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Analysis of Protein-Protein Interaction in Zebrafish Kidney Stem Cells

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ABSTRACT

The kidney is a vital organ that filters metabolic waste from the blood. Injury to the kidney can eventually require replacement therapy. Current therapies include dialysis and transplantation, but both have severe limitations. A possible alternative approach is a stem cell/regenerative therapy. However, further research is needed before this approach becomes a viable option. Zebrafish are a powerful model system for studying stem cells and regeneration. Recently, a specific stem cell population was identified in the adult zebrafish kidney. Transplantation of these stem cells into recipient fish with kidney disease led to the regeneration of new kidney tissue. Therefore, a similar therapeutic approach may be possible for human patients. The kidney stem cells in zebrafish express the *lhx1a* gene, and it likely plays an important role in regulating these stem cells. A better understanding of the molecular function of *lhx1a* would provide insight into developing future stem cell therapies. In this study, the *lhx1a* gene was cloned into a yeast plasmid for protein-protein interaction studies. The success of this cloning was confirmed by DNA sequencing and protein expression analysis. With this new plasmid carrying the *lhx1a* gene, it is possible to identify new proteins that interact with the *lhx1a* protein using a yeast two-hybrid selection approach. Discovering new proteins that interact with *lhx1a* would provide a better understanding of how kidney stem cells are regulated and may contribute to developing new regenerative therapies for treating kidney disease.

Keywords: kidney; *lhx1a*; stem cells; yeast; two-hybrid; zebrafish

The kidney filters metabolic waste from the blood, excreting it through the urine, and maintaining fluid and electrolyte balance (Dressler 2006). Chronic Kidney Disease (CKD) is prevalent worldwide and approximately 20 million people in the United States, 10% of the population, are affected [National Kidney and Urologic Diseases Information Clearinghouse (NKUDIC) 2012]. The primary causes of CKD are diabetes and high blood pressure. These conditions result in the overwork and premature damage of nephrons (the functional units of the kidney).

CKD can eventually lead to end stage kidney disease, where kidney replacement therapy is necessary for survival. Current therapies include dialysis and kidney transplantation. Unfortunately, the chance of receiving a kidney transplant is small with only approximately 26% of those patients on a three-year waiting list receiving a transplant. Dialysis is not an ideal treatment either, as it has a high death rate (about 20%) and a poor life style (NKUDIC 2012). Thus, examining kidney stem cells as a regenerative therapy could provide a possible alternative for treatment of CKD and end stage kidney disease (Hopkins et al. 2009).

Stem cells have great potential for regenerative therapies (Cohen and Melton 2011). These unspecialized cells can undergo self-renewal to replenish themselves, and they also have the ability to specialize into many different mature cell types. Research using human embryonic stem cells can be controversial and have limitations. Therefore, animal models are needed to complement the research of human stem cells.

Zebrafish have been an important model for developmental biology for several decades (Lieschke and Currie 2007). Recently, it has become more prevalent in stem cell and regeneration research (Gemberling et al. 2013). A population of stem cells within the adult

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zebrafish kidney has been identified and when transplanted into recipient fish with kidney failure, the stem cells were able to regenerate new functional kidney tissue. During kidney development, these same stem cells also contributed to forming the adult kidney (Diep et al. 2011). Therefore, a better understanding of how these stem cells are able to make new kidney tissue will provide insight into a regenerative therapy.

The adult kidney stem cells in zebrafish express the LIM homeobox 1a (*lhx1a*) gene (Diep et al. 2011), which is a transcription factor important for kidney development (Kobayashi et al. 2005). Deletion of this gene in mice led to kidney malformation (Shawlot and Behringer 1995). Therefore, understanding the molecular mechanism of how *lhx1a* regulates kidney stem cells may provide insight into designing future regenerative therapies. One approach is to identify new proteins that interact with the *lhx1a* protein. A powerful genetic method to do this is the yeast two-hybrid (Brückner et al. 2009). This system allows one to screen a library of thousands of "prey" proteins and determine which ones would bind to the protein of interest (the "bait" protein).

Since *lhx1a* is a transcription factor, it cannot be used in the conventional yeast two-hybrid system, in which the protein-protein interaction takes place in the nucleus of the cell. However, new versions of the system allow for the interaction to take place in the cytosol of the cell, thus allowing the use of transcription factors as bait proteins. One example is the split ubiquitin system (Möckli et al. 2007) (Figure 1). In this system, the bait protein is fused with C-terminal half of the ubiquitin (ub) protein (the Cub) and the prey protein is fused with the N-terminal half of ub (the NubG). When the bait and prey protein interact, the two halves of ub come together to reconstitute a functional ub protein. This causes the proteasome to cleave off the artificial transcription factor LexA-VP16, thus turning on reporter genes to allow yeast growth on selective media.

Prior to performing a yeast two-hybrid selection, the *lhx1a* gene must be cloned into the appropriate plasmid. In this report, the *lhx1a* gene successfully cloned into the DHB1 plasmid (Figure 2). This was confirmed by restriction enzyme digest and DNA sequencing. Finally, the DHB1-*lhx1a* plasmid was able to properly produce the *lhx1a* protein in yeast cells. The DHB1-*lhx1a* plasmid can now be used in split ubiquitin system to identify new proteins that interact with *lhx1a*.

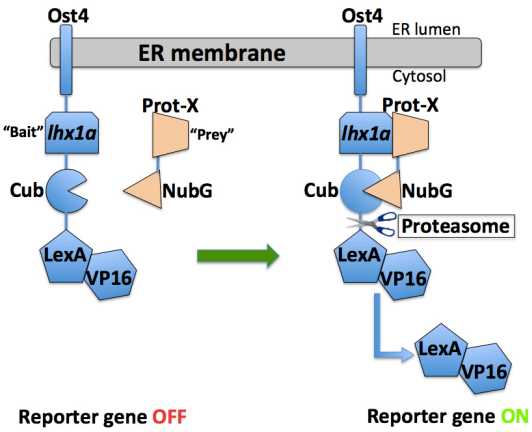

Figure 1. The split ubiquitin system allows for the use of a transcription factor in a yeast two-hybrid selection. The protein-protein interaction between the "bait" and "prey" occurs in the cytoplasm, not in the nucleus. Interaction between the bait and prey results in the reconstitution of a native ubiquitin protein. This causes the proteasome to cleave off the artificial transcription factor LexA-VP16, which shuttles into the nucleus to active expression of reporter genes. Therefore, the protein interaction can be selected for by growing yeast cells on the appropriate selective media.

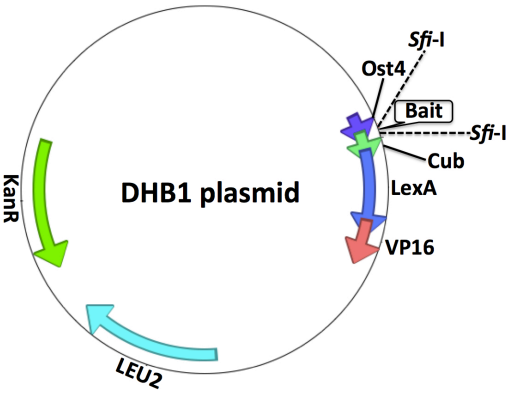

Figure 2. The DHB1 plasmid is used to clone any gene of interest (the bait) for the yeast two-hybrid system. The gene is inserted into the *Sfi*-I restriction enzyme sites in the proper reading frame. This results in one fusion protein consisting of Ost4-bait-Cub-LexA-VP16. The LEU2 gene is for

selection in yeast, and the KanR gene is used for bacterial selection using the antibiotic kanamycin.

METHODS

Culture Conditions

Bacteria were cultured in Lysogeny Broth supplemented with 100 ug/mL ampicillin at 37°C. Cultures were grown for 16 hours prior to harvesting. Yeast cells were grown in either rich media (Yeast Extract Adenine Dextrose) or Synthetic Complete media (supplemented with dextrose) with the proper amino acid dropout at 30°C (Sherman 1991). Yeast cultures were grown for 24 hours prior to experimentation.

Cloning of *lhx1a*

The pCS2 plasmid carrying the *lhx1a* gene was provided by Dr. Neil Hukriede (University of Pittsburgh), and polymerase chain reaction (PCR) was used to amplify *lhx1a*. Primers CQDp128 (5'-AGCAGCAGC GGCCATTACGGCCatggtccactgtgcgggctgcga gaggcctatattgg), CQDp151 (5'-GGCCGAGG CGGCCCCcagactgtgccctcgttcatttctgacggagg atggg) (*Sfi*-I recognition sequences underlined), and the pCS2-*lhx1a* template were used with the Expand High Fidelity PCR System (Roche Diagnostics Corporation). The PCR cycle parameters are described in Table 1. The PCR product was isolated from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and ligated to the GEMTE intermediate plasmid (Promega) by TA-cloning. The ligation was used to transform TOP10 electrocompetent cells (Life Technologies). *lhx1a* was released from GEMTE-*lhx1a* by digestion with *Sfi*-I, purified from the agarose gel, and ligated (T4 DNA ligase, New England BioLabs) to the DHB1 plasmid (Dualsystems Biotech) digested with *Sfi*-I. The ligation was used to transform cells as described above, and the final plasmid candidates were digested with *Age*-I to confirm proper insertion of the *Sfi*-I fragment. The 5-prime and 3-prime junctions were confirmed by DNA sequencing (SimpleSeq, Eurofins).

Western Blot Analysis

The NMY51 yeast strain (Dualsystems Biotech) was transformed with the appropriate plasmid and grown to mid-log phase. Protein extracts were prepared using the post-alkaline lysis method (Kushnirov 2000). The proteins were resolved using sodium dodecyl polyacrylamide gel electrophoresis (10% acrylamide gel). Primary antibody (from rabbit) against the LexA protein (Sigma) was used to detect the bait proteins fused to LexA. Peroxidase conjugated antibodies (from goat) (Sigma) was used as the secondary antibody. The chemiluminescent blot was analyzed by the C-DiGit scanner (LI-COR).

Table 1. PCR cycle parameters for amplification of the *lhx1a* gene.

Cycle #	Denature	Anneal	Elongate
1	94° C 2 min		
2-30	94° C 30 s	56° C 30 s	72° C 90 s
31			72° C 5 min

RESULTS

Cloning of the *lhx1a* Gene into the Intermediate GEMTE Plasmid

PCR was used to isolate the *lhx1a* gene from the plasmid CS2-*lhx1a*. Primers CQDp128 and CQDp151 were used, which incorporated the recognition sites for the restriction enzyme *Sfi*-I in both forward and reverse primers. The PCR product was analyzed by agarose gel electrophoresis and a band was observed between the 1,000 and 1,650 base pair DNA markers (Figure 3). This is consistent with the expected size of the *lhx1a* gene at 1,254 base pairs. This band was cut out of the gel for purification. The purified DNA was ligated to the GEMTE intermediate plasmid. The ligation reaction was used to transform electrocompetent bacterial cells. Four colonies from the selection plate were selected for analysis by inoculation into selective media and their plasmids were isolated for restriction enzyme digestion. All four candidate plasmids were digested with the *Sfi*-I restriction enzyme and the digestions were analyzed by agarose gel electrophoresis. If *lhx1a* successfully ligated with the GEMTE plasmid, we would expect bands of 1,230 and 3,039 base pairs. We did observe these expected results (Figure 4), indicating that

lhx1a was successfully cloned into the GEMTE plasmid.

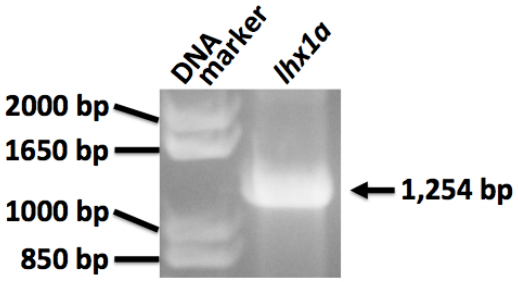

Figure 3. Forward primer CQDp129 and reverse primer CQDp151 were used to isolate the full-length *lhx1a* gene from the pCS2-*lhx1a* template. The *Sfi*-I recognition sites were incorporated into both primers to facilitate insertion into the final DHB1 plasmid.

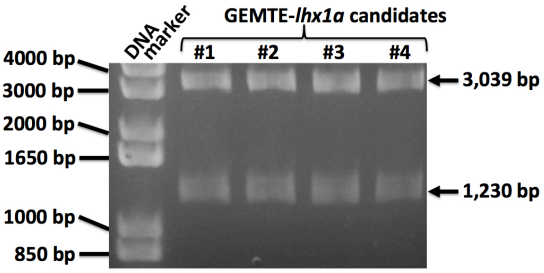

Figure 4. Candidates from the *lhx1a* ligation into the GEMTE plasmid were digested with *Sfi*-I, which should release the *lhx1a* insert.

Cloning of the *lhx1a* Gene into the Yeast Two-Hybrid DHB1 Plasmid

The 1,230 base pair *lhx1a* band (Figure 4) was cut out of the gel for purification. The purified DNA was ligated to the DHB1 plasmid, where the DHB1 plasmid was also pre-digested with *Sfi*-I and purified. The ligation reaction was used to transform electrocompetent bacterial cells. Four colonies from the selection plate were chosen for analysis and their plasmids were isolated for digestion with the restriction enzyme *Age*-I. If the ligation was successful, we would expect bands of 3,174 and 6,984 base pairs. Candidate plasmid number one and number three had the expected fragments (Figure 5), suggesting that the ligation was successful for these two candidates. Because the *Sfi*-I recognition sites at the 5-prime and 3-prime ends of the PCR amplified *lhx1a* gene contained different internal sequences, and therefore produce incompatible overhangs, the ligation of *lhx1a* would occur in only one direction. This directionality should be in frame with the existing coding sequence in DHB1.

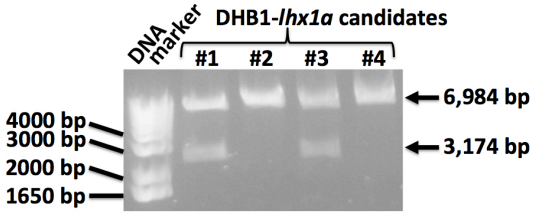

Figure 5. Candidates from the *lhx1a* ligation into DHB1 were digested with *Age*-I. Insertion of *lhx1a* in the correct orientation should give the expected fragment sizes shown.

DNA Sequencing of the DHB1-*lhx1a* Plasmid

The DHB1-*lhx1a* plasmid was sent for sequencing to confirm that the ligation junctions were correct. The observed sequence was compared to the expected sequence using the BLAST server (National Center for Biotechnology Information). Both of the 5-prime end and 3-prime end of *lhx1a* were correctly ligated into DHB1. The 5-prime end contained the ATG start codon of *lhx1a*, while the 3-prime end contained the last codon of *lhx1a* (Figure 6). This confirmed that we had successfully cloned *lhx1a* into the DHB1 plasmid.

Figure 6. Sequencing showed that the 5-prime junction consisted of the *lhx1a* start codon (ATG, highlighted), and the 3-prime junction contained the last codon of *lhx1a* (TGG, highlighted). This confirmed that *lhx1a* was properly inserted into the DHB1 plasmid.

Western Blot Analysis of the *lhx1a* Fusion Protein

The DHB1-*lhx1a* plasmid was transformed into the NMY51 yeast two-hybrid strain to confirm that the *lhx1a* fusion protein was properly produced. A colony from the selection plate was inoculated into liquid selective media and grown to mid-log phase. The cells were then collected for protein extraction. Protein extraction from cells carrying a positive (DHB1-large-T) and a negative control (pPR3-N) was also carried out in parallel. Equal amounts of all three protein extracts were run on a polyacrylamide gel for Western blot analysis. Since the bait proteins (large-T and *lhx1a*) were fused to the LexA protein, an antibody against LexA was used for the Western blot. The data in Figure 7 confirmed that the *lhx1a* protein was properly produced in the yeast strain. The presence of a nonspecific protein band indicates that equal amounts of proteins were loaded for each sample.

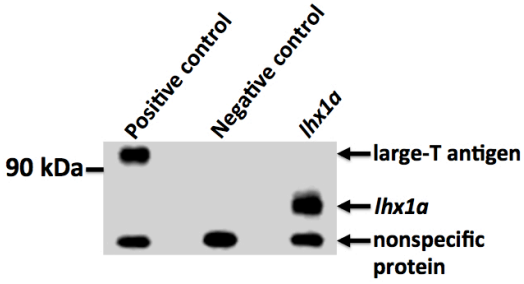

Figure 7. Western blot analysis was performed to show that the *lhx1a* bait protein was properly produced in yeast cells. The NMY51 yeast strain (used for the split ubiquitin system) was transformed with a positive control bait (DHB1-large-T antigen), an empty negative control plasmid (pPR3-N), and DHB1-*lhx1a*. The data confirmed that the *lhx1a* bait was produced at an equivalent level as the positive control. The presence of the nonspecific band suggests that an equal amount of protein extract was loaded for each sample.

DISCUSSION

With kidney disease on the rise and a dire need for alternative therapies, it is important to understand the function and regulation of kidney stem cells in order to develop alternative therapies. One way of doing this is to determine what genes are expressed in kidney stem cells and analyze their protein-protein interaction network (Wang and Orkin 2008).

Zebrafish serve as an important model for kidney regeneration because their adult kidneys have stem cells that perpetually make new kidney tissue during homeostasis and in response to injury (Diep et al. 2011). We recently found that zebrafish kidney stem cells express the transcription factor *lhx1a*. Although it is known that *lhx1a* is essential for kidney development, its molecular function is still not completely known. Therefore, identifying proteins that interact with the *lhx1a* protein might reveal its molecular function.

Because *lhx1a* is a transcription factor, it cannot be used with the classical yeast two-hybrid, since it will activate reporter genes without a prey present (Fashena et al. 2000). However, newer versions of the yeast two-hybrid have solved this problem (Hirst et al. 2001; Laser et al. 2000). In this report, we have successfully cloned *lhx1a* into the DHB1 plasmid. In the future, we will use this DHB1-*lhx1a* bait to identify proteins that interact with *lhx1a* using the split ubiquitin yeast two-hybrid. The identity of these proteins will provide insight into how *lhx1a* works to regulate kidney stem cell and regeneration, therefore providing insight into future regenerative therapies.

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