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Wnt5a Is Necessary for Normal Kidney Development in Zebrafish and Mice

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Abstract

Background—Wnt5a is important for the development of various organs and postnatal cellular function. Little is known, however, about the role of *Wnt5a* in kidney development, although *WNT5A* mutations were identified in patients with Robinow syndrome, a genetic disease which includes developmental defects in kidneys. Our goal in this study was to determine the role of *Wnt5a* in kidney development.

Methods—Whole-mount in situ hybridization was used to establish the expression pattern of *Wnt5a* during kidney development. Zebrafish with *wnt5a* knockdown and *Wnt5a* global knockout mice were used to identify kidney phenotypes.

Results—In zebrafish, *wnt5a* knockdown resulted in glomerular cyst formation and dilated renal tubules. In mice, *Wnt5a* global knockout resulted in pleiotropic, but severe, kidney phenotypes, including agenesis, fused kidney, hydronephrosis and duplex kidney/ureter.

Conclusions—Our data demonstrated the important role of *Wnt5a* in kidney development. Disrupted Wnt5a resulted in kidney cysts in zebrafish and pleiotropic abnormal kidney development in mice.

Keywords

Wnt5a; Kidney development; Pronephros; Mesonephros; Metanephros

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Disclosure Statement

There are no competing financial interests.

Introduction

Congenital kidney and urinary tract abnormalities have been described in patients with Robinow syndrome [1, 2], but the molecular mechanisms are unclear. *WNT5A* mutations were found in patients with Robinow syndrome and *Wnt5a*, and its receptor *Ror2* knockout mice show phenotypes similar to these patients [3]. Wnt5a is a noncanonical glycoprotein of the Wnt family that regulates a wide range of developmental processes. It is known that Wnt5a is essential for proper skeletal, urogenital and gonad development [4, 5], but the role of Wnt5a in kidney development is unknown.

The mammalian kidney originates from intermediate mesoderm (IM). The metanephros, which develops into the permanent kidney, forms when the ureteric bud (UB) grows out of the nephric (wolffian) duct (ND) and contacts the metanephric mesenchyme (MM). The UB then elongates and branches to form the collecting duct system. The metanephric mesenchymal cells condense around the tip of the UB, aggregate, epithelialize and differentiate to podocytes and proximal and distal tubular cells.

Our goal in this study was to determine the role of Wnt5a in kidney development. We used two models, zebrafish and mice. The simple nature of the zebrafish pronephric kidney makes it a suitable system to study the early developmental events that lay the foundation for genesis of more complex kidneys, while mouse metanephric kidney development is similar to human kidney development.

Methods

Zebrafish Maintenance

Wild-type and *Tg(wt1b:GFP)* (green fluorescent protein) transgenic zebrafish [6] were reared and maintained as described [7]. Embryos were collected after natural spawn, kept at 28.5°C, and staged as described [8]. Embryos from 48-hpf *Tg(wt1b:GFP)* zebrafish were anesthetized in 0.016% tricaine solution and embedded in 3% methyl cellulose with dorsal side facing up and imaged using a fluorescence microscope (Leica M205C).

Zebrafish Injections and Morpholino Oligonucleotides

Wnt5a morpholino oligonucleotides (MOs) were generated by Gene Tools, LLC (Philomath, Oreg., USA). MOs were injected into embryos at the one-to-four-cell stage as described [9]. Capped mouse *Wnt5a* full-length mRNA was synthesized using the mMessage mMachine T7 kit (AM1344, Ambion). For the rescue experiments, 40 pg of *Wnt5a* mRNA was coinjected with the AUG-MOs into one-to-four-cell stage embryos.

Wnt5a$^{-/-}$ Null Mice

Wnt5a$^{-/-}$ null mice were generated by mating CMV-Cre mice (Jackson Lab stock No. 006054) with *Wnt5a*$^{fl/fl}$ mice [10] and were similar in phenotype to the commercially available *Wnt5a* global knockout mice [11]. The day of vaginal plug was considered E0.5. The *Wnt5a*$^{-/-}$ embryos were identified visually by a reduced size phenotype, and the absence of *Wnt5a* was confirmed by PCR as described [10, 11]. IM from E10.5, E11.5, and metanephros from E13.5 and E15.5 were dissected out and fixed for whole-mount in situ

hybridization (wmISH). E11.5–18.5 metanephros were dissected out, and images were taken with a Leica M205C dissecting microscope. MRI of E16.5 *Wnt5a*$^{-/-}$ mouse embryos was performed at the Penn Small Animal Imaging Facility using a 9.4-tesla (400 MHz) vertical bore spectrometer with 55-mm gradients.

Whole-Mount in situ Hybridization

Mouse *Wnt5a* was subcloned from MSCV2.2Wnt5aIRES-GPF (kindly provided by Dr. Stephen Jones at University of Massachusetts Medical School) into the pEGM-T Easy Vector (Promega). The plasmid for the mouse Ret gene was kindly provided by Dr. Mendelsohn, Columbia University. The zebrafish *wnt5a* template was prepared by TA cloning. Digoxigenin-labeled RNA probes were prepared by in vitro transcription (DIG RNA Labeling kit, Roche). wmISH in mouse or zebrafish embryos was performed as previously described [5, 12].

Statistical Analyses

Phenotypes for the rescue experiments were classified as abnormal after MO injection by the presence of any combination of the following features: edema, short body size and curled tails. For comparison of means, a t-test was performed using SPSS software (v.15.0; SPSS Inc., Chicago, Ill., USA). For all tests, $p < 0.05$ was considered to represent statistically significant differences.

Results

Wnt5a Localizes to the Developing Zebrafish Kidney

wmISH of wild-type zebrafish embryos at 72 h after fertilization (hpf) confirmed *wnt5a* expression in the pronephric kidney (fig. 1a, b). The *wt1a* probe served as a positional marker to confirm *wnt5a* expression in the pronephric glomerulus (fig. 1c).

Wnt5a Is Necessary for Zebrafish Pronephros Development

We knocked down *wnt5a* with a translation blocking morpholino (AUG-MO), which targets the start codon and affects both maternal and zygotic *wnt5a* mRNA, and an exon/intron border splice morpholino (splice-MO), which targets the third splice donor site and affects only the zygotic transcript of *wnt5a* (fig. 2a). Both the AUG and the splice morphants phenocopied each other, with multiple defects at 72 hpf, including: reduced axis length, curly tail down body axis, and pericardial edema (fig. 2b). HE staining of the transverse histological sections of morphants revealed disrupted and dilated glomerular structure and dilated proximal tubules (fig. 3a). The *Tg(wt1b:GFP)* transgenic zebrafish [7], in which GFP is driven by the *wt1b* promotor so that GFP recapitulates endogenous expression of *wt1b*, was used to examine the pronephric structure. The *Tg(wt1b:GFP)* fish showed glomerular cyst formation after *wnt5a* knockdown (fig. 3b). Cilia staining showed disordered pronephric cilia in *wnt5a* knockdown zebrafish compared to the injection controls (fig. 3c).

Wnt5a Localizes to the Developing Mouse Kidney

To determine whether *Wnt5a* plays a role in mouse metanephric kidney development, we performed wmISH of *Wnt5a* in E10.5 and E11.5 IM, and E13.5 and E15.5 metanephros. wmISH of the *Ret* gene expression was used to identify the ND and UB structure (fig. 4a–d). At E10.5, *Wnt5a* mRNA was diffusely expressed in the entire IM with higher expression level caudally (fig. 4e, g). At E11.5 when the UB starts to branch, *Wnt5a* expression increased significantly in the gonad and mesonephric region, however, not in the metanephric region where the UB starts to initiate (fig. 4f, h). At E13.5 and E15.5, *Wnt5a* was expressed in the metanephros (fig. 4i, j) with a pattern similar to *Ret* expression (fig. 4c, d), indicating uretic bud expression of *Wnt5a*.

Wnt5a$^{-/-}$ Mice Have Abnormal Kidney Development

Wnt5a$^{-/-}$ mice were used to assess the loss of *Wnt5a* expression on kidney development. Since *W nt5a*$^{-/-}$ mice are perinatal lethal, an MRI was performed on E16.5 embryos, and showed kidneys in the wild-type mice (fig. 5a), but not in the *Wnt5a*$^{-/-}$ embryo (fig. 5b). We then dissected multiple wild-type and *Wnt5a*$^{-/-}$ embryos and identified several different, but severe, phenotypes (fig. 5c–f, online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000368411, and table 1). Histology (fig. 5g) of the *W nt5a*$^{-/-}$ kidneys showed markedly reduced kidney development.

wmISH of the *Ret* gene was used to identify the ND and UB structure. In wild-type embryos, UB initiated at the caudal region of the IM, and started to branch at E11.5 (fig. 6a, d). The mutant embryos had truncated IM and abnormal UB initiation, such as medial dislocation of the outgrowth sites (fig. 6e), delayed UB branching (fig. 6e) and outgrowth at more than two sites (fig. 6c, f). By E15.5, the *Ret* expression was localized at the branched UB (fig. 6h); unilateral hypoplasia and duplex kidney and ureter were seen in *Wnt5a*$^{-/-}$ mutant embryos (fig. 6i, j).

Discussion

Pleotropic kidney defects, including cystic kidney disease [2], hypoplastic kidney [13], hydronephrosis and nephrocalcinosis [14] have been reported in patients with Robinow syndrome. *Wnt5a* has been found to play important roles in these patients' craniofacial and skeletal abnormalities. Our study utilized two different animal models, zebrafish and mouse, to demonstrate the role of *Wnt5a* during normal kidney development, and to help identify the causes of kidney abnormalities in Robinow syndrome patients.

Wnt5a knockdown in zebrafish resulted in glomerular cyst formation. *Wnt5a* functions in planar cell polarity (PCP) regulation in mice [15], and disruption in the PCP pathway has been shown to result in kidney cyst formation [16]. The abnormal body axis curvature with downward- curving tail in our *wnt5a* morphants is also seen in zebrafish with defects in cilia motility [17, 18] or assembly [19]. *Wnt5a* morphants had disordered pronephric cilia, suggesting that *wnt5a* might work through ciliogenesis and PCP to control normal kidney development. It is also possible that the pronephric tubular dilation could have played a role

in the ciliary phenotype. Further investigations will be conducted to establish the relationship among *wnt5a*, cilia and PCP.

We found pleiotropic abnormal renal phenotypes in *Wnt5a*$^{-/-}$ mutant mice (table 1). There are many examples of pleiotropic renal phenotypes in knockout mice. For instance, only one third of newborn *Ret* knockout mice showed complete absence of ureters and kidneys, and one tenth had bilateral kidney rudiments [20].

The cause of the duplex kidney and ureter was likely due to *Wnt5a* controlling IM extension [21] and regulating the interaction of ND and MM [22]. Besides duplex ureter/kidney, our data also demonstrated bilateral and unilateral kidney agenesis in *Wnt5a* knockout mice. Our Wnt5a knockout mice had truncated IM, medial dislocation of the UB outgrowth sites and delayed UB branching. Dislocated UB outgrow sites can cause a dysregulated interaction between ND and MM and result in kidney agenesis. Delayed UB branching results in kidney hypoplasia. It is interesting that *Wnt5a* strongly expresses at the mesonephros, but does not strongly express in the UB outgrowth area, indicating an important role of the mesonephros in the development of metanephros. *Wnt5a* may play a role in UB branching morphogenesis, but is likely not involved in the mesenchymal epithelial transformation or S-shaped body cell differentiation because these structures develop relatively normally in the *Wnt5a* null kidneys. Another notable phenotype of *Wnt5a* global knockout mice is fused kidneys. The fused kidney is secondary to defects in capsule formation, which prevents the kidneys from detaching from the body wall. The reason why loss of *Wnt5a* expression results in fused kidneys is not clear, though the medial dislocation of the UB outgrowth sites might be one of the causes of fused kidneys. FoxD1, a transcription factor expressed in the stromal mesenchymal region of the developing kidney, plays a role in controlling cellularity in the renal capsule [23]. Whether *Wnt5a* works through a Fox signaling pathway or is downstream of FoxD1 signaling remains to be seen.

Wnt5a regulates skeletal morphogenesis, and MRI of the mouse embryo showed significant skeletal abnormalities. Our previous work showed that *Wnt5a*$^{-/-}$ mice had significant urogenital abnormalities, which may be the reason for the hydronephrosis phenotype seen in the kidney. It is difficult to determine cyst formation in this *Wnt5a* mutant model because of perinatal lethality and urogenital abnormalities; therefore, kidney-specific *Wnt5a* knockout mice are needed, which we are currently generating.

In summary, the present study establishes a key role for *Wnt5a* during early kidney development, and allows it to join the large group of Wnts that are involved in different stages of metanephrogenesis. Whether *Wnt5a* is involved in ciliogenesis and PCP signaling needs further investigation. Our finding helps us to understand the mechanism of kidney and urinary anomalies in patients with Robinow syndrome [1, 3]. Interestingly, defective *wnt5a* signaling causes renal cyst formation in our zebrafish model and cystic kidney disease has also been reported in patients with Robinow syndrome [24]. Our future efforts will be directed towards studying kidney-specific *Wnt5a* knockout mice, which should allow the mice to survive long enough to fully manifest renal defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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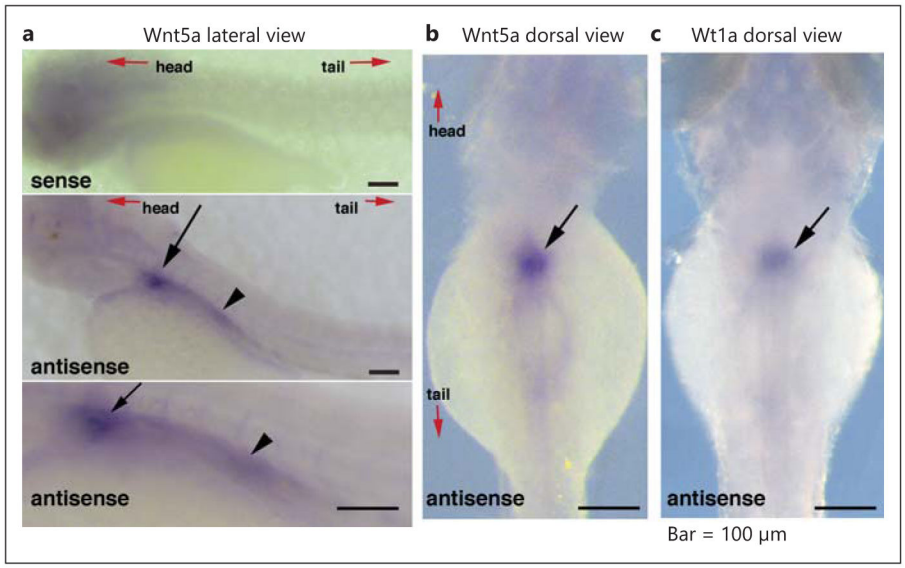

Fig. 1.

wnt5a expression localizes in the zebrafish kidney. Lateral view (**a)** and dorsal view (**b)** of wmISH of zebrafish embryos at 72 hpf with the staining of *wnt5a* probes. The Sense probe (upper panel in **a)** shows no signal. **c** Dorsal view of zebrafish embryos at 72 hpf with the *wt1a* antisense probe, used as a positional marker, showing *wt1a* expression in the pronephric glomerulus. *Wnt5a* is expressed in the zebrafish pronephric glomerulus (arrow). Wnt5a can also be seen in the proximal tubules in the lateral view (**a,** arrowhead), but not in the dorsal view (**b)**.

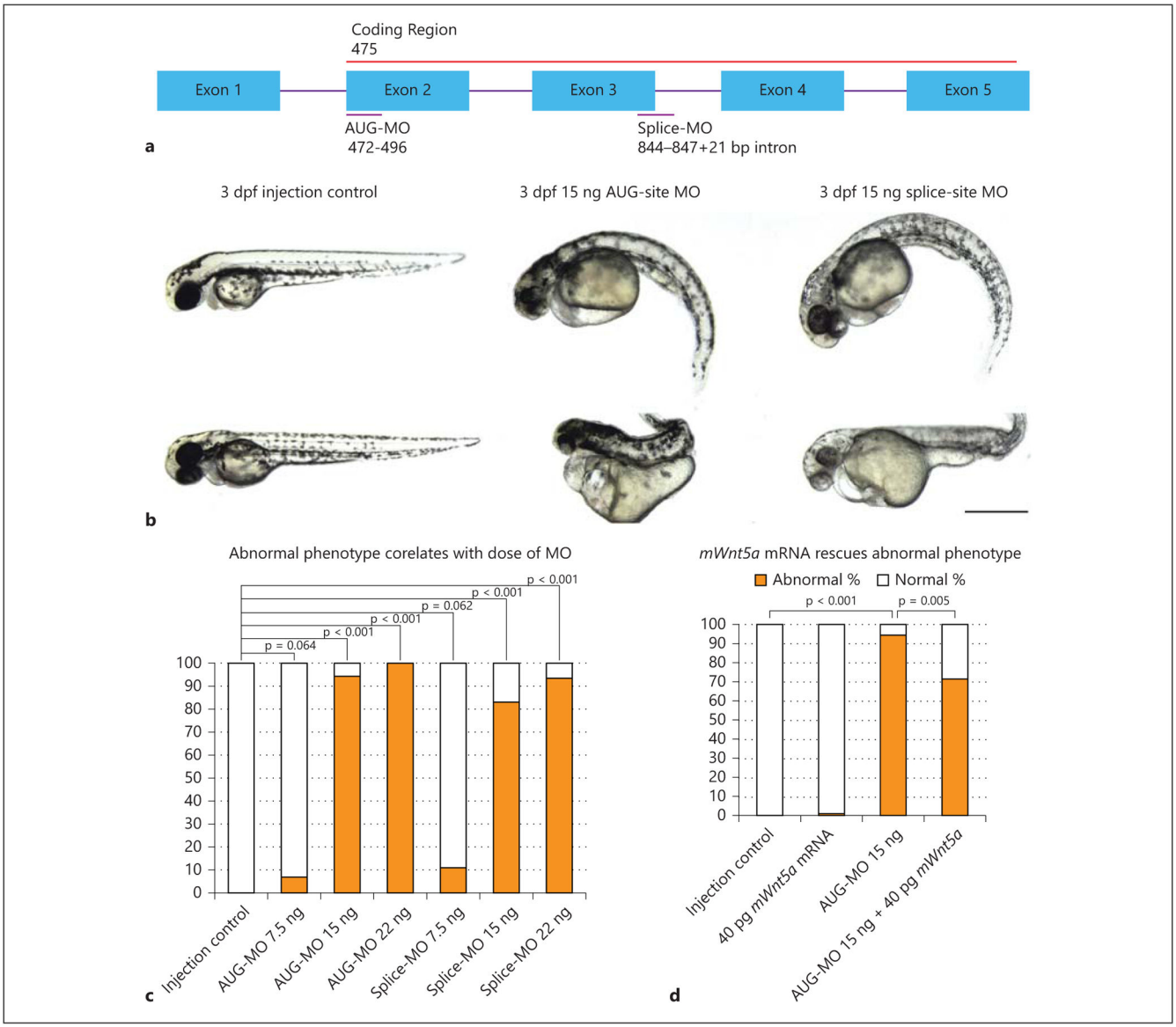

Fig. 2.

wnt5a knockdown by antisense morpholinos results in abnormal body curvature. **a** Diagram showing the zebrafish *wnt5a* gene, the coding region, and the regions targeted by the AUG- and splice-MO. The sequences of MOs complementary to the gene target were: AUG-MO: 5′-ACTTCAGCTTCAGCAGCATCATAAC-3′ (GI_BC150218) and splice-MO: 5′-gtgtttttgcggcgtacatacCGAT-3′ (GI_BC150218; capitalized letters: targeted exon; non-capitalized letters: intron). **b** Phenotype of injection control (phenol red) embryos and AUG- and splice-site *wnt5a* morphants at 3 dpf with dorsal to the top and anterior to the left. The AUG- and splice-site morphants phenocopied each other with a downward curved tail, small eyes, and pericardial edema. ‘Class 1’ morphants exhibits a ‘curly tail down’ body shape and pericardial edema (90% of total abnormal morphants, first row middle and right column). ‘Class 2’ morphants exhibits more severe defects including a reduced and abnormally shaped body axis and significant pericardial edema (10% of total abnormal

morphants, second row middle and right column). Bar = 2 mm. **c** A dose-response occurs, with increasing amounts of morpholino resulting in a worsening phenotypes for both the AUG- and splice-MOs ($p = 0.064$ in AUG-MO 7.5 ng vs. control, $p < 0.001$ in AUG-MO 15 ng vs. control, $p < 0.001$ in AUG-MO 22 ng vs. control, $p = 0.062$ in splice-MO 7.5 ng vs. control, $p < 0.001$ in splice-MO 15 ng vs. control, $p < 0.001$ in splice-MO 22 ng vs. control). The use of two MOs with unrelated sequences demonstrates the specificity of the observed phenotype. **d** The AUG-MO phenotype can be partially rescued with mouse *Wnt5a* mRNA, which is resistant to the AUG-MO due to a difference in primary base pair structure, thereby demonstrating that the phenotypes are not due to off-target effects. Abnormal phenotypes as described above are counted at 72 hpf in different injection groups (we tried mouse mRNA dose from 20 to 120 pg and identified 40 pg RNA to be the dose at which nonspecific or toxic effects of the RNA do not occur). The difference is statistically significant at $p < 0.05$ ($p < 0.001$ in AUG-MO 15 ng vs. control group, $p = 0.005$ in AUG-Mo 15 ng + 40 pg *mWnt5a* mRNA group vs. AUG-MO 15 ng group).

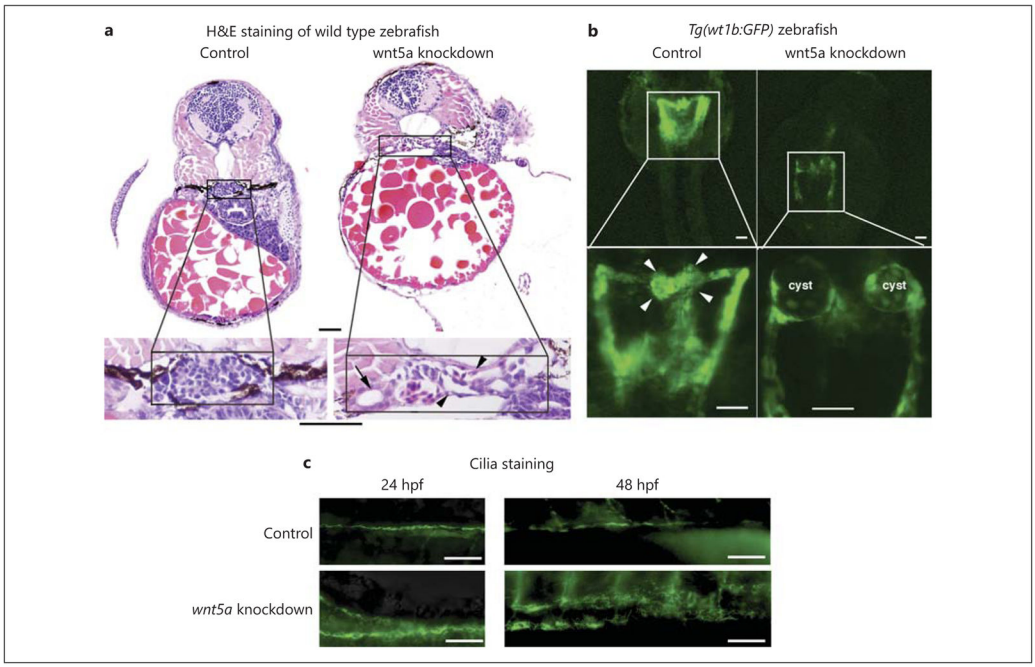

Fig. 3.

wnt5a knockdown by antisense morpholinos results in pronephric cyst formation. **a** HE staining of the transverse histological sections of 72-hpf morphant embryos reveals disrupted glomerular structures and dilated glomeruli (arrowhead) and renal tubules (arrow) after *wnt5a* knockdown by both AUG- and splice-MOs (image is from control and AUG-injected zebrafish; splice-MO injection showed similar results, but image is not shown here). Bar = 20 μm. **b** Representative images of the pronephros of 48-hpf *Tg(wt1b:GFP)* transgenic zebrafish embryos injected with phenolred control and *wnt5a* AUG-MO. Cystic glomeruli are clearly visible in the *wnt5a* morpholino-injected animals but not in the control (phenol-red) ones. Arrowheads indicate glomeruli. (Splice- MO injection showed similar results, which is not shown here.) Bar = 50 μm. **c** Representative images of the ciliary staining in pronephros at 24 and 48 hpf. *wnt5a* knockdown results in disordered ciliary structure compared with injection control. Bar = 50 μm.

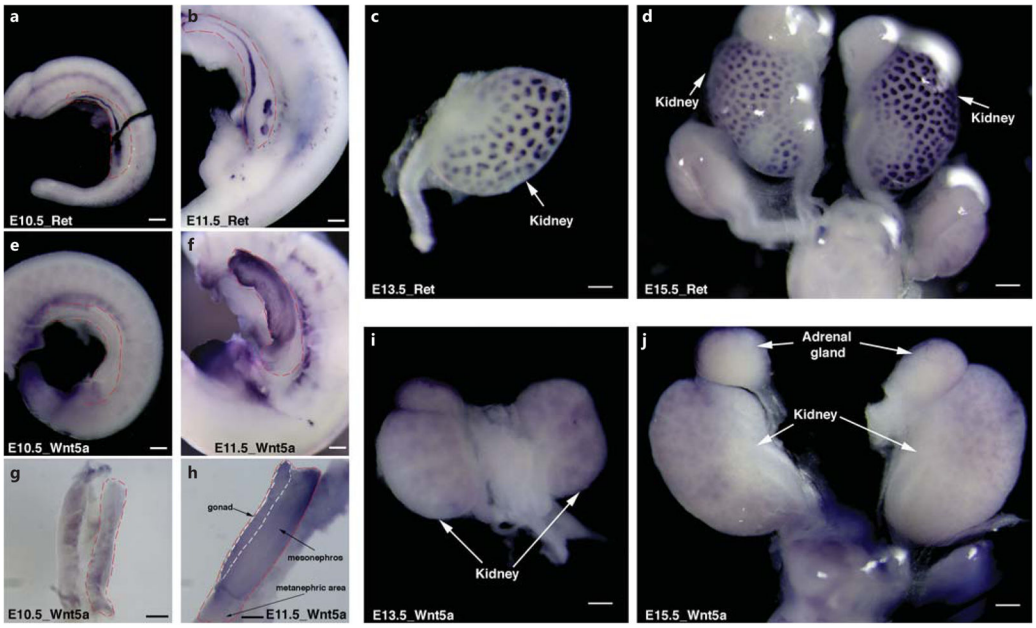

Fig. 4.

wmISH demonstrates *W nt5a* expression in the mouse IM, mesonephros and metanephric kidney. wmISH of *Ret* gene expression **(a–d)** is used to identify IM and UB location at E10.5 (**a**), E11.5 (**b**), E13.5 (**c**) and E15.5 (**d**). Red dashed lines outline the IM tissues. At E10.5, while *Ret* is expressed in the ND and at the site of UB outgrowth (**a**), *Wnt5a* is diffusely expressed at a lower level in the entire IM region with higher expression caudally (**e** and **g**). **f, h** At E11.5, *Wnt5a* expression level increases significantly in the gonad and mesonephric kidney, but not in the metanephric region where the UB initiates. At E13.5 (**i**) and E15.5 (**j**), *Wnt5a* is expressed in the metanephric kidney, with a pattern similar to *Ret* expression. Bar = 200 μm.

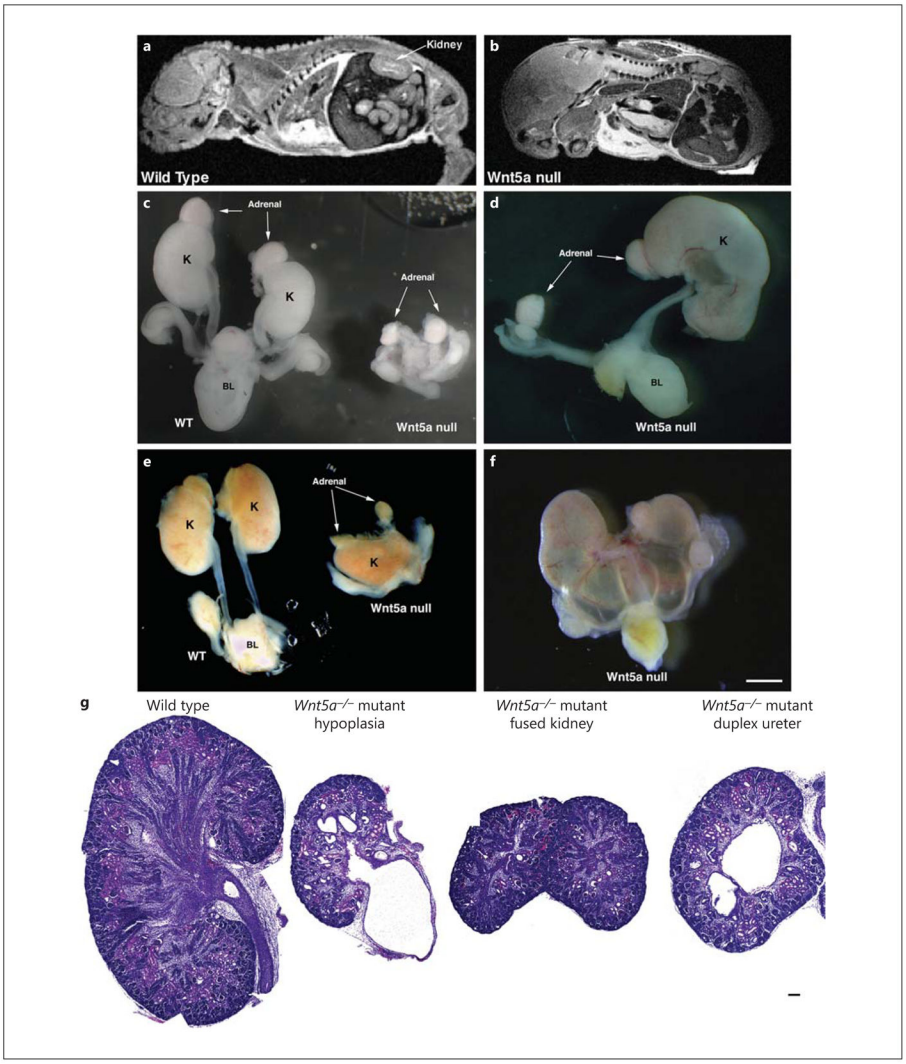

Fig. 5.

Metanephric kidney development is severely compromised in *Wnt5a* global knockout mice as determined by MRI, fine dissection, and HE staining. **a, b** MRI of E16.5 mouse embryos. A normal-sized kidney is seen in the wild-type mouse embryo (**a)**, whereas a *Wnt5a*$^{-/-}$ mouse embryo shows absence of kidney formation (**b)**. Due to the skeletal abnormalities, it was difficult to identify identical sections in control and *Wnt5a*$^{-/-}$ mice. **c–f** Wild-type and *Wnt5a*$^{-/-}$ mouse kidneys (when tissue was present) and urinary tracts were dissected at E16.5. The kidneys from *Wnt5a*$^{-/-}$ mutants shows pleiotropic, but severe, kidney phenotypes, including bilateral kidney agenesis (**c)**, unilateral kidney agenesis (**d)**, fused kidneys (**e)** and hydroureteronephrosis (**f). c–f** Bar = 1 mm. **g** HE staining of the embryonic kidneys from wild-type and *Wnt5a*$^{-/-}$ mice. Histologic sections of E16.5 wild-type (left) and *Wnt5a*$^{-/-}$ (right) embryonic kidneys shows markedly reduced kidney tubules and glomeruli in *Wnt5a*$^{-/-}$ embryos. There is also significant dilatation of the renal tubules and hydronephrosis in the *Wnt5a*$^{-/-}$ mutant embryos. **g** Bar = 50 μm.

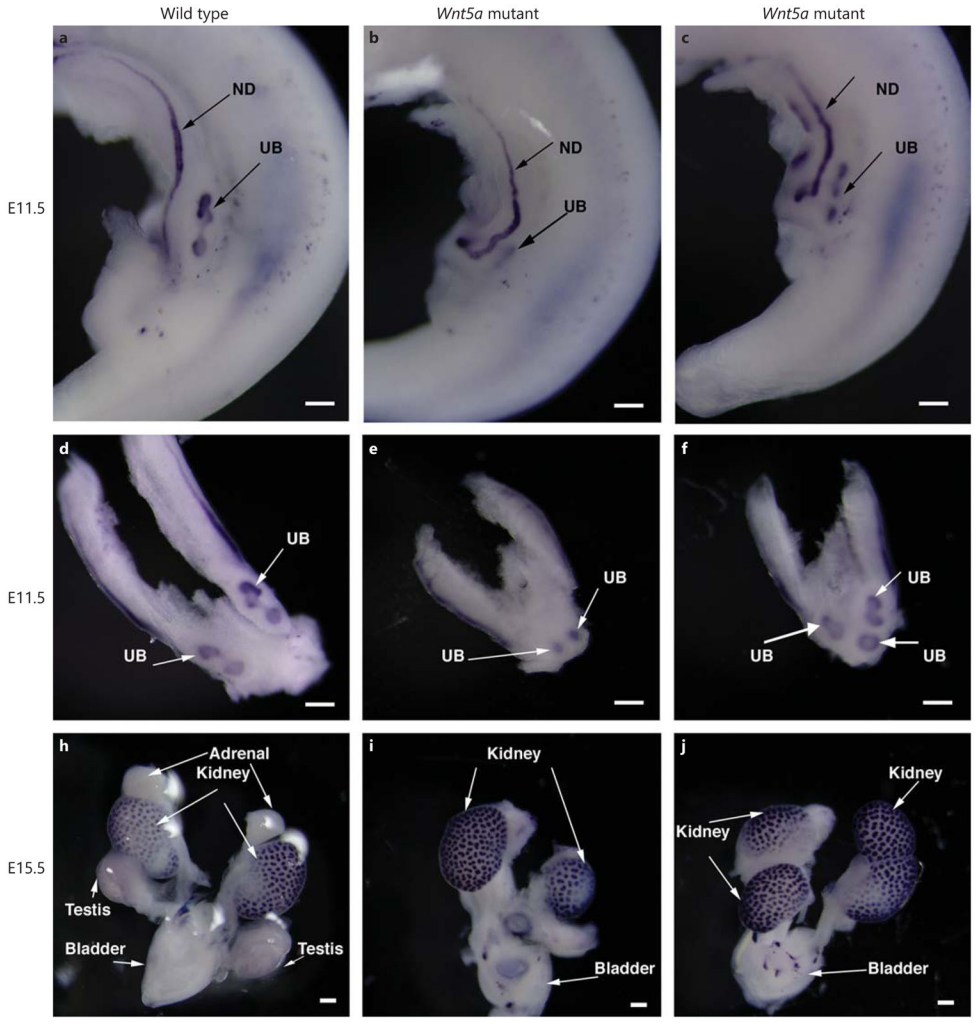

Fig. 6.

wmISH of Ret gene expression in wild-type and *Wnt5a* knockout mouse embryos. **a, d** At E11.5, the UB forms at the metanephric region and starts to branch in the wild-type embryo. **b, e** In some *Wnt5a* knockout embryos, the regions containing *Ret*-expressing IM are limited, the site of UB outgrowth occurred at the medial region and the branching is delayed. **c, f** In other *Wnt5a* knockout embryos, there are additional UB outgrowth sites. At E15.5, some *Wnt5a* knockout embryos developed unilateral kidney hypogenesis **(i)**, while others have duplex kidneys/ureter **(j)**.

Table 1

Percentage of abnormal kidney phenotypes in *Wnt5a* knockout embryos

Kidney phenotype	Percentage
Bilateral kidney agenesis	21
Unilateral kidney agenesis	28
Hydronephrosis	71
Hydroureterosis	50
Duplex kidney	14
Duplex ureter	29
Fused kidney	36