Video Article

# A Possible Zebrafish Model of Polycystic Kidney Disease: Knockdown of *wnt5a* Causes Cysts in Zebrafish Kidneys

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## Abstract

Polycystic kidney disease (PKD) is one of the most common causes of end-stage kidney disease, a devastating disease for which there is no cure. The molecular mechanisms leading to cyst formation in PKD remain somewhat unclear, but many genes are thought to be involved. Wnt5a is a non-canonical glycoprotein that regulates a wide range of developmental processes. Wnt5a works through the planar cell polarity (PCP) pathway that regulates oriented cell division during renal tubular cell elongation. Defects of the PCP pathway have been found to cause kidney cyst formation. Our paper describes a method for developing a zebrafish cystic kidney disease model by knockdown of the *wnt5a* gene with *wnt5a* antisense morpholino (MO) oligonucleotides. *Tg(wt1b:GFP)* transgenic zebrafish were used to visualize kidney structure and kidney cysts following *wnt5a* knockdown. Two distinct antisense MOs (AUG - and splice-site) were used and both resulted in curly tail down phenotype and cyst formation after *wnt5a* knockdown. Injection of mouse *Wnt5a* mRNA, resistant to the MOs due to a difference in primary base pair structure, rescued the abnormal phenotype, demonstrating that the phenotype was not due to “off-target” effects of the morpholino. This work supports the validity of using a zebrafish model to study *wnt5a* function in the kidney.

## Video Link

The video component of this article can be found at https://www.jove.com/video/52156/

## Introduction

Zebrafish (*Danio rerio*) embryos have been widely used as a model for studying kidney development and polycystic kidney disease. There are many advantages to using zebrafish as an animal model: the feasibility of studying genetic interactions, the ability to use antisense morpholinos (MO) for protein knockdown, the opportunity to quickly assay large numbers of embryos, and the ease of viewing organ phenotypes in living larvae [1]. The pronephros is the first kidney to develop in vertebrates and is functional in larval zebrafish [2]. The structure of the zebrafish pronephros is relatively simple compared to the mammalian metanephros, the third and final kidney to develop in mammals. The nephron is the working unit of the kidney, with each human kidney containing between 500,000-1,000,000 nephrons[3,4] and each mouse kidney having approximately 13,000 nephrons[5], making it difficult to observe single nephron structure in human or mouse kidneys. The zebrafish has only two nephrons, and each zebrafish nephron contains all the major components found in the glomerulus and tubules of mice and humans[6]and similar specialized renal cell types. Compared to other vertebrate models such as *Xenopus*, the zebrafish nephron more closely resembles the mammalian nephron because it has a closed system[7].

In recent years, the zebrafish genome has been sequenced, allowing the wide introduction of genetic tools, extensive mutant resources, and collections of transgenic reporter lines in zebrafish models. The zebrafish pronephros forms between 12-72 hr post fertilization (hpf) and can be visualized easily in the transparent embryos. The Wilm’s tumor protein WT1 is an essential factor for kidney development. Transgenic zebrafish lines expressing green fluorescent protein (GFP) under the control of the *wt1b* promoter *Tg(wt1b:GFP)* show GFP expression specifically located in pronephric regions in zebrafish embryos, starting from 17 hpf [8]. Nephronophthisis (NPHP), an autosomal recessive cystic kidney disease, is caused by mutations of NPHP genes [9]. NPHP4 knockdown by morpholino caused cyst formation in the *Tg(wt1b:GFP)* fish. [10] Therefore, this transgenic fish is a suitable model for observing kidney structures and cyst formation during kidney development. Importantly, the influence of modulators of kidney development can be studied using this strain in a time and labor efficient manner.

Our paper describes the use of *Tg(wt1b:GFP)* fish as a model to visualize kidney cyst formation after gene modulation. We used start- and splice-site anti-sense MOs to knock down the *wnt5a* gene in zebrafish. Wnt5a is a non-canonical secreted glycoprotein of the Wnt family that plays an important role in the development of various organs and postnatal cellular function[11]. Wnt5a works through non-canonical Wnt pathways, including the planar cell polarity (PCP) pathway, which has been found to play a role in oriented cell division during renal tubular elongation. Wnt5a regulates the Wnt/PCP pathway by forming a complex with the receptor like tyrosine kinase (Ryk), which further transduces Wnt5a signaling by forming a complex with the VANGL planar cell polarity protein 2 (Vangl2), thereby promoting Vangl2 stability [12]. Defects in the PCP pathway can result in random cell division and cause renal cyst formation. We used the *Tg(wt1b:GFP)* zebrafish line to observe kidney

 

cyst formation following *wnt5a* knockdown. The *Tg(wt1b:GFP)* zebrafish model allows live imaging and timely observation of kidney structure. After *wnt5a* knockdown, kidney cyst formation was found beginning at 24 hpf; at 72 hpf, cysts could be found in the glomeruli and the proximal tubules. This method could also be used to screen other genes that might cause kidney cyst formation.

## Protocol

NOTE: Ethics Statement: All zebrafish experiments were approved by the Institutional Animal Care and Use Committee at the Eastern Virginia Medical School.

## 1. Morpholino Preparation

1. Design and synthesize translation-blocking (AUG-) and splice-inhibiting (Splice-) anti-sense morpholino (MO) oligonucleotides for the gene of interest as per manufacturer's instructions (**Figure 1A**). Please see manufacturer's information in **Table 1**.
   NOTE: MOs are shipped as lyophilized stocks in glass bottles.
2. Add high-grade sterile water to the glass bottles to re-suspend MOs to a final concentration of 25 µg/µl. Make sure that the oligonucleotide is completely dissolved. If some solid remains, heat the vial containing the stock oligonucleotide solution at 65 °C for 5 to 10 min and vortex briefly.
3. Store the MO stock solution at RT. Do not store them at 4 °C or -20 °C because lower temperatures could cause the MO oligonucleotide to bind to the container wall. Measure the concentration of the stock solution using a spectrophotometer (please refer to **Table1**) each time a new MO stock solution is made.
4. Prepare the MO working solution on the day of injection by diluting the stock solution with high-grade sterile water to the desired dose. Add 0.5% phenol-red to reach a concentration of 0.05%. For example, to make 5 µl working solution with a concentration of 15 ng/nL, add 3 µl MO stock solution and 0.5 µl 0.5% phenol-red to 1.5 µl of water.
   NOTE: With this working concentration, each drop (500 pl) of injection contains 7.5 ng of MO and two drops contain 15 ng of MO.

## 2. Preparation of the Injection Apparatus

1. Purchase glass pre-pulled needles (please see **Table 1** for detailed information). Alternatively, pull the glass injection needle with a needle puller.
2. Turn on the air compressor and adjust the pressure setting to 50 psi. Turn on the dissecting microscope light source and Pico micro-injection pump and adjust the settings as follows: hold pressure 20 psi, eject pressure 10 psi, period value of 2.5 and 100 µsec range of gating.
3. Load the glass pre-pulled needle with 5 µl of the injection solution and place the needle in a vertical position with the tip pointing down. Wait until all the solution reaches the needle tip with no visible air bubbles. Place the needle holder in an appropriate position next to the microscope and insert the glass needle into the holder. Adjust the injection angle to 45 degrees.
4. Bring the needle tip into view under the microscope, high off the stage, and focus on the thinnest region of the tip. Break off the needle tip with fine point tweezers.
5. Place a ruler and a capillary tube with inner diameter of 0.15 mm side by side under the microscope; inject the solution into one end of the capillary tube to make a liquid column. Adjust the eject time to reach every 17 drops per 1 mm length liquid column, then the volume of every drop is 1 nl (drop volume = $\pi$ x radius$^2$ x length (of liquid column) / count (of drops).
   NOTE: A drop with a diameter of 0.1 mm gives a injection volume of about 500 pl (drop volume = 4/3 x $\pi$ x radius$^3$).

## 3. Preparation of Fertilized Zebrafish Embryos and Injection with Morpholinos

1. Set up *Tg(wt1b:GFP)* transgenic zebrafish breeding pairs according to published protocols for standard zebrafish husbandry and maintenance. Collect embryos after natural spawn, and keep them in a 10 cm Petri dish filled with E3 water (5mM NaCl, 0.17mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). Start MO injection right away [13]. Monitor embryo morphology under the microscope and make sure MO injection is at the one-cell stage, or no later than the four-cell stage.
   1. Transfer the embryos to a 10 cm petri dish with wedge-shaped agar troughs. Aspirate the extra E3 water and gently press the embryos into the troughs.
   2. Manipulate the embryos with the micropipette to visualize 1-cell stage embryos under the dissecting microscope. Penetrate the chorion and then the yolk to inject one or two drops of MO into the yolk (1 drop = 500 pl). Transfer the injected embryos to a 10 cm petri dish with E3 water and incubate at 28.5 °C.
2. After the injections, remove the dead embryos and record the number of injected embryos. Replace the E3 water in the dish every 24 to 48 hr.

## 4. Phenotype Rescue Experiments

1. Select an orthologue from another species that has a different primary base pair structure (usually 3-7 base pair mismatches) and is, therefore, resistant to the MO, for rescue. For example, in this experiment, we chose mouse because the mouse *Wnt5a* mRNA sequence is different from the zebrafish sequence.
2. Design a primer set with the forward primer starting at the translational site and the reverse primer at close to the end of the mRNA coding region. Add a T7 promoter sequence at the 5'- end of the forward primer sequence. In this experiment, we used the following the primer sequences; forward: *5'- taa tac gac tca cta tag gga cta tga tgc tgc tga agc tga a- 3*' and reverse: *5'- tca ctt gca gac gta ctg gtc- 3'.*
3. Perform a 50 µl Polymerase Chain Reaction (PCR) with the primer set (0.5 µM of each primer) designed above and 0.1 µg template DNA containing the mouse *Wnt5a* cDNA sequence with the following PCR program: 1 cycle at 95 °C for 5 min; 35 cycles of 95 °C (30 sec), 58.5 °C (30 sec), 72 °C (1 min); and final extension step at 72 °C for 7 min.

4. After finishing the cycle, run 2 µl of the PCR product on 1% agarose gel to make sure the band is the right size. Purify the PCR product with a PCR purification kit according to manufacturer's instructions. Check DNA concentration with a spectrophotometer. Store the purified PCR product at -20 °C or directly use in capped RNA transcription in the next step.
5. Synthesize *in vitro* capped mRNA with a capped RNA synthesis kit according to the manufacturer's instructions. Dilute the mRNA in 20 µl RNase free water and determine the concentration. Aliquot the RNA and store at -80 °C.
6. On the day of injection, prepare a working solution of the mRNA with RNase free sterile water. Note that 40 pg is generally the highest RNA dose at which nonspecific or toxic effects of the RNA do not occur. Keep the working solution on ice. Inject the embryo with MO and then the rescue mRNA, or co-inject both together. For example, if the concentration of the mRNA prepared in step 4.5 is 0.6 µg/µl, add 0.67 µl of mRNA (0.4 µg) to 4.33 µl of RNase free water to make a final concentration of 0.08 µg/µl, then one drop (500 pl) of each injection contains 40 pg of mRNA. Prepare MO as described in step 3.

## 5. Preparation of Injected Embryos for Fluorescent Imaging

1. After incubation at 28.5 °C O/N, add 0.003% N-Phenylthiourea (PTU) to the E3 water to prevent melanization, which affects observation of pronephros structure using fluorescence microscopy. However, do not add PTU before gastrulation, because it affects early embryonic development.
2. At 48 hpf, manually dechorionate the embryos with fine tweezers. Take images of 48 and 72 hpf embryos under a light dissection microscope. NOTE: These pictures can be taken without anesthesia.
3. Approximately 10 min prior to imaging under the fluorescence microscope, anesthetize the embryos by placing them into a 10 cm petri dish containing 160 µg/ml buffered Tricaine. Wait for 5-10 min, and then lightly touch the zebrafish to confirm that they do not move and therefore the anesthesia is working.
4. After the embryos are anesthetized, mount them in 3% methyl cellulose and orient them in a prone position under a dissecting microscope (please refer to **Table 1**).
5. Observe the pronephros structure under a fluorescence microscope (please refer to **Table 1**) and take pictures at different magnifications (10X and 20X).

## Representative Results

*Wnt5a* knock down was achieved by introducing translation blocking MO (AUG-MO) or exon/intron border splice MO (splice-MO) to zebrafish embryos at the one cell stage. The AUG-MO targets the start codon and, therefore, inhibits both maternal and zygotic *wnt5a* message. The splice-MO targets the third splice donor site and inhibits only the zygotic transcript of wnt5a (**Figure 1A**). The AUG- and splice- morphants phenocopied each other with multiple defects, including curly tail down body axis and pericardial edema (**Figure 1B**). Mouse Wnt5a mRNA partially rescues the morphant phenotype (**Figure 4B**), demonstrating that the phenotype is not due to off-target effects. The *Tg(wt1b:GFP)* transgenic zebrafish, which expresses GFP driven by the wt1b promoter so that the GFP recapitulates endogenous expression of *wt1b*, was used to outline the pronephric structure. The *Tg(wt1b:GFP)* fish showed glomerular cyst formation after wnt5a knockdown at 48 hpf, whereas control embryos injected with phenol-red alone displayed normal pronephric development, forming a fused glomerulus in a "U" shape with tubules that extend laterally (**Figure 2**). At 72 hpf, glomerular structure further developed with easily visualized vascular loops, with both AUG- and splice-MOs developed cysts in the glomeruli and proximal tubules (**Figure 3**). H&E staining of the transverse histological sections of 72 hpf morphant embryos revealed cyst formation, disrupted glomerular structures and dilated renal tubules (**Figure 4A**).

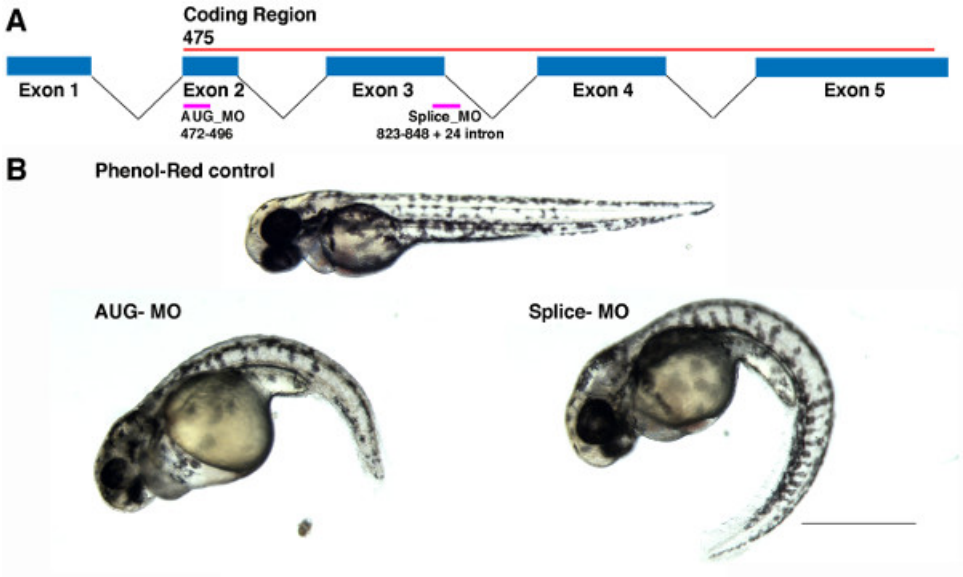


**Figure 1: Morpholino design and light microscopic appearance of zebrafish embryos at 72 hr post fertilization (hpf) following injection with phenol-red control, AUG-MO, or splice-MO. (A)** Diagram showing the zebrafish *wnt5a* gene, the coding region, and the regions targeted by the AUG- and splice- MO. The wnt5a AUG-MO was designed to target the ATG start codon to block protein translation; the splice-MO was designed to target the border of exons 3 and 4. Splice-MO oligo directed against any splice junction is expected to generate either a complete or partial single exon deletion or a complete or partial single intron insertion. We chose to knock down wnt5a with both an AUG-MO, which affects maternal and zygotic message, and a splice-MO, which affects only the zygotic transcript, in order to enhance specificity. **(B)** The embryo injected with phenol-red (injection control) showed normal body curvature, length and pericardium. The embryo injected with the AUG-MO showed downward curly tail structure and pericardial edema, and the embryo injected with the splice-MO phenocopied the appearance of the embryo injected with AUG-MO. Bar = 2mm.

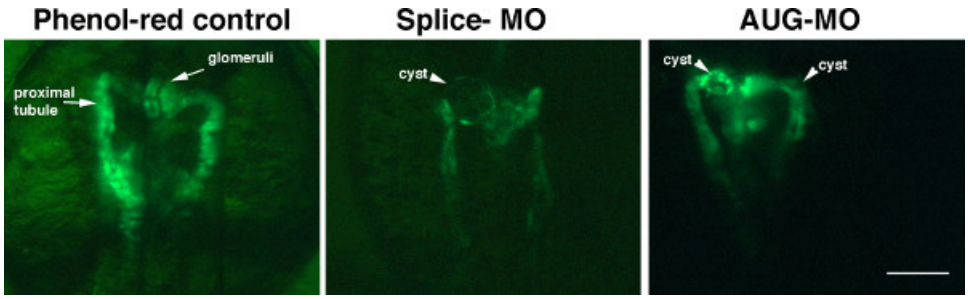


**Figure 2: Fluorescence microscopic appearance of *Tg (wt1b:GFP)* zebrafish embryos at 48 hpf after injection with phenol-red control, *wnt5a* AUG-MO or *wnt5a* Splice-MO.** In the phenol-red injection control, pronephros development was complete at 48 hpf. Two fused glomeruli and the proximal tubules can be seen. Cyst formation can be observed in glomeruli in both the AUG- and splice-MO embryos at 48 hpf by the end of zebrafish pronephros development (bar = 100 µm).

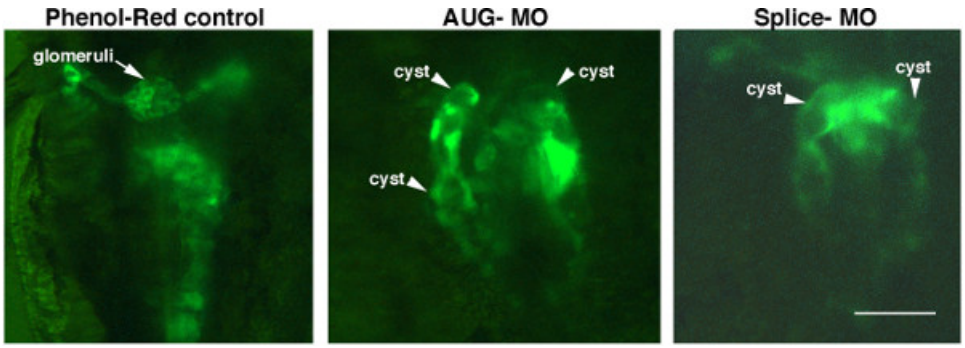


**Figure 3: Fluorescence microscopic appearance of *Tg(wt1b:GFP)* zebrafish embryos at 72 hpf after injection with phenol-red control, *wnt5a* AUG-MO or *wnt5a* splice-MO.** At 72 hpf, glomerular structure can be clearly visualized. In the phenol-red injection control, two fused glomeruli and vascular loops can be observed. *Wnt5a* knockdown resulted in cyst formation in all the nephron structures that could be visualized, including the glomeruli and proximal tubular region (bar = 100 µm).

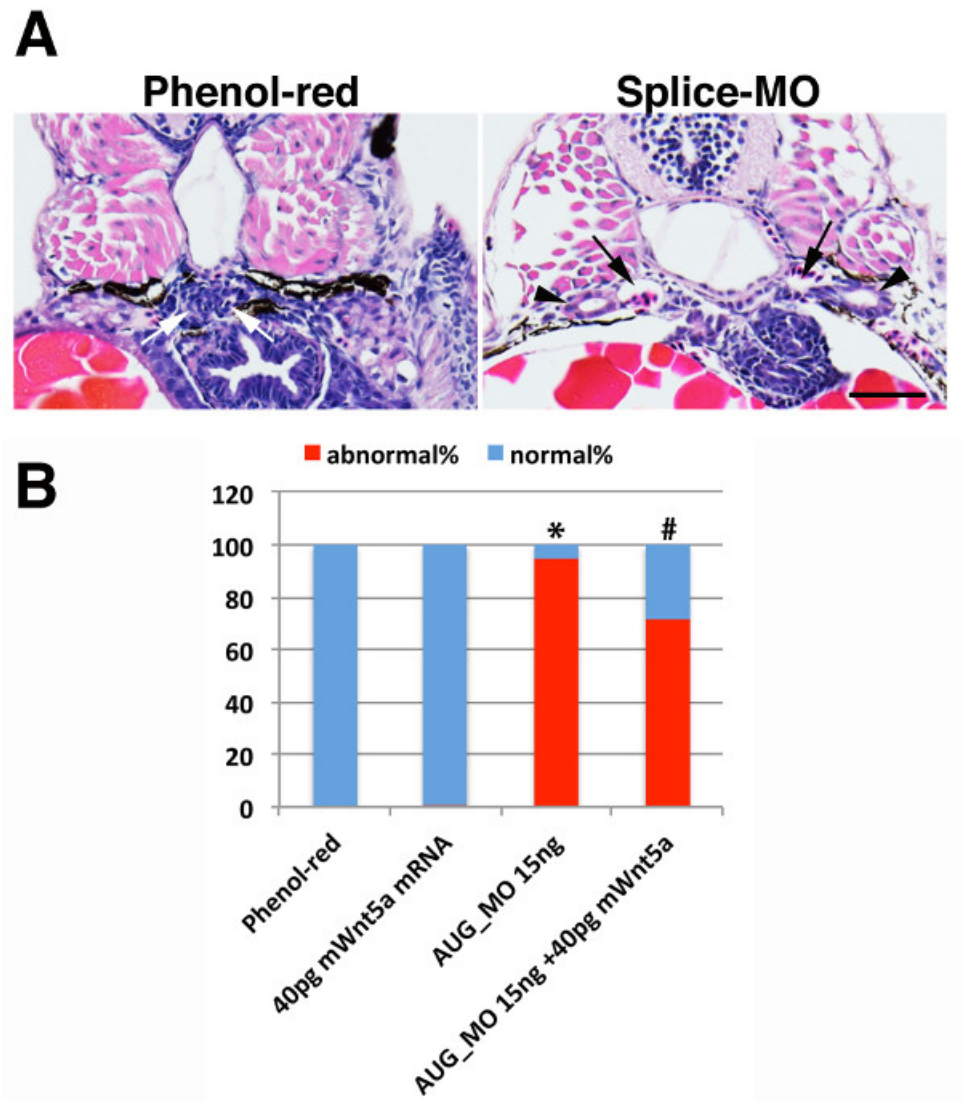


**Figure 4: (A)** H&E staining of the transverse histological sections of 72 hpf morphant embryos revealed cyst formation, disrupted glomerular structures and dilated renal tubules. Arrow: pronephric glomeruli; arrowhead: dilated renal tubule. Bar = 20 µm. **(B)** The AUG-MO phenotype could be partially rescued with mouse Wnt5a, resistant to the MO due to a difference in primary base pair structure, thereby demonstrating that the phenotype was not due to off-target effects. The difference was statistically significant at $p < 0.05$ (* $P < 0.05$ vs Phenol-red group; # $P < 0.05$ vs AUG-MO 15 ng group).

## Discussion

Polycystic kidney disease (PKD) is one of the leading causes of end-stage renal disease in humans and is characterized by progressive cyst formation, renal enlargement, and abnormal tubule development [14]. Autosomal dominant PKD (ADPKD) is a genetic disease in which mutation of either PKD1, encoding polycystin-1 (PC1), or PKD2, encoding polycystin-2 (PC2), results in polycystic kidneys. Many other genes, especially those encoding proteins found in the primary cilium, are thought to be involved in the development of renal cysts, and so far there is no ideal tool to screen these genes. We describe a fast and easy way to observe kidney cyst formation after gene knockdown in zebrafish. This method can likely be used to screen other candidate PKD genes.

One of the strengths of this technique is its simplicity. In *Tg(wt1b:GFP)* zebrafish, the expression of GFP is in glomeruli, tubules and ducts, which makes it a particularly suitable model to study the entire pronephric kidney structure. The *Tg(wt1b:GFP)* zebrafish allows direct visualization of pronephric structure and kidney cyst formation after gene knock down. It was suspected that *Wnt5a* knockdown could cause PCP pathway defects and kidney cyst formation in mice. However, global knockout of *Wnt5a* is postnatal lethal in mice and could not be used to observe kidney cyst formation after birth. By using the method described here, in a short period of time it was determined that *wnt5a* knockdown in zebrafish resulted in kidney cyst formation. With this result, the next step will be to generate kidney specific *Wnt5a* knockout mice.

This technique also has a few limitations. It is difficult to estimate the efficacy of MOs without a good antibody, and to rule out the possibility that the MO might inhibit the function of an irrelevant gene to cause the phenotype. Extensive control experiments are required to overcome these problems. Two distinct MOs, the AUG-MO and the splice- MO were used to evaluate wnt5a knockdown. Both morphants phenocopied

 

each other, and injection of mouse *Wnt5a* mRNA, resistant to the MOs due to a difference in primary base pair structure, rescued the abnormal phenotype, demonstrating that the phenotype was not due to “off-target” effects of the MO.

Our model demonstrates that *wnt5a* knockdown causes kidney cyst formation in zebrafish embryos. This methodology can be employed to test many other genes that are suspected to cause renal cyst formation, as it is simple and less time consuming. Once it is demonstrated that the gene mutation results in kidney cyst formation in zebrafish, further experiments can be conducted to investigate the detailed mechanisms of kidney cyst formation. In this experiment, since the *Wnt5a* global knockout mice are postnatal lethal and cannot be used to observe kidney cyst formation after birth, the next step will be to generate kidney-specific *Wnt5a* knockout mice using the Cre-lox system.

## Disclosures

The authors have nothing to disclose.

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