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Synergistic role of β-catenin1 and 2 in ovarian differentiation and maintenance of female pathway in Nile tilapia

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ABSTRACT

Two *β-catenin* (*β-cat*) genes exist in teleosts but little is known about their expression and function in ovarian development. We identified *β-cat1* and *β-cat2* from the *Nile tilapia*. *β-cat1* and *β-cat2* displayed a similar expression pattern in the ovary during development, and were mainly expressed in the oogonia and oocytes. In luciferase assays, β-cat1 activated the TOPFlash reporter dose-dependently, whereas β-cat2 failed to do so. Cotransfection of *β-cat1* and *β-cat2* synergistically enhanced the expression of the reporter. A specific interaction between β-cat1 and β-cat2 was also observed in a mammalian two-hybrid assay. Furthermore, tilapia recombinant Dkk1, an inhibitor of the β-cat pathway, decreased *β-cat1* and *β-cat2*, while increased *sox9*, *dmrt1*, *cyp11b2* and *foxl2* expression in the *in vitro* cultured tilapia ovary, which could be abolished by simultaneous treatment with Bio, an agonist of β-cat. Consistently, *β-cat1* or *β-cat2* knockdown in XX fish by TALENs caused the retardation of ovarian differentiation and masculinization, as reflected by the upregulation of *dmrt1*, *cyp11b2*, *sox9*, and serum 11-KT level. On the contrary, serum E2 level was unchanged even though *foxl2* transcription was upregulated. These data suggestes that both *β-cat1* and *β-cat2* are important members and play synergistic roles in the canonical Wnt signal pathway in fish. Independent of Foxl2-leading estrogen pathway, they might be involved in ovarian differentiation and repression of the male pathway gene expression in tilapia.

1. Introduction

Sex determination/differentiation is a unique developmental process. A bipotential gonad develops into an ovary or testis, two highly specialized organs that differ morphologically and physiologically, despite sharing common reproductive and endocrine functions (Chassot et al., 2014). The sex determination in the mammalian bipotential gonads is controlled by an antagonistic process between the male (*Sry/Sox9/Fgf9*) and female (*Rspo1/Wnt/β-cat* and *Foxl2*) signaling pathways (Wilhelm and Koopman, 2006; Cool and Capel, 2009; Eggers and Sinclair, 2012). In XY individuals, Sry activates transcription factor Sox9, and either of these two proteins is necessary and sufficient to allow testicular development through promoting Sertoli cell differentiation (Chassot et al., 2014). In XX individuals, Rspo1/Wnt4-activated canonical Wnt/β-cat pathway and Foxl2-leading estrogen pathway, which act independently and complementarily to promote the ovarian fate and block testis development (Kocer et al., 2008; Garcia-Ortiz et al., 2009). The activation of the β-cat signaling pathway by Rspo1 and/or Wnt4 is required to allow granulosa cell differentiation and thus ovarian differentiation (Maatouk et al., 2008; Chassot et al., 2014). The heterozygous mutation of the human *RSPO1* gene leads to XX sex reversal (Parma et al., 2006). Mice lacking *Rspo1* exhibit partial female-to-male sex reversal, which is similar to the phenotype of mice lacking *Wnt4*, suggesting that these genes act within the same pathway to activate β-cat and play key roles in ovarian differentiation (Chassot et al., 2008; Chassot et al., 2014). Moreover, in the

Abbreviations: β-cat, β-catenin; dah, days after hatching; *ISH*, *In situ* hybridization; IHC, immunohistochemistry; KD, knock down; Cyp19a1a, cytochrome P450, family 19, subfamily A, polypeptide 1a (aromatase); Cyp11b2, cytochrome P450, family 11, subfamily B, polypeptide 2 (11β-hydroxylase); Dmrt1, doublesex- and mab-3-related transcription factor-1; E2, estradiol-17β; 11-KT, 11-ketotestosterone.

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gonads of *Rspo1*$^{-/-}$ XX mice, germ cell proliferation, Stra8 expression, and entry into meiosis are all impaired. Therefore, Rspo1/β-cat signaling is proposed to be involved in meiosis (Chassot et al., 2011). The loss of *β-cat* in fetal mouse ovaries leads to the development of a testis-specific coelomic vessel and the androgen-producing adrenal-like cells (Liu et al., 2009). Overexpression of β-cat in the XY gonads of mice causes male-to-female sex reversal (Maatouk et al., 2008). All these studies indicate that Rspo1/Wnt/β-cat signaling is essential for ovarian differentiation in mammalian species.

In contrast to mammals, estrogen plays an important role in ovarian differentiation and maintenance in noneutherian vertebrates (Smith and Sinclair, 2004). Estrogen is regarded as a natural inducer of ovarian differentiation in fish during early female sex determination/differentiation (Nagahama, 2005). Gonadal aromatase (encoded by the *cyp19a1a* gene), an enzyme that catalyzes the conversion of androgens to estrogens, increased its expression rapidly in the ovary from 5 dah, plays a crucial role in ovarian differentiation in tilapia (*Oreochromis niloticus*) (Nagahama et al., 2004; Ijiri et al., 2008). Treatment of tilapia XX fry with fadrozole (an aromatase inhibitor) or tamoxifen (an estrogen receptor antagonist) results in complete sex reversal to functional males, which could be rescued by simultaneous treatment with estradiol-17*β* (E2) (Kobayashi et al., 2003; Wang et al., 2007). Both *in vitro* and *in vivo* experiments have demonstrated the roles of Foxl2 and Dmrt1 in tilapia gonadal differentiation, through their transcriptional regulation of the aromatase gene (*cyp19a1a*) (Wang et al., 2007; Li et al., 2013). Expression data from the protandrous black porgy (*Acanthopagrus schlegeli*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) indicate that Rspo1/Wnt/β-cat signaling might also play a role in fish gonadal differentiation (Wu and Chang, 2009; Zhang et al., 2011; Zhou et al., 2012). Two *β-cat* genes, *β-cat1* and *β-cat2* were identified in zebrafish (Bellipanni et al., 2006). Knockdown experiment showed that they play essential roles in the formation of dorsal axial structures and the neurectoderm, through partially overlapping mechanisms (Zhang et al., 2012). However, up to now there have been no reports provided direct evidence for the roles of Wnt/β-cat in female sex determination/differentiation in nonmammalian vertebrates. In addition, it is unclear whether both of the two *β-cats* in fish are involved in the ovarian development.

Nile tilapia is an excellent model for the study of fish gonadal differentiation because of the availability of mono sex fry, genome sequences and genome editing tools (Soler et al., 2010; Li et al., 2013; Brawand et al., 2014). In this study, we identified two paralogous *β-cat* genes from the tilapia genome (http://www.ensembl.org/Oreochromis_niloticus/Info/Index) and examined their expression pattern in the developing ovaries using real-time PCR, *in situ* hybridization (*ISH*) and immunohistochemistry (IHC). We also validated their functions by knocking out *β-cat1* and *β-cat2* in XX tilapia using the transcription activator-like effector nucleases (TALENs) system. Here we demonstrate the importance of the Wnt/β-cat pathway in ovarian differentiation and development in teleosts.

2. Materials and methods

2.1. Fish

Nile tilapia, *O. niloticus*, were reared in large tanks with recirculating aerated freshwater systems. The fish were maintained at ambient temperature (26 °C) under a 14 h (h) light: 10 h dark photoperiod. XX males and XY females were generated by treating fry from 5 to 30 dah with aromatase inhibitor fadrozole (100 μg/g) (Novartis Company, Switzerland), and 17β-estradiol (E2, 50 μg/g) (Sigma, USA), respectively, as described previously (Kobayashi et al., 2003; Sun et al., 2012). Super males (YY) were obtained by crossing normal XY males with an XY female. All-XX progenies were obtained by crossing a pseudomale (XX male) with a normal female (XX). All-XY progenies were obtained by crossing a supermale (YY) with a normal female. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Committee for Laboratory Animal Experimentation at Southwest University, China.

2.2. Identification of two β-cats

Two *β-cat* genes (*β-cat1*: ENSONIT00000009116; *β-cat2*: ENSONIT00000002594) were retrieved from the genome database of tilapia (http://www.ensembl.org/Oreochromis_niloticus/Info/Index). Gene-specific primers were designed to amplify the ORFs (β-cat1-oF,-oR; β-cat2-oF,-oR) from the ovary cDNA. RNA isolation and cDNA synthesis were carried out according to the manufacturer's instructions (TaKaRa, Japan). All PCR products were ligated into the pGEM-T Easy Vector (Promega, USA) and sequenced by Life Technologies Corporation (Shanghai, China).

2.3. Real-time PCR and statistical analysis

The primer sets used for real-time PCR were designed using Primer Express software (Applied Biosystems, USA) with at least one primer in each set flanking an intron-exon boundary to prevent sequence amplification from the genomic DNA. Linear standard curves building and all real-time PCRs were performed in the ABI-7500 fast real-time PCR system (Applied Biosystems, USA) following the manufacturer's protocol. All real-time PCRs were carried out in a 20 μl reactions with a mixture of 10 μl 2×SYBR Premix ExTaq (TaKaRa, Japan), 2 μl of diluted cDNA or PCR-grade water as negative control, 6 μl of PCR-grade water, and 1 μl of each 10 μM primer. The PCR reactions were initiated by denaturation at 95 °C (5 min), followed by 40 amplification cycles at 95 °C (15 s) and 60 °C (30 s), and then 95 °C (15 s), 60 °C (60 s) and 95 °C (15 s) to get the melt curve. The relative expression level (RNA abundance) was calculated by dividing the copy number of target gene by the geometric mean of three reference genes (*eef1a1*, *actb*, and *gapdh*). Data are expressed as means ± SD. Significance ($P < 0.05$) in the results was determined using the Student's t-test or one-way ANOVA followed by Bonferroni post hoc test as appropriate for single or multiple comparisons respectively.

2.4. Tissue distribution and ontogenic analysis by real-time PCR

For tissue distribution analysis, samples of different tissues from three female adult fish and the testis from three male adult fish were prepared to evaluate the expression of two *β-cat* genes. Briefly, total RNA was extracted from the head kidney, kidney, brain, gill, heart, intestine, liver, muscle, ovary and testis of the adult tilapia, according to the manufacturer's instructions (TaKaRa, Japan). Total RNA (200 ng) from each tissue was reverse transcribed into first-strand cDNA using PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, Japan) following the manufacturer's instructions. Real-time PCR and statistical analysis were carried out according to the aforementioned method.

Three independent gonadal samples from female fish at 5, 10, 20, 30, 60, 90 and 180 dah were used for ontogenic expression analysis. Gonads from 50 to 100 fish were pooled for each sample collected from 5 to 60 dah. One ovary was used for each sample collected from 90 to 180 dah. RNA extraction, cDNA synthesis, real-time PCR and statistical analysis were carried out as described above.