UTRIP Written Report
August 20 2013

Cadherin-17 mRNA Expression in Wild-Type and ktu Medaka Embryos

Student: Amy Robbins, University of Michigan USA
Lab: PI Hiroyuki Takeda PhD, Laboratory of Embryology, Department of Biological Sciences, University of Tokyo Graduate School of Science
Contributing Lab Members: Atsuko Shimada PhD, Sayaka Tayama, Kota Abe, Yusuke Inoue, Takayoshi Yamamoto

Abstract

In the medaka *kintoun* (*ktu*) mutant, loss of cilia motility leads to polycystic kidney disease, among other symptoms[1]. However, the role of cilia motility in kidney development and function is largely unknown. In mammalian kidneys, primary (non-motile) cilia have been shown to regulate calcium levels[2]. Our lab plans to design a calcium-specific transgenic medaka in order to study the relationship between cilia motility and calcium regulation. This report describes how cadherin-17 was identified as an ideal promoter for such a transgenic. Cadherin-17 is known to be expressed in the renal tubule in zebrafish[3], and our lab has verified its presence in adult medaka. Cadherin-16, a marker of the mammalian kidney[4], was also tested. By performing *in situ* hybridizations at two developmental stages, cadherin-17, but not cadherin-16, was found to be equivalently expressed in wild-type and *ktu* medaka embryos. Moreover, expression patterns of cadherin-17 suggest the presence of medaka-specific posterior gut structures and a relationship with the rate of cilia formation.

Introduction

Cilia are small, microtubule-based hair-like structures that coat a variety of tissue surfaces in vertebrae such as the lungs, trachea, inner ear, brain epithelium, and kidney epithelium. Cilia are essential for functions such as fluid transport, cell motility, and body patterning. Defects in cilia can lead to a wide array of diseases collectively known as ciliopathies[5].

In the human kidney, primary (non-motile) cilia are found on renal tubule epithelial cells. These cilia bend in response to urine flow, which causes glycoprotein polycystin-1 (*PKD1*) located in the axoneme to interact with glycoprotein ploycytin-2 (*PKD2*) located in the basal body, forming a polycystin protein complex that regulates intracellular calcium levels. Disruptions in this complex, primarily in *PKD1*, lead to polycystic kidney disease (PKD) in humans[6]. PKD is characterized by deregulated epithelial tissue growth resulting in multiple fluid-filled cysts in a grossly enlarged kidney. Tight regulation of calcium within the polycystin complexes is therefore necessary for proper kidney development and function.

In teleost fish such as zebrafish and medaka, cilia of the kidney epithelium are motile and believed to only direct urine flow, though studies are ongoing. In the *kintoun (ktu)* medaka mutant, complete or partial loss of the dynein arms in the cilia axoneme results in a complete loss of cilia motility. Proteins encoded by the ktu gene are now understood to be involved in the cytoplasmic pre-assembly of cilia dynein arms. (Takeda and Shimada 2010). Loss of motility in the *ktu* mutant leads to a host of complications including randomization of the left-right body patterning axis, *situs inversus* (a mirror image-like directional inversion) of the heart, and PKD. Cilia motility is therefore required for normal development and function of the kidney in medaka, but the underlying mechanisms are unknown.

In order to examine the relationship between cilia motility and kidney development and function, the Takeda lab aims to create a kidney-specific calcium-indicating transgenic medaka line. Such a line would allow for comparison of calcium levels at various stages of development between wild-type and *ktu* medaka, and provide insight into the still unknown mechanisms of of calcium regulation in the context of the teleost kidney. This could also lead to identification of homologous pathways in humans.

To create such a transgenic, a kidney-specific promoter equally expressed in both wild-type and *ktu* medaka is needed to link to a calcium indicator. In the current study, two genes encoding glycoproteins belonging to the calcium-dependent adhesion (cadherin) protein superfamily found at specific cell-cell junctions were selected as potential promoters. mRNA expression levels for these genes were compared in two developmental stages of medaka embryos – at stage 25 before renal tubule formation, and at stage 30 after renal tubule formation[7] using whole mount *in situ* hybridization.

Cadherin-17 (cdh17) is one of few markers shown to be specifically expressed in the zebrafish renal tubule, and the Takeda lab has verified that cdh-17 is also expressed in the renal tubule of adult medaka (Horsfield et al 2002). Therefore, cdh17 was an attractive candidate as a potential promoter to test for creating the calcium-specific transgenic. Cadherin-16 (cdh16), a marker of the adult mammalian kidney, was also selected for testing (Shao et al 2002). Our lab has confirmed the presence of cdh16 in adult medaka, but no previous attempt for verification of cdh16 presence in embryos had been attempted[8].

After designing and synthesizing RNA-oligonucleotide probes for cdh16 and cdh17, whole mount *in situ* hybridizations revealed that cdh17 mRNA was expressed at similar levels at both selected stages of medaka embryogenesis, while cdh16 mRNA was not expressed at all. Surprisingly, expression patterns of cadherin-17 were not limited to the renal tubule but also included some posterior regions of the gut, suggesting the presence of medaka-specific structures in this region. Finally, changes in the amount of cdh17 along the pronephros suggest a correlation between cdh17 and the rate of cilia formation. We conclude that cdh17 would make an ideal promoter for a kidney-specific calcium-indicating medaka transgenic, and also gained new research directions regarding medaka morphology and cilia formation.

Materials and Methods

Medaka Husbandry and Mutants. Medaka (*Oryzias latipes*) were raised in a laboratory breeding colony under standard conditions with a 13.5 hour light/ 10.5 hour dark cycle maintained at 28°C. Wild-type d-rR and homozygous mutant *ktu* (-/-) strains of closed colonies were used. Embryos were staged to approximately stage 25 and 30 as outlined in *Iwamatsu et al 2004* morphological criteria. Embryos were fixed in 4% PFA and stored in 100% methanol at -20°C until needed.

Probe Design and Synthesis. cdh16 and cdh17 sense and antisense RNA probes were designed using genome information from Ensembl Genome Browser and BLAST databases. cdh16 probe was designed to be 480 base pairs (bp) in length and cdh17 probe was designed to be 440 bp. DNA templates were created by selection and amplification through PCR, followed by plasmid amplification with DHalpha Competent Cells. After checking for relative sizes by plasmid linearization and gel electrophoresis, selected colonies were mini-prepped using Qiagen Prep Kit and correct insertion of probe region verified using DYEnamic ET Terminator Cycle Sequencing Kit. RNA probes were *in vitro* transcribed with RNA SP6 and T7 Polymerases and labeled with digoxigenin, purified using Qiagen RNeasy Mini Kit, and relative sizes verified by gel electrophoresis.

Whole Mount In Situ Hybridization. Embryos were rehydrated on a MeOH – PBS + 0.1% Tween20 gradient, and permeability optimized by incubating in 10ul/ml proteinase-K. Hybridization was performed using 30ng/ml of antisense probe in hybridization buffer and incubated > 15 hours at 65°C. Immunoreaction was performed using PBS+0.1% Tween20 + 0.2% Blocking Reagent (Roche Molecular Biochemicals, Indianapolis, IN) and anti-DIG IgG antibody in a 1:7000 dilution and incubated at 4°C overnight. Staining was conducted using 4-nitroblue tetrazolium/5-bromo- 4-chloro-3-indolyl phosphate (NBT/BCIP; Roche).

Microscopy. Stained embryos were post-fixed in 4% PFA and mounted in 2% methyl cellulose. All imaging was conducted using a Leica M165 FC microscope and images acquired using LAS V3.8 software.

Results

Cadherin-16 mRNA is not expressed by stage 30

To verify functionality of probes, both cdh16 sense and antisense probes were tested alongside a previously made and verified cdh17 antisense probe as a control using stage 30 wild-type embryos. Stage 25 embryos were not tested under the reasoning that if cdh16 mRNA was not present at stage 30, the developmental stage preceded its emergence. The cdh16 antisense probe yielded no staining whatsoever. The cdh17 antisense control probe yielded renal-tubule specific staining, eliminating the possibility of experimental conditional error. Interestingly, the cdh16 sense probe yielded staining in the head region, but the staining was

nonspecific. Since specific staining was unable to be produced using stage 30 wild-type embryos, cdh16 probes were not tested on stage 25 or *ktu* embryos.

Cadherin-17 mRNA is strongly expressed at stages 25 and 30

cdh17 sense and antisense probes were also tested against the previously made cdh17 antisense probe control on stage 30 wild-type embryos. The staining from the newly synthesized cdh17 antisense probe matched the specific staining pattern exhibited in the control cdh17 antisense probe. However, the intensity was lower in the new cdh17 antisense staining, most likely due to a less concentrated probe. The cdh17 sense probe did not produce any staining. Since specific staining was produced in wild-type stage 30 embryos, *in situs* were also conducted on stage 25 embryos using the cdh-17 antisense probe. Staining specific to the same region in the stage 30 embryos was produced.

Cadherin-17 mRNA expression appears to be unaffected in *ktu* mutants

In situ hybridizations were conducted using the cdh17 antisense probe on wild-type and *ktu* embryos at stages 25 and 30. *ktu* embryos exhibited staining of the same specific regions as wild-type. Though the concentration of staining was not quantified, qualitatively the intensity of staining appears to have no marked difference between wild-type and *ktu* embryos.

Cadherin-17 mRNA expression in medaka differs from zebrafish

Cadherin-17 expression in zebrafish has been shown to be localized to the renal tubule. Interestingly, in stage 30 medaka embryos the staining for cdh17 mRNA extends to regions of the posterior gut. In stage 25 medaka, the lower abdominal morphology is not as well defined but the staining also extends further up the body than in comparative zebrafish *in situs* (Horsfield et al 2002).

Notably, in stage 30 embryos there was a clear difference in the level of staining along the pronephros. Regardless of strain, the posterior end of the pronephros was most heavily stained, followed by the anterior end, and the intermediate areas contained the lightest levels of staining.

Discussion

Cadherin-17 is an ideal promoter for a kidney-specific calcium-indicating transgenic

The lack of staining produced by *in situs* using the cdh-16 antisense probe indicates that cdh16 mRNA is not present by stage 30 of wild-type medaka embryos. In mammals, cdh-16 is also known as kidney-specific cadherin (Ksp-cdh) and is exclusively found in adult kidney cells[9]. Since we examined cdh-16 expression in embryonic medaka, the lack of expression is reasonable. An extension from this result would be to look more closely at cdh-16 mRNA expression in adult medaka, which our lab has previously verified through western blotting but never through *in situ*. We can conclude that due to the lack of mRNA expression, cdh-16 can be

eliminated as a potential promoter for making a kidney-specific, calcium-indication transgenic medaka.

By tracking mRNA expression with *in situ* hyrbidizations, cdh17 mRNA expression was shown to be both specific to regions of the kidney and without observable difference between wild-type and *ktu* medaka at stages 25 and 30. These results indicate that cdh17 would make an excellent promoter for a kidney-specific, calcium-indication transgeneic medaka. Follow-up work entails isolating the promoter region of cdh17 or using a back construct of cdh17 to begin work on constructing a cdh17-TG line.

However, since the cadherin superfamily is named for being 'calcium-dependent adhesion' proteins, the lack of change in mRNA expression between wild-type and *ktu* embryos was surprising(SOURCE). The possibility that cdh17 is being regulated or modified post-transcriptionally is noteworthy. Additionally, quantifying the amount of mRNA may reveal subtle differences between wild-type and *ktu* expression.

Medaka posterior gut region contains unique structures

An unexpected result of the cdh17 specificity was that the staining region included structures other than the anterior renal tubule region of the pronephros as exhibited in zebrafish (Horsfield et al 2002). The staining observed started at the posterior tip of the pronephric tube, included the pronephric ducts and glomeruls, and extended to some posterior regions of the gut. While zebrafish and medaka morphology is largely complimentary, this marked difference suggests that the posterior gut region of medaka contains yet unstudied unique structures.

Cadherin-17 mRNA expression is linked to the rate of cilia formation

Using cdh17 as a marker, a differentiation pathway was revealed between developmental stages by differences in staining patterns. At stage 25, the level of staining along the pronephros was fairly consistent. By stage 30, the anterior and posterior ends contained more cdh17 mRNA than the middle region. Between these two stages, significant cell migration and differentiation must have occurred.

In zebrafish, similar pathways have been identified in ion transporters. The Na-K-Cl symporter *slc12a1* is found along the entire early pronephros but only in the distal tubule in later stages[10]. This pattern may be related to the rate of kidney epithelial cilia formation, which form first on the ends of the proneprhos[11]. The differentiation pathway shown by cdh17 patterning suggest The processes underlying the development of the pronephros

Conclusions

This project identified cdh17 as an ideal promoter to construct a kidney-calcium indicating transgenic medaka line. New research directions stemming from this project include cdh16 expression in adult medaka, identification of posterior gut structures unique to medaka, and in the pathways underlying cilia formation along the pronephros.

[1] Takeda, Hiroyuki, and Atsuko Shimada. "The Art of Medaka Genetics and Genomics: What Makes Them So Unique?" *Annual Review of Genetics* 44.1 (2010): 217-41.

[2] I-Chun Tsai, Nicholas Katsanis, Renal cystic disease: from mechanisms to drug development, Drug Discovery Today: Disease Mechanisms (2013)

[3] Julia Horsfield et al, Cadherin-17 is required to maintain pronephric duct integrity during zebrafish development, Mechanisms of Development, Volume 115, Issues 1–2 (2002): 15-26

[4] Shao, X., Johnson, J.E., Richardson, J.A., Hiesberger, T., Igarashi, P. A minimal Ksp-cadherin promoter linked to a green fluorescent protein reporter gene exhibits tissue-specific expression in the developing kidney and genitourinary tract. *J. Am. Soc. Nephrol.* (2002)

[5] Takeda, Hiroyuki, and Daisuke Kobayashi. "Chapter 9: Primary Cilia Dyskinesia in Fish: The Analysis of a Novel Medaka Mutant Kintoun." *Medaka: A Model for Organogenesis, Human Disease, and Evolution*. By Kiyoshi Naruse, Minoru Tanaka, and Hiroyuki Takeda. Tokyo: Springer, 2011. 131-56.

[6] Hanaoka, K. et al. "Co-assembly of Polycystin-1 and -2 Produces Unique Cation-permeable Currents." *Nature* 408 (2000): 990-94.

[7] Iwamatsu, Takashi. "Stages of Normal Development in the Medaka Oryzias Latipes☆"*Mechanisms of Development* 121.7-8 (2004): 605-18.

[8] Takeda laboratory unpublished data (2013)

[9] Thedieck, C., M. Kuczyk, K. Klingel, I. Steiert, C. A. Müller, and G. Klein. "Expression of Ksp-cadherin during Kidney Development and in Renal Cell Carcinoma."*British Journal of Cancer* 92.11 (2005): 2010-017.

[10] Drummond, Iain A. "The Zebrafish Pronephros: A Genetic System for Studies of Kidney Development." *Pediatric Nephrology* 14.5 (2000): 428-35.

[11] Kramer-Zucker, A. G. "Cilia-driven Fluid Flow in the Zebrafish Pronephros, Brain and Kupffer's Vesicle Is Required for Normal Organogenesis." *Development* 132.8 (2005): 1907-921.