MOLECULAR CLONING OF WT1a AND WT1b AND THEIR POSSIBLE INVOLVEMENT IN FISH SEX DETERMINATION AND DIFFERENTIATION

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Introduction:

The Nile tilapia is considered as a good experimental animal for the sex determination and differentiation, owing to its monosex offsprings availability and a short (14 days) spawning period. Feral Southern catfish, an endemic fish to China, has a sex ratio 1:1, while the fry obtained by artificial propagation were all female, thus making it a good experimental fish to be studied. It is well demonstrated that estrogens play decisive role in the ovarian differentiation of the teleosts. It has been reported earlier that Wt1 is involved in the mammalian sex determination and differentiation *via* regulation of *Sry*, *Sox9*, *Amh* and *Cyp19a1*. Mice carrying homozygous knockout mutations of Wt1 lack kidneys, gonads and adrenal glands, and have defectively formed heart and spleen. Mutation in Wt1 changes the +KTS/-KTS ratio and causes male to female sex reversal (Frasier syndrome) in mice. However, +KTS and -KTS ablated mice show clearly different phenotypes, indicating distinct functions of the two splicing variants, in particular, in the sex determination pathway. Although *Wt1* duplication [designated as *Wt1a* and *Wt1b*] were found in different teleosts, but their role in early gonadal development is poorly understood. To understand the role of Wt1 in fish sex determination/differentiation, the two Wt1 genes were cloned, both in tilapia and Southern catfish, and the expression pattern in the developing gonad and kidney was studied. The ability of Wt1 in transcriptional regulation of *Cyp19a1a* and *Dmrt1* gene expression was also investigated.

Methods:

All genetic female (XX) and male (XY) tilapia (*Oreochromis niloticus*) were obtained by artificial fertilization of eggs from normal females (XX) with sperm from either sex-reversed males (XX) or super males (YY), respectively. Southern catfish (*Silurus meridionalis*), collected from Hechuang to Beibei section of the Jialing River, was used for the experiment. Gonads of adult tilapia and Southern catfish were dissected and total RNA was extracted. Different alternatively spliced isoforms (cDNAs) of Wt1a and Wt1b were cloned using RT-PCR and RACE. The body (after the removal of the yolk sac, intestine, head and tail) of the 3, 5, 20, 40 dah (days after hatching) fish and the gonads of the 8 month old fish were fixed and embedded in paraffin. Digoxigenin- labeled sense and antisense probes of Wt1a and Wt1b were used for *in situ* hybridization which was performed as previously described.

Approximately, 2kb promoter region of *Cyp19a1a* and *Dmrt1* were amplified from the tilapia genome and cloned into pGL3-basic vector. Different isoforms of Wt1 a/b were cloned into pcDNA3.1 plasmid. pRL-TK was used as an internal control plasmid. HEK293/TM4 cells were used for transcription analysis. Luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Results and Discussion:

Both the *Wt1a* and *Wt1b* cDNAs were cloned from tilapia and Southern catfish. 5 variants of *Wt1a* and 3 variants of *Wt1b* were isolated from tilapia, which were derived from alternative splicing within the ORFs, including either a +KTS or –KTS isoforms. *Wt1a* of tilapia and Southern catfish comprises an ORF of 1245 and 1257bp, encoding 415 and 419aa, respectively. Tilapia Wt1b has a 1272bp ORF, encoding 424aa while the Southern catfish Wt1b has a 1209bp ORF, encoding 402aa. Multiple alignment analysis revealed that Wt1a was more conserved than Wt1b, as it shared a higher similarity to other teleosts (85-90%) as compared to mammals (70%). On the contrary, Wt1b shared a homology of 66% and 61% to other fishes and mammals, respectively. However, Wt1a and Wt1b of both the species showed less homology (<70%) among themselves. Phylogenetic analysis also revealed that fish Wt1a and Wt1b clustered into two different clads, due to the fish specific genome duplication.

In situ hybridization results showed that until 40dah, Wt1 homologues were found to express in the gonadal somatic cells and pronephrons of the kidney. Wt1a showed higher expression in the XY gonad, while Wt1b expression was found to be highest in both the XX and XY gonad. Wt1a and Wt1b expression was found to be down regulated with the development from 3dah to 40dah. In adulthood, (8 months after hatching) Wt1a/b expression was concentrated in the theca and interstitial cells of the ovary and Sertoli cells of the testis. Interestingly, Wt1b expression was seen to be higher in the testicular Sertoli cells that colocalizes with *Dmrt1*, a

gene critical for male sex determination, in testis. Promoter analysis showed that in various transcription factor tested, only the -KTS form of Wt1b activated *Dmrt1* in a dose dependent manner. Addition of Foxl2 and Sf1 could not change the Wt1b (-KTS) dependent *Dmrt1* activation. This shows the potential role of Wt1b in testis development via *Dmrt1* regulation. On the other hand, we failed to correlate *cyp19a1a* activation with either Wt1a or Wt1b homologues, which is different from the situation found in mammals.

Conclusion:

In the present study, transcription factor Wt1a and Wt1b were cloned in the Southern catfish and tilapia. Both genes were found to be expressed higher in the XY gonad compared with the XX gonad in the critical period for tilapia sex determination and differentiation. Furthermore, –KTS isoform of Wt1b can activate the *Dmrt1* transcription. Taken together, our data highlight the role of Wt1 in testicular differentiation. Even though several alternatively spliced forms of Wt1a and Wt1b were isolated, future detail analysis is necessary to understand the roles of individual homologue of Wt1 in the gonad developmental process.