Activation of NF-κB Protein Prevents the Transition from Juvenile Ovary to Testis and Promotes Ovarian Development in Zebrafish 

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Background: NF-κB is a key regulator of anti-apoptotic processes and plays a role in gonad formation in mammals. Results: NF-κB activation leads to female-biased sex differentiation in zebrafish. Conclusion: Anti-apoptotic signaling during the juvenile ovarian stage is needed for the maintenance of oocytes in zebrafish. Significance: Unraveling the regulation of apoptotic processes during gonadal transformation will facilitate understanding the molecular mechanism of zebrafish sex differentiation.

Testis differentiation in zebrafish involves juvenile ovary to testis transformation initiated by an apoptotic wave. The molecular regulation of this transformation process is not fully understood. NF-κB is activated at an early stage of development and has been shown to interact with steroidogenic factor-1 in mammals, leading to the suppression of anti-Müllerian hormone (Amh) gene expression. Because steroidogenic factor-1 and Amh are important for testis development, NF-κB-mediated induction of anti-apoptotic genes could, therefore, also play a role in zebrafish gonad differentiation. The aim of this study was to examine the potential role of NF-κB in zebrafish gonad differentiation. Exposure of juvenile zebrafish to heat-killed Escherichia coli activated the NF-κB pathways and resulted in an increased ratio of females from 30 to 85%. Microarray and quantitative real-time-PCR analysis of gonads showed elevated expression of NF-κB-regulated genes. To confirm the involvement of NF-κB-induced anti-apoptotic effects, zebrafish were treated with sodium deoxycholate, a known inducer of NF-κB or NF-κB activation inhibitor (NAI). Sodium deoxycholate treatment mimicked the effect of heat-killed bacteria and resulted in an increased proportion of females from 25 to 45%, whereas the inhibition of NF-κB using NAI resulted in a decrease in females from 45 to 20%. This study provides proof for an essential role of NF-κB in gonadal differentiation of zebrafish and represents an important step toward the complete understanding of the complicated process of sex differentiation in this species and possibly other cyprinid teleosts as well.

The molecular mechanism of sex determination is unknown in zebrafish (Danio rerio). Earlier, neither cytogenetic studies (1, 2) nor comparative analysis of recombination rates between the two sexes (3) nor an array-based genome screen (4) have led to the identification of sex chromosomes. Recently, analysis of genetic linkage maps have revealed the presence of regions associated with sex determination on four chromosomes (5, 6), indicating polygenic sex determination in zebrafish (4–7).

On the other hand, a number of candidate genes with potential role in sexual development have been identified, including Sry-related HMG box gene 9 (sox9), anti-Müllerian hormone (amh), cytochrome P450, family 19, subfamily A, polypeptide 1a and b (cyp19a1a and b), nuclear receptor subfamily 5, group A (nr5a1 a and b), forkhead box protein L2 (foxl2), dead end (dnd), and factor in germ line a (figa) (7–10). Nr5a1 or steroidogenic factor-1 controls the expression of sox9, cyp19a1a, and amh (7, 10). In mammals, SOX9 is involved in the regulation of Amh (4) (11). In mice, homozygous mutation of Sox9 binding site in Amh leads to lack of its transcription and development of pseudohermaphrodites (12), whereas mutations in the sox9 gene result in sex reversal in XY campomelic dysplasia patients (13).

Zebrafish gonadal differentiation starts with the formation of a juvenile ovarian structure that either matures into adult ovaries or transforms into testes (14, 15). The testis transformation process has been suggested to depend on apoptosis (16). This...
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**EXPERIMENTAL PROCEDURES**

**Breeding**—Adult zebrafish were maintained in a recirculating system (Aquaneering) with a 14-h light/10-h dark cycle. The fish were fed twice a day with newly hatched *Artemia salina* nauplii and commercial flake food (*Tetrarubin*). The male and female brooders were kept in separate aquaria at 26–27 °C, and they were allowed to breed once a week. The fish handling procedures were approved by the Swedish Ethical Committee in Linköping (Permit 32-10).

**Preparation of Heat-killed Bacteria**—*E. coli* MG1655 was grown on Luria-Bertani (LB) agar and incubated at 37 °C overnight. One colony was inoculated into 10 ml of LB broth and incubated on a shaker (200 rpm) at 37 °C overnight. The bacteria were then centrifuged and washed with 5 ml of phosphate-buffered saline (Sigma). The bacterial pellet was resuspended in 2 ml of PBS and killed by heating to 70 °C for 1 h. To ensure 100% bacteria death, 10 μl of the heat-killed suspension was plated and incubated overnight at 37 °C.

**NFκB-pGL4 Plasmid Construction**—The pGL4 plasmid with promoter-less Luciferase gene and neomycin selection marker was purchased from Promega, and the NFκB cis-element insert was obtained from commercially available pNFκB-Luc plasmid (BD Biosciences). The pGL4 luciferase plasmid and the pNFκB-Luc plasmid were cut with HindIII and KpnI (Fermentas) and gel-purified by using Wizard SV Gel and PCR Clean-Up System (Promega). The NFκB cis-element and the pGL4 luciferase plasmid were ligated using T4 DNA ligase (Fermentas).

**Cell Culture and Development of Stably Transfected Cells**—ZFL cells were maintained at 28 °C and 3% CO2 in a humidified incubator in a complex media containing 50% L15 (Invitrogen), 35% DMEM-high glucose (PAA Laboratories), and 15% Ham’s F-12 (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone), 15 mm Hepes buffer (Lonzza), 0.15 g/liter sodium bicarbonate (Biochrom AG), 50 ng/ml mouse EGF, and 0.01 mg/ml bovine insulin. For stable transfection, ZFL cells were plated in 24-well plates (BD Biosciences) (80,000 cells/well). The next day the cells were transfected with the NFκB-pGL4 plasmid (500 ng/well) using Lipofectamine 2000 (Invitrogen). After 48 h the cells were treated with 1 mg/ml G418 antibiotic (Duchefa Biochemie). Antibiotic-resistant colonies were selected, and the cells were screened for NFκB activity using the Luciferase assay kit (Promega).

**ZFL Cell-based Experiments**—Before exposure the stably transfected ZFL cells (nZFL) were plated in 24-well plates (80,000 cells/well) and incubated for 16–18 h at 28 °C and 3% CO2. Wild type ZFL cells were plated in 12-well plates (BD Biosciences) (2 × 105 cells/well) and incubated for 16–18 h at 28 °C and 3% CO2. The experiments were started by adding heat-killed bacteria at 5 × 106, 1 × 107, 5 × 107, or 1 × 108 colony forming units (cfu)/ml, sodium deoxycholate (DOC) at 200 or 300 μM (Sigma), or 40 nM NFκB activation inhibitor (NAI) (Calbiochem) prepared in fresh media to the cells. The cells were incubated for 12 h (Luciferase assays) or 24 h (quantitative RT-PCR (qRT-PCR) assays) at 28 °C and 3% CO2. Detection of luciferase activity was performed using Luciferase assay kit, whereas cells used for qRT-PCR analysis were lysed with TRI-Reagent (Sigma).
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Maintenance and Exposure of Juvenile Zebrafish—In the evening, pairs of adult zebrafish breeders were transferred to mating containers (Aquateering). The next morning fertilized embryos were collected and divided in groups of 50 in 115-mm diameter crystallization dishes containing 100 ml of water and maintained at 28 °C. At 4 dpf, the juveniles were transferred to the circulating system. The water flow was adjusted to 20–30 drops/min, 14-h light/10-h dark cycle and fed twice with newly hatched *A. salina* nauplii and commercial flake food (Larval AP 100).

At the time of exposure, juvenile zebrafish were transferred back to crystallization dishes of 115-mm diameter with 100 ml of water (15 or 20 dpf) or 1-liter containers (180/100 mm) with 500 ml of water (41 or 70 dpf) with 20 individuals in each container. The juveniles were exposed to heat-killed *E. coli* (1 × 10⁶, 5 × 10⁶, 1 × 10⁷, 5 × 10⁷, 5 × 10⁸), DOC (200 μM), or NAI (20 nM). The fish were fed twice a day, and 50% of the water was changed every third day and heat-killed *E. coli*, DOC, or NAI was replenished to maintain the original concentrations. The water quality was monitored over the course of studies. The temperature was maintained at ±0.2 °C. Water pH averaged 7.3 ± 0.2, and salinity averaged 0.08 ± 0.01%. The nitrite level averaged 0.04 ± 0.05 mg/liter, whereas nitrate and ammonia level remained undetectable. Survival through the experimental exposures averaged 85 ± 5%. There was no effect of the different treatments on these parameters.

The exposures were terminated at different time points depending on the experiment and assay. For sample collection at 21 dpf, zebrafish juveniles were sacrificed by snap-freezing in liquid nitrogen and stored at −80 °C until further use. Juveniles of 35, 42, and 71 dpf of age were dissected under a stereomicroscope, and their gonads were isolated, snap-frozen in liquid nitrogen, and stored at −80 °C until further use. For sex ratio determination, the fishes were transferred back to the circulating system at the end of exposure (at 35 dpf), and the sex ratios were determined after dissection and microscopic observation of the gonads at 70 dpf.

RNA Extraction and qRT-PCR—Cells, juvenile zebrafish, and isolated gonads were homogenized in 200 μl Trizol Reagent (Sigma) and RNA was isolated according to manufacturer’s instruction. cDNA synthesis was performed using qScript cDNA synthesis kit (Quanta Biosciences). Primers were designed for listed genes (supplemental Table S1). SYBR Green (Quanta) was used to determine the expression levels of all genes. Thermocycling conditions for SYBR Green consisted of a denaturation step for 5 min at 95 °C followed by 40 cycles of 95 °C for 2 s and 60 °C for 30 s. Data analysis was performed using standard curve method and ΔΔCt method (32).

Microarray-based Transcriptomics of Gonadal Samples—The gonads of 35 dpf juvenile zebrafish were isolated, and RNA was extracted from individual samples using TRIzol reagent according to manufacturer’s instruction. Amplification and labeling of total RNA was performed as described previously (33) with the following modifications: 5 μg of antisense RNA was labeled with Alexa Fluor 647, whereas 20 μg of a common reference consisting of pooled antisense RNA from one individual in each treatment group (a total of 4 individuals) was labeled with Alexa Fluor 555. Microarray printing, hybridization, scanning, and data processing were performed as described previously (33). A total of 11 samples were analyzed: 6 control individuals, 3 individuals from the intermediate treatment, and 2 individuals from the highest treatment dose.

Analysis of Microarray Data—Raw expression values obtained from the GenePix Pro 6.0 software were background-corrected and normalized using R 2.9.0 (34) and the BioC limma 2.18.2 package (35, 36). Briefly, array intensity values were background corrected using the normexp method (37) followed by print-tip loess normalization within arrays and
quantile normalization between arrays. Differential expression was calculated by fitting a linear model to a group means parameterization. Multiple testing was corrected by controlling the false discovery rate (38). Principal component analysis of the normalized expression values was conducted in R using the prcomp function. The gene symbol and putative function for each EST sequence was obtained based on the best zebrafish BLAST hit against the RefSeq RNA database (as of April 12, 2011) unless indicated otherwise. The full set of microarray expression data has been deposited in ArrayExpress under the accession number E-MEXP-3249.

**Testis Culture**—Adult zebrafish were euthanized, and their testes were isolated. The testes were sterilized with 0.5% v/v commercial bleach in PBS (Sigma) for 2 min and washed 3 times in PBS. The testes were transferred to 24-well plate (BD Biosciences) and maintained at 28 °C and 3% CO2 in a humidified incubator in ZFL cell media containing antibiotic-antimycotic solution (Invitrogen). The explants were cultured in parallel and exposed to heat-killed bacteria (5 × 10⁷ cfu/ml) for 24 h.

**Western Blot Analysis**—The ovary samples from 71 dpf individuals were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8) containing protease inhibitor mixture (Sigma). Protein content was quantified using Bradford reagent (Bio-Rad), and the immunoreactive complex was detected by Super Signal West Pico chemiluminescent substrate (Thermo Scientific). The bands were quantified using a two-tailed non-paired Student’s t test for two-group comparison or one-way analysis of variance followed by multiple testing was corrected by controlling the false discovery rate (38). Principal component analysis of the normalized expression values was conducted in R using the prcomp function. The gene symbol and putative function for each EST sequence was obtained based on the best zebrafish BLAST hit against the RefSeq RNA database (as of April 12, 2011) unless indicated otherwise. The full set of microarray expression data has been deposited in ArrayExpress under the accession number E-MEXP-3249.

**Results**

**Heat-killed Escherichia coli Activate Zebrafish NF-κB Resulting in Female-biased Sex Ratios**—ZFL cells stably transfected with the NF-κB-pGL4 vector (nZFL) were exposed to heat-killed E. coli to determine whether this would result in activation of NF-κB. A dose-dependent activation of NF-κB was observed after exposure of nZFL cells to heat-killed E. coli (Fig. 1A). The qPCR analysis also showed up-regulation of inflammatory genes like p65, p50, prostaglandin-endoperoxide synthase 2α (ptgs2α), interleukin 8 (i88), and tumor necrosis factor a (tnfa) in response to heat-killed E. coli after exposure of ZFL cells (Fig. 1, B–F).

Zebrafish juveniles are known to enter a juvenile ovary stage between 14 and 28 dpf (14, 15). This transition was indicated by the elevated levels of female-specific zona pellucida (zp2) gene at 19 dpf (Fig. 2A). Based on this information, all the exposures were started before or at the time of entry into the juvenile ovary stage.

Exposure to heat-killed bacteria from 15 to 21 dpf resulted in dose-dependent up-regulation of the zp2 gene expression and showed a bell-shaped curve with no effect at the highest dose (Fig. 2B). Next, zebrafish juveniles were exposed to 1 × 10⁷ cfu/ml heat-killed bacteria for 24 h at 20 dpf to determine
whether this would result in in vivo up-regulation of NF-κB. The transcript level of p65 was significantly up-regulated, whereas sox9α and amh expression were down-regulated (Fig. 2, C–E). This demonstrates that heat-killed bacteria induce the NF-κB pathway, similar to what is reported in mammals (28), NF-κB also down-regulates amh expression in zebrafish juveniles. To confirm if NF-κB activation occurred in the gonads, juveniles at 41 dpf were exposed to heat-killed bacteria for 24 h, and the gonads were excised. qRT-PCR analysis showed significant up-regulation of gonadal p65 expression after the treatment (Fig. 2F). Together, these results demonstrate that treatment with heat-killed E. coli led to activation of NF-κB and induction of female-specific gene expression.

To determine whether treatment with heat-killed bacteria would lead to alterations in sex ratio, zebrafish juveniles were exposed to three concentrations of heat killed bacteria (1 \times 10^6, 5 \times 10^6 or 1 \times 10^7 cfu/ml) during the critical period of sex differentiation (15–35 dpf). The sex ratio was determined at 70 dpf and demonstrated an increase in the proportion of females from 30 to 85% (Fig. 2G). These results indicated that activation of NF-κB and the inflammatory signaling pathways interfere with testis formation in zebrafish.

Microarray-based Transcriptomic Analysis Indicate the Involvement of NF-κB Pathway in the Feminization Process—A custom-printed cDNA array comprising 6370 unique gonad-derived clones (33) was used to assess changes in the gene expression landscape of juvenile zebrafish gonads in response to two different concentrations of heat-killed E. coli (5 \times 10^6 and 1 \times 10^7 cfu/ml) between 15 and 35 dpf. Principal component analysis of the normalized array expression values illustrated that exposure to heat-killed bacteria defined the main treatment effect seen in the expression data (Fig. 3A). Each
treatment group formed a tight cluster indicating no outliers among the individual transcriptomes analyzed. The results showed that individuals treated with an intermediate dose of heat-killed bacteria clustered more tightly with control females (and further apart from the control males) than the high dose group, and this may be due to the bell-shaped dose response curve observed with heat-killed bacteria (Fig. 2B). Sexually dimorphic expression of a selected set of genes between the control males and females is shown in the form of a heatmap (Fig. 3B; -fold change ≥q value <0.05). Comparative analysis of the sex-specific gene expression in the treated groups revealed a striking similarity between the gonadal transcriptomic profiles of those treated with the intermediate dose and the control females, highlighting that treatment with heat-killed *E. coli*-enhanced female gonad differentiation and/or inhibited male gonad differentiation (supplemental Table S2).

### TABLE 1

| Gene symbol | I vs. CM | Gene name | RefSeq ID |
|-------------|----------|-----------|-----------|
| sinup       | 6.38572  | Sia-z-activating nuclear protein | NM_197937 |
| qui1a       | 5.77982  | Trisphosphatase isomerase 1a | NM_153667 |
| stau2       | 5.27945  | Stau, RNA-binding protein, homolog 2 (Drosophila) | NM_209025 |
| zp3         | 5.13024  | Zona pellucida glycoprotein 3 | NM_131331 |
| zp2,2       | 5.508029 | Zona pellucida glycoprotein 2.2 | NM_131827 |
| cldnb       | 5.502843 | Claudin g | NM_180965 |
| surbp       | 5.379775 | Small nuclear ribonucleoprotein polypeptides B and B1 | NM_205667 |
| scg5        | 5.368851 | Secretogranin V | NM_200726 |
| mid1ip1     | 5.338822 | MIDI-interacting protein 1 | NM_213439 |
| bik5b       | 5.314676 | Baculoviral IAP repeat-containing 5B | NM_215195 |
| met2        | 5.266296 | Metallothionein 2 | NM_00131053 |
| cycl1       | 5.200272 | Cyclin A1 | NM_212818 |
| ccdn        | 5.179989 | Cyclin E | NM_130995 |
| slc16a3     | 5.150553 | Solute carrier family 16 member 16 | NM_212708 |
| retat1      | 5.095099 | Retinol dehydrogenase 1 (all-trans-retinol 13,14-reductase)-like | NM_001000400 |
| cca1        | 5.044201 | Cyclin A1 | NM_212818 |
| zp2         | 4.998241 | Zona pellucida glycoprotein 2 | NM_131330 |
| cldnd       | 4.871266 | Claudin d | NM_180964 |
| lph         | 4.835238 | Thy-1 cell surface antigen | NM_190865 |
| zp2l1       | 4.824493 | Zona pellucida glycoprotein 2, like-1 | NM_00105104 |
| zp2,1       | 4.691868 | Zona pellucida glycoprotein 2.2 | NM_131827 |
| zar1        | 4.598525 | Zygote arrest 1 | NM_194381 |
| col1a2      | −6.30784 | Collagen, type I, α2 | NM_182968 |
| col1a3      | −5.40109 | Collagen, type I, α1b | NM_182968 |
| tpa5        | −5.1099  | Carboxypeptidase A5 | NM_199271 |
| acta2       | −4.77678 | Actin, α2, smooth muscle, aorta | NM_212620 |
| rdh11       | −4.74052 | Dehydrogenase/reductase (SDR family) member 9 | NM_199609 |
| scp2        | −4.74032 | Sterol carrier protein 2 | NM_200865 |
| bbe2        | −4.72664 | Hemoglobin β embryonic-2 | NM_212846 |
| rtn1a       | −4.58485 | Retinol dehydrogenase/reductase (SDR family) member 9 | NM_00102986 |
| gpx4a       | −4.42594 | Glutathione peroxidase 4a | NM_00072722 |
| aldob       | −4.35434 | Aldolase B, fructose-bisphosphate | NM_200865 |
| sparc       | −4.2209  | Secreted acidic cysteine rich glycoprotein | NM_001001942 |
| hhae1       | −4.19334 | Hemoglobin α embryonic-1 | NM_182940 |
| cebp        | −3.81485 | CCAAT/enhancer binding protein (C/EBP), β | NM_131884 |
| tby         | −3.78749 | Tryptophan 2, smooth muscle, aorta | NM_194367 |
| acta2       | −3.71892 | Actin, α2, smooth muscle, aorta | NM_212620 |
| lpl         | −3.69103 | Lipoprotein lipase | NM_131127 |
| bg1         | −3.69101 | B-cell translocation gene 1 | NM_200020 |
| fabp3       | −3.65859 | Fatty acid-binding protein 3, muscle and heart | NM_152961 |
| kre4        | −3.62196 | Keratin 4 | NM_131708 |
| vcf4        | −3.59129 | Replication factor C (activator 1) 4 | NM_214737 |
| gxl1a       | −3.55498 | Glutathione peroxidase 1a | NM_00072721 |
| knapl2      | −3.53922 | Keratin 4 | NM_131759 |

*Fold change values between the intermediate and control male groups (I vs. CM) have been log2-transformed.

The q value represents the false discovery rate corrected p-value derived from the linear fit to a group means parameterization.

### TABLE 2

| Gene name | RefSeq ID |
|-----------|-----------|
| cyp19a1a  | NM_197937 |
| amh       | NM_153667 |
| cyp19a1b  | NM_197937 |
| sox9a     | NM_153667 |
| fox2l     | NM_205667 |
| cyp11c1   | NM_205667 |
| nr5a1b    | NM_205667 |
| nr5a5     | NM_205667 |
| nr5a1a    | NM_205667 |

| Gene name | RefSeq ID |
|-----------|-----------|
| cyp19a1a  | NM_197937 |
| amh       | NM_153667 |
| cyp19a1b  | NM_197937 |
| sox9a     | NM_153667 |
| fox2l     | NM_205667 |
| cyp11c1   | NM_205667 |
| nr5a1b    | NM_205667 |
| nr5a5     | NM_205667 |
| nr5a1a    | NM_205667 |
Venn analysis was performed on the microarray data to identify genes specifically expressed in treated fish in comparison to control males and females (Fig. 3, C and D). Comparison of the gonadal transcriptome profile of the group treated with an intermediate dose with that of control females (I versus CF) showed that only 11 genes differed in their expression pattern between these groups (Fig. 3 C). Two of the genes that showed differential expression were dead (Asp-Glu-Ala-Asp) box poly-peptide 3 (ddx3), a RNA helicase involved in spermatogenesis (39), and chordin (chd), which plays an important role in the specification of dorsal-ventral axis during zebrafish development (40). On the other hand, 1809 genes showed significant differential expression between the gonads of individuals treated with the intermediate dose and those of the control males (I versus CM) (Fig. 3). Similar results were observed with the individuals treated with high dose of heat-killed bacteria, as they have also demonstrated increased female bias (Fig. 2 G). The expression level of 354 genes (supplemental Table S3) differed between individuals treated with intermediate and high dose, as well as between control and treated individuals.

### TABLE 3

Analysis of differential expression of seven genes by qRT-PCR to validate microarray data

| Gene symbol | CF vs. CM | I vs. CM | H vs. CM |
|-------------|-----------|----------|----------|
| bcl2        | -1.34     | 1.53     | -1.31    | 2.41b    | 1.09 | 0.64 |
| dap3        | 1.57−     | 13.04d   | 1.67d    | 21.00d   | 1.17 | 5.49d |
| tgfβ3       | 1.12      | 7.52     | 1.20     | 14.94d   | 1.01 | 3.85d |
| pycard      | −1.55b    | −0.85    | −1.77b   | −0.99    | 1.29 | −0.57 |
| casp3a      | 1.4b      | 42.84d   | 1.42d    | 89.61d   | 1.05 | 10.82b |
| wta1        | −1.01     | 5.91     | −1.05    | 7.54     | 1.07 | 3.22 |
| zp2         | 40.59     | 388.56   | 31.96    | 450.17e   | 17.50 | 102.64b |

* Fold changes in gene expression for CF, I, and H compared to CM were calculated according to the formula, fold change = ((CF, I, or H) − CM)/CM, using gonads from 35-dpf-old juveniles. Statistical significant difference from CM within a gene was determined using two-tailed Student’s t-test.

a, p < 0.05.

b, p < 0.001.

c, p < 0.001.

d, p < 0.01.

**FIGURE 4. Alteration of apoptotic/anti-apoptotic signaling by heat-killed E. coli treatment.** Exposure of juvenile zebrafish to an intermediate concentration (5 × 10⁶ cfu/ml) of heat-killed E. coli was performed between 15 and 35 dpf. Gonads were dissected at 35 dpf. After RNA extraction, microarray was used to evaluate apoptotic and anti-apoptotic gene expression in individual gonads. Several critical apoptotic genes were suppressed (cflar, dedd1, pycard, bnip3l, and smac), whereas anti-apoptotic (bcl210, adaf, ap5l, and mcl1a) and NF-κB-target genes (birc5a and birc5b) were up-regulated.
treated with the high dose of heat-killed *E. coli* and the control females (*H* versus *CF*) compared with 1138 array features differentially expressed between the high dose group and control males (*H* versus *CM*; Fig. 3D). Taken together, this indicated that the gene expression pattern elicited by heat-killed *E. coli* treatment closely resembled that of the control females as opposed to control males.

Genes showing the largest differential expression levels between individuals treated with the intermediate dose of heat-killed *E. coli* and control males (*I* versus *CM*) are listed in Table 1. Several of these genes, including *zpa2*, *zpa3*, and zygote arrest 1 (*zar1*) as well as the anti-apoptotic gene baculoviral IAP repeat-containing 5B (*birc5b*) were shown to be expressed in a female-specific manner in the treated individuals. Analysis of the differences in gene expression between control females and males and between the group treated with intermediate dose of heat-killed *E. coli* and control males showed that the expression level of *amh*, *cyp19a1a* and *b*, and cytochrome P450, family 11, subfamily *C*, polypeptide 1 (*cyp11c1*) did not differ between these groups at 35 dpf (Table 2). However, *zpa2* showed higher expression in females and treated individuals than in males (Table 3), whereas *nrsat1a* showed higher expression in males (Table 2).

**Apoptotic Signaling Pathways Are Involved in the Survival of Oocytes**—To perform a more detailed assessment of the potential role of apoptotic signaling pathways in zebrafish gonad differentiation, we analyzed the expression of those genes involved in these processes that were present on the microarray. The resulting gene expression pattern was consistent with the activation of anti-apoptotic pathways in zebrafish ovary differentiation, we analyzed the expression of those genes involved in these processes that were present on the microarray. The resulting gene expression pattern was consistent with the activation of anti-apoptotic pathways and the inhibition of apoptotic pathways in response to treatment with heat-killed *E. coli* (Fig. 4; Table 4). However, a number of genes involved in apoptosis remained up-regulated in both female and those treated with heat-killed bacteria. Several crucial pro-apoptotic genes, including direct IAP-binding protein with low phosphatidylinositol/second mitochondria-derived activator of caspase (*diablo/smac*), CASP8 and FADD-like apoptosis regulator (*cflar*) were suppressed in treated individuals compared with controls, whereas genes involved in proliferation, including two *birc5* genes (*birc5a* and *birc5b*), were shown to be expressed in a female-specific manner in the treated individuals (Table 4).
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FIGURE 5. DOC activates NF-κB and up-regulates p65 and ptgs2a expression in vitro. nZFL were exposed to DOC (200 μM, 300 μM) for 12 h, and luciferase activity was analyzed to check NF-κB activation (A). ZFL cells were exposed to DOC (200 μM, 300 μM) alone and combination (200 μM DOC) with NAI (40 nM) for 24 h, and total RNA was extracted followed by qRT-PCR analysis of p65 (B and D) and ptgs2a (C and E) expression. The statistically significant difference between groups was determined using Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). n = 4. Error bars represent the mean ± S.D.

- b), were up-regulated (Table 4). To confirm the microarray data, the expression patterns of a selected group of genes, including B-cell leukemia/lymphoma 2 (bcl2), death associated protein 3 (dap3), tp53, pyCARD, Wilms tumor 1a (wt1a), and zp2 were validated by qRT-PCR (Table 3). The obtained results confirmed the transcriptional activity profiles observed by the microarray for these genes.

Treatment with Sodium Deoxycholate Induces NF-κB Pathway Resulting in Female-biased Sex Ratios—To confirm the involvement of NF-κB in gonad differentiation, we exposed zebrafish cells and juveniles to a known NF-κB activator, DOC (41). Exposure of nZFL and ZFL cells to DOC resulted in NF-κB activation (Fig. 5 A) and up-regulation of both p65 and ptgs2a expression (Fig. 5, B and C). Exposure of zebrafish to 200 μM DOC from 15 to 21 dpf also caused an up-regulation of p65 expression (Fig. 6 A), whereas the levels of sox9a, amh, and wt1a transcripts were not significantly reduced (data not shown), suggesting that DOC triggers a weaker response than heat-killed bacteria. However, significantly up-regulated p65 and ptgs2a expression (Fig. 6, B and C) was detected in the gonads of zebrafish juveniles exposed to DOC for 24 h at 41 dpf. The expression of p65 expression was also up-regulated in testis explants in response to heat-killed bacteria and DOC (Fig. 6 D), whereas Western blot analysis of ovary samples showed a decrease in the level of caspase 3a protein (Fig. 6 E), confirming that the treatment inhibited gonadal apoptosis. The sex ratio was determined at 70 dpf and revealed an increased percentage of females from 25 to 45% after exposure to 200 μM DOC from 15 to 35 dpf (Fig. 6 F).

NAI Exposure Down-regulates p65 and ptgs2a Expression Resulting in Male-biased Sex Ratios—NAI is a 6-amino-quinazoline-derived compound that is reported to inhibit NF-κB activation and TNF-α production (42). ZFL cells exposed to 200 μM DOC alone or in combination with 40 nM NAI were analyzed for p65 and ptgs2a expression. NAI treatment significantly down-regulated DOC-induced ptgs2a and p65 gene expression (Fig. 5, D and E). A significant down-regulation of p65 and up-regulation of caspase 8, apoptosis-related cysteine peptidase (casp8), cyclin-dependent kinase inhibitor 1A (p21), and smac (Fig. 7, A–D) expression has been observed in juveniles exposed to 20 nM NAI for 24 h at 20 dpf. Six days of NAI exposure of zebrafish juveniles (15–21 dpf) resulted in decreased expression of ptgs2a, X-linked inhibitor of apoptosis (xiap), and zp2 (Fig. 8, A–C). Exposure of juveniles to NAI for 24 h at 41 dpf resulted in decreased gonadal ptgs2a gene expression (Fig. 8 D), confirming the effect of NF-κB inhibition on gonadal gene expression. Furthermore, exposure of zebrafish juveniles to 20 nM NAI from 15 to 35 dpf resulted in a decreased percentage of females from 45 to 20% (Fig. 8 E). These results further support a role of NF-κB in the maintenance of oocyte development during zebrafish sex differentiation.

DISCUSSION

Although the molecular mechanism of zebrafish sex determination remains unknown, several genes, including sox9, amh, nr5a, and doublesex and mab-3 related transcription factor 1 (dmrt1), have been proposed to be involved in the initial stages of testis and ovary differentiation (7, 8). Testis differentiation in zebrafish starts with a juvenile ovary stage followed by a transformation involving apoptotic loss of oocytes leading to the eventual development of male sex organs (14, 16). Analysis of temporal expression profile of the zp2 gene suggests that the juvenile ovary stage is initiated before 19 dpf, indicating that all of our treatments were started before the initiation of the juve-
nile ovary stage. In this study we show that induction of gonadal inflammation, through treatment with heat-killed *E. coli* or DOC during the critical stage of gonad differentiation, activates NF-κB and anti-apoptotic signaling, resulting in maintenance of oocyte development and inhibition of testis formation. In contrast, inhibition of NF-κB activity by exposure to NAI results in an induction of testis differentiation. These results demonstrate that NF-κB is involved in the regulation of the gonadal differentiation process in zebrafish. Identifying the mechanisms of gonadal differentiation in zebrafish as well as interlinks and alterations by apoptotic and inflammatory (anti-apoptotic) responses are key factors in the understanding of possible endogenous and environmental influences during this delicate process.

NF-κB is a potent inducer of inflammatory responses as well as a modulator of apoptotic signaling (23). Several critical pro-apoptotic genes, including smac, pycard, BCL2/adenovirus E1B interacting protein-like b (*bnip3l*), and Tax1 (human T-cell leukemia virus type I)-binding protein 1b (*tax1bp1*) and cflar, were repressed by exposure to heat-killed *E. coli*. The decrease of caspase 3a protein levels in the ovaries confirms the anti-apoptotic effect of the treatment. The pro-apoptotic activity of these genes is well documented, and their increased expression leads to the activation of several caspases, including *casp3*, *casp8*, and *casp9* (43). Their roles in the activation and translocation of proteins associated with the mitochondrial apoptotic pathway have also been demonstrated (44). Furthermore, the expression of several genes with anti-apoptotic functions associated with...
NF-κB signaling, including tnfrsf1a-associated via death domain (tradd), birc5a, birc5b, apoptosis inhibitor 5 (api5), apoptosis antagonizing transcription factor (aatf), and myeloid cell leukemia sequence 1a (mcl1a), was significantly upregulated in response to induced inflammation. In addition, expression of the NF-κB inhibitory gene nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ab (nfkbiab) was suppressed. These genes have been reported to alter apoptotic signals transduced through the extrinsic TNF receptor (45) and the intrinsic (mitochondrial) (46) pathways. This is in agreement with the observed feminization after exposure to heat-killed bacteria and DOC. To confirm the involvement of NF-κB in this process, we used NAI to block NF-κB activation.

Short term exposure of larvae to NAI down-regulated p65, ptgs2a, and xiap expression, whereas the activity of apoptotic genes, smac, casp8, and p21 was significantly increased. The expression of female-specific zp2 gene was also downregulated, and this correlated with male-biased populations after long term exposure. Down-regulation of zp2 and the male bias sex ratio, therefore, involves NF-κB inhibition and induction of apoptotic signals leading to oocyte apoptosis and initiation of testis differentiation. It was interesting to note that gonadal ptgs2a was up-regulated in DOC-treated individuals and down-regulated in NAI-treated individuals. At present, data linking prostaglandins to a direct role in sex differentiation in zebrafish are lacking. Therefore, further studies are clearly needed to elucidate the role of cyclooxygenase-2 and prostaglandins in these processes. There have been indications of the involvement of nr5a genes in zebrafish sex determination and gonad differentiation (8). The induction of Amh by Sox9, via steroidogenic factor-1, is well documented in mammals (47), but the molecular basis for gonad differentiation in zebrafish remains unclear. In this study only nr5a1a expression was found to be up-regulated in male fish at 35 dpf, whereas we did not observe any differential expression pattern for cyp19a1a, cyp19a1b, or cyp11c1 in either control fish or after exposure to heat-killed E. coli from 15 to 35 dpf. This suggests that altered 17β-estradiol and 11-ketotestosterone levels are a consequence of gonad differentiation. The recent discovery of sex-associated genomic regions on chromosomes 3, 4, 5, and 16 (5, 6) makes it unlikely that zebrafish would have a classical sex determination system. Instead, the interplay between different loci may be instrumental to the development of testis or ovary in the species (4, 7). As the identified regions account only for some of the variance, it is likely that other genes are also involved in the process.

Zebrafish gonad differentiation involves a juvenile ovary stage after which the gonad either receives signals to maintain differentiating oocytes or to induce oocyte apoptosis and trigger the transformation into a testis structure. The correlation between NF-κB activity and sex differentiation shown in this study is in good agreement with earlier studies suggesting the involvement of apoptosis and tp53 in testis development (16, 17). Further research is needed to elucidate the sex-specific signaling pathways leading to the regulation of NF-κB activity in zebrafish during the juvenile ovary stage, thereby controlling the further development into the ovary and testis.
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