Developmental Biology 428 (2017) 63–73

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

CRISPR/Cas9-induced disruption of *wt1a* and *wt1b* reveals their different roles in kidney and gonad development in Nile tilapia

Dongneng Jiang[a,b], Jinlin Chen[a], Zheng Fan[a], Dejie Tan[a], Jiue Zhao[a], Hongjuan Shi[a], Zhilong Liu[a], Wenjing Tao[a], Minghui Li[a], Deshou Wang[a,*]

[a] *Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Sciences, Southwest University, Chongqing 400715, China*

[b] *Key Laboratory of Marine Ecology and Aquaculture Environment of Zhanjiang, Key Laboratory of Aquaculture in South China Sea for Aquatic Economic Animal of Guangdong Higher Education Institutes, Fisheries College, Guangdong Ocean University, Zhanjiang 524088, China*

ARTICLE INFO

Keywords:
Wilms tumor 1
Nile tilapia
CRISPR/Cas9
Kidney
Gonad
Sex determination and differentiation
Reproduction

ABSTRACT

Wilms tumor 1 (Wt1) is an essential factor for urogenital system development. Teleosts have two *wt1s*, named as *wt1a* and *wt1b*. In this study, the expression pattern of *wt1a* and *wt1b* and their functions on the urogenital system were analyzed by *in situ* hybridization and CRISPR/Cas9. *wt1a* was found to be expressed in the glomerulus at 3 dah (days after hatching), earlier than *wt1b*. *wt1a* and *wt1b* were simultaneously expressed in the somatic cells of gonads at 3 dah, while their cell locations were similar, but not identical in adult fish gonads. The $wt1a^{-/-}$ fish displayed pericardial edema and yolk sac edema at 3 dah and subsequently expanded as general body edema at 6 dah, failed to develop glomerulus and died during 6–10 dah, whereas the $wt1b^{-/-}$ fish were phenotypically normal. Immunohistochemical analyses revealed that the germ cell marker Vasa was expressed, while somatic cell genes Cyp19a1a, Amh, Gsdf and Dmrt1 were not expressed in the $wt1a^{-/-}$ gonads at 6 dah. The sex phenotypes of XX and XY in the $wt1b^{-/-}$ fish were not affected. Real-time PCR revealed that the ovarian *cyp19a1a* expression was up-regulated in XX $wt1b^{-/-}$ fish, compared with XX control at 90 dah. Serum estradiol-17β level was also up-regulated in XX $wt1b^{-/-}$ fish at 90 and 180 dah. The XY $wt1b^{-/-}$ fish had normal serum estradiol-17β and 11-ketotestosterone levels and remained fertile. These results suggest that Wt1a and Wt1b have different functions in the kidneys and gonads of tilapia.

1. Introduction

Urinary system and genital system are closely related during embryogenesis. The urogenital apparatus includes the kidneys, gonads, and urinary and reproductive ducts (Kuure et al., 2000; Nakamura et al., 2006; Massé et al., 2009; Gerlach and Wingert, 2013). The Wilms' tumor 1 (*WT1*) gene encodes a zinc finger protein that is mutated in a proportion of nephroblastomas, an embryonic kidney tumor found in 1:10,000 newborns (Gessler et al., 1990; Pritchard-Jones et al., 1990; Menke et al., 1998). In addition to being a candidate tumor suppressor gene, WT1/Wt1 is also known to play critical roles in the regulation of the urogenital system development, including the kidney and gonad (Kreidberg et al., 1993).

In vitro and *in vivo* studies suggest that WT1/Wt1 acts as both a transcriptional activator and repressor (Shimamura et al., 1997; Palmer et al., 2001; Sim et al., 2002; Gurates et al., 2002, 2003; Bradford et al., 2009). A combination of alternative splicing, alternative translational start sites and RNA editing of *WT1* leads to the expression of at least 24 different isoforms in human (Scholz and Kirschner, 2005). Of particular interest are two alternative splice donor sites at the end of exon 9, leading to the insertion or omission of three amino acids (KTS) between zinc finger domains 3 and 4 (designated as +KTS and -KTS isoforms). *In vitro* studies (Caricasole et al., 1996) and computer modeling (Kennedy et al., 1996) suggest that +KTS isoform can bind to RNA, and it has been speculated that it may play a role in posttranscriptional modification. NMR relaxation experiments demonstrate that the insertion of the KTS sequence leads to an increased linker flexibility and loss of DNA binding of zinc finger 4 (Laity et al., 2000). The KTS alternative splice site and zinc finger domain are highly conserved in all vertebrates (Spotila and Hall, 1998; Carmona et al.,

Abbreviations: Amh, Anti-Müllerian hormone; Cyp19a1a, cytochrome P450, family 19, subfamily A, polypeptide 1a; Cyp11b2, cytochrome P450, family 11, subfamily B, polypeptide 2; dah, days after hatching; Dmrt1, double sex- and mab-3-related transcription factor-1; Gsdf, gonadal soma-derived factor; ISH, *in situ* hybridization; MD, Müllerian duct; PGC, primordial germ cell; Sf-1, nuclear receptor subfamily 5, group a, member 1; Vasa, DEAD-box helicase 4; WD, Wolffian duct; Wt1, Wilms tumor 1

* Corresponding author.
E-mail address: wdeshou@swu.edu.cn (D. Wang).

http://dx.doi.org/10.1016/j.ydbio.2017.05.017
Received 29 March 2017; Received in revised form 6 May 2017; Accepted 17 May 2017
Available online 18 May 2017
0012-1606/ © 2017 Published by Elsevier Inc.

2001; Yamamura et al., 2005). In teleosts, two *wt1* gene (*wt1a* and *wt1b*) have been identified, and +KTS and -KTS splice variants exist in both genes (Bollig et al., 2006; Klüver et al., 2009).

Analysis of *Wt1* expression in mice showed a developmentally dynamic expression pattern with an onset at 9.5 days postcoitum (dpc) within the urogenital ridge. During kidney development, *Wt1* is expressed at low levels within the un-induced metanephric blastema, and increases during condensation and formation of comma- and S-shaped bodies (Armstrong et al., 1993). In adult kidneys, Wt1 is expressed within the glomerular podocytes (Gao et al., 2004). During gonadal development, *Wt1* expression can be found within the coelomic epithelial cell layer, as well as the developing Sertoli cells of males and the granulosa cells of females (Mundlos et al., 1993; Gao et al., 2006, 2014). In zebrafish and medaka embryos, expression of *wt1a* occurs earlier and more broadly than *wt1b* in the kidney (Bollig et al., 2006; Perner et al., 2007; Klüver et al., 2009). In medaka, expression of *wt1a* also occurs earlier than *wt1b* in the genital ridge. In adult fish, *wt1* genes also express in the kidneys and gonads. In adult medaka, *wt1a* and *wt1b* are both expressed in Sertoli cells of the testes, and expression of *wt1a* is more widespread than *wt1b* (Klüver et al., 2009). These expression profiles suggest Wt1s have conserved role in the urogenital system in teleosts.

The importance of WT1 during the development of the urogenital system is reflected by mutations, which lead to two developmental syndromes in man: Denys-Drash syndrome (DDS) and Frasier syndrome (Little and Wells, 1997). DDS patients suffer from diffuse mesangial sclerosis, urogenital abnormalities, and a high risk of developing Wilms' tumors, whereas Frasier patients have focal segmental glomerular sclerosis, show male-to-female sex reversal, however, do not usually develop tumors. Notably, *WT1* mutations in Frasier patients are found in intron 9 and affect recognition of the second alternative splice donor site in the KTS region. As Frasier syndrome is caused by dominant mutation, it has been proposed that splice site mutation causes a shift in the ratio of KTS isoforms toward the -KTS forms (Barbaux et al., 1997; Klamt et al., 1998).

Mice carrying homozygous mutations of *Wt1* lack kidneys, gonads and adrenal glands (Kreidberg et al., 1993). Most recent evidence suggests that *Wt1* participates in multiple functions in both testes and ovaries in mammals (Ji et al., 2013; Chen et al., 2014; Gao et al., 2014; Takasawa et al., 2014; Zhang et al., 2015a; Liu et al., 2016). In zebrafish, knockdown of *wt1a* led to failure of glomerular differentiation and morphogenesis, resulting in a rapidly expanding general body edema, while inactivation of *wt1b* was compatible with early glomerular development (Perner et al., 2007). In medaka, *wt1a*, *wt1b* and *wt1a/b* morphants die shortly before or after hatching, probably due to non-functional pronephros and additional embryonic deformities, while only double knockdown of *wt1a* and *wt1b* displayed strong influence on the number of primordial germ cells (PGC) during gonad development (Klüver et al., 2009). These results suggest that Wt1a and Wt1b are involved in different functions in kidney and gonad development in fish. However, the role of Wt1a and Wt1b in urogenital system development has not been well elucidated owing to the lack of knockout approach in teleosts.

Nile tilapia (*Oreochromis niloticus*) is an important aquaculture fish that has an XX-XY sex-determination system (Sun et al., 2014a). Nile tilapia is also a good model fish as the genomic sequence has been determined and it gene editing methods have been established (Li et al., 2013, 2014; Brawand et al., 2014). In this study, *wt1a* and *wt1b* were identified in Nile tilapia. Their expression profiles in the kidney and gonad of Nile tilapia were analyzed by *in situ* hybridization (ISH). *wt1a* and *wt1b* knockout lines were separately established using CRISPR/Cas9 technology. Our data suggested that *wt1a* and *wt1b* have different functions in kidney and gonad development in Nile tilapia. The $wt1a^{-/-}$ and $wt1b^{-/-}$ fish may be good models for revealing Wt1 function in vertebrates.

2. Materials and methods

2.1. Animals

Nile tilapia, *Oreochromis niloticus*, were kept in recirculating, aerated freshwater tanks at 26 °C with a natural photoperiod. All XX progenies were obtained by crossing a pseudomale (XX male, producing sperm after hormonal sex reversal) with a normal female (XX). All XY progenies were obtained by crossing a supermale (YY) with a normal female. Animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

2.2. Data mining and phylogenetic and syntenic analyses

The complete cDNA sequences of tilapia *wt1a* and *wt1b* were obtained from the National Center for Biotechnology Information (NCBI) databases with the accession numbers XM 005449373 and XM 019359281, respectively. The cDNA sequences were available from previous transcriptome data reported by our group (Tao et al., 2013). We also designed primers based on the predicted open reading frames (ORF) to confirm the cDNA sequence (Supplementary Table S1).

Genome databases, including the UCSC Genome Bioinformatics (http://genome.ucsc.edu/), Ensembl (http://www.ensembl.org/) and the NCBI databases (http://blast.ncbi.nlm.nih.gov/), were searched for *wt1*-like sequences in available representative vertebrate genomes.

Multiple alignments of Wt1a and Wt1b amino acid sequences were performed using ClustalX (http://www.clustal.org/). The neighbor-joining method, as implemented in MEGA 5.0 (http://www.megasoftware.net/), was used to construct the phylogenetic tree. For syntenic analysis, the orientation and chromosomal positions of *wt1a* and *wt1b* and their adjacent genes were determined. The gene loci flanking the *wt1* genes in the genomes of different vertebrates were ascertained with the ensemble genome browser using tilapia *wt1a* (ENSONIG00000011043) and *wt1b* (ENSONIG00000005545) as query sequences. Genomic location data were then used to rebuild the synteny maps surrounding the *Wt1* genes in vertebrates.

2.3. Reverse transcription PCR (RT-PCR)

Total RNA (2.0 μg) was isolated from various tissues of adult XX and XY fish. The quality and concentration of the RNA were assessed using a NanoDrop 2000 (Thermo Scientific, USA). Total RNA was treated with RNase-free DNase I (Thermo Scientific Corp, Waltham, MA, USA). M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) was used for cDNA synthesis with oligo-dT (12–18) primers. cDNA was subjected to amplification using specific primers based on *wt1a* and *wt1b*. *actb* (accession number: EU887951) expression was used as an internal reference for normalization. The *wt1a*, *wt1b* and *actb* primers for tissue distribution are listed in Supplementary Table S1. Amplification consisted of 35 cycles (*actb* 28 cycles) of 30 s at 95 °C, 60 °C for 30 s, and 72 °C for 30 s; followed by further extension at 72 °C for 10 min. PCR products were separated on a 2.0% agarose gel and visualized with ethidium bromide.

2.4. In situ hybridization (ISH)

The whole bodies (after removal of the yolk and gut) of XX and XY tilapia fry, at 3, 5, 20 and 40 days after hatching (dah) and gonads of tilapia at 90, 120 and 180 dah were fixed in 4% paraformaldehyde (Sangon Biotech, Shanghai, China) in 0.85×PBS at 4 °C to determine the expression of *wt1a* and *wt1b* in the gonad and kidney by *ISH,* as described previously (Yu et al., 2014). Digoxigenin (DIG) -labeled riboprobes of both *wt1a* and *wt1b* lacked the highly-conserved zinc-finger encoding region to avoid cross-hybridization. The *wt1a* and

wt1b gene-specific primers for *ISH* are listed in Supplementary Table S1. Sense and antisense DIG-labeled riboprobes for *wt1a* and *wt1b* were transcribed *in vitro* with an RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) from linearized pGEM-T-easy plasmid (Promega, USA) containing partial ORFs of tilapia *wt1a* and *wt1b*.

2.5. Establishment of wt1a and wt1b mutant lines by CRISPR/Cas9

The tilapia *wt1a* and *wt1b* mutants were generated by CRISPR/Cas9-induced gene knockout, as described previously (Li et al., 2014). Briefly, gRNA and *Cas9* mRNA were co-injected into one-cell stage embryos at a concentration of 250 ng/μl and 500 ng/μl. Twenty embryos were collected 72 h after injection. Genomic DNA was extracted from pooled control and injected embryos and used for the mutation assays. DNA fragments spanning the target site were amplified using the primers listed in Supplementary Table S1. The mutated sequences were analyzed by restriction enzyme digestion and Sanger sequencing.

F0 fish were screened by restriction enzyme digestion, as outlined above. Males reach sexual maturity at 180 dah, much earlier than females (at 240 dah or even later) in Nile tilapia and mature males produce sperm daily while females spawn only once in a 14 day spawning cycle. Heterozygous F1 offspring were obtained by a F0 male founder mated with a wild type XX female fish. The F1 fish were genotyped by fin clip assay and the individuals with frame-shift mutations were selected. XY male and XX female siblings of F1 generation, carrying the same mutation, were mated to generate homozygous F2 mutants.

2.6. Genotyping

Genomic DNA of fin clips was isolated by phenol/chloroform extraction following proteinase (Sigma, Germany) digestion. The genotypic sex of the fish was determined by sex-linked DNA Marker-5 (Sun et al., 2014a).

The *wt1a* and *wt1b* F2 mutants were detected using the heteroduplex motility assay (HMA), as previously reported (Zhang et al., 2015b). In brief, fragments including the target sites of *wt1a* (159 and 179 bp) and *wt1b* (92 and 109 bp) were amplified by gene specific primers (Supplementary Table S1). A three-step PCR was carried out as follows: 95 °C for 3 min, 37 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 20 s, followed by 10 min at 72 °C; and 5 μl of PCR reaction was electrophoresed on 12% polyacrylamide gels at 250 V for 30 min, after which the gel was stained with ethidium bromide and visualized using a Gel Doc XR System (Bio-Rad, USA).

2.7. Sampling and histological examination

The whole bodies of *wt1a* and *wt1b* F2 generation fries, at 3 and 6 dah (after removal of the yolk and gut), and gonads of *wt1a* and *wt1b* F2 generation fish, at 90 and 180 dah, were sampled. Samples were fixed in Bouin's solution for 24 h at room temperature, dehydrated and embedded in paraffin. Tissue blocks were sectioned at 5 μm and stained with hematoxylin and eosin.

2.8. Immunohistochemistry (IHC)

The whole bodies of XX/XY $wt1a^{-/-}$ and $wt1a^{+/+}$ fry, at 6 dah, and gonads of XX/XY $wt1b^{-/-}$ and control fish, at 90 dah, were sampled. Sections were prepared as outlined above. Production of polyclonal antibodies and IHC analysis were performed, as described previously (Li et al., 2015). Cyp19a1a (cytochrome P450, family 19, subfamily A, polypeptide 1a) and Vasa (DEAD-box helicase 4) antibodies were provided by Professor Yoshitaka Nagahama, the National Institute for Basic Biology, Okazaki, Japan (Kobayashi et al., 2003; Zhang et al., 2014). Amh (Anti-Müllerian hormone), Dmrt1 (double sex- and mab-3-related transcription factor-1) and Gsdf (gonadal soma-derived factor) antibodies were prepared in our laboratory (Sun et al., 2014b; Jiang et al., 2016; Xie et al., 2016). Antibodies against Cyp19a1a, Vasa, Amh, Dmrt1, and Gsdf were diluted at 1:3000, 1:100, 1:1000, 1:100 and 1:1000, respectively. Goat anti-rabbit antibody conjugated with horseradish peroxidase (SV0002, Boster, China) was used to detect primary antibodies.

2.9. Real-time PCR

Ovaries and testes were dissected from the $wt1b^{-/-}$ and control XX/XY fish at 90 dah. Total RNA extraction and cDNA synthesis were carried out as mentioned above. Real-time PCR was performed on an ABI-7500 real-time PCR machine (Applied Biosystems, USA), with the SYBR® Premix Ex Taq™ II (Takara, Japan) according to the manufacturer's instructions. The relative abundance of *wt1a*, *cyp19a1a*, *amh* and *cyp11b2* (cytochrome P450, family 11, subfamily B, polypeptide 2) transcripts was evaluated using the formula: $R = 2^{-\Delta\Delta Ct}$. The reference gene *actb* was used to normalize the expression values. Primer sequences used for real-time PCR are listed in Supplementary Table S1.

2.10. Serum steroid hormone assay

Blood samples were collected from the caudal veins of $wt1b^{-/-}$ and control XX/XY fish at 90 and 180 dah and stored at 4 °C overnight. Serum was collected after centrifugation and stored at −20 °C until use. Serum estradiol-17β and 11-ketotestosterone levels were measured using enzyme immunoassay kits (Cayman, USA), according to the manufacturer's instructions.

2.11. Statistical analysis

All data are expressed as means ± SD. Significant differences in the data between groups were analyzed by one-way ANOVA with Duncan's post-hoc test using a threshold of $P < 0.05$. Paired-samples T test was also used with the significance level at $P < 0.05$. All statistical calculations were performed using SPSS 16.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Sequence analysis

We identified two *wt1* genes in the tilapia genome comparable to those found in zebrafish and medaka (Bollig et al., 2006; Klüver et al., 2009). The two tilapia *wt1* genes were designated *wt1a* (NCBI accession number: KY432302) and *wt1b* (NCBI accession number: KY432303). The reconstructed full-length ORFs of *wt1a* and *wt1b* are encoded by nine exons. The KTS is located between zinc finger domains 3+4 (Supplementary Fig. S1). Additionally, in certain clones of *wt1a,* we detected a splice isoform lacking exon 4 (84 bp) (Schnerwitzki et al., 2014), which was also present in the +/− KTS isoform, while a *wt1b* sequence lacking exon 4 was never detected.

The *wt1a* and *wt1b* ORFs encode proteins of 442 and 423 amino acids, respectively. Alignment of the Wt1a and Wt1b sequences revealed proteins with an overall amino acid identity of 71%, but with a much higher identity of 93% in the zinc-finger regions. The overall sequence of Wt1a shares 65% and 67% identity with Wt1/WT1 of mouse and human, respectively, while Wt1b shares 63% identity with Wt1/WT1 of both mouse and human. However, in the zinc-finger region, there is high sequence homology among them. The zinc-finger region of Wt1a shares 91% and 93% identity with Wt1/WT1 of mouse and human, respectively, and Wt1b shares 90% identity with Wt1/WT1 of both mouse and human. Multiple alignments showed that tilapia Wt1a was highly homologous to Wt1a from medaka (94%), tetraodon (*Tetraodon nigroviridis*) (87%) and zebrafish (81%) at the amino acid level. Tilapia Wt1b showed a high degree of similarity to the Wt1b of

medaka (83%), tetraodon (78%) and zebrafish (69%) (Supplementary Fig. S1).

3.2. Phylogenetic and syntenic analyses

Phylogenetic analysis also revealed that fish Wt1a and Wt1b clustered into two different clades. The Wt1a group contained the fish Wt1a and the Wt1 from tetrapods, indicating that this ortholog may have evolved from the ancient Wt1 protein. In contrast, the Wt1b group only contained teleost Wt1, indicating that this ortholog may have resulted from a recent fish-specific genome duplication (Supplementary Fig. S2). *wt1a* and its upstream genes, *tcpiil1* and *hipk3a,* were conserved in synteny in both teleosts and tetrapods, as did *wt1b* along with its upstream genes, *prrg4, cstf3* and *hipk3b,* and its downstream genes, *rcan1, pax6b, elp4* and *immp1l* (Supplementary Fig. S3).

3.3. Tissue distribution

In adult female fish, *wt1a* was highly expressed in the spleen, ovary and heart, but only weakly expressed in the gill, liver, kidney, stomach, hypothalamus and pituitary. *wt1b* was highly expressed in the spleen, ovary and heart, moderately expressed in the liver, but weakly expressed in other tissues. In adult male fish, *wt1a* was highly expressed in the spleen, testis and heart, but weakly expressed in the gill, liver and kidney. *wt1b* was highly expressed in the spleen, testis, liver and kidney, moderately expressed in the heart and hypothalamus, and only weakly expressed in other tissues (Supplementary Fig. S4).

3.4. Expression of wt1a and wt1b in larval kidney

ISH analysis showed that *wt1a* was found to be expressed in the pronephros at 3 dah, while *wt1b* was not detected at the same time point. *wt1b* was expressed more laterally and weakly in the glomerulus than *wt1a* in the area of the glomerular-tubular junction at 5 dah (Fig. 1A, a-d). Both *wt1a* and *wt1b* were expressed in the glomerulus of the kidney at 20 and 40 dah (Fig. 1A, e-h).

3.5. Expression of wt1a and wt1b in larval and adult gonads

ISH analysis showed that *wt1a* and *wt1b* were expressed in somatic cells of both sexes during embryonic stages from 3 dah onward (Fig. 1B, a-h). In the adult stage, *wt1a* was expressed in the interstitial cells, theca cells and granulosa cells, while *wt1b* was expressed in the interstitial cells of the ovary. In the testis, *wt1a* was expressed in the somatic cells, likely to be Sertoli cells by their location, while *wt1b* was expressed in myoid cells and Sertoli cells in adult stage (Fig. 1B, i-l).

3.6. Efficient disruption of wt1a and wt1b by CRISPR/Cas9

The gRNA sites containing *Fok* I and *Hp166* II adjacent to PAM, for mutation analysis, were selected in the first exon of *wt1a* and *wt1b*, respectively. Genomic DNA from 20 pooled injected embryos was used as a template for PCR amplification and mutation assays. Complete digestion with a selected restriction enzyme produced two fragments in the control groups, while an intact DNA fragment was observed in embryos injected with both Cas9 mRNA and target gRNA. The percentage of uncleaved bands was measured using Quantity One Software. Representative Sanger sequencing results from the uncleaved bands are listed (Fig. 2A; Supplementary Fig. S5).

3.7. Establishment of homozygous wt1a and wt1b mutant lines

The XY F0 *wt1a* or *wt1b* deficient fish were screened by enzyme digestion. The XY F0 *wt1a* and *wt1b* deficient fish were able to produce sperm at adult stages. The mutation types of *wt1a* and *wt1b* in the F1 generation were screened by enzyme digestion and sequencing. The *wt1a* and *wt1b* heterozygous F1 offspring selected to breed the F2 generation were found to have 20 and 17 bp deletions, respectively. Frame-shift mutation resulted in premature termination of *wt1a* and *wt1b* (Fig. 2B; Supplementary Fig. S6). HMA (heteroduplex motility assay) identified the heterozygous $wt1a^{+/-}$ and $wt1b^{+/-}$ individuals with both heteroduplex and homoduplex amplicons, while the $wt1a^{+/+}$, $wt1a^{-/-}$, $wt1b^{+/+}$ and $wt1b^{-/-}$ individuals possessed only the homoduplex amplicons (Fig. 2C).

3.8. Effects of wt1a and wt1b mutation on kidney development

The F2 $wt1a^{-/-}$ fish developed pericardial edema and yolk sac edema at 3 dah and subsequent general body edema at 6 dah. All $wt1a^{-/-}$ fish died before 10 dah, while $wt1b^{-/-}$ fish were normal and viable (Fig. 3A, B). Only $wt1a^{+/+}$ and $wt1a^{+/-}$ fish (12:24) in the *wt1a* F2 generation survived into adulthood, proving that the *wt1a* homozygous mutation in tilapia is lethal (Table 1). Histological observations revealed that the glomerulus did not form in the pronephros of $wt1a^{-/-}$ fish, while glomerulus of $wt1b^{-/-}$ and wild type fish were not affected at 6 dah (Fig. 3C).

3.9. Effects of wt1a and wt1b mutation on gonad development

Histology observation showed that the gonad morphology of both male and female $wt1a^{-/-}$ fish was abnormal. The diameters of the gonads and PGCs were smaller compared with the control at 6 dah, and the shapes of the gonads were irregular. $wt1a^{-/-}$ fish died during 6–10 dah, as mentioned above. We analyzed the gene expression in F2 $wt1a^{-/-}$ fish gonads at 6 dah. IHC was performed to detect germ cell marker Vasa and somatic cell genes (Cyp19a1a, Dmrt1, Gsdf and Amh). IHC revealed that Vasa was expressed in the PGC of both XX and XY $wt1a^{+/+}$gonads. Cyp19a1a was expressed in somatic cells of the XX $wt1a^{+/+}$, while Amh, Gsdf and Dmrt1 were expressed in somatic cells in XY $wt1a^{+/+}$ gonads at 6 dah. Vasa was expressed in the PGC of both XX and XY $wt1a^{-/-}$ gonads, while Amh, Gsdf, Dmrt1 and Cyp19a1a were detected in neither XX nor XY $wt1a^{-/-}$ gonads at 6 dah (Fig. 4).

We compared the phenotype *versus* genotype of 91 *wt1b* F2 offspring at 90 and 180 dah. Three *wt1b* genotypes ($wt1b^{+/+}$, $wt1b^{+/-}$ and $wt1b^{-/-}$) were present in Mendelian ratios. The phenotype sexes of the *wt1b* F2 were consistent with the genotype sexes (Table 1). IHC analysis showed that Cyp19a1a was expressed in the interstitial cells of $wt1b^{-/-}$ and $wt1b^{+/+}$ ovaries of XX fish, but was not expressed in $wt1b^{-/-}$ and $wt1b^{+/+}$ testes of XY fish at 90 dah (Fig. 5A). *cyp19a1a* was significantly up-regulated in XX $wt1b^{-/-}$ ovaries compared with the $wt1b^{+/+}$ ovaries, while *amh* expression was significantly down-regulated in XY $wt1b^{-/-}$ testes compared with the $wt1b^{+/+}$ testes at 90 dah. *wt1a* and *cyp11b2* expression in XX and XY $wt1b^{-/-}$ gonads was not influenced at 90 dah (Fig. 5B). Serum estradiol-17β levels in XX $wt1b^{-/-}$ fish were significantly higher than those of the XX $wt1b^{+/+}$ fish at 90 and 180 dah. The serum estradiol-17β and 11-ketotestosterone levels in the XY $wt1b^{-/-}$ fish were not changed (Fig. 5C). The $wt1b^{-/-}$ male were fertile as they produce 100% $wt1b^{+/-}$ progenies when mated with $wt1b^{+/+}$ wild-type females (Supplementary Fig. S7).

4. Discussions

Wilms tumor protein Wt1 is an essential factor for kidney and gonad development. Teleosts possess two *wt1* co-orthologs (*wt1a* and *wt1b*) due to fish-specific genome duplication. Functional studies of Wt1a and Wt1b have only been carried out in model fish species (Perner et al., 2007; Bollig et al., 2009). In this study, we identified two *wt1* co-orthologs in Nile tilapia and analyzed their expression and function during kidney and gonad development using ISH and CRISPR/Cas9 knockout approaches. Our results showed functional

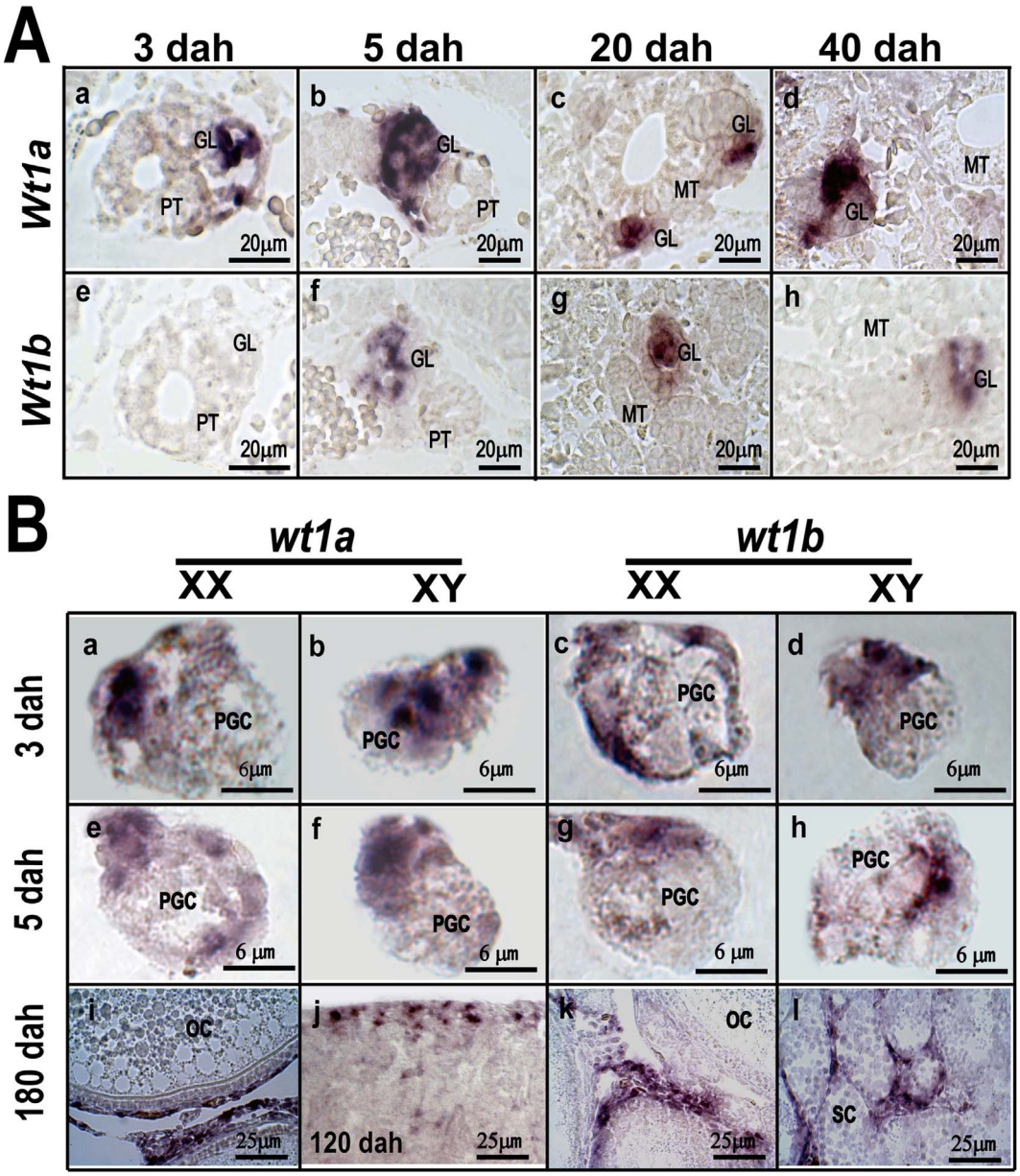

Fig. 1. Expression of *wt1a* and *wt1b* in tilapia kidney (A) and gonads (B) analyzed by *in situ* hybridization. (A) a-d, *wt1a* was expressed in the glomerulus of kidney at 3, 5, 20 and 40 dah. e, *wt1b* was not expressed in the kidney at 3 dah; f-h, *wt1b* was expressed in the glomerulus of kidney at 5, 20 and 40 dah. GL, glomerulus; PT, pronephric tubule; MT, mesonephric tubule. (B) a-h, *wt1a* and *wt1b* were expressed in the somatic cells of gonads at 3 and 5 dah. In most case, a single PGC will be observed in one cross section of gonads at 3 and 5 dah. PGCs were distinguishable morphologically from somatic cells by their larger size. If the cross sections are in the center of the PGCs, the PGCs may occupy larger area of the cross section and the boundaries between them are relatively clear (Fig. 1B, a, c, e, f, g). On the other hand, if the cross sections are on the edges of the PGCs, the PGCs and somatic cells may overlap with each other on some cross sections to make the boundaries between them unclear (Fig. 1B, b, d, h). i, *wt1a* was expressed in the interstitial cells, thecal cells and granulosa cells of ovary at 180 dah; j, *wt1a* was expressed in the Sertoli cells of testis at 120 dah. k and l, *wt1b* was expressed in the myoid cells and Sertoli cells of testis, and in the interstitial cells of the ovary at 180 dah. PGC, primordial germ cells; OC, oocytes; SC, spermatocyte. A purple color corresponds to a positive signal.

divergence at the transcriptional level, as well as functional redundancy at the protein level, and compensation between tilapia *wt1a* and *wt1b*.

4.1. Conservation of wt1a and wt1b during teleost evolution

In this study, only one copy of the *wt1* genes were successfully isolated from spotted gar (*Lepisosteus oculatus*), coelacanth (*Latimeria chalumnae*) and elephant shark (*Callorhinchus milii*). Synteny analysis revealed that the genes surrounding *wt1* from teleost species (including tilapia) and genes surrounding *wt1* from other bony fishes (coelacanth and spotted gar) and tetrapods were quite conserved. These results confirmed that *wt1b* is a teleost-specific gene and originated from the fish-specific genome duplication (3R) which took place approximately 350 million years ago (Volff, 2005). Both *wt1* genes in tilapia are expressed in the +KTS and −KTS isoforms, which are highly conserved throughout vertebrate evolution (Haber et al., 1991; Kent et al., 1995; Hammes et al., 2001). In addition, alternative splicing of *wt1a* exon 4, which is a common event in teleosts, is also found in tilapia (Schnerwitzki et al., 2014). Tilapia Wt1a and Wt1b sequences are evolutionarily conserved with those of other teleosts.

4.2. Function of Wt1a and Wt1b on kidney development

The expression profiles of *wt1a* and *wt1b* in tilapia embryonic kidneys were temporally and spatially similar to those of zebrafish and medaka (Bollig et al., 2006, 2009; Klüver et al., 2009). A highly

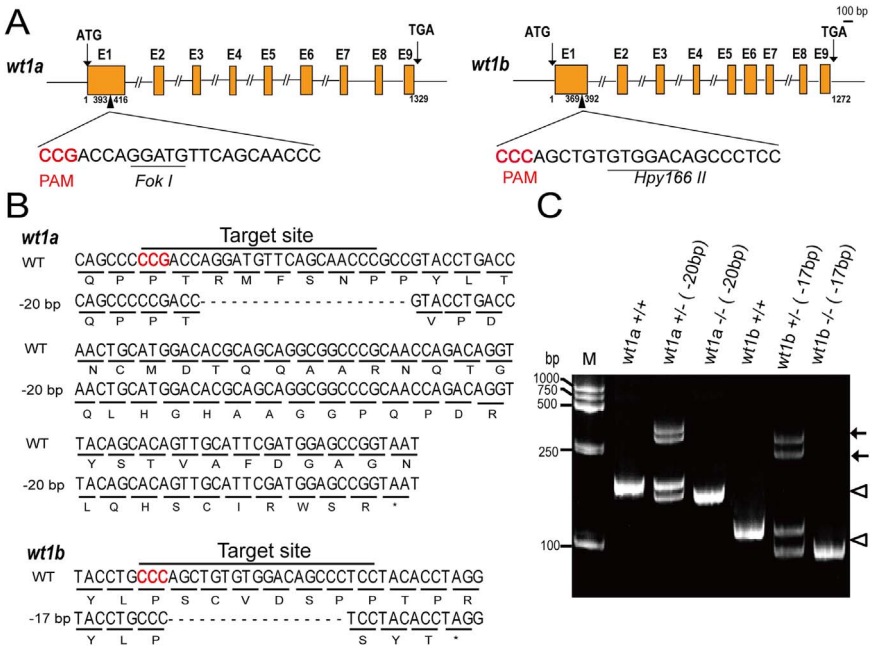

Fig. 2. Design of CRISPR/Cas9 targeting *wt1a* and *wt1b* and establishment of homozygous mutation lines. (A) *wt1a* and *wt1b* genes and target sites of CRISPR/Cas9 (arrowheads). The numbers indicate the base positions starting at the adenine nucleotide of the initiation codon. Nucleotide sequences of the CRISPR/Cas9 target sites and PAM (in red) listed. (B) The F1 fish selected for breeding F2. Nucleotide and amino-acid sequences of CRISPR/Cas9 target sites (arrowheads), PAM (red) and the resultant deletions. Deletion of 20 bp form the first exon of *wt1a* and deletion of 17 bp in the first exon of *wt1b* were obtained. Predicted amino-acid sequences of wild type (WT) and deletion alleles are listed. Asterisks indicate stop codons. (C) The genotypes were identified by heteroduplex motility assay (HMA) on PCR amplicons, which shows homoduplexes (open arrowhead) in WT (+/+) and homozygous mutant (-/-) and both homoduplexes and heteroduplexes (arrow) in the heterozygous mutant (+/-), M, DNA marker.

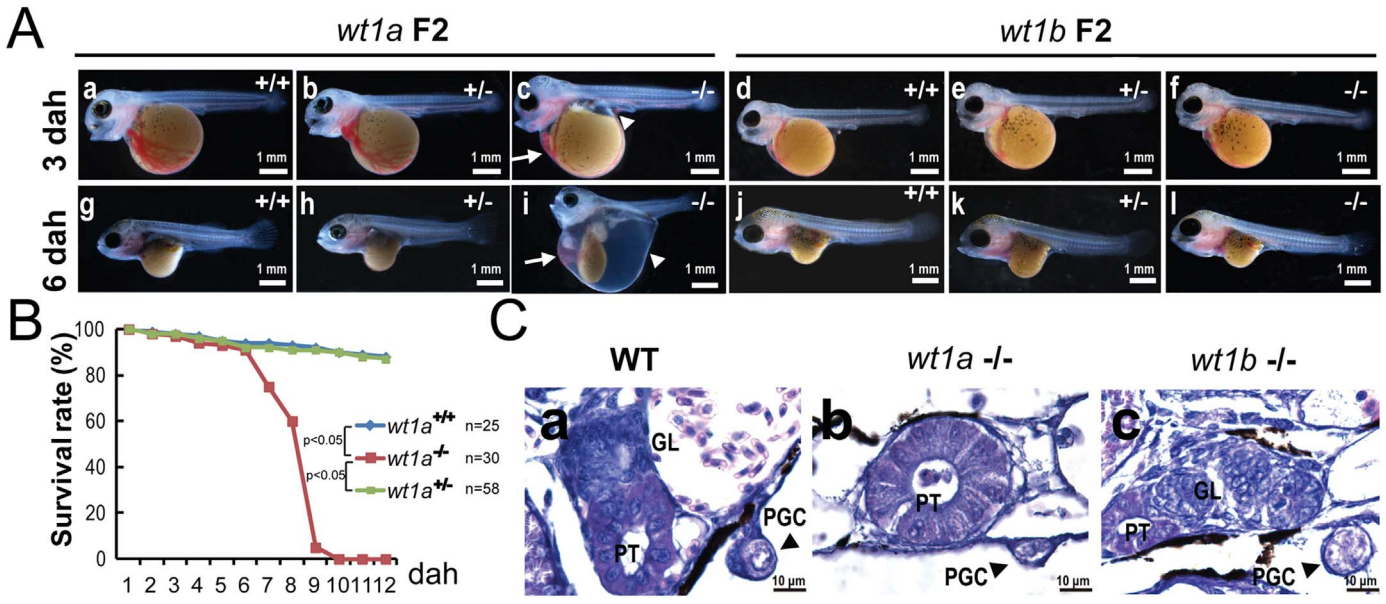

Fig. 3. Effects of homozygous mutation of *wt1a* and *wt1b* on embryonic and larval development of Nile tilapia. (A) Observation of the embryonic and larval development of *wt1a* and *wt1b* mutant fish. $wt1a^{+/+}$ and $wt1a^{+/-}$ in F2 fish, $wt1b^{+/-}$ and $wt1b^{-/-}$ F2 fish is normal at 3 and 6 dah, while $wt1a^{-/-}$ fish developed pericardial edema and yolk sac edema at 3 dah, which became more serious at 6 dah. Pericardial edema is marked by arrows and yolk sac edema by arrowheads. (B) Survival rate of the *wt1a* mutant F2 embryos. The survival rate of $wt1a^{-/-}$ fish is significantly lower than that of wild type and $wt1a^{+/-}$ fish. Paired-samples *T*-test was carried out ($P < 0.05$). (C) Histological observation of cross sections of kidney of the $wt1a^{-/-}$, $wt1b^{-/-}$ and the wild type fish at 6 dah. The glomerulus and pronephric tubule were observed in the $wt1b^{-/-}$ and the wild type fish, but not in the $wt1a^{-/-}$ fish. In addition, PGCs in the $wt1b^{-/-}$ and the wild type fish are much larger than that of $wt1a^{-/-}$ fish. GL, glomerulus; PT, pronephric tubule; PGC, primordial germ cell.

conserved retinoic acid responsive element (5`-AGTTGA-$(N)_5$-GGGTCA-3`), which controls zebrafish *wt1a* expression in the pronephros (Bollig et al., 2009), was also found 8.3 kb upstream of Nile tilapia *wt1a*. The regulatory element and expression profiles divergences provide evidence for different functions of *wt1a* and *wt1b* in the development of the pronephric kidney in teleosts.

Inactivation of Wt1 in mice caused embryonic lethality and multiple organ defects, most notably agenesis of the kidneys (Kreidberg et al., 1993). The kidney defects seen in *Wt1* mutation mice were mimicked by the *wt1a* homozygous mutation in tilapia and *wt1a* morphant zebrafish embryos. *wt1b* morphant embryos were compatible with early glomerular development, however, they subsequently developed cysts in the glomeruli, tubules and pericardial edema suggesting that Wt1b functions at later stages of nephrogenesis in zebrafish (Perner et al., 2007). In medaka, *wt1a*, *wt1b* and *wt1a/b* morphants were embryonic lethal suggesting that both *wt1a* and *wt1b* are essential for

Table 1
Genotype, genotypic and phenotypic sex of *wt1a* and *wt1b* F2 generation fish at 90 and 180 days after hatching.

Generation	Genotype	XX		XY	
		Male	Female	Male	Female
	+/+	0	5	7	0
wt1a	+/-	0	11	13	0
	-/-	0	0	0	0
	+/+	0	12	10	0
wt1b	+/-	0	24	25	0
	-/-	0	10	10	0

Note, the *wt1a* and *wt1b* genotype, genotypic and phenotypic sexes were determined using heteroduplex motility assay (HMA), sex specific Marker-5 and gonad histology observation at 90 and 180 days after hatching.

embryonic pronephros development (Klüver et al., 2009). In contrast to medaka and zebrafish, the *wt1b*$^{-/-}$ tilapia fish were viable, with normal glomerulae suggesting that Wt1b was redundant for pronephric kidney development in tilapia. Taken together, Wt1a alone or together with Wt1b perform the functions of mammalian Wt1 during kidney development in teleosts. Double-homozygous knockouts need to be established in the future to judge whether double mutants would be more severely affected than the *wt1a* mutants and whether there is compensation or redundancy of Wt1 subtypes.

4.3. Function of Wt1a and Wt1b on sex determination and differentiation

In the bipotential gonads of XY mice, *Wt1* was expressed at 9.5 dpc within the urogenital ridge, while the master male sex-determining gene *Sry* was expressed later at 10.5 dpc, suggesting that *Wt1* is the upstream gene of *Sry* (Armstrong et al., 1993; Karl and Capel, 1998; Kim et al., 1999). *SRY*/*Sry* initiates male differentiation by triggering the expression of *SOX9*/*Sox9* and *AMH*/*Amh* in mammals (Josso and Clemente, 2003; Joseph et al., 2009). Amh is essential for Müllerian duct (MD) regression in mammals, while the MD does not exist in teleosts (Pfennig et al., 2015). In the genital ridge of medaka, *wt1a* was expressed in somatic cells at stage 33, earlier than the master male sex-determining gene *dmy,* which is detectable at stage 37. *wt1b* was not detectable in embryonic developing gonads, suggesting that Wt1a independently regulated *dmy* in the gonads during the early stages of development (Kobayashi et al., 2004; Klüver et al., 2009). In addition, *in vitro* promoter analyses revealed that medaka Wt1a was able to regulate *dmy* (Chakraborty et al., 2016). In Nile tilapia, *amhy* is the master male sex-determining gene and was detectable in the XY gonads 5 dah (Li et al., 2015), while both *wt1a* and *wt1b* were simultaneously expressed in somatic cells surrounding the PGCs of both sexes at 3 dah, suggesting that *amhy* might be regulated by Wt1a and Wt1b. The gonads of the larval fish are very small and it is very hard to reveal the boundaries between the PGCs and somatic cells by ISH. IHC is probably a better way to show the expression pattern of these genes. The Nile tilapia Wt1a and Wt1b polyclonal antibodies are in preparation.

In the present study, based on gene expression we concluded that the sex of the *wt1a*$^{-/-}$ fish was undifferentiated at 6 dah. In Nile tilapia, the molecular sex determination occurred (judged by the expression of the sex determining gene *amhy* in XY fish) before 5 dah (Li et al., 2015). The female somatic cell marker Cyp19a1a was expressed in the gonad of XX *wt1a*$^{+/+}$ control fish at 6 dah (Sun et al., 2014b), but not in the gonad of XX *wt1a*$^{-/-}$ fish; while the male somatic markers Dmrt1 and Gsdf were expressed in the gonad of XY *wt1a*$^{+/+}$ control fish (Sun et al., 2014b; Jiang et al., 2016; Shi et al., 2017), but not in the gonad of the XY *wt1a*$^{-/-}$ fish. All *wt1a*$^{-/-}$ fish died before 10 dah preventing us from doing functional studies on sex determination and differentiation. The effects of the *wt1a* homozygous mutation on somatic cell protein expression may be caused by secondary effects resulting from multiple organ defects in the *wt1a*$^{-/-}$ fish. Furthermore, based on both gene expression and morphology, the sex phenotypes of the XX and XY *wt1a* F0 fish with high mutation rates (Supplementary Table S2) and *wt1b*$^{-/-}$ fish (Table 1) were not affected after 90 dah. Taken together, our results suggested that *wt1a* and *wt1b* may compensate one another in sex determination and differentiation in tilapia. Disruption of *wt1a* by CRISPR/CAS9 in the *wt1b*$^{-/-}$ fish might give insight of Wt1s function on teleost sex determination and differentiation.

4.4. Function of Wt1a and Wt1b in testes

In the testes of adult mammals, Wt1 is specifically expressed in Sertoli cells. It has been shown that Wt1 and Sf-1 (nuclear receptor subfamily 5, group a, member 1) can synergistically promote Amh expression (Nachtigal et al., 1998). In *Wt1* conditional knockout mouse testes, Sertoli cells were not maintained and the genes in the Sertoli cells were not expressed, including Amh and Sox9 (Gao et al., 2006). Wt1 was also required for steroidogenesis in Leydig cells in mouse testes, deletion of the Wt1 gene resulted in a defect in testosterone biosynthesis and downregulation of steroidogenic gene expression (Chen et al., 2014). In the testes of adult medaka, both *wt1a* and *wt1b* mRNA transcripts were detected in Sertoli cells (Klüver et al., 2009). Similarly, in tilapia testes, *wt1a* and *wt1b* were expressed in Sertoli cells indicating the functional conversion of Wt1s in fish testis. The *wt1a*$^{-/-}$ fish are lethal and therefore it was difficult to determine the expression of genes in the gonads after Wt1a inactivation. The *wt1a* conditional knockout fish model should be established to overcome the limitation of the homozygous mutants in the future. In XY *wt1b*$^{-/-}$ fish, testicular *amh* expression was down-regulated instead of completely abolished at 90 dah, suggesting that *amh* was probably regulated by both Wt1a and Wt1b in tilapia. Despite partial down-regulation of *amh*, serum estradiol-17β and 11-ketotestosterone levels in XY *wt1b*$^{-/-}$ fish were normal. In addition, the adult XY *wt1b*$^{-/-}$ fish were normal and fertile. Taken together, our results suggested that Wt1b is redundant for testicular function and male reproduction in tilapia.

4.5. Function of Wt1a and Wt1b in ovaries

In the ovaries of adult mammals, Wt1 is specifically expressed in the granulosa cells (Gao et al., 2014). In tilapia, *wt1a* was expressed in the interstitial cells, thecal cells and granulosa cells, while *wt1b* was dominantly expressed in the interstitial cells of the ovaries, suggesting functional diversification of Wt1s in fish ovaries (Fig. 1B). To our knowledge, this is the first report of *wt1s* expression in adult fish ovaries. However, it is still hard to tell which cell types were positive for Wt1a and Wt1b in the gonads. These problems will also be resolved by IHC procedure with Nile tilapia Wt1a and Wt1b polyclonal antibodies in our further studies.

At present, the physiological significance of Wt1 in the ovary remains elusive in non-mammalian vertebrates. XX *Wt1*$^{+/R394W}$ female mice were subfertile, follicle development was aberrant and some genes, including *Fshr*, *3β-HSD* and *Cyp19a1a*, were up-regulated (Gao et al., 2014). Likewise, stimulation of *CYP19A1* promoter activity by SF-1 in cell lines is inhibited by WT1 and DAX1 *in vitro* (Gurates et al., 2002, 2003). At 90 dah, the XX *wt1b*$^{-/-}$ ovarian *cyp19a1a* expression was significantly up-regulated, compared with the XX *wt1b*$^{+/+}$, and serum estradiol-17β levels were also up-regulated, suggesting that Wt1b may inhibit *cyp19a1a* expression in tilapia (Fig. 5). The influence of the *wt1b* homozygous mutation on female fertility remains to be tested in future studies. If fertility of the XX *wt1b*$^{-/-}$ female can be influenced by the increase of serum sex hormone levels, following the up-regulation of *cyp19a1a* expression, the XX *wt1b*$^{-/-}$ female fish may be a good model for understanding the influence of Wt1 mutations on female fertility in mammals.

However, despite the *wt1a* F0 XX fish (knockout rate =80%) being

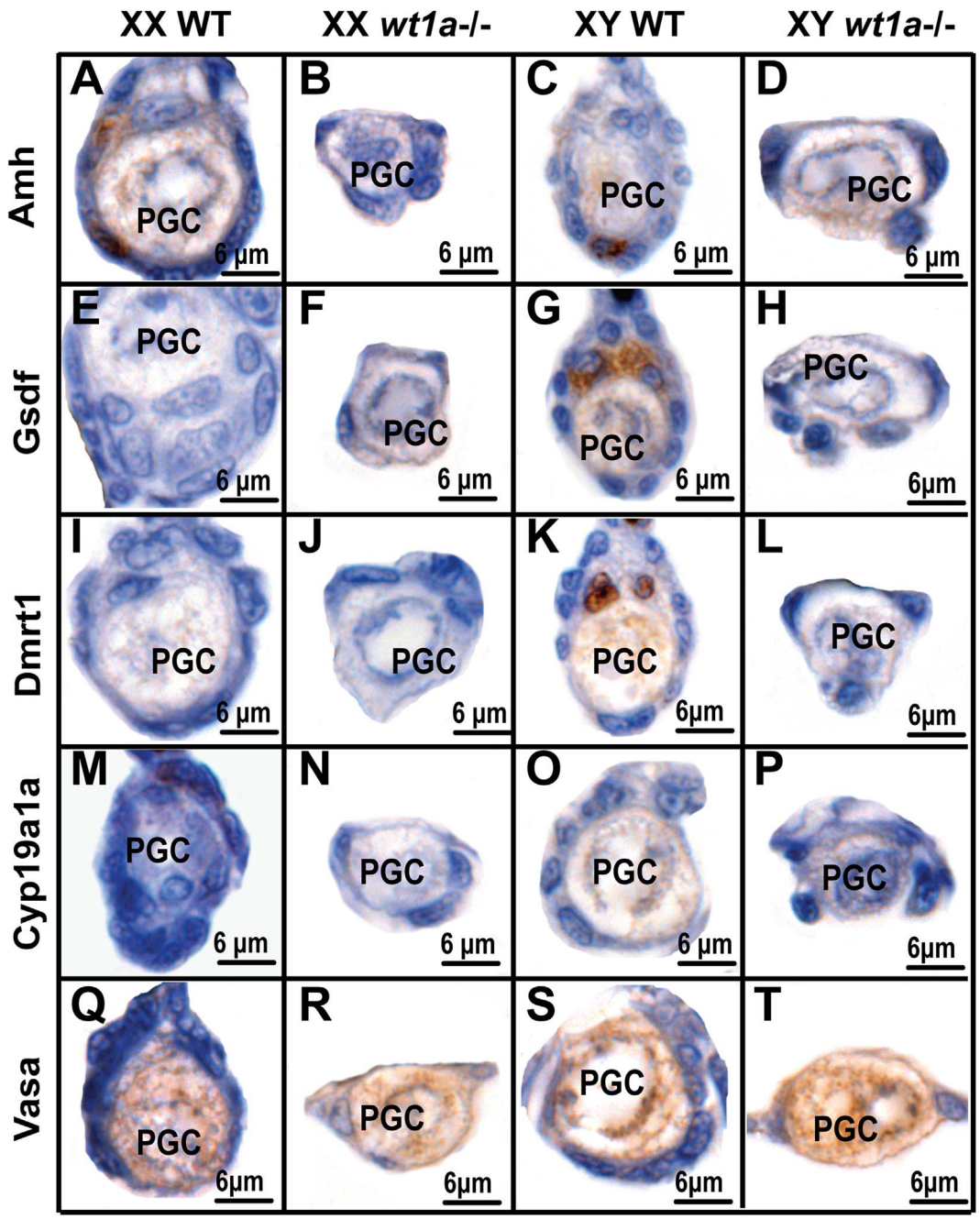

Fig. 4. Effects of *wt1a* mutation on gene expression in Nile tilapia gonads at 6 days after hatching by immunohistochemistry. Amh and Cyp19a1a were expressed in the somatic cells, Vasa was expressed in the germ cells, while Gsdf and Dmrt1 were not expressed in XX $wt1a^{+/+}$ gonads. Amh, Gsdf and Dmrt1 were expressed in somatic cells, Vasa was expressed in germ cells, while Cyp19a1a was not expressed in XY $wt1a^{+/+}$ gonads. Vasa was expressed in germ cells, while Amh, Gsdf, Dmrt1, and Cyp19a1a were not expressed in XX and XY $wt1a^{-/-}$ gonads. The PGCs and the somatic cells can be distinguished by their sizes. The PGCs are much bigger than the somatic cells. They may also overlap with each other on some cross sections to make the boundaries between them unclear. The germ cell marker and somatic cell marker can help us to localize the respective cell types. WT, wild-type. PGC, primordial germ cell. The positive signal corresponds to the brownish color.

fertile (data not shown), the possibility that the residual Wt1a in the F0 mosaic fish is essential for ovarian function cannot be excluded. Together, our results suggest that both Wt1a and Wt1b are important for ovarian function in tilapia.

5. Conclusions

We identified *wt1a* and *wt1b* sequences in Nile tilapia. The sequences and alternative splice sites of tilapia *wt1a* and *wt1b* are conserved with other teleosts. The expression profiles of tilapia *wt1a* and *wt1b* in the kidney and gonads are similar, but not identical with other teleosts. A functional study by CRISPR/CAS9 demonstrated that Wt1a is essential, while Wt1b is redundant for tilapia pronephros development. The $wt1a^{-/-}$ fish were larval lethal and the function of Wt1a on sex determination and differentiation was difficult to determine, while the sex of $wt1b^{-/-}$ fish was not affected in tilapia. Wt1b is redundant in male reproduction, while its function in female reproduction remains to be elucidated.

Declaration of interest

The authors declare that there is no financial or other potential conflict of interests.

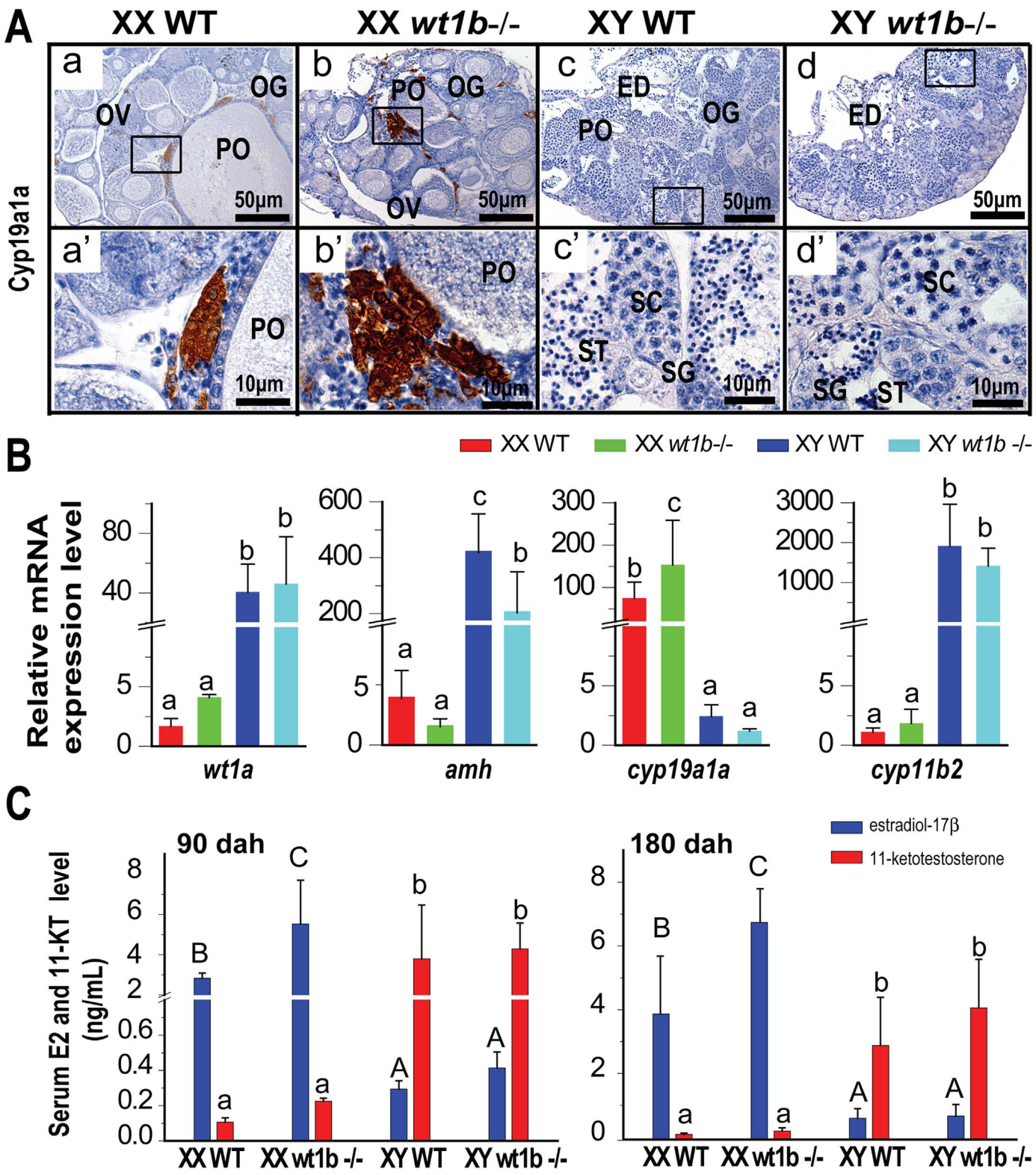

Fig. 5. Effects of *wt1b* mutation on gene expression in F2 tilapia gonads and serum hormone levels at 90 and 180 dah. (A) Effects of *wt1b* homozygous mutation on Cyp19a1a expression in gonad at 90 dah by immunohistochemistry. Cyp19a1a was expressed in the interstitial cells of XX $wt1b^{+/+}$ and $wt1b^{-/-}$ ovaries, but was not expressed in the XY $wt1b^{+/+}$ and $wt1b^{-/-}$ testes. OG, oogonia; PO, previtellogenic oocytes; OV, ovarian cavity; SG, spermatogonia; SC, spermatocytes; ST, spermatids; ED, efferent duct. A brown color corresponds to a positive signal. (B) *wt1a*, *amh*, *cyp19a1a* and *cyp11b2* expression in the $wt1b^{-/-}$ and $wt1b^{+/+}$ gonads at 90 dah by real-time PCR. *wt1a*, *amh* and *cyp11b2* expression in XY $wt1b^{-/-}$ and $wt1b^{+/+}$ testes was significantly higher than that of XX $wt1b^{-/-}$ and $wt1b^{+/+}$ ovaries, while *cyp19a1a* expression in XX $wt1b^{-/-}$ and $wt1b^{+/+}$ ovaries was significantly higher than that of XY $wt1b^{-/-}$ and $wt1b^{+/+}$ testes. *cyp19a1a* expression in XX $wt1b^{-/-}$ ovaries was significantly higher than that in XX $wt1b^{+/+}$ ovaries. *amh* expression in the XX $wt1b^{-/-}$ testes was significantly lower than that in XY $wt1b^{+/+}$ testes. The reference gene *actb* was used to normalize the expression values. (C) Effects of *wt1b* mutation on serum hormone levels at 90 and 180 dah. The serum estradiol-17β levels in the XX $wt1b^{-/-}$ and $wt1b^{+/+}$ fish were significantly higher than that of XY $wt1b^{-/-}$ and $wt1b^{+/+}$ fish, while serum 11-ketotestosterone levels in the XX $wt1b^{-/-}$ and $wt1b^{+/+}$ fish were significantly lower than that in XY $wt1b^{-/-}$ and $wt1b^{+/+}$ fish at 90 and 180 dah. Serum estradiol-17β levels in the XX $wt1b^{-/-}$ fish were significantly higher than that in XX $wt1b^{+/+}$ fish at 90 dah. Data are expressed as the mean ± SD of triplicates. Different letters above the error bar indicate statistical differences at $P < 0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test. WT, wild-type.

Acknowledgements

The authors cordially thank Prof. Yoshitaka Nagahama from the National Institute for Basic Biology, Okazaki, Japan for providing the Vasa and Cyp19a1a antibodies. We are grateful to Mr. Mustapha UF, Fisheries College, Guangdong Ocean University, China for his English correction of the manuscript.

Grant Support: This work was supported by grants 31630082, 91331119, 31602134 and 31502147 from the National Natural Science Foundation of China; grant 20130182130003 from the Specialized Research Fund for the Doctoral Program of Higher Education of China; grants cstc2015jcyjB80001 from the Natural Science Foundation Project of Chongqing, Chongqing Science and Technology Commission; grants 2016T90830, 2015M570765 and 2013M542239 from National Science Foundation for Post-doctoral Scientists of China; grant Xm2015028 from Chongqing Postdoctoral Science Foundation; grant SWU112027 from Doctor Foundation Project of Southwest University; grants XDJK2016E090, XDJK2016E091, XDJK2016C157, XDJK2013D019 and XDJK2013C039 from the Fundamental Research Funds for the Central Universities.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.05.017.

References

Armstrong, J.F., Pritchard-Jones, K., Bickmore, W.A., Hastie, N.D., Bard, J.B., 1993. The expression of the Wilms' tumour gene, *WT1*, in the developing mammalian embryo. Mech. Dev. 40, 85–97.

Barbaux, S., Niaudet, P., Gubler, M.C., Grünfeld, J.P., Jaubert, F., Kuttenn, F., Fékété, C.N., Souleyreau-Therville, N., Thibaud, E., Fellous, M., McElreavey, K., 1997. Donor splice-site mutations in *WT1* are responsible for Frasier syndrome. Nat. Genet. 17, 467–470.

Bollig, F., Mehringer, R., Perner, B., Hartung, C., Schäfer, M., Schartl, M., Volff, J.N., Winkler, C., Englert, C., 2006. Identification and comparative expression analysis of a second *wt1* gene in zebrafish. Dev. Dyn. 235, 554–561.

Bollig, F., Perner, B., Besenbeck, B., Köthe, S., Ebert, C., Taudien, S., Englert, C., 2009. A highly conserved retinoic acid responsive element controls wt1a expression in the zebrafish pronephros. Development 136, 2883–2892.

Bradford, S.T., Wilhelm, D., Bandiera, R., Vidal, V., Schedl, A., Koopman, P., 2009. A cell-autonomous role for WT1 in regulating *Sry* in vivo. Hum. Mol. Genet. 18, 3429–3438.

Brawand, D., Wagner, C.E., Li, Y.I., Malinsky, M., Keller, I., Fan, S., Simakov, O., Ng, A.Y., Lim, Z.W., Bezault, E., Turner-Maier, J., Johnson, J., Alcazar, R., Noh, H.J., Russell, P., Aken, B., Alföldi, J., Amemiya, C., Azzouzi, N., Baroiller, J.F., Barloy-Hubler, F., Berlin, A., Bloomquist, R., Carleton, K.L., Conte, M.A., D'Cotta, H., Eshel, O., Gaffney, L., Galibert, F., Gante, H.F., Gnerre, S., Greuter, L., Guyon, R., Haddad, N.S., Haerty, W., Harris, R.M., Hofmann, H.A., Hourlier, T., Hulata, G., Jaffe, D.B., Lara, M., Lee, A.P., MacCallum, I., Mwaiko, S., Nikaido, M., Nishihara, H., Ozouf-Costaz, C., Penman, D.J., Przybylski, D., Rakotomanga, M., Renn, S.C., Ribeiro, F.J., Ron, M., Salzburger, W., Sanchez-Pulido, L., Santos, M.E., Searle, S., Sharpe, T., Swofford, R., Tan, F.J., Williams, L., Young, S., Yin, S., Okada, N., Kocher, T.D., Miska, E.A., Lander, E.S., Venkatesh, B., Fernald, R.D., Meyer, A., Ponting, C.P., Streelman, J.T., Lindblad-Toh, K., Seehausen, O., Di Palma, F., 2014. The genomic substrate for adaptive radiation in African cichlid fish. Nature 513, 375–381.

Caricasole, A., Duarte, A., Larsson, S.H., Hastie, N.D., Little, M., Holmes, G., Todorov, I., Ward, A., 1996. RNA binding by the Wilms tumor suppressor zinc finger proteins. Proc. Natl. Acad. Sci. USA 93, 7562–7566.

Carmona, R., González-Iriarte, M., Pérez-Pomares, J.M., Muñoz-Chápuli, R., 2001. Localization of the Wilm's tumour protein WT1 in avian embryos. Cell Tissue Res. 303, 173–186.

Chakraborty, T., Zhou, L.Y., Chaudhari, A., Iguchi, T., Nagahama, Y., 2016. Dmy initiates masculinity by altering Gsdf/Sox9a2/Rspo1 expression in medaka (*Oryzias latipes*). Sci. Rep. 6, 19480.

Chen, M., Wang, X., Wang, Y., Zhang, L., Xu, B., Lv, L., Cui, X., Li, W., Gao, F., 2014. Wt1 is involved in leydig cell steroid hormone biosynthesis by regulating paracrine factor expression in mice. Biol. Reprod. 90, 71.

Gao, F., Maiti, S., Sun, G., Ordonez, N.G., Udtha, M., Deng, J.M., Behringer, R.R., Huff, V., 2004. The Wt1+/R394W mouse displays glomerulosclerosis and early-onset renal failure characteristic of human Denys-Drash syndrome. Mol. Cell Biol. 24, 9899–9910.

Gao, F., Maiti, S., Alam, N., Zhang, Z., Deng, J.M., Behringer, R.R., Lécureuil, C., Guillou, F., Huff, V., 2006. The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. Proc. Natl. Acad. Sci. USA 103, 11987–11992.

Gao, F., Zhang, J., Wang, X., Yang, J., Chen, D., Huff, V., Liu, Y.X., 2014. Wt1 functions in ovarian follicle development by regulating granulosa cell differentiation. Hum. Mol. Genet. 23, 333–341.

Gerlach, G.F., Wingert, R.A., 2013. Kidney organogenesis in the zebrafish: insights into vertebrate nephrogenesis and regeneration. Wiley Interdiscip. Rev. Dev. Biol. 2, 559–585.

Gessler, M., Poustka, A., Cavenee, W., Neve, R.L., Orkin, Sh, Bruns, G.A., 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343, 774–778.

Gurates, B., Sebastian, S., Yang, S., Zhou, J., Tamura, M., Fang, Z., Suzuki, T., Sasano, H., Bulun, S.E., 2002. WT1 and DAX-1 inhibit aromatase P450 expression in human endometrial and endometriotic stromal cells. J. Clin. Endocrinol. Metab. 87, 4369–4377.

Gurates, B., Amsterdam, A., Tamura, M., Yang, S., Zhou, J., Fang, Z., Amin, S., Sebastian, S., Bulun, S.E., 2003. WT1 and DAX-1 regulate SF-1-mediated human P450arom gene expression in gonadal cells. Mol. Cell Endocrinol. 208, 61–75.

Haber, D.A., Sohn, R.L., Buckler, A.J., Pelletier, J., Call, K.M., Housman, D.E., 1991. Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc. Natl. Acad. Sci. USA 88, 9618–9622.

Hammes, A., Guo, J.K., Lutsch, G., Leheste, J.R., Landrock, D., Ziegler, U., Gubler, M.C., Schedl, A., 2001. Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell 106, 319–329.

Ji, S.Y., Hao, J.X., Li, L., Zhang, J., Zheng, Q.S., Li, X.X., Wang, X.N., Han, C.S., Gao, F., Liu, Y.X., 2013. Expression of inhibin-alpha is regulated synergistically by Wilms' tumor gene 1 (Wt1) and steroidogenic factor-1 (Sf1) in sertoli cells. PLoS One 8, e53140.

Jiang, D.N., Yang, H.H., Li, M.H., Shi, H.J., Zhang, X.B., Wang, D.S., 2016. *gsdf* is a downstream gene of dmrt1 that functions in the male sex determination pathway of the Nile tilapia. Mol. Reprod. Dev. 83, 497–508.

Joseph, A., Yao, H., Hinton, B.T., 2009. Development and morphogenesis of the Wolffian/epididymal duct, more twists and turns. Dev. Biol. 325, 6–14.

Josso, N., Nd, Clemente, 2003. Transduction pathway of anti-Müllerian hormone, a sex-specific member of the TGF-beta family. Trends Endocrinol. Metab. 14, 91–97.

Karl, J., Capel, B., 1998. Sertoli cells of the mouse testis originate from the coelomic epithelium. Dev. Biol. 203, 323–333.

Kennedy, D., Ramsdale, T., Mattick, J., Little, M., 1996. An RNA recognition motif in Wilms' tumour protein (WT1) revealed by structural modelling. Nat. Genet. 12, 329–331.

Kent, J., Coriat, A.M., Sharpe, P.T., Hastie, N.D., van Heyningen, V., 1995. The evolution of WT1 sequence and expression pattern in the vertebrates. Oncogene 11, 1781–1792.

Kim, J., Prawitt, D., Bardeesy, N., Torban, E., Vicaner, C., Goodyer, P., Zabel, B., Pelletier, J., 1999. The Wilms' tumor suppressor gene (*wt1*) product regulates Dax-1 gene expression during gonadal differentiation. Mol. Cell Biol. 19, 2289–2299.

Klamt, B., Koziell, A., Poulat, F., Wieacker, P., Scambler, P., Berta, P., Gessler, M., 1998. Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1+/-KTS splice isoforms. Hum. Mol. Genet. 7, 709–714.

Klüver, N., Herpin, A., Braasch, I., Driessle, J., Schartl, M., 2009. Regulatory back-up circuit of medaka Wt1 co-orthologs ensures PGC maintenance. Dev. Biol. 325, 179–188.

Kobayashi, T., Kajiura-Kobayashi, H., Nagahama, Y., 2003. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. Cytogenet. Genome Res. 101, 289–294.

Kobayashi, T., Matsuda, M., Kajiura-Kobayashi, H., Suzuki, A., Saito, N., Nakamoto, M., Shibata, N., Nagahama, Y., 2004. Two DM domain genes, DMY and DMRT1, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. Dev. Dyn. 231, 518–526.

Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., Jaenisch, R., 1993. WT-1 is required for early kidney development. Cell 74, 679–691.

Kuure, S., Vuolteenaho, R., Vainio, S., 2000. Kidney morphogenesis: cellular and molecular regulation. Mech. Dev. 92, 31–45.

Laity, J.H., Dyson, H.J., Wright, P.E., 2000. Molecular basis for modulation of biological function by alternate splicing of the Wilms' tumor suppressor protein. Proc. Natl. Acad. Sci. USA 97, 11932–11935.

Li, M.H., Yang, H.H., Li, M.R., Sun, Y.L., Jiang, X.L., Xie, Q.P., Wang, T.R., Shi, H.J., Sun, L.N., Zhou, L.Y., Wang, D.S., 2013. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. Endocrinology 154, 4814–4825.

Li, M., Yang, H., Zhao, J., Fang, L., Shi, H., Sun, Y., Zhang, X., Jiang, D., Zhou, L., Wang, D., 2014. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. Genetics 197, 591–599.

Li, M., Sun, Y., Zhao, J., Shi, H., Zeng, S., Ye, K., Jiang, D., Zhou, L., Sun, L., Tao, W., Nagahama, Y., Kocher, T.D., Wang, D., 2015. A tandem duplicate of anti-Mullerian hormone with a missense SNP on the Y chromosome is essential for male sex determination in Nile tilapia, *Oreochromis niloticus*. PLoS Genet. 11, e1005678.

Little, M., Wells, C., 1997. A Clinical Overview of WT1 Gene Mutations. Hum. Mutat. 9, 209–225.

Liu, C., Rodriguez, K., Yao, H.H., 2016. Mapping lineage progression of somatic progenitor cells in the mouse fetal testis. Development 143, 3700–3710.

Massé, J., Watrin, T., Laurent, A., Deschamps, S., Guerrier, D., Pellerin, I., 2009. The developing female genital tract: from genetics to epigenetics. Int. J. Dev. Biol. 53, 411–424.

Menke, A., McInnes, L., Hastie, N.D., Schedl, A., 1998. The Wilms' tumor suppressor WT1: approaches to gene function. Kidney Int. 53, 1512–1518.

Mundlos, S., Pelletier, J., Darveau, A., Bachmann, M., Winterpacht, A., Zabel, B., 1993.

Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. Development 119, 1329–1341.
Nachtigal, M.W., Hirokawa, Y., Enyeart-VanHouten, D.L., Flanagan, J.N., Hammer, G.D., Ingraham, H.A., 1998. Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 93, 445–454.
Nakamura, S., Kobayashi, D., Aoki, Y., Yokoi, H., Ebe, Y., Wittbrodt, J., Tanaka, M., 2006. Identification and lineage tracing of two populations of somatic gonadal precursors in medaka embryos. Dev. Biol. 295, 678–688.
Palmer, R.E., Kotsianti, A., Cadman, B., Boyd, T., Gerald, W., Haber, D.A., 2001. WT1 regulates the expression of the major glomerular podocyte membrane protein Podocalyxin. Curr. Biol. 11, 1805–1809.
Perner, B., Englert, C., Bollig, F., 2007. The Wilms tumor genes *wt1a* and *wt1b* control different steps during formation of the zebrafish pronephros. Dev. Biol. 309, 87–96.
Pfennig, F., Standke, A., Gutzeit, H.O., 2015. The role of Amh signaling in teleost fish–Multiple functions not restricted to the gonads. Gen. Comp. Endocrinol. 223, 87–107.
Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., Heyningen, V.V., Hastie, N., 1990. The candidate Wilms' tumour gene is involved in genitourinary development. Nature 346, 194–197.
Schnerwitzki, D., Perner, B., Hoppe, B., Pietsch, S., Mehringer, R., Hänel, F., Englert, C., 2014. Alternative splicing of Wilms tumor suppressor 1 (Wt1) exon 4 results in protein isoforms with different functions. Dev. Biol. 393, 24–32.
Scholz, H., Kirschner, K.M., 2005. A role for the Wilms' tumor protein WT1 in organ development. Physiology 20, 54–59.
Shi, H., Gao, T., Liu, Z., Sun, L., Jiang, X., Chen, L., Wang, D., 2017. Blockage of androgen and administration of estrogen induce transdifferentiation of testis into ovary. J. Endocrinol. 233, 65–80.
Shimamura, R., Fraizer, G.C., Trapman, J., Lau, Yf.C., Saunders, G.F., 1997. The Wilms' tumor gene WT1 can regulate genes involved in sex determination and differentiation: SRY, Müllerian-inhibiting substance, and the androgen receptor. Clin. Cancer Res. 3, 2571–2580.
Sim, E.U., Smith, A., Szilagi, E., Rae, F., Ioannou, P., Lindsay, M.H., Little, M.H., 2002. Wnt-4 regulation by the Wilms' tumour suppressor gene, WT1. Oncogene 21, 2948–2960.
Spotila, L.D., Hall, S.E., 1998. Expression of a new RNA-splice isoform of WT1 in developing kidney-gonadal complexes of the turtle, *Trachemys scripta*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 119, 761–767.
Sun, Y.L., Jiang, D.N., Zeng, S., Hu, C.J., Ye, K., Yang, C., Yang, S.J., Li, M.H., Wang, D.S., 2014a. Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile tilapia (*Oreochromis niloticus*). Aquaculture 433, 19–27.
Sun, L.N., Jiang, X.L., Xie, Q.P., Yuan, J., Huang, B.F., Tao, W.J., Zhou, L.Y., Nagahama, Y., Wang, D.S., 2014b. Transdifferentiation of differentiated ovary into functional testis by long-term treatment of aromatase inhibitor in Nile tilapia. Endocrinology 155, 1476–1488.
Takasawa, K., Kashimada, K., Pelosi, E., Takagi, M., Morio, T., Asahara, H., Schlessinger, D., Mizutani, S., Koopman, P., 2014. FOXL2 transcriptionally represses Sf1 expression by antagonizing WT1 during ovarian development in mice. FASEB J. 28, 2020–2028.
Tao, W.J., Yuan, J., Zhou, L.Y., Sun, L.N., Sun, Y.L., Yang, S.J., Li, M.H., Zeng, S., Huang, B.F., Wang, D.H., 2013. Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) reveals differentially expressed genes. PLoS One 8, e56006.
Volff, J.N., 2005. Genome evolution and biodiversity in teleost fish. Hered. 94, 280–294.
Xie, Q.P., He, X., Sui, Y.N., Chen, L.L., Sun, L.N., Wang, D.S., 2016. Haploinsufficiency of SF-1 causes female to male sex reversal in Nile tilapia, *Oreochromis niloticus*. Endocrinology 157, 2500–2514.
Yamamura, Y., Aoyama, S., Oshima, Y., Kato, T., Osawa, N., Nakamura, M., 2005. Molecular cloning and expression in gonad of *Rana rugosa* WT1 and Fgf9. Zool. Sci. 22, 1045–1050.
Yu, X., Wu, L., Xie, L., Yang, S., Charkraborty, T., Shi, H., Wang, D., Zhou, L., 2014. Characterization of two paralogous StAR genes in a teleost, Nile tilapia (*Oreochromis niloticus*). Mol. Cell Endocrinol. 392, 152–162.
Zhang, X., Wang, H., Li, M., Cheng, Y., Jiang, D., Sun, L., Tao, W., Zhou, L., Wang, Z., Wang, D., 2014. Isolation of doublesex- and mab-3-related transcription factor 6 and its involvement in spermatogenesis in tilapia. Biol. Reprod. 91, 1–10.
Zhang, L., Chen, M., Wen, Q., Li, Y., Wang, Y., Wang, Y., Qin, Y., Cui, X., Yang, L., Huff, V., Gao, F., 2015a. Reprogramming of Sertoli cells to fetal-like Leydig cells by Wt1 ablation. Proc. Natl. Acad. Sci. USA 112, 4003–4008.
Zhang, Z., Zhu, B., Ge, W., 2015b. Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. Mol. Endocrinol. 29, 76–98.