae where lhx1a promoter drives GFP expression, and FGF signaling has been reported to be regulated by dusp6 (a dual specificity phosphatase) (Diep et al., 2011; Znosko et al., 2010). Further, zebrafish are sexually mature after three months, cost effective in management, and are highly affordable and feasible for large scale screens.

**slc20a1a Gene as a Proximal Convoluted Tubule Marker**

The proximal convoluted tubule (PCT) is part of the nephron that connects from the Bowman’s capsule to the loop of Henle. It is an essential nephron component that regulates the pH of glomerular filtrate by exchanging hydrogen ions in the interstitial space for the filtrate’s bicarbonate ions (Beck, Neuhofer, Dorge, Giebisch, & Wang, 2003; Dantzler, 2003). While water is reabsorbed passively to maintain the osmolarity of the proximal tubule, the PCT is also highly involved in the reabsorption (Figure 5) of sodium ions (Na\(^+\)), potassium ions (K\(^+\)), calcium ions (Ca\(^{2+}\)) and magnesium ions (Mg\(^{2+}\)), sodium bicarbonate (NaHCO\(_3\)) and organic solutes (glucose and amino acids) (Beck et al., 2003; Dantzler, 2003). Filtrate reabsorption in the
zebrafish PCT is primarily driven by the Na\(^+\)/K\(^+\) ATPase located in the basolateral membrane of the epithelial cells (Dantzler, 2003).

**Figure 5.** The nephron depicting reabsorption and solute transport at the proximal convoluted tubule. (a) Shows the glomerulus (GL), proximal convoluted tubule (PCT), distal tubule (DT) and collecting duct (CD) of the nephron; (b) Enlarged view of the proximal convoluted tubule showing the reabsorption and transport of solutes. Source: Katzung BG et al.: Basic & Clinical Pharmacology, http://basic-clinical-pharmacology.net/chapter%2015_%20diuretic%20agents.htm (Katzung et al., 11\(^{th}\) edition)

Investigations have revealed that the slc20a1a gene, a sodium/phosphate co-transporter and symporter is specifically expressed in the PCT (Figure 6) (Cianciolo Cosentino et al., 2010; Wingert et al., 2007). Also, the injection of gentamycin in adult zebrafish resulted in nephron damage and failure to reabsorb filtered fluorescent dextran, which corresponded with the downregulation of slc20a1a gene (Diep et al., 2011). These suggest that slc20a1a gene
expression serves as a guide in PCT functionality and a transgenic line where the slc20a1a promoter drives the expression of GFP, will be a potential PCT monitor.

Figure 6. Embryonic kidney expression of known renal genes and solute transporters isolated using functional genomic. In 24 hpf pronephros, slc20a1a is depicted to be specifically expressed in the PCT (columns 5 – 8). Similarly, 48 hpf pronephros showed slc20a1a-PCT specificity (columns 3 – 7). Adapted from Wingert et al., PLoS Genetics, 2007.
**Bacterial Artificial Chromosome and *E. coli* Recombineering Strain, **SW102**

Bacterial artificial chromosome (BAC) is a DNA construct that has been widely applied in the study of gene regulation and generation of animal models of human diseases, especially with transgenic mice (Chandler et al., 2007). Because BACs can contain DNA fragments between the ranges of 150kb – 300kb, it has become very useful in researches that involve complex genes with numerous regulatory sequences in the upstream of the encoding sequence (Bussmann & Schulte-Merker, 2011; M. L. Suster et al., 2011). Unlike other forms of smaller cloning vectors, the BACs are very stable and can resist rearrangements. BAC constructs can be made in recombineering *Escherichia coli* strain (such as SW102), where the reporter gene and other sequences of interest are incorporated into the BAC transgene via homologous recombination. Consequently, the BAC transgene is injected into the mice to visualize expressions in particular tissue or cells in live mice (Maximiliano L. Suster, Gembu Abe, Anders Schouw, & Koichi Kawakami, 2011). Apart from mice, BAC transgenesis has been successfully applied in zebrafish (Higashijima, 2008; Z. Yang et al., 2006). This approach has further enhanced the efficacy of the optically transparent zebrafish as a model of interest in visualizing and monitoring live events in transgenic lines. While transgenic zebrafish lines expressing fluorescent proteins have been generated with the BAC, genetic manipulations based on site-specific recombination through *Gal4/UAS, Cre/lox, ΦC31* integrase, *TetON* and *LexA/Op* are also possible (Hu, Goll, & Fisher, 2011; M. L. Suster et al., 2011).

Furthermore, it has been reported that BAC transgenes have been effectively integrated into the zebrafish genome using the transposon mediated transgenesis as discussed previously (Maximiliano L. Suster et al., 2011). The medaka *Tol2*- mediated integration is independent of the BAC size and endogenous expression in germline of founder fish is ~15% when compared to
other methods such as microinjection of naked DNA (low integration rates), injection of 18-bp I-SceI meganuclease recognition site with I-SceI protein (works better with ~5 kb) and retroviral vectors (limited DNA construct of < 8 kb) (Rembold, Lahiri, Foulkes, & Wittbrodt, 2006; Maximiliano L. Suster et al., 2011; Thomas, Ehrhardt, & Kay, 2003). Therefore, we expect that our final BAC construct of ~250 kb will be integrated into the zebrafish genome using the Tol2-mediated transgenesis.

The SW102 is a modified DH10B strain containing the defective lambda prophage. The strain has heat-inducible recombinations. The cassette to be recombined is a linearized PCR product with a donor sequence and 50 bp homologies (primers designed to fit) to the target sequence on each end (Maximiliano L. Suster et al., 2011). The cassette is incorporated into the BAC-containing cells by electroporation. The recombination proteins are turned on by heating the cultures to 42°C for 15 min (Warming, Costantino, Court, Jenkins, & Copeland, 2005). While the λ-Red–encoded Gam function prevents degradation of the PCR product, Exo or Redα and Beta or Redβ mediate recombination of the cassette (Warming et al., 2005).
CHAPTER 3
MATERIALS AND METHODS

The materials and methods were adapted from M. L. Suster et al. (2011) and Bussmann & Schulte-Merker (2011).

Materials

Wild-type stains of Tübingen/AB were obtained from the aquatic facility in the Department of Biology, Indiana university of Pennsylvania. SW102 bacteria harboring a defective λ-prophage came from Donghun Shin (University of Pittsburgh). sle20a1a BAC clone (ZK90N12) was purchased from Source BioScience (United Kingdom). The iTol2 cassette plasmid (pCR8GW-iTol2-amp), Kan cassette plasmid (pBSK-GFP-pA-FRT-kan-FRT) and zebrafish codon-optimized Tol2 transposase (TP) plasmid (pCS2-zT2TP) were supplied by Maximiliano Suster (Sars International Centre for Marine Molecular Biology, Bergen, Norway). Plasmid #802 (GFP-2A-CreER) was donated by Michael Parsons (Department of Surgery, Johns Hopkins University). Plasmid miniprep kit (27106), QIAquick gel extraction kit (28706) and PCR purification kit (28106) were obtained from Qiagen. Other reagents include nuclease-free water (Ambion, 9939), 1Kb+ DNA (Invitrogen, 15615-016), mMMESSAGE mMACHINE SP6 kit (Ambion, AM1340), ethidium bromide (10 mg/ml; Sigma, E1510), dimethylsulphide (DMSO; Sigma, D8418), glycerol (OmniPur, EM-4760), restriction enzymes (EcoRI, AgeI, DpnI, NcoI,) from NEB, and expand high-fidelity PCR system (Roche, 11681834001).

Oligonucleotides (Primers)

All the primers used in this study were synthesized by Eurofins and the list is shown in Table 1.
Table 1

List of primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5' – 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>iTol2-amp</td>
<td>CQDp95-fw</td>
<td>TTCTCTGTITIT ITGCCGTTGGAATGAAATGAAATGGTTCCGCAATCTC</td>
</tr>
<tr>
<td></td>
<td>CQD96-rev</td>
<td>ATCGCTCTCTGCTGACCGGCGCAGCCGAGCTATTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCAGGCAAACACGTCGAGCGCAACCCCTTGCCGGCGCATATTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGATCCCTTAGATCAGATCT</td>
</tr>
<tr>
<td>iTol2 recombination</td>
<td>CQDp131-fw</td>
<td>CTCAAGTACTTTACACCTCTGg</td>
</tr>
<tr>
<td>confirmation</td>
<td>CQDp132-rev</td>
<td>ACTCAAAGTATACCTG GAGATCAGATCT</td>
</tr>
<tr>
<td>polyA-KanR</td>
<td>CQDp142-fw</td>
<td>gaggggggcctttaataaacctaggCGGCCGCGGATCGATCGCGA</td>
</tr>
</tbody>
</table>
|                       | CQDp143-rev  | CTCTAACTGATATCGACCGCAGGCCAACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCAC
digital camera (Olympus; model DP72) dissecting stereomicroscope (Leica; model S6E), injection system (WPI; model PV820 Pneumatic PicoPump), micromanipulator apparatus (Narishege) and benchtop centrifuge (Labnet; C2400-R).

Reagents

Ampicillin sodium salt (Fisher, BP1760-25): 50 mg/ml in water, stored at −20°C.

Tetracycline hydrochloride (Sigma, T3383): 12.5 mg/ml in 50% (vol/vol) ethanol, stored at −20°C.

Kanamycin sulfate (Invitrogen; 11815): 25 mg/ml in water, stored at −20°C.

Chloramphenicol (OmniPur, 3130): 12.5 mg/ml in 99.7% (vol/vol) ethanol, stored at −20°C.

LB-agar plates: Luria-Bertani (LB) agar (Difco, 244510), prepared using manufacturers instruction and with one or several antibiotics: chloramphenicol, 12.5 μg/ml; kanamycin, 25 μg/ml; ampicillin, 50 μg/ml; and tetracycline, 12.5 μg/ml and stored at 4°C.

500 ml “low-salt” LB broth with 5 g/1 L of NaCl: Prepared with 2.5 g yeast extract (Fluka, 70161), 2.5 g sodium chloride (OmniPur, 7760) and 5 g tryptone (Fisher Scientific, BP1421)

TAE (50×): 242 g of Tris base, 57.1 ml of glacial acetic acid and 18.6 g of EDTA in 1 liter of water.

E3 (50X): 14.61 g of NaCl (250 mM), 2.43g CaCl2.2H2O (16.5 mM), 4.07g MgSO4.7H2O (16.5 mM) and 0.63 g KCl (8.5 mM) in 1 L of Milli-Q water.

Phenol red solution (Sigma, P0290): 2.5% of (wt/vol) phenol red in nuclease-free water.
10X Tricaine solution (Aldrich, E10521-50G): 0.2% tricaine in E3 and make to pH7 with Tris (pH9.5).

Methods

Fish Husbandry

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana University of Pennsylvania. The wild-type Tü zebrafish strain (Danio rerio) was maintained in the fish facility of the Department of Biology, Indiana University of Indiana at a pH7 – 8 and ~28ºC. The facility has a recirculating rack system (Aquateering Inc) and adult fish were raised ~8 fish/1.8 L tank. The babies (6 days – 14 weeks) were fed with spirulina and hatchfry, juveniles (14 days – 30 days) with spirulina, hatchfry and live brine shrimp, and adults (≥ 30 days) were fed with live brine shrimp and TetraMin flake food.

Identification and Preparation of *slc20a1a* Bacterial Artificial Chromosomes (BAC) Clone

**Identification and purchase.** The http://zfin.org/ site was used to identify the BAC containing *cis*-regulatory elements of interest. On the page, “Genes/Markers/Clones” was clicked and the gene name, *slc20a1a* was typed into the “Name/Symbol” field. Under “Types”, “Gene” was selected and “search” button was clicked to display the page of gene information. Under the “SEGMENT (CLONE AND PROBE) RELATIONSHIPS” section, “*slc20a1a* contained in BAC” was clicked. The schematic was clicked to view genomic sequences of interest and thereafter, the order was placed on ZK90N12 clone using the provided link on the page.

**Preparation of slc20a1a-BAC DNA.** The bacteria culture from the supplied BAC agar stab (Source BioScience) was streaked out on an LB-chloramphenicol (*cam*) plate and grown
overnight to generate single colonies. Two single colonies were picked using sterile inoculation sticks and transferred into an 18 mm round-bottom tube of 5 ml LB-cam. This was grown overnight in a roller drum (knob was at value 8) connected inside a 32°C incubator. 920 µL of overnight culture was used to make DMSO (80 µl DMSO) stock and stored immediately at -80°C.

To prepare slc20a1a BAC DNA using quick miniprep, the remaining culture was transferred to a 50 ml falcon tube (falcon tube size depends on the available centrifuge) and centrifuged at 2500 g /4500 rpm for 5 min at room temperature (RT). The supernatant was removed and resuspended in 250 µl buffer P1 by gently tapping (did not vortex to avoid breaking the BAC DNA). The mixture was transferred to a 1.7 ml microcentrifuge tube and 250 µl of buffer P2 was added, mixed by inverting gently and incubated at RT for 5 min. 250 µl of buffer N3 was added, mixed by inverting gently and incubated on ice for 5 min. The mixture was centrifuged at 16000 g /13300 rpm for 5 min at RT for two rounds and the supernatant was transferred to a new 1.7 ml tube each time. The BAC DNA was precipitated by adding 750 µl of isopropanol, mixed gently by inverting gently and incubated on ice for 10 min. The mixture was centrifuged at 16000 g /12000 rpm for 10 min at RT and supernatant was removed. The pellet was washed by adding 1 ml of 70% (vol/vol) ethanol. The mixture was spinned for 3 min and air dried for 20 min. The resulting pellet was dissolved in 50 µl of sterile water (~2.5 – 3.0 µg of DNA) and stored at -20°C for future use.

**Restrictions digest to confirm slc20a1a-BAC DNA.** 10 µl of nuclease-free water was added to a 1.7 ml microcentrifuge tube followed by the addition of 25 µl of slc20a1a-BAC DNA. To the resulting mixture, 4 µl of NEB buffer 1 and 1 µl of AgeI were added respectively. The mixture was incubated for 2 hours at 37°C and 4 µl of 6x loading dye was added and mix by
tapping gently. The sample was run on 1% (wt/vol) agarose gel for 45 min at 98 Volts (V) (The 1% agarose gel was prepared with 0.75 g of agarose and 75 mL of 1x TAE. 2 µL of ethidium bromide was added into the melted agarose). The digestion pattern was observed under a UV light and the picture taken with a camera (Canon DS126191) attached to a gel box. The pattern was compared on http://tools.neb.com/NEBcutter2/.

**Preparation of SW102 electrocompetent cells.** The SW102 cells were streaked from the -80°C stock onto an LB-tetracycline (tet) plate and grown overnight at 32°C. A single colony was picked and inoculated in 18mm round bottom tube of 5 ml LB-tet (20 mg/ml tet) and placed in a roller drum connected to a 32°C incubator. 2.5 ml of the overnight culture was inoculated in 500ml LB-tet and grown in a shaker incubator till OD$_{600}$ of ~0.58 (0.55 – 0.6 is recommended and should be ~3 – 4hours). (While the culture was in the shaker incubator, the following were ready, 500 ml LB, 2 autoclaved & prechilled 250 ml centrifuge tubes (Nalgene), 800 ml of prechilled sterile water, prechilled 50 ml falcon tube (the tube depends on the centrifuge bucket that is available), styrofoam box with ice and ice cold sterile 10% glycerol were ready).

The cells were transferred to two 250 ml prechilled centrifuge tubes and chilled on ice for 20 min. The tubes were transferred to a 4°C prechilled centrifuge (Sorvall) and spun at 4500 g for 15 min. The supernatant was removed from each tube and the cells were resuspended in 200 ml ice-cold sterile water. The tubes were spun at 4500 g for 15 min at 4°C the supernatant was removed from each tube and the pellet was resuspended in 200 ml ice-cold sterile water. The spinning was repeated at 4500 g for 15 min at 4°C. All supernatant from each tube were removed and the cells were resuspended in 5 ml ice cold sterile 10% glycerol. The resulting 10 ml was transferred to a prechilled 50 ml falcon tube and 50 µl aliquots were made in 1.7 ml Avant tubes and stored at -80°C.
Transformation of electrocompetent SW102 cells with *slc20a1a-BAC* DNA by electroporation. As previously described by (Maximiliano L. Suster et al., 2011), the electrocompetent SW102 from the -80°C freezer was thawed on ice and 25µl was transferred to a prechilled 1.7 ml tube. 5 µl (~300 ng) of the BAC DNA was added to the cells and mixed gently by tapping. The mixture was incubated on ice for 10 min and transferred to a prechilled 0.1 cm gap cuvette. The cuvette was immediately transferred to the electroporation holder while ensuring that the outside of the cuvette is dry (cleaned with a paper towel) before applying a current. The pulse button under the Ec1 setting in the BioRad MicroPulser electroporator was pressed and a double beep sound confirmed that electroporation has been done. 1 ml of LB (5 g NaCl/1 L) was immediately added to the cuvette and the cells were gently pipetted up and down with a P1000 pipette. The mixture was transferred to a 16 mm round bottom tube and the cells (tubes placed in a roller drum) were recovered by incubating at 32°C for 1 hour. After incubation, the cells were transferred to a 1.7 ml Avant tube and 100 µl was spread on an LB-cam plate. The remaining culture was spun down at 14000 g for 30 s. The supernatant was reduced to leave ~100 µl. The leftover (~9/10 th of starting culture) was spread on an LB plate. The plates were incubated overnight at 32°C. The next day, two single colonies (SW102-BAC) were respectively picked and incubated at 32°C in LB-cam overnight. Stocks were prepared in DMSO as previously described (Preparation of *slc20a1a-BAC* DNA).

Recombineering the *Tol2-amp* Cassette into SW102-BAC Cells

Polymerase Chain Reaction (PCR) to isolate *Tol2-amp* cassette (*from pCR8GW-iTol2-amp plasmid*). The PCR reaction was performed using the expand high-fidelity PCR system. The reaction was performed in a total volume of 100 µl containing 1 µl *piTol2-amp* plasmid DNA (1 - 1.9 ng), 10 µl of 10x expand high-fidelity buffer with MgCl₂ (1x), 2 µl 10 mM
dNTP mixture (0.3 mM), 2 µl CQDp95 (Table 1) (1 µM), 2 µl CQDp96 (Table 1) (1 µM) and 83 µl nuclease-free water (to make up 100 µl). The samples were placed in a thermal cycler and the parameters were set as recommended by M. L. Suster et al. (2011), which is shown on Table 2.

Table 2

**PCR parameters to isolate iTol2-amp cassette**

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Anneal</th>
<th>Extend</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94ºC, 2 min</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2 - 30</td>
<td>94ºC, 30 sec</td>
<td>55ºC, 30 sec</td>
<td>72ºC, 1 min 15 sec</td>
</tr>
<tr>
<td>31</td>
<td>-</td>
<td>-</td>
<td>72ºC, 5 min</td>
</tr>
</tbody>
</table>

To remove the template plasmid DNA from the PCR product, 10 µl NEB buffer 4 and 2 µl *DpnI* were added. The mixture was incubated at 37ºC for ~6 – 8 hours. 10 ul of 6x DNA loading dye was added and the mixture was split into 50 µl each and run on 1% agarose TAE gel (as previously described under, Restrictions digest to confirm *slc20a1a*-BAC DNA) for 30min. The expected bands were cut with clean and new razor blade and product purification followed the procedure on the Qiagen Gel Extraction kit instructions. The *i Tol2-amp* was reconfirmed by running 1 µl of the extracted product on 1% agarose TAE gel as previously described. The rest of the product was stored at -20ºC.

**Preparation of electrocompetent cells and electroporation.** From the *SW102*-BAC stock stored at -80ºC, cells were streaked out on LB-*cam* plate and incubated at 32ºC overnight. Two single colonies of *SW102*-BAC were picked and inoculated in 5 ml LB-*cam* (5 g NaCl/L) in an 18 mm round tube. They were grown overnight in a roller drum (knob was at value 8) connected inside a 32º C incubator. 500 µl of the overnight culture was transferred into 25 ml
LB-cam (prewarmed to RT) in a 50 ml flat-bottom flask. The preparation was made for two 50 ml flat-bottom flasks since we were having two different controls for the experiment. The flasks were placed in a shaker incubator and cells were grown at 32°C at 210 rpm until OD$_{600}$ reaches 0.55–0.6 (~3 – 4 h). (The following were placed in a styrofoam of ice for each of the flask, 50 ml of autoclaved Milli-Q water, two 50 ml Falcon tubes (size of tube was dependent on the available centrifuge), two 1.7 ml Avant tubes. Two 10 ml pipettes and 2 electroporation cuvettes were also kept in a 4ºC refrigerator for each flask).

When the expected OD$_{600}$ was reached, 10 ml of the culture was pipetted out from one of the flasks into a prechilled 50 ml falcon tube and placed at 4ºC. This sample served as control 1 in this experiment. The rest of the cultures in the 2 flasks were heat-shocked in a water bath at 42°C for exactly 15 min (not more or less than this). In place of a shaker-water bath, shaking was done by hand every 4 min to ensure even transfer of heat. The flasks containing the cells were immediately transferred to ice and cooled for 5 min. The cells were gently swirled and 10ml was transferred into each of the two prechilled 50 ml falcon tubes. One of the tubes was used for test experiment while the other served as control 2. The tubes were centrifuged (including control 1) at 4500 g for 5 min in a 0ºC-precooled centrifuge. The tubes were quickly transferred to ice while the supernatant were carefully drained. The pellet was resuspended by adding 10 ml of the prechilled Milli-Q water using a chilled 10 ml pipette. The pellets were allowed to resuspend while on ice as vortexing could hamper cells from recombineering. The tubes were centrifuged at 4500 g for 5 min in a 0ºC-precooled centrifuge. The draining and resuspension was repeated as earlier described. For the third time, the tubes were centrifuged and drained as previously described. However, complete drainage was ensured at this time by inverting the tubes on a paper towel as long as the ~50 µl cells at the bottom of the tubes were intact. The ~50 µl
electrocompetent cells were respectively transferred to new prechilled 1.7 ml Avant tubes on ice. 1 – 2 µg Tol2-amp cassette was added to the experimental tube (previously heat-shocked) and mixed by tapping gently. 1 – 2 µg Tol2-amp cassette was also added to control 1 tube (was not previously heat-shocked) and mixed. Tol2-amp DNA was not added to the control 2 tube (previously heat-shocked). The electroporation, plating (on LB-cam-amp) and incubation were performed as previously described (Transformation of electrocompetent SW102 cells with slc201a-BAC DNA by electroporation). Besides, the electroporation was performed within 30 min after making electrocompetent cells.

**Confirmation of iTol2 cassette recombination into SW102-BAC.** Replica plating was performed by picking 10 single-colony candidates from the experimental plate and mixing them respectively in 30 µl LB in a 1.7 ml Avant tubes. The LB-cam-amp, LB-cam-kan and LB-cam-spect plates were respectively marked with spots numbered from 1 – 10. Consequently, 5 µl was pipetted from each of the tubes and gently dispensed on the corresponding spot on the respective plates. The inocula were allowed to dry and the plates were incubated at 32ºC overnight. The next day, stocks of iTol2-BAC were prepared (as previously described) from candidates that only grew on LB-cam-amp but did not grow on either or both LB-cam-kan and LB-cam-spect plates.

The iTol2 cassette integration was also confirmed by PCR. The iTol2-BAC DNA was extracted as previously described (Preparation of slc20a1a-BAC DNA). The primers, CQDp131 and CQDp132 (Table 1) were specifically designed for the expected junctions of the cassette in the BAC DNA. The concentrations of the PCR components were as previously described (Polymerase Chain Reaction to isolate iTol2-amp cassette) except that 5 – 10 µg iTol-BAC DNA was used in 50 µl total volume. The parameters were set as shown on Table 2.
Recombineering the *EGFP* Reporter Gene Cassette into the *iTol2-BAC*

In order to have a selection marker to confirm the integration of the reporter gene into the *iTol2-BAC*, SW102 cells were first transformed with #802 plasmid (*attP1*-*GFP*-2A-*CreER*-*attP2*) which harbors the reporter gene cassette. The electrocompetent SW102 cells stored at -80°C were used as previously described (Transformation of electrocompetent SW102 cells with *slc201a*-BAC DNA by electroporation) in the transformation and 50 – 100 ng of #802 applied. The next day, two single colonies (*SW102-#802*) were picked and incubated at 32°C in LB-amp overnight. Stocks were prepared in DMSO as previously described (Preparation of *slc20a1a*-BAC DNA).

**Polymerase Chain Reaction (PCR) to isolate polyA-KanR cassette from *pbs SK-GFP-pA-FRT-kan-FRT* plasmid.** After making *SW102-#802*, next was to isolate the kanamycin selection marker (*KanR*) to be recombined into it. A total volume of 100 ul was used for the PCR. Apart from the added 5% DMSO and 2.5 mM MgCl₂, the concentrations of other PCR components were as previously described (Polymerase Chain Reaction (PCR) to isolate *iTol2-amp* cassette). The CDQp142 and CQDp143 primers (Table 1) were designed to anneal to the polyA sequence of the template. The PCR parameters are depicted on Table 3.

**Table 3**

*PCR parameters to isolate FRT-kan-FRT cassette*

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denature</th>
<th>Anneal</th>
<th>Extend</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C, 2 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 - 30</td>
<td>94°C, 30 sec</td>
<td>52.6°C, 30 sec</td>
<td>72°C, 1:50 sec</td>
</tr>
<tr>
<td>31</td>
<td>-</td>
<td>-</td>
<td>72°C, 5 min</td>
</tr>
</tbody>
</table>
Following the PCR, the digest of the template plasmid, the KanR cassette extraction and purification followed previous description (*Polymerase Chain Reaction (PCR) to isolate iTol2-amp cassette*).

**polyA-KanR cassette recombineering into SW102-#802.** This recombineering was to generate the CQD29 (*GFP-2A-CreER-pA-FRT-Kan-FRT*) which would eventually be isolated and recombined into iTol-BAC. From the SW102-#802 stock stored at -80ºC, cells were streaked out on LB-cam plate and incubated at 32ºC overnight. Two single colonies of SW102-#802 were picked and inoculated in 5 ml LB-cam (10 g Nacl/L) in an 18 mm round tube. The remaining steps followed previous description (*Preparation of electrocompetent cells and electroporation*) and inoculation was performed on LB-amp-kan agar plates. The next day, stocks of putative CQD29 were prepared as previously described (*Preparation of slc20a1a-BAC DNA*).

From the stock (harboring CQD29 cassette) cells were streaked out on LB-amp-kan plate and incubated at 32ºC overnight. Two single colonies from the resulting growths were picked and inoculated in 5 ml LB-amp-kan (10 g Nacl/L) in an 18 mm round tube. They were grown overnight in a roller drum (knob was at value 8) connected inside a 32ºC incubator. The plasmid DNA was subsequently extracted using the procedure on manufacturer’s (Qiagen miniprep kit) instruction and stored at -20ºC.

**Confirmation of polyA-KanR cassette recombination into SW102-#802.** The resulting plasmid DNA was digested with restriction enzymes, *Xcm-I* and *Nco-I*. The template DNA (#802) was also digested to serve as control. Each of the mixtures in 1.7 ml Avant tubes had 10 µl total volumes constituting of 1 µl test DNA, 1 µl NEB corresponding buffer, 0.4 µl restriction enzyme and 7.5 µl sterile water respectively. The tubes were incubated for 1hour at 37ºC and ran
on agarose gel as previously described (*Restrictions digest to confirm slc20a1a-BAC DNA*). The gel bands were compared using the Ape program (*A plasmid editor; http://biologylabs.utah.edu/jorgensen/wayned/ape/*).

To further confirm the orientation of the recombined cassette (*polyA-KanR*) in the plasmid, *CQD29* was sent to Eurofins for sequencing using reverse primer CQDp166 (Table 1) which anneals to the *KanR* sequence.

Again, we confirmed the functionality of *CQD29* by performing microinjection of *CQD29* plasmid into the zebrafish embryos at one-cell stage. First, *pCS2-zT2TP* (sequence of medaka *Tol2* transposase optimized for zebrafish codon) was linearized by digesting with *NotI* and purified by phenol-chloroform-isoamyl and chloroform extractions respectively. The *Tol2* transposase was precipitated with 70% (vol/vol) ethanol and the pellets were dissolved to a concentration of ~1 µg/µl. 1 µg of the resulting *Tol2* transposase was used to synthesize *Tol2* transposase mRNA according the manufacturer’s instruction on the mMESSAGE mMACHINE SP6 kit. 1 µl aliquots of *Tol2* transposase mRNA were made and stored at -80°C.

Prior to the day of injection, the three males and five females were placed in different 1 liter mating tanks in the afternoon. The needles for microinjection were prepared by pulling a glass capillary and breaking the tips with fine forceps to obtain fine ends. On the morning of the microinjection, the *CQD29/RNA* injection mixture (Table 4) was prepared in an RNase-free 1.7 ml tube, vortexed, quick-spinned and kept at 4°C. A P20 pipette was inserted into the microloader tip and 3 µl of the injection mixture was pipetted and loaded into the fine glass capillary needle. The pressurized air injection system and the micromanipulator device were arranged at a stereomicroscope station.
Table 4

*Components of the injection mixture*

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQD29 DNA (310 ng/μl)</td>
<td>0.8 μl</td>
<td>~0.25 μg</td>
</tr>
<tr>
<td>Tol2 transposase RNA (250 ng/μl)</td>
<td>0.6 μl</td>
<td>0.15 μg</td>
</tr>
<tr>
<td>KCL (0.4M)</td>
<td>5 μl</td>
<td>0.2 M</td>
</tr>
<tr>
<td>Phenol red solution (2.5%, wt/vol)</td>
<td>1 μl</td>
<td>0.5%</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>1.6 μl</td>
<td>Make up</td>
</tr>
</tbody>
</table>

The capillary glass was then fixed into the holder of the injection system (WPI) with the pressure set at 20 psi and timed duration of 850 ms. A male and female fish were placed together in one 1 liter mating tank and watched pending the laying of eggs. The eggs were collected using a strainer and transferred into a Petri dish containing E3 solution. Using a 2ml-transfer pipette, the eggs were transferred into E3 medium in a Petri dish and lined under the agarose gel (prepared by pouring melted 2% agarose over stacked thin-film glass slides in a Petri dish). The embryos (~15/injection set) were set with the one-cell part facing the injection needle. Injection was quickly performed by penetrating the chorion and pressing the pedal to release the DNA/RNA mix when the needle is situated between the yolk and cytoplasm. The quantity and location of the DNA/RNA mix was monitored with the color of the phenol red. The injected embryos as well as the uninjected (control to monitor embryo viability) were incubated at 28ºC in E3 medium. On day three after injection, 1 ml of 0.2% tricaine was added to the Petri dish and gently swirled in order to anesthetize the injected embryos (now larvae). Five larvae were transferred to a concave microscope glass slide using a 2 ml transfer pipette and viewed under the fluorescent microscope with an attached digital camera for image capture. The candidates were viewed under the bright field, red and green channels of the microscope respectively. After
taking the pictures, the larvae were transferred to the Petri dish and recovered by replacing the tricaine medium with fresh E3.

**Polymerase Chain Reaction (PCR) to isolate CQD29 cassette (GFP-2A-CreER-pA-FRT-Kan-FRT).** After confirmation of CQD29 functionality, we proceeded to do PCR to isolate CQD29 cassette from the template plasmid using CQDp137 and CQDp154 primers (Table 1) which both anneal to CQD29. A total volume of 100 µl was used for the PCR. Apart from the added 5% DMSO and 1.5 - 2.5 mM MgCl₂, the concentrations of other PCR components were as previously described (*Polymerase Chain Reaction (PCR) to isolate iTol2-amp cassette*). The PCR parameters are depicted on Table 5.

Table 5

<table>
<thead>
<tr>
<th>PCR parameters to isolate CQD29 cassette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle number</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2 - 30</td>
</tr>
<tr>
<td>31</td>
</tr>
</tbody>
</table>

Following the PCR, the digest of the template plasmid, the CQD29 cassette extraction and purification were performed as previously described (*Polymerase Chain Reaction (PCR) to isolate iTol2-amp cassette*). For CQD29 cassette recombineering into iTol2-BAC, streaks were made from the iTol2-BAC stock (stored at -80ºC) on LB-cam-amp plate and incubated at 32ºC overnight. Two single colonies of iTol2-BAC were picked and inoculated in 5 ml LB-cam-amp (5 g NaCl/L) in an 18 mm round tube. The remaining part of the procedure was performed as previously described (*Preparation of electrocompetent cells and electroporation*) and plating was carried out on LB-cam-amp-kan plate. Next day, two single colonies were picked and
inoculated to make stocks as previously described. Also, the streaks were made on LB-\textit{cam-amp-kan} agar plate and sent to Eurofins for DNA extraction and sequencing.
CHAPTER 4

RESULTS

Restriction Digest of slc20a1a-BAC DNA

The restriction digest of the BAC DNA with AgeI is shown in Figure 7. The patterns of the two candidates that were digested were consistent with the expected pattern.

![Figure 7. Agarose gel electrophoresis showing results of the restriction digests of slc20a1a BAC DNA with AgeI. Lanes 1 and 2 show 1kb ladder and the uncut slc20a1a BAC respectively while lanes 3 and 4 show the slc20a1a BAC candidates digested with AgeI.]

Polymerase Chain Reaction (PCR) to Isolate iTol2-amp Cassette

In order to facilitate the integration of the final BAC construct into the zebrafish genome, a Tol2 cassette having the minimal sequences of 200 bp (L200) on the left and 150 bp (R150) on the right is required in the BAC DNA for transposon mediated transgenesis. So, we cloned the inverted L200 and R150 Tol2 cassette (iTol2) from pCR8GW-iTol2-amp plasmid. There was no amplified product with 1 ng of template, pCR8GW-iTol2-amp in the final mixture when ran on agarose gel electrophoresis. We got the expected 1416 bp band (Figure 8) when the template DNA was increased to 1.9 ng final under the same parameters (Table 2).
**iTol2-amp Cassette Recombination into SW102-BAC and Confirmation of Tol2-BAC**

Having linearized the *iTol2-amp* cassette by PCR, we proceeded with its integration by homologous recombination into SW102 harboring the *slc20a1a* BAC clone. The linearized *iTol2* has been designed to have the 50 bp homologies to both ends of the target sequence in the *slc20a1a* BAC clone. The cassette is introduced into the SW102 harboring the *slc20a1a* BAC by electroporation after heat-shocking SW102 cells to induce recombinase functions. Because the *Tol2* cassette harbors an ampicillin resistant sequence, the cells were selected on LB-*amp* plates.

The experimental plates had single colonies but with more on the 9/10th plate as expected. As also predicted, the control plates (both with and without heat-shock) had no colonies after overnight incubation. To confirm that the *Tol2-amp* recombination (Figure 9) was successful, we did replica plating for ten candidates. All the five candidates grew on LB-*cam-amp* (Figure 10) but did not grow on LB-*cam-spect* and LB-*cam-kan*. The *Tol2* template (*pCR8GW-iTol2-amp*) has the Spectinomycin resistant gene and the result suggests that the *DpnI*-template digest was

*Figure 8. Agarose gel electrophoresis showing PCR isolation of Tol2-amp cassette. Lane 1: 1kb ladder, Lane 2: 1416 bp of Tol2-amp.*
successful. These suggest that the integration of the *Tol2* cassette may have occurred at a reasonable efficient rate.

![Diagram](image)

**Figure 9.** Schematic integration of *Tol2*-amp cassette into the *slc20a1a* BAC starting from A to B.

![Image](image)

**Figure 10.** Replica plating of five candidates showing the growth of *Tol2*-BAC cells on LB-cam-amp plate but no growth on LB-cam-spec and LB-cam-kan plates.

To further confirm that the integration of the *Tol2*-amp cassette into the *slc20a1a* BAC was responsible for the result of the replica plating, we amplified the *iTol2* sequence using PCR. The PCR product was ran on agarose gel and the band corresponded to the expected 1153 bp (Figure 11). Also, the amplification was optimized when the forward and reverse primer concentrations was increased to 1 µM in the final reaction respectively. At this concentration, any annealing temperature within the ranges of 45 – 65°C was good for amplification.
Figure 11. Agarose gel electrophoresis showing the 1153 bp (lane 2) from PCR to confirm the Tol2 cassette integration into the slc20a1a BAC.

**Polymerase Chain Reaction (PCR) to Isolate polyA-KanR Cassette**

Since we have already generated a slc20a1a BAC clone having the ampR, the integration of a reporter gene that does not have a different antibiotic resistant sequence makes the media selection of the final BAC construct a difficult task. Therefore, we cloned the kanR cassette (Figure 12) from pBSK-GFP-pA-FRT-kan-FRT plasmid in order to generate an intermediate construct (using SW102 transformed with #802 plasmid) that contains the GFP-reporter gene and kanR.

![Figure 12. Agarose gel electrophoresis showing the PCR isolation of the 1649 bp (lane 2) KanR cassette.](image)

The isolation of the kanamycin resistant gene (KanR) from the plasmid was only optimized under the PCR conditions of 2.5% DMSO and 2.5 mM MgCl₂. Under these conditions
and at an annealing temperature of 52.6°C, we were able to generate our expected band of 1649 bp (Figure 12).

**KanR Cassette Recombination into SW102-#802 (GFP-F2A-CreER) and Confirmation**

With the linearized kanR having the 50 bp homologies to the #802-target sequence on each end, the cassette was introduced by electroporation after the recombinase functions have been induced by 42°C heat-shock for 15 min and cells made electrocompetent.

After an overnight incubation, the experimental plates had single colonies while the two sets of controls (with and without heat-shock) had no colonies. Our restriction digest on CQD29 and #802 plasmid with XcmI and NcoI (Figure 13) corresponded to “A plasmid Editor” (ApE, a DNA sequence editor) results. On ApE, #802 template had three bands of 10481 bp, 2190 bp and 1676 bp while CQD29 had four bands of 10274 bp, 2190 bp, 1676 bp and 1236 bp. The extra band of 1236 bp corresponded to the integrated KanR cassette on ApE. Similarly, XcmI showed (picture not shown) one band (14347 bp) for the template and the predicted two bands (9381 bp and 5995 bp) for CQD29. To confirm that the integration of the KanR occurred in the correct orientation, we sequenced (Eurofins) ten candidates from the resulting CQD29 using a reverse primer, CQD166 (Table 1) that anneals at the KanR. The result showed that two candidates (C1-3 and C2-3) had the KanR integration in the expected orientation. Also, figure 14 shows that the sequencing of the 5’ end of the PolyA part of the KanR insert and 3’ end within the KanR matched the expected orientation using primers (Table 1) CQDp146 and CQDp147 respectively.

Apart from this confirmation, we tested the functionality of the reporter gene by injecting the intermediate-CQD29 construct into zebrafish embryos at the one-cell stage. The reporter gene expression in the intermediate-CQD29 is driven by an ubiquitous gene promoter while the KanR
is controlled by another bacterial promoter. After three-day post injection, the larvae were viewed under the fluorescent microscope and the result (Figure 15) showed mosaic expression of green fluorescent protein especially at the muscle cells (based on morphology and location).

*Figure 13.* Agarose gel electrophoresis to confirm KanR integration into the intermediate CQD29. Lane 1: 1 Kb ladder, Lane 2: #802 plasmid, Lane 3: CQD29 with extra band of KanR integration.

*Figure 14.* Confirmation of sequencing result of CQD29 using NCIB BLASTN program. CQDp146 forward primer was used for the 5’ end and CQDp147 reverse primer for the 3’ end.
Isolation of attP1-GFP-F2A-CreER-pA-Kan-attP2 from CQD29 and Recombineering into Tol-BAC

Upon our conviction of the functionality of our intermediate construct, we designed forward and reverse primers (Table 1) that contained the 50 bp homologies, integrase sequences and eventually anneals to CQD29 respectively. Consequently, we have a reporter gene that not only has the CreER sequence, but also has both kanR sequence to aid selection and a “switchable” integrase sites. Following the cloning of attP1-GFP-F2A-CreER-pA-Kan-attP2 from CQD29 we proceeded with its introduction into Tol2-BAC to generate our final BAC construct.

The amplification of the cassette was not optimized at 1.5 mM and 2.5 mM MgCl₂ respectively. However, at 1.5 mM MgCl₂ and 5% DMSO, PCR was optimized resulting in a specific and the expected 4633 bp band (Figure 16).

Figure 15. Mosaic expression of GFP-2A-CreER-KanR in 3 dpi larvae under the green channel of a fluorescence. The were no such expressions in the bright and red channels which serve as control.

Figure 16. Agarose gel electrophoresis showing in lane 2 the result of the PCR isolation of the 4633 bp cassette (attP1-GFP-F2A-CreER-pA-Kan-attP2) from CQD29. Lane 1 shows the 1 kb ladder.
The homologous recombination of *CQD29* cassette into the *Tol2*-BAC yielded colonies in the experimental plates but none in the control plates (with and without heat-shock), suggesting that our final construct, *CDQ30* (Figure 17) successful.

We are currently processing the sequencing of the *CQD30* and this will be performed as soon as the sequencing company resolves their technical challenge.

*Figure 17.* A sketch of our final *slc20a1a* BAC construct containing the *Tol2* cassette for transposon mediated transgenesis and integrase bound reporter gene for future genetic switch.
CHAPTER 5

DISCUSSION

The enhancement of the existing methods of inducing kidney injury in zebrafish remains indispensable in our understanding of cellular and molecular events leading to kidney regeneration. Several methods have been applied to induce kidney injury such as injection of nephrotoxic compounds and laser ablation (Cianciolo Cosentino et al., 2010; Hentschel et al., 2005). It is obvious that these approaches have increased our current understanding in terms of development. For example, Diep et al. (2011) were able to report the landmark expression of \(lhx1a\) progenitor cells which contributes to neonephrogenesis in developing zebrafish using a nephrotoxin-induced kidney injury model. While noting the success of these methods in bringing about kidney injuries, one cannot overlook the inconsistencies that may be associated with the resulting cellular damages especially in large scale studies. Apart from being time-consuming, laser ablation could likely affect neighboring cells (Pisharath, Rhee, Swanson, Leach, & Parsons, 2007; C. T. Yang, Sengelmann, & Johnson, 2004). Under such conditions, false positive results and conclusions become imminent.

Here, we have generated a construct, \(CQD30\) which when injected into the zebrafish embryos (at the one-cell stage) can be used to induce kidney injury, monitor and identify cells involved in kidney regeneration. Our construct is built within a \(slc20a1a\) BAC that contains essential regulatory sequences and flanked by the \(Tol2\), which will be mediating the integration of our construct into the zebrafish genome. The \(slc20a1a\) is integral to the PCT and as such will label the proximal segment of the nephron with \(GFP\). Subsequently, the resulting generation of the transgenic zebrafish can be characterized for endogenous expression.
The use of transgenic zebrafish models has expounded studies aimed at monitoring cellular and molecular interactions in tissues and organs using fluorescent protein that is driven by promoter of a specific gene of interest (Moon et al., 2013; W. Zhou & Hildebrandt, 2012). However, most of the transgenic lines lack site-specific recombinases such as integrase which can allow cassettes to be switched in-vivo (without needing to generate new transgenic line) once proper transgenic line is isolated and characterized. Besides, our DNA construct (Figure 16) has the integrase sites (attP) flanking the reporter gene, the CreER and the KanR bound FRT. While site-specific recombinases such as LoxP and FRT are reversible in their reactions, the integrase is directional and irreversible (Hu et al., 2011). Based on its efficiency, the attP-flanked EGFP-2A-CreER cassette can be “switched out” for a toxic gene product (such as nitroreductase) for cell ablation (Pisharath et al., 2007). Since the EGFP-2A-CreER cassette is flanked with the attP1 and recombination sequences, injecting ΦC31 integrase mRNA into transgenic embryos can mediate intermolecular recombination between the genomically integrated cassette and the co-injected plasmid carrying the bacterial nitroreductase (NTR) gene flanked by corresponding recombination sequences (Hu et al., 2011). This approach is advantageous because it allows for the generation of a “new” transgenic line expressing the toxic gene product within the genetic background of a previously characterized transgenic line. Once the second transgenic line is generated, it can be used for genetic ablation of the proximal nephron (via exposure to chemicals such as metronidazole which is converted to a cytotoxin by NTR) and allow us to characterize kidney injury, repair, and regeneration.

Moreover, the use of NTR-dependent cells ablation does not affect neighboring cells populations. In their investigation to determine the effects of NTR-dependent beta cell ablation in zebrafish embryos, Pisharath et al. (2007) reported that the level of transcripts of major
pancreatic indicators such as trypsin gene and glucagon were similar to controls. Also, Pisharath et al. (2007) and W. Zhou & Hildebrandt, 2012 reported regeneration of pancreatic beta cells and podocytes respectively following NTR-induced cell death.

Again, the Cre recombinase site within characterized zebrafish lines will be vital in future fate mapping in order to detect sources of kidney cells repair and regeneration (Duffield & Humphreys, 2011). When a transgenic line expressing CQD30 is crossed with another transgenic line that harbors a reporter gene and a “STOP” cassette positioned in-between loxP sites, the “STOP” cassette is removed which results to the activation of the reporter gene. Consequently, the descendants from such a recombination will express the reporter gene which becomes a lineage analytical tool for kidney repair and regeneration.

In addition, we generated an intermediate CQD29 that has the inserted FRT-flanked resistance cassette. The CQD29 harbors the GFP reporter gene, the Cre and KanR. With the insertion of KanR selection marker, CQD29 will be an important tool in generating transgenic lines. More so, since the KanR is bound by the FRT sites, it allows subsequent removal by flippase recombinase as the need arise. The functionality of the CQD29 was also tested by injecting into embryos where they showed mosaic expression of GFP cells (Figure 14).

In conclusion, although we would need to sequence the junctions of the inserts within the final slc20a1a BAC (CQD30) before proceeding to inject into the embryos, all our test results and controls through the process suggest that we have successfully generated the final slc20a1a BAC construct (Figure 16). The CQD30 is a genetic switch model that promises to be a high-throughput and consistent method of inducing kidney injury to study regeneration in zebrafish. The GFP expression will be specific to the PCT which makes the model an ideal one to detect...
cell proliferation. Consequently, potential chemicals that can enhance regeneration can be screened at a large scale.
REFERENCES


developing pronephros in Xenopus and zebrafish embryos. *Gene Expression Patterns, 6*(7), 667-672.


The University of Kansas Medical Centre. *Early Stem Cells*. (© 2011 The University of Kansas Medical Center; cited December 4, 2011). Available at


