Gonad Differentiation in Zebrafish Is Regulated by the Canonical Wnt Signaling Pathway[1]

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ABSTRACT

Zebrafish males undergo a "juvenile ovary-to-testis" gonadal transformation process. Several genes, including nuclear receptor subfamily 5, group A (nr5a) and anti-Müllerian hormone (amh), and pathways such as Tp53-mediated germ-cell apoptosis have been implicated in zebrafish testis formation. However, our knowledge of the regulation of this complex process is incomplete, and much remains to be investigated about the molecular pathways and network of genes that control it. Using a microarray-based analysis of transforming zebrafish male gonads, we demonstrated that their transcriptomes undergo transition from an ovary-like pattern to an ovotestis to a testis-like profile. Microarray results also validated the previous histological and immunohistochemical observation that there is high variation in the duration and extent of commitment to the juvenile ovary phase among individuals. Interestingly, global gene expression profiling of diverging zebrafish juvenile ovaries and transforming ovotestes revealed that some members of the canonical Wnt/beta-catenin signaling pathway were differentially expressed between these two phases. To investigate whether Wnt/beta-catenin signaling plays a role in zebrafish gonad differentiation, we used the Tg (hsp70l:dkk1b-GFP)w32 line to inhibit Wnt/beta-catenin signaling during gonad differentiation. Activation of dkk1b-GFP expression by heat shock resulted in an increased proportion of males and corresponding decrease in gonadal aromatase gene (cyp19a1a) expression. The Wnt target gene, lymphocyte enhancer binding factor 1 (lef1), was also down-regulated in the process. Together, these results provide the first functional evidence that, similarly to mammals, Wnt/beta-catenin signaling is a "pro-female" pathway that regulates gonad differentiation in zebrafish.

INTRODUCTION

Zebrafish is an undifferentiated gonochorous species with a polygenic sex determination system [1–3]. It has been described as a juvenile hermaphrodite because males undergo a "juvenile ovary-to-testis" gonadal transformation process [4–6]. Use of transgenic reporter lines combined with histological analysis revealed a variation among males in the onset of transformation and in the number of oocytes in the juvenile ovary prior to the transformation [7–10]. Despite accumulating histological, toxicological, and gene expression data, there is little information available on the molecular mechanisms that regulate the gonadal differentiation process [11–15].

Sex differentiation of teleosts, including the zebrafish, is known to be highly pliable and can be easily affected by exposure to steroids and endocrine disruptors (for review see references [16–19]). Of pivotal importance is the gonadal aromatase Cyp19a1a, an enzyme involved in the conversion of androgens to estrogens. It is down-regulated during zebrafish gonadal transformation, and its expression remains sexually dimorphic in the adults: high in ovary and low in testis [11, 20, 21]. Cyp19a1a is, hence, a key physiological effector of ovarian differentiation, and its down-regulation has been additionally hypothesized to be the only step necessary for testis differentiation in fish [18, 22].

Activators of cyp19a1a expression include Nr5a1a and Foxl2, whereas Amh has been proposed as the candidate suppressor (see Supplemental Table S1 for full gene names; all Supplemental Data are available online at www.biolreprod.org) [20, 23–26]. These genes show sexually dimorphic expression in the zebrafish [27, 28]. In mammals, beta-catenin has been

shown to regulate the transcription of *Cyp19a1* via interactions with NR5A1 in rat granulosa cells [29]. Beta-catenin is also the mediator of the canonical Wnt signaling pathway, and this points to a potential role for Wnt signaling in teleost gonad differentiation.

Wnt signaling has long been known to be involved in mammalian sex development. XX mice carrying homozygous mutations of the *Wnt4* gene were masculinized and experienced loss of oocytes, demonstrating the requirement of the Wnt4 protein for ovary development [30]. In humans, XY sex reversal is associated with overexpression of *WNT4,* and it has been shown that WNT4 up-regulates NR0B1, which in turn represses *SRY*, the mammalian male sex-determining gene [31]. Wnt/beta-catenin has also been shown to repress expression of *Sox9,* an Nr5a1 and Sry target gene, in mouse by displacing Nr5a1 from the upstream regulatory region of *Sox9* [32]. RSPO1, a canonical Wnt signaling ligand, is also suggested to be an active ovarian determinant in mammals, as SRY was found to repress Rspo1/beta-catenin signaling [33].

Recent gene expression studies have pointed to a potential reproductive role for Wnt signaling in teleosts. In the protandrous black porgy (*Acanthopagrus schlegelii*), increased *wnt4* expression is associated with ovarian growth during early sex change [34]. In the zebrafish, *rspo1* showed a higher expression in the ovary than in the testis [35]. On the other hand, although there are no sexually dimorphic expressions of *wnt4* and *rspo1* in the rainbow trout (*Oncorhynchus mykiss*), there are other Wnt signaling genes which show increased expression in either the testis or ovary at different stages of development, indicating the potentially complex functions of Wnt signaling during gonad differentiation [36, 37]. It has also been further demonstrated in rainbow trout that the expression levels of both *fst*, which is involved in ovarian differentiation, and *cyp19a1a* are inhibited when Wnt signaling pathway is down-regulated [38]. Interestingly, the expression of two antagonists of the canonical Wnt signaling pathway (*dkk1* and *dact1*) was found to be up-regulated in the ovary compared to that in testis of shovelnose sturgeon (*Scaphirhynchus platorynchus*) [39].

We have previously shown that the intensity of enhanced green fluorescent protein (Egfp) signals in transgenic *Tg (vasa:vasa-EGFP)zf45,* from here onwards known as *Tg*(*vas*), zebrafish juveniles can be a reliable tool for differentiating between a juvenile ovary (strong signal) and a transforming juvenile ovotestis (weak or no signal) [9]. Using this knowledge in combination with a customized microarray containing 6.3 K unique zebrafish gonadal cDNAs, we were able to perform a detailed transcriptomic analysis of these two gonad types earlier in adults and now during ovary-to-testis transformation [27].

We found that the transition from a juvenile ovary to testis during gonadal differentiation is also reflected at the transcriptomic level and that Wnt signaling genes were differentially expressed during this process. We also demonstrated that the transgenic inhibition of Wnt signaling could result in male-biased sex ratios, with a concurrent decrease in *cyp19a1a* expression levels in the gonads of treated fish, indicating the involvement of Wnt signaling in zebrafish gonad differentiation.

MATERIALS AND METHODS

Ethics

Experiments performed at Temasek Life Sciences Laboratory (TLL) were approved by TLL Institutional Animal Care and Use Committee (approval ID: TLL [F]-10-001).

Fish Stocks and Collection of Gonad Samples

Wild-type zebrafish of the AB strain and the transgenic zebrafish strains *Tg(vas)* and *Tg (hsp70I:dkk1-GFP)w32*, from here onwards known as *Tg(dkk)*, were kept at our fish facility under 26°C–28°C and 14L:10D cycle conditions in an AHAB (Aquatic Habitats) recirculation system.

For the microarray experiments, three 32-days-postfertilization (dpf) juvenile ovaries (JO), four 32-dpf juvenile ovotestes (JOT), four 34-dpf JOT, three 35-dpf JO, three 35-dpf JOT, and six 36-dpf JOT gonads were removed using fine forceps from *Tg(vas)* zebrafish with the aid of Egfp visualization through a dissecting fluorescence microscope. The JO had much stronger Egfp signal than the ovotestes (Supplemental Fig. S1). Total RNA was extracted from these samples using TRIzol reagent (Life Technologies). RNA quantity and quality were assessed using agarose gel electrophoresis and a spectrophotometer (Nanodrop).

For the heat-shock experiments, trunk sections (without head and tail sections) of 36-dpf zebrafish from heat-shocked wild-type and heat-shocked transgenic F_1 offspring were collected, because in the absence of *Tg(vas)*, direct gonad collection was impossible.

Microarray Target Labeling, Hybridization, and Statistical Analysis

All microarray studies were carried out using the Gonad Uniclone Microarray system containing 6370 unique cDNA clones derived from zebrafish gonads at adult and differentiating (3, 4, and 5 wk postfertilization) stages (ArrayExpress identification [ID]: E-MEXP-3879) [27]. Target amplification and target labeling were performed as described previously with the following modifications: 5-μg samples of amplified RNA (aRNA) from all samples were labeled with Alexa Fluor 647, whereas a 20-μg common reference sample derived from pooled gonad, brain, kidney and remainder of the body of one male and one female adult zebrafish was labeled with Alexa Fluor 555 [27]. Frequency of incorporation of dye molecules into the targets ranged from 17–45 dye molecules/1000 nucleotides. Hybridization, scanning, data processing, and statistical analysis of the microarrays were performed as described previously [27]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) was used to assign genes that were differentially expressed between the juvenile ovary and juvenile ovotestis to KEGG pathways. The FASTA sequences of expressed sequence tags and nucleotides shown in Supplemental Table S2 were compared using Basic Local Alignment Search Tool to the entire ''for Eukaryotes'' gene data set by using the single-directional best hit (SBH) assignment method [40].

Immunohistochemistry and Histology

Immunohistochemistry was carried out with cryosections of heat-shocked juvenile *Tg(dkk)* testes and ovaries. Tissue sections were first incubated with primary antibody (chicken polyclonal anti-green fluorescent protein [GFP] antibody; Abcam) in blocking solution overnight at 4°C, followed by a 1-h incubation at room temperature in secondary antibody (Alexa Fluor 488 goat anti-chicken antibody [Invitrogen]) and 4′,6-diamidino-2-phenylindole (Sigma-Aldrich).

Histological examination was carried out using gonads from adult zebrafish that were fixed in 10% formalin overnight at room temperature. After being dehydrated, samples were embedded in plastic resin (HistoResin; Leica Biosystems). Serial cross-sections of 5 μm were cut by microtome (Leica Biosystems), dried on slides at 42°C overnight, stained with hematoxylin and eosin, and then mounted in Permount (Thermo Fisher Scientific).

Real-Time RT-PCR

We carried out real-time RT-PCR using the BioMark HD system (Fluidigm Corp). Total RNA from zebrafish gonads (15–30 ng) or trunk sections (800 ng) were reversed transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories) following the manufacturer's instructions. The cDNA was subsequently used for specific target amplification by using TaqMan PreAmp Master Mix (product no. 4361128; Applied Biosystems) and loaded onto dynamic array integrated fluidic circuits (IFC; Fluidigm) according to EvaGreen DNA binding dye protocols (Fluidigm). The primer sequences used are listed in Supplemental Table S2.

Triplicates were analyzed for each biological sample. For the validation of microarray results, we used one 96.96 dynamic array IFC to analyze JO collected from five *Tg(vas)* zebrafish and juvenile ovotestes collected from five other *Tg(vas)* zebrafish at 35 dpf. For the heat-shock experiments, we used three 48.48 dynamic array IFC to analyze trunk segments collected from 13 heat-shocked hemizygous transgenic [*Tg(dkk)*/-] F_1 offspring and 13 heat-
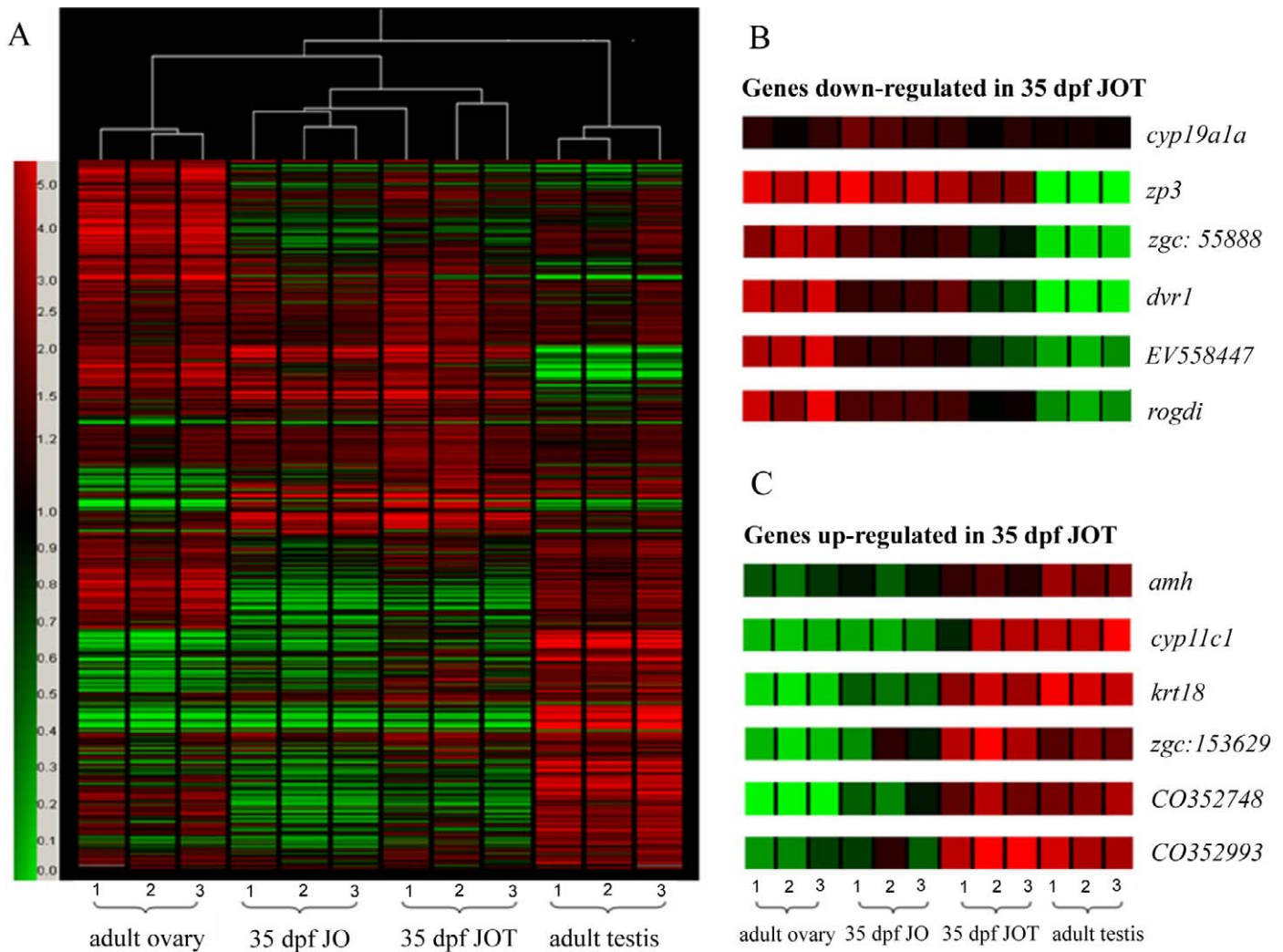
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FIG. 1. Transcriptomic analysis of zebrafish gonads at 35 dpf confirmed a variable gonadal transformation process in future males. Individuals transgenic to *Tg(vas)* were sorted according to their gonadal Egfp signals. Gonadal total RNAs were isolated from three juvenile ovaries (JO) and three juvenile ovotestes (JOT). RNA samples were labeled and subjected to microarray-based analysis using a homemade 6.3-K cDNA array and adult gonadal samples as references. **A**) Hierarchical cluster analyses of 35-dpf JO and JOT samples with adult ovaries and testes are shown. Each column represents the gonad sample collected from a single individual, and each row represents genes probed by the microarray. Genes in red were highly expressed, whereas genes in green showed low expression level. **B**) Selected genes were down-regulated in 35-dpf JOT samples. **C**) Selected genes were up-regulated in 35-dpf JOT samples.

shocked wild-type F_1 individuals. Three samples (adult ovary, juvenile ovary, and juvenile ovotestes) were used as interplate controls.

We included *18S*, *actb1*, *eef1a1l1*, *GAPDH*, *rpl13*, and *tbp* for the qPCR experiments as potential endogenous reference genes, using geNorm [41]. Of these, *rpl13*, *eef1a1l1*, and *actb1* had the highest gene expression stability and were used as reference genes for the validation of microarray results. For the heat-shock experiments, we used *rpl13*, *eef1a1l1*, and *GAPDH* as reference genes.

Chemical Treatments

IWR-1-endo [42] was used as the treatment to inhibit Wnt signaling during the zebrafish gonad differentiation period from 15–40 dpf. IWR-1-endo was dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution. For the treatment groups, 0.2 ml of the 10 mM stock was added to 1 L of egg water to make 2 µM of IWR-1-endo. An equal volume of DMSO was added to the control group. The egg water with DMSO or IWR1-endo was changed daily.

Heat-Shock Experiments

A single hemizygous transgenic [*Tg(dkk)*/-] zebrafish was crossed with a single wild-type AB strain partner. The resulting offspring, consisting of approximately 50% wild-type and 50% hemizygous transgenic [*Tg(dkk)*/-] F_1 siblings, were reared together. After approximately 2 weeks, the larvae were

transferred to a wire gauze cage measuring 15 cm × 10 cm × 5 cm and kept within an 800-ml water tank.

At 20 dpf, all the F_1 fish were heat shocked for 2 h by transferring the wire gauze cage containing the fish from the original water tank to another 800-ml water tank that had been preheated overnight in an incubator set at 39.0°C. After 2 h, the entire water tank together with the wire gauze cage and fish were transferred to another incubator set at 28.5°C for gradual cooling to normal temperature. This process was repeated every day until 60 dpf, including and well beyond the period when zebrafish gonad differentiation is known to occur normally [9].

At the end of the heat-shock treatment, the fish were transferred to an AHAB recirculation systems and subsequently reared together until approximately 70–80 dpf, at which point they were sorted according to size to prevent stunting. At approximately 100–120 dpf, the fish were sorted according to the phenotype presented by the dorsal fin and then sexed by visual analysis of the dissected gonads.
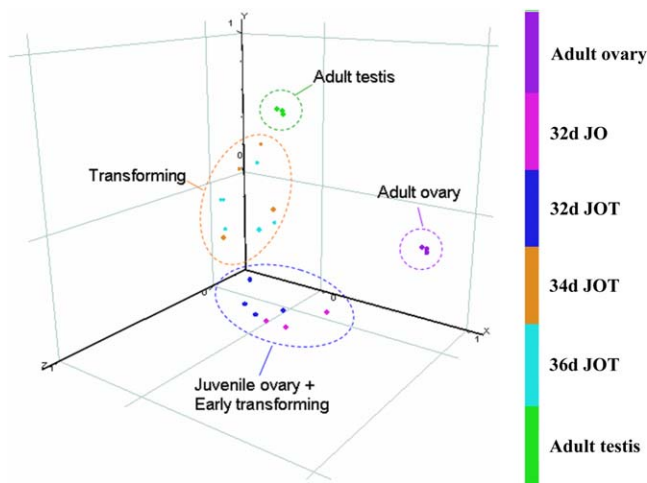
FIG. 2. The transcriptome of transforming zebrafish gonad undergoes transition from an ovary-like to a testis-like organ. Principal component analysis plot shows the transcriptomes of the adult testes were tightly clustered and distinct from the adult ovaries, which similarly formed a tight group. On the other hand, the transcriptomes of JO and JOT were scattered widely, indicating the wide variation in transcriptome profiles. The 32-dpf JO and early transforming ovotestes (32 dpf JOT) were scattered nearer to the adult ovaries and farther from the adult testes, whereas the transforming ovotestis obtained from older juveniles (34 and 36 dpf) clustered closer to the adult testes. This indicated that the transcriptomes of JOs and early JOTs were more similar to those of adult ovaries, whereas the late transforming JOTs assumed transcriptome profiles that were more similar to those of adult testes.

RESULTS

Changes During the Juvenile Ovary-to-Testis Transformation of Zebrafish Gonad Are Reflected at the Transcriptomic Level

To understand how juvenile ovary-to-testis transformation in zebrafish is genetically regulated, we first compared the transcriptomes of JO and JOT samples at 35 dpf (Fig. 1) with those of the adult ovaries and adult testis determined in our previous work [27]. While JO samples were individuals with strong gonadal Egfp signal, JOTs were isolated from *Tg(vas)* individuals that showed a decreasing level of reporter expression in the gonad during development, an indication that they were in the early stages of the transformation process leading eventually to maleness [9]. Although in general both the 35-dpf JO and JOT transcriptomes were more similar to that of the adult ovary than the adult testis, expression levels of several ''pro-male'' genes (e.g., *cyp11c1* and *CO352993*) in JOT samples have a tendency to shift toward the testicular level. Both *cyp11c1* and *CO352993* were shown by microarray analysis to be significantly up-regulated in JOT compared to JO samples (see Supplemental Table S3).

In addition, one of the 35-dpf ovotestes (JOT1) clustered together with the 35-dpf ovaries, suggesting that this particular individual was a relatively late transformer compared to the other two JOT individuals (JOT2 and JOT3) (Fig. 1A). It could also be observed that the three JOT samples showed a more varied expression pattern among themselves than the adult testes or adult ovaries (Fig. 1, B and C), indicating that the three individuals were transforming at variable rates.

The above observations were further strengthened by the fact that several genes that showed variations between replicates in the 35-dpf JOT group had been implicated earlier in gonad differentiation (e.g., amh, *cyp11c1*, *cyp19a1a*, and

TABLE 1. Pathways potentially involved in the zebrafish gonad transformation process as identified by the differentially expressed genes detected by microarray analysis.

KEGG pathway	Name	No. of genes
map00140	Steroid hormone biosynthesis	5
map04310	Wnt signaling pathway	6
map0411	Cell cycle	21
map04114	Oocyte meiosis	14
map04914	Progesterone-mediated oocyte maturation	10

zp3) (Fig. 1, B and C). Both the higher expression of pro-female genes (*cyp19a1a* and *zp3*) (Fig. 1B) and the lower transcript levels of pro-male genes (*amh* and *cyp11c1*) (Fig. 1C) in JOT1 than in JOT2 and JOT3 indicated that gonad transformation in the former may be at an earlier stage than in the latter two.

We then further expanded our study by analyzing the transcriptomes of 32-, 34-, and 36-dpf transforming gonads from a different experiment by using the same Gonad Uniclone Microarray (Fig. 2) and again compared their profiles to those of adult gonads [27]. A principal component analysis (Fig. 2) of the array data showed that the transcriptome of the differentiating zebrafish gonad underwent a transition from an adult ovary-like profile to an adult testis-like one. This was revealed by the grouping of the transcriptomes of the early differentiating gonads (32-dpf JO and JOT) close to that of the adult ovaries, whereas the late transformers (34- and 36-dpf JOT) tended to group closer to that of the adult testes.

Genes in Several Pathways, Including Wnt Signaling, Are Differentially Expressed During Gonad Transformation

In order to identify genes that were potentially involved in the gonad transformation process, we combined JO (32 and 35 dpf) and JOT (32, 34, 35, and 36 dpf) samples into two groups. We then compared the transcriptomes of the two groups and identified a total of 2086 genes that were differentially expressed by at least 1.5-fold ($P < 0.01$) between them. Of these, 877 genes were down-regulated in the juvenile ovary compared to the juvenile ovotestis, whereas 1209 were up-regulated (see Supplemental Dataset S1).

We further analyzed the 2086 differentially expressed genes by using the internet-based KEGG KASS (http://www.genome.jp/tools/kaas/) and were able to identify KEGG pathways whose member genes were differentially expressed between juvenile ovary and juvenile ovotestis (Table 1) [40]. Among these were genes involved in cell cycle, oocyte meiosis, progesterone-mediated oocyte maturation, and Wnt signaling. We identified six Wnt signaling pathway genes that were differentially expressed, four of which belonged to the canonical pathway (see Supplemental Fig. S2).

We retested 57 genes from the microarray by real-time RT-PCR with a separate set of gonadal samples collected at 35 dpf. Of these 57 genes, 44 were differentially expressed genes and 13 were nondifferentially expressed genes on the microarray results. Among the 57 genes, 41 were validated as they had the direction of differential expressions or nondifferential expressions similarly indicated by real-time RT-PCR (see Supplemental Table S3).

The 41 validated genes included eight with known sex-related functions and three (*dkk3*, *psen1*, *ctnnbip1*) that were involved in Wnt/beta-catenin signaling (Table 2 shows a selected set of 11 validated genes, and Supplemental Table S3 shows the full list). Of the sex-related genes, pro-female genes

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TABLE 2. Genes with reproductive function and Wnt-related genes that were differentially expressed between JOT and JO during zebrafish gonad transformation.

Gene symbol*	GenBank accession no.	Fold-change (JOT/JO)[†]	
		Microarray results	qPCR results
Genes with known sex-related functions			
cyp11c1	NM_001080204.1	12.1	69.9
star	NM_131663.1	3.6	36.7
esr2b	NM_174862.3	4.5	9.6
ts1	EF554575.2	1.9	3.7
dmrt1	NM_205628.1	2.6	2.9
sycp3	NM_001040350.1	2.0	2.4
zp2	NM_131330.1	−5.1	−5.8
zp3	NM_131331.1	−8.2	−7.4
Genes involved in Wnt signaling			
dkk3	NM_001089545.1	2.4	5.1
psen1	NM_131024.1	−1.9	−1.6
ctnnbip1	NM_131594.1	−1.7	−1.7

* See Supplemental Table S1 for full gene names.
[†] Conditions for differential expression, where microarray results showed ≥1.5-fold difference ($P < 0.01$); and real-time RT-PCR showed ≥1.5-fold difference ($P < 0.05$).

(e.g., zp2 and zp3) showed higher expression levels, whereas pro-male genes (e.g., dmrt1, sycp3, and cyp11c1) had lower expression levels in JO than in JOT samples.

Transgenic Inhibition of Canonical Wnt Signaling Pathway Promoted Male Gonad Formation in Zebrafish

In order to provide functional evidence for the involvement of canonical Wnt signaling in zebrafish gonad differentiation, we first tried to use a small-molecule inhibitor of Wnt signaling, IWR-1-endo, which promotes beta-catenin destruction by stabilizing axin, hence down-regulating the Wnt/beta-catenin pathway [42]. The treatment interfered with fin development in all treated groups and down-regulated cyp19a1a expression by 2.02-fold ($P = 0.04$) compared to that in controls in one group that was euthanized at 30 dpf, after 15 days of IWR-1-endo treatment. However, although we observed increased proportion of males in 3 of 7 groups, we did not obtain consistent male bias as a consequence of inhibitor treatment (see Supplemental Table S4).

Subsequently, we used the heat-inducible, transgenic Tg(dkk) zebrafish line to promote ubiquitous expression of the Wnt antagonist dickkopf 1b (dkk1b) [43]. The Dkk1b protein is known to inhibit canonical Wnt signaling by binding to the LRP5/6 coreceptors, preventing downstream signaling [44]. We heat shocked 44-dpf Tg(dkk) zebrafish for 2 h at 39°C and carried out immunohistochemistry analysis against dkk1b-GFP. The result confirmed that the transgene is expressed in the somatic cells of both the juvenile ovary and testis following the heat-shock treatment (see Supplemental Fig. S3).

We crossed hemizygous transgenic [Tg(dkk)/-] zebrafish with wild-type partners in order to obtain F1 progeny containing equal proportions of wild-type and transgenic offspring. To induce dkk1b expression from the transgene, F1 progeny were incubated daily for 2 h at 39°C from 20–60 dpf, including and well beyond the period when zebrafish gonad differentiation is known to occur normally [9].

The transgenic F1 individuals could be easily distinguished from their wild-type siblings via the Egfp expression after heat shock, or by partial deformation of their caudal and dorsal fins (Supplemental Fig. S4). The effect on the caudal and dorsal fins remained permanent even after the termination of heat-shock treatment.

The heat-shock treatment resulted in a significant increase in the proportion of males in the Tg(dkk) F1 progeny compared to their wild-type full siblings (Fig. 3). There was also more mortality in the transgenic F1 progeny as equal numbers of Tg(dkk) and wild-type offspring were expected. However, even if we were to hypothetically increase the number of surviving Tg(dkk) F1 individuals to match that of the wild types, two of
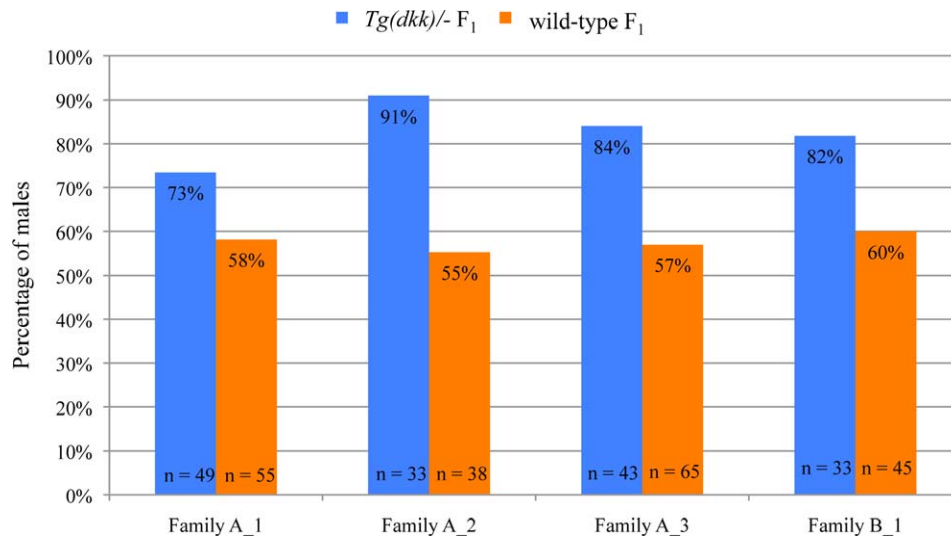

FIG. 3. Heat-induced expression of Dkk1b, a Wnt inhibitor, resulted in a significant increase in males compared to heat-shocked controls. A mixture of hemizygous transgenic [Tg(dkk)/-] individuals and their nontransgenic siblings were heat shocked at 39°C for 2 h on a daily basis between 20 and 60 dpf. At 100–120 dpf, fish were sexed by dissection, and gonads were observed with wet-mount microscopy to confirm sex. Within each family, the heat-shocked transgenic progenies always had a higher percentage of males (15%–36% higher) than their wild-type full siblings. Three batches from different spawnings were tested from family A, and a consistent male-bias was seen in the transgenic progenies following heat shock, whereas a second family (family B; one clutch) also showed the same result. A paired t-test with one-tailed distribution showed that the difference between transgenics and controls was significant ($P = 0.005$).
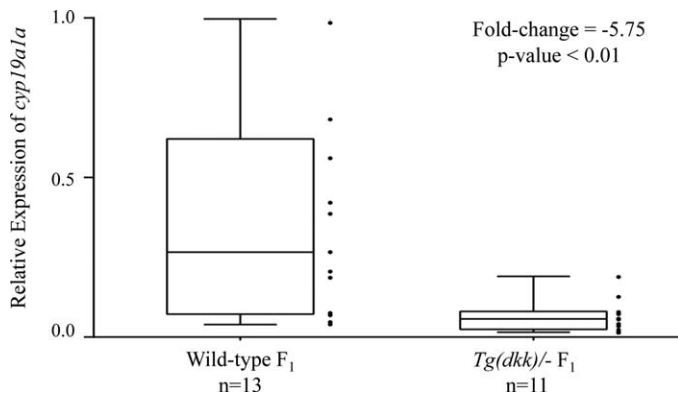
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FIG. 4. The relative expression of gonadal aromatase (*cyp19a1a*) was significantly decreased in heat-shocked transgenics compared to that of their control siblings (also heat treated). Altogether, 13 wild-type F_1 and 11 hemizygous transgenic [*Tg(dkk)/-*] F_1 offspring originating from the same family were tested. These F_1 offspring had undergone the daily heat-shock treatment for 16 days and were killed at 36 dpf for gene expression study. Real-time RT-PCR was performed with trunk segments. The wild-type F_1 samples showed a greater variation in *cyp19a1a* expression than that of their *Tg(dkk)/-* siblings. The whiskers of the box plots indicate the minimum and maximum values.

the families (A1 and A2) would still show male-biased sex ratios. Hence, it is unlikely that the observed male-bias was caused exclusively or even primarily by the preferential mortality among females. This indicates that Wnt signaling plays a role in regulating zebrafish gonad differentiation. On the other hand, we did not observe any significant morphological differences between the gonads of heat-shocked *Tg(dkk)* individuals and those of heat-shocked wild-type individuals at 3.5 mo postfertilization (Supplemental Fig. S5). Furthermore, motile sperm could be observed under the microscope in the testes of both heat-shocked groups. This indicated that overexpression of *dkk1b* during the treatment period did not affect the subsequent functionality of the ovaries and testes of the adults.

To identify the genes that were affected by the transgenic inhibition of Wnt signaling, we quantified the expression levels of 42 genes by real-time RT-PCR in wild-type and transgenic siblings from one family at 36 dpf (see Supplemental Table S5). These siblings comprised a mixture of JO and JOT individuals as they could not be distinguished. Overall, we found that *cyp19a1a* expression was 5.75-fold down-regulated in the transgenic individuals compared to wild-types (Fig. 4). This showed that heat-induced overexpression of *dkk1b* resulted in reduced transcript levels of *cyp19a1a* in the gonads of the transgenic individuals. We also observed decreased variation in the expression of *cyp19a1a* among the transgenics (range: 0.015-0.190) compared to their wild-type siblings (range: 0.040-1.000). This indicates that even the gonads of those transgenics that would eventually be able to resist the "pressure to transform" would have reduced *cyp19a1a* levels compared to their wild-type siblings due to the inhibition by *dkk1b*.

Members of the Wnt signaling pathway, *wnt4a* and *lef1*, were also significantly down-regulated in the heat-shocked *Tg(dkk)* offspring (Table 3). Additionally, *lef1* is known to be a downstream target of canonical Wnt signaling and has been shown to be up-regulated by Wnt/beta-catenin signaling in HEK293 cells and in human colon cancers [45, 46]. Although Wnt/beta-catenin signaling has been shown to inhibit *sox9b* in regenerating zebrafish fins, the down-regulation of *sox9b* in the heat-shocked *Tg(dkk)* offspring was expected as *sox9b*

TABLE 3. Differentially expressed genes between individuals with transgenic *dkk1b* overexpression and controls detected by real-time RT-PCR.

Gene symbol*	GenBank accession no.	P value	Fold-change
Up-regulated genes			
dkk1b	NM_131003.1	0.000	323.7
esr2b	NM_174862.3	0.003	1.4
Down-regulated genes			
cyp19a1a	NM_131154.2	0.006	−5.8
lef1	NM_131426.1	0.008	−2.0
sox9b	NM_131644.1	0.000	−1.6
inhbb	NM_131068.2	0.048	−1.5
wnt4a	NM_001040387.1	0.023	−1.3

* See Supplemental Table S1 for full gene names.

expression is found in the oocytes [11, 47]. The down-regulation of *inhbb* appeared contradictory as *Inhbb* is inhibited by Wnt/beta-catenin signaling in mouse ovaries [48, 49]. However, zebrafish have three inhibin-beta paralogs: *inhbaa*, *inhbab*, and *inhbb*. The regulation of all three paralogs is not fully understood, and there is currently no study that would show the regulation of *inhbb* by the Wnt pathway in zebrafish. Interestingly, *esr2b* was up-regulated in the transgenic F_1 individuals. This corresponded to our microarray and real-time RT-PCR data that showed *esr2b* levels were higher in juvenile ovotestes than in JO (Table 2).

In a separate experiment, we also heat-shocked 35-dpf *Tg(dkk)* zebrafish (which had not been heat-shocked before) to investigate the responsiveness of *cyp19a1a* down-regulation to transgene expression. We found that as early as 4 h post-treatment, *dkk1b* was more than 700-fold up-regulated compared to transgenic *Tg(dkk)* zebrafish, without any heat shock (control). There was also a corresponding 7.2-fold decrease in *cyp19a1a* expression (Fig. 5). Then, at 8 h post-treatment, the magnitude of *dkk1b* up-regulation compared to control was reduced (338-fold up-regulation), and the magnitude of *cyp19a1a* down-regulation was also similarly reduced (4.7-fold down-regulation) (Fig. 5).

DISCUSSION

The path to testis differentiation in zebrafish involves a juvenile ovary-to-testis transformation that all future male individuals must undergo [4–6]. The molecular mechanisms regulating this process are still not fully known (for a review see ref. [28]). In our study, we use a microarray-based approach to provide the first glimpse into the transcriptome of differentiating zebrafish gonads in order to identify components and pathways that could be implicated in this process.

Our microarray analysis of 35-dpf JO and JOTs provided the molecular support to our earlier data showing that the zebrafish gonad transformation process is highly variable, with early and late transformers [9]. The variation in gonadal transformation process among different individuals, as reflected in the histological and reporter expression data, implied that a larger sample size is required for gene expression analysis in such studies to allow for distinguishing between the gonad types at any point in time. A time course microarray dissection of transforming juvenile ovotestis also demonstrated that the transcriptomes of the differentiating male zebrafish gonads progressively undergo a transition from an adult ovary-like one to an adult testis-like one, similar to what we had observed earlier through histological analysis [9].

The role of Wnt/beta-catenin signaling in mammalian sex determination and differentiation is well known, and gene
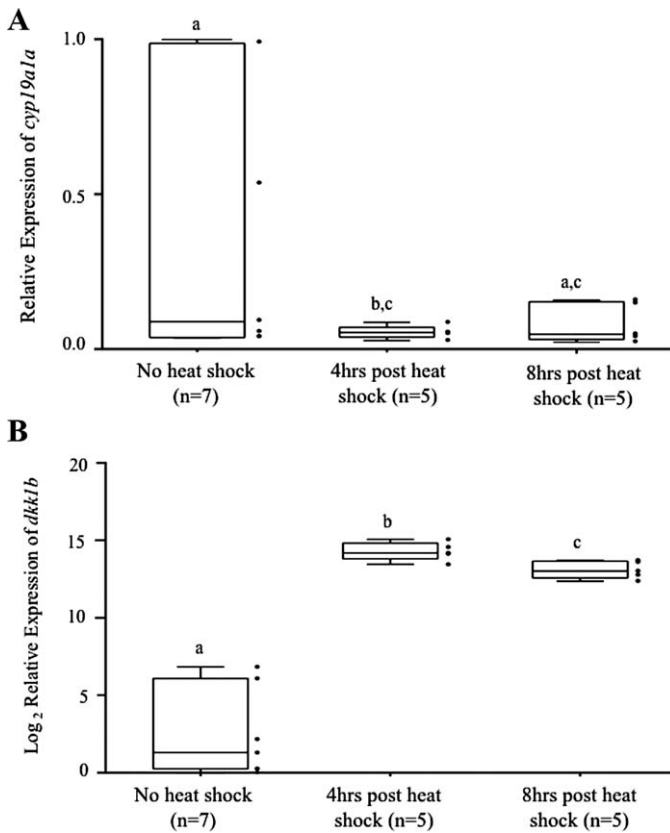
FIG. 5. Effect of heat-shock treatment on expression of *cyp19a1a* (**A**) and *dkk1b* (**B**) in 35-dpf transgenic *Tg(dkk)* zebrafish. As early as 4 h post-treatment, *cyp19a1a* was 7.2-fold down-regulated whereas *dkk1b* was more than 700-fold up-regulated. At 8 h post-treatment, the magnitude of *dkk1b* up-regulation, compared to control, was reduced (338-fold up-regulation), and the magnitude of *cyp19a1a* down-regulation was similarly reduced (4.7-fold down-regulation). Real-time RT-PCR was performed with eviscerated trunks, and *rpl13* and *eef1a1l1* were used as reference genes. a, b, and c indicate $P < 0.05$. The whiskers of the box plots indicate the minimum and maximum values.

expression studies of other teleosts such as the black porgy and rainbow trout have also implicated Wnt signaling in teleost gonad differentiation [30–34, 37, 39, 50]. In this study, we extended the role of Wnt/beta-catenin signaling in gonad differentiation to zebrafish.

Through microarray analysis of the zebrafish gonad transformation process, we identified three members of the Wnt/beta-catenin signaling pathway (*ctnnbip1*, *psen1*, and *dkk3*) that were found to be differentially expressed and which were also validated by real-time RT-PCR. In the Wnt/beta-catenin pathway, beta-catenin (*ctnnb1*) is the key transcriptional coactivator and is degraded by proteosomes in the absence of Wnt ligands, leading to the repression of Wnt target genes [51]. Ctnnbip1 inhibits Wnt/beta-catenin signaling by interfering with beta-catenin binding to the Tcf/Lef family of transcription factors [52–54]. Psen1 also functions as a negative regulator of the signaling pathway by associating with and facilitating the phosphorylation of beta-catenin and hence its subsequent degradation [55–58]. Dkk3 is a member of the Dickkopf family of secreted glycoproteins, whose members Dkk1 and Dkk2 have been shown to bind to the Wnt coreceptors, LRP5/6, and inhibit Wnt/beta-catenin signaling [44, 59]. Nevertheless, Dkk3 has not been shown to interact with LRP5/6 and, like Dkk2, may inhibit or promote Wnt

signaling depending on the specific cell types and corresponding factors involved [60–64].

In the juvenile ovary, compared to the transforming ovotestis, both the microarray and real-time RT-PCR analyses showed that the Wnt/beta-catenin signaling inhibitors *ctnnbip1* and *psen1* were up-regulated. This observation seems to indicate that canonical Wnt signaling was repressed in the juvenile ovary. However, the much larger magnitude of down-regulation of *dkk3* (5.1-fold in real-time RT-PCR) in the juvenile ovary may indicate otherwise, given its unknown effect on Wnt signaling in the zebrafish gonad.

We also tried unsuccessfully to use the *Tg(TOP:dGFP)w25* zebrafish to provide additional proof that Wnt signaling acts as a pro-female process in zebrafish (data not shown). The TOPdGFP construct expresses a destabilized GFP (dGFP) under the control of the beta-catenin-responsive promoter (*lef*). This facilitates the visualization of regions where canonical Wnt signaling is active [65]. However, the dGFP could not be detected either directly or via immunohistochemistry in the developing zebrafish gonads. A possible explanation is that the TOPdGFP construct may not be expressed sufficiently for it to be detected in the gonads.

Functionally, it has been shown in rainbow trout that the use of the Wnt signaling chemical inhibitor IWR-1-endo could result in temporary down-regulation of the expression of *fst* and *cyp19a1a* in the differentiating ovary [38]. Similarly, our initial attempt to inhibit Wnt signaling using IWR-1-endo in zebrafish also resulted in the down-regulation of *cyp19a1a* (*fsta* was not tested). However, we could not achieve consistent male-biased sex ratios by these treatments. Neither were we able to increase the concentration of IWR-1 beyond 2 μM nor extend treatment beyond 40 dpf as both would result in excessive mortality. Thus, a reason for the inconclusive result could be attributed to the insufficient repression of Wnt/beta-catenin signaling and/or the potential reversibility of the transformation process during the gonad differentiation period.

Henceforth, we used a transgenic system that allowed us to repress Wnt/beta-catenin signaling from 20 dpf until 60 dpf without incurring significant mortalities. Although the process of gonad transformation could have already been initiated at 20 dpf, this treatment period allowed us to investigate the role of Wnt signaling in regulating zebrafish gonad transformation [9]. Using *Tg(dkk)* zebrafish, we showed that inhibition of canonical Wnt signaling during the gonad differentiation period could lead to male-biased sex ratios. We also found that the transgenic inhibition of Wnt signaling during this period down-regulated *cyp19a1a* expression significantly. In addition, expression of *lef1*, a Wnt signaling target gene, was also down-regulated.

Given the ubiquitous expression of the *dkk1b-GFP* transgene, there is a possibility that the male-biased sex ratios could be due to the effect of *dkk1b-GFP* on other tissues involved in reproduction (e.g., brain) and that the down-regulation of *cyp19a1a* is hence a consequence of the male-biased sex ratio. Nevertheless, we have also shown that *cyp19a1a* down-regulation is responsive to changes in levels of *dkk1b-GFP* transgene expression and that the down-regulation could be detected after the very first heat shock. Immunohistochemical analysis has also confirmed that *dkk1b-GFP* was expressed in the somatic cells of the developing gonads upon heat shock, which coincides with the known localization of *cyp19a1a* [20]. In addition, through computational prediction using PRO-MO (open web-based software), LEF-1 and TCF-4 binding sites could be found at the upstream region of zebrafish *cyp19a1a* [66, 67]. The mammalian *CYP19A1* proximal region could also be amplified from DNA obtained from chromatin

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immunoprecipitation assays performed with beta-catenin antibodies [29]. These assays indicated that *cyp19a1a* is a direct target of canonical Wnt signaling. Furthermore, although chemical inhibition of the canonical Wnt pathway using IWR-1 did not conclusively change the sex ratios in all treated groups, we also found that *cyp19a1a* was 2.02-fold down-regulated. In another study on the rainbow trout, the inhibition of Wnt pathway for 5 days using IWR-1 during ovarian differentiation similarly resulted in down-regulation of *cyp19a1a* [38].

Taken together, all the evidence strongly suggest that the decreased *cyp19a1a* expression is largely a direct consequence of the inhibition of Wnt signaling resulting from *dkk1b-GFP* transgene expression. Hence, it is likely that the male-biased sex ratios are due to the decreased *cyp19a1a* expression.

We have provided evidence that Wnt/beta-catenin signaling is involved in teleost gonad differentiation and the first functional proof that manipulation of the pathway during that period resulted in altered sex ratios that lasted to adulthood. The inhibition of canonical Wnt signaling also resulted in partial deformation of the caudal and dorsal fins. This observation thus supports existing studies showing that Wnt signaling is required for vertebrate fin development [68, 69].

We believe Wnt/beta-catenin signaling could also potentially affect the sex determination and differentiation process at even earlier developmental stages, for example, by affecting the number of primordial germ cells (PGCs). The presence of germ cells is required for the development of the zebrafish ovary, and an empty somatic gonadal shell forms in knock-down morphants injected with anti-*dnd* morpholino [70, 71]. In the medaka and stickleback, higher PGC numbers can lead to ovarian differentiation [72, 73]. In mouse, Wnt4 has been shown to maintain female germ cell survival by inhibiting *Inhbb* expression via beta-catenin in the somatic cells [48]. Aberrant activation of Wnt/beta-catenin signaling in mammalian PGCs is also deleterious for their normal development [74].

Wnt/beta-catenin signaling may also have complex relationships with other pathways to form differentiated gonads, as demonstrated in mammalian species. In human cell lines, TP53 was shown to be able to repress the activity of canonical Wnt signaling pathway through up-regulation of *DKK1* expression [75]. On the other hand, depletion of beta-catenin levels in HeLa cells resulted in increased activity of several apoptotic pathways, including the one regulated by TP53 [76]. In addition, expression of the aromatase gene (*CYP19*) is regulated not only by beta-catenin but also by Tp53 [29, 77]. Beta-catenin is also found to exert regulatory effects not only on *AMH* but on its receptor too [78].

Earlier studies have already identified the involvement of several proteins and pathways in zebrafish gonad differentiation, for example, *nr5a* has been linked to roles in steroidogenesis; *amh* and *cyp19a1a* have shown sexually dimorphic expression in zebrafish juveniles; and *fancl* and *tp53*, where Tp53-mediated germ-cell apoptosis in zebrafish *fancl* mutant resulted in female-to-male sex reversal [11, 20, 28, 79]. Recent studies have also shown that higher temperatures during gonad differentiation phase can result in male-biased sex ratios or sex reversal in genetic zebrafish females [80, 81]. High temperatures have been shown to result in male bias in European seabass (*Dicentrarchus labrax*) due to increased DNA methylation of aromatase promoter or due to increased cortisol, as shown in pejerrey (*Odontesthes bonariensis*), Japanese flounder (*Paralichthys olivaceus*), and Japanese medaka (*Oryzias latipes*) [82–85].

Other pathways with proven or proposed connection to zebrafish gonad differentiation include the bone morphogenetic proteins (BMP), which have been shown to regulate germ cell differentiation and oocyte maturation in zebrafish [86, 87]. It was also recently shown that during induced inflammatory response in developing zebrafish, NF-kappaB-induced anti-apoptotic effects resulted in inhibition of juvenile ovary-to-testis transformation [88]. Our results add canonical Wnt signaling to this growing list.

In conclusion, we have provided the first evidence that canonical Wnt signaling regulates zebrafish gonad differentiation. The inhibition of the Wnt/beta-catenin pathway by transgenic *dkk1b* expression resulted in decreased *cyp19a1a* expression and male-biased sex ratios. Hence, canonical Wnt signaling is a "pro-female" pathway whose down-regulation is required for testis differentiation in zebrafish.

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