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Germline Development of Genetically Female Nile Tilapia (*Oreochromis niloticus*) Reared under Different Temperature Regimes

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Keywords

Genetically female fish · Germline stem cells · Nile tilapia · Ovary · PCNA · Sex determination · Temperature · Vasa

Abstract

In teleosts, elevated temperature during embryogenesis can act on germline cell development, which in turn plays a role for sexual fate. In Nile tilapia, a species with high-temperature-induced masculinization, little is known about the effects of increased temperature on gonadal development in non-masculinized females. The aim of the present work was to investigate persistent effects on the germline of genetically female (XX) Nile tilapia reared at normal (28°C) or elevated temperature (36°C) during the critical time of gonadal sex differentiation at 10 to 20 days post fertilization. Non-sex-reversed females were compared to control females to determine persistent effects of temperature on subsequent ovarian development using histological approaches. Germline stem cells were identified using the germline marker Vasa in combination with the proliferation marker PCNA. Vasa- and PCNA-positive germline stem cells were found in ovaries of both high-temperature-treated and control females. In both groups, ovarian germline stem cells were located at the germinal epithelium of the ovigerous lamellae.

Although no detrimental effects of high temperature on gonadal development in female Nile tilapia were observed, implications on the reproductive fitness caused by elevated temperature need to be investigated in greater depth.

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Given the poikilothermic metabolism in fish, external temperatures have profound effects on gonadal and reproductive development within the species-specific normal thermal range. Albeit, temperatures close to a species' physiological limits might have detrimental effects, specifically on the germ cells. Heat-induced detrimental effects on the germ cells and the germinal epithelium have been reported in several fish species [de Alvarenga and de França, 2009; Lee et al., 2009]. Elevated temperatures applied during sex differentiation might affect germ cells in both sexes and lead to germ cell death [Strüssmann et al., 1998; Ito et al., 2003; Majhi et al., 2009]. The same holds true in higher vertebrates. Exposure to elevated temperature in scrotal mammals results in loss of germ cells [Socher et al., 1997]. As observed in males, heat stress can lead to impaired development and oocyte dysfunction in females, too, with most evidence for dairy cattle [reviewed in Hansen, 2009]. In birds, heat

exposure can have significant detrimental effects on reproductive function in terms of egg number and relative fecundity as well as the number of follicles [Rozenboim et al., 2007]. In the pejerrey (*Odontesthes bonariensis*), germ cells of both sexes, male and female, exhibited abnormalities or even features of degeneration after exposure to elevated temperature [Strüssmann and Patiño, 1999; Strüssmann et al., 1998; Ito et al., 2003]. High-temperature exposure times of 7 days resulted in apoptosis, whereas longer treatments even induced sterility [Strüssmann et al., 1998; Strüssmann and Patiño, 1999]. Yet, the authors reported that high-temperature-induced sterility was reversible. Baroiller et al. [2009] argued that not all germ cells were negatively affected by the temperature treatment, or that “neo”-germ cells were able to repopulate the gonads. Pandit et al. [2015] showed that heat treatment at 37°C, starting at 9, 50, 100, or 160 days post hatching (dph) and lasting for 45 days, resulted in thread-like pairs of gonads without germ cells in an all-female population of Nile tilapia. The authors confirmed the absence of germ cells in heat-treated fish by immunohistochemistry using the antibody Vasa. A reduced treatment period of 30 days was less effective in induction of sterility [Pandit et al., 2015].

A unique characteristic of the female teleost germline is the capability of proliferating throughout the entire lifespan [Wildner et al., 2013; Hanna and Hennebold, 2014]. Grier et al. [2007] stated that the germinal epithelium is a source of germline stem cells both in male and female individuals. In rainbow trout (*Oncorhynchus mykiss*), they identified the germinal epithelium in spent females and found that it contained oogonia, mitotic oogonia, early diplotene oocytes, and prefollicle cells sheathed within nests. During embryonic development, primordial germ cells are located outside the gonad primordia [Yoshizaki et al., 2002; Kobayashi et al., 2013]. During normal gonad development in tilapia, primordial germ cells are located in the area between gut and mesonephric duct and migrate to the gonad via the dorsal mesentery [Devlin and Nagahama, 2002]. After reaching the gonad, the germ cells divide [Nakamura and Takahashi, 1973]. In female tilapia, proliferation of germ cells starts at 9 dph, whereas in males it occurs between 9 and 14 dph [Kobayashi et al., 2013], i.e., up to 19 days post fertilization (dpf). The ovarian cavity forms after the first germ cells enter meiosis and proliferate [Devlin and Nagahama, 2002; Kobayashi et al., 2011]. In females of Nile tilapia, gonadal differentiation is evident at 20 dph, indicated by formation of the ovarian cavity. In contrast, male tilapia gonads differentiate at 25 dph, equivalent to 29 dpf,

indicated by the formation of efferent ducts [Kobayashi et al., 2013].

There is a wealth of literature showing that elevated temperature regimes can induce sex reversal in Nile tilapia, an economically important aquaculture species which also exhibits genetic sex determination [Baroiller et al., 1995, 2009; Tessema et al., 2006; Wessels and Hoerstgen-Schwark, 2007, 2011]. However, there is a lack of knowledge concerning the effect of elevated temperature treatments on the development of the ovarian germline and the non-determinate lifetime proliferation of the female Nile tilapia germline. Therefore, the present study aimed at investigating germline stem cell development of a genetically all-female population of Nile tilapia, subjected to elevated temperature during gonadal differentiation, using histological and immunohistochemical techniques.

Material and Methods

Experimental Design, Fish Maintenance, and Sampling

All experiments with live fish were carried out in the warm water recirculation unit of the Division of Aquaculture and Water Ecology of the Department of Animal Sciences, Goettingen University. Parental fish from a Lake Manzala population, Egypt, were kept at 28°C in a warm water recirculation system. Genetic females were hand-stripped upon identification of reproductive behavior (swollen urogenital papilla and aggressive behavior). Sperm from a temperature-treated pseudo male (XX) was used to fertilize eggs derived from 5 genetic females (XX) by way of artificial fertilization. Thereafter, eggs of half-sib families were incubated individually for 9 days in 2-cL jars with continuous flow of water at 28°C. On day 10, all-female (XX) fry were randomly divided into a treatment group kept at 36°C and a control group kept at 28°C, each containing 330 fry. The temperature treatment was carried out according to Tessema et al. [2006]. After 10 days of treatment, fish were carefully re-acclimatized to 28°C and raised to adulthood. To investigate the female ovary and germ cell development after the temperature treatment, 2–6 female fish from the control and treatment group were sacrificed at 60 (completion of first meiosis), 90, 120, 150, 180, and 210 dpf (Fig. 1). After 240 dpf, the remaining fish in both groups were sexed in order to assess the overall sex ratio among the total number of 330 fish per group. Female fish were dissected, and the excised gonads were directly transferred to phosphate-buffered saline (PBS). Paraformaldehyde at a concentration of 4% was used as fixative when applying embedding in PolyFreeze-tissue-freezing-medium (Polysciences, Inc., Germany) as well as for paraffin-embedding. Gonad samples that were to be paraffin-embedded were fixed in 4% paraformaldehyde overnight, dehydrated in increasing concentrations of ethanol (70, 80, 90, 100, and 100%), followed by 2 xylene baths and embedding in paraffin. Samples for the PolyFreeze-tissue-freezing-medium-embedded approach were divided into 2 groups, one that was fixed in 4% paraformaldehyde and one that was not fixed for comparison. Fixation of samples for the PolyFreeze-tissue-freezing-medium-

Fig. 1. Schematic diagram of the experimental design illustrating the set-up of control and treatment group, temperatures, the duration of treatment, and sampling points (red dashed lines). The upper bar (C) represents the control group kept at 28°C throughout the experiment. The lower bar (T) represents the temperature treatment group kept at 36°C from 10 to 20 dpf (red bar). Surplus fish were sexed at 240 dpf. *n*, number of sampled females.

embedding approach was performed in 4% paraformaldehyde for 15 min at room temperature. Then, they were rinsed for 5 min in PBS pH 7.3 at room temperature, followed by submersion in 10% sucrose solution for 2 h and 30% sucrose solution overnight at 4°C. Samples were finally embedded in PolyFreeze tissue freezing medium and adjusted to the respective section orientation. Samples that were not fixated were directly rinsed with PBS pH 7.3 for 5 min at room temperature and then processed in the same way as fixated samples.

Sectioning

Paraffin-embedded samples were sectioned at 7 µm with a microtome (Leica Wetzlar, Germany), whereas the PolyFreeze-tissue-freezing-medium-embedded samples were sectioned at 12 µm with a cryostat (Leica). Samples to be immunostained were either stored at room temperature (paraffin-embedded sections) or at 4°C (PolyFreeze-tissue-freezing-medium-embedded).

Immunostaining of Paraffin-Embedded Samples

Sections were successively passed through 2 xylene baths, a decreasing ethanol series (100, 90, 80, and 70%), and aquades followed by antigen-unmasking in citrate buffer (pH 6) in a microwave (4 × 5 min at 600 W). After cooling to room temperature for 20 min, the samples were transferred from Tris-buffered saline/0.1% Tween (TBST), 2× with agitation, into 3% H₂O₂ in methanol (30 min), followed by triple washing in TBST, and finally into 1% BSA in PBS and the primary antibody: zebrafish-Vasa 1:100 (rabbit-polyclonal [Knauf et al., 2000]) and PCNA 1:100 (mouse-monoclonal; DAKO, Glostrup, Denmark). Staining was performed overnight at 4°C. On the following day, the samples were washed 3 times for 3 min in TBST, incubated with secondary anti-rabbit antibody conjugated with rhodamine 1:1,000 (Jackson ImmunoResearch, No. 111-295-003) and anti-mouse-Alexa 488 (1:250; Cell Signaling Technology, No. 4408) for 1 h, and rinsed 3 times for 5 min each in TBST. Nuclei were counterstained with DAPI (1:10,000 from the stock solution 5 mg/ml). Negative control immunostaining was performed by omitting the primary antibody.

Immunostaining of PolyFreeze-Tissue-Freezing-Medium-Embedded Samples

Sections were kept at room temperature for 1 h before processing. Negative controls for immunostaining were performed by

omission of the primary antibody. Blocking of protein binding sites was carried out with 1% BSA for 1 h, followed by overnight Vasa and PCNA staining as the primary antibodies (see above). After washing twice in 0.1 M K-phosphate buffer, samples were incubated with secondary antibody conjugated with rhodamine red and Alexa-fluor 488 for 1 h at room temperature, followed by counterstaining with DAPI (1:10,000).

Immunostained sections were kept at 4°C until inspection. A fluorescence microscope (AxioImager; Zeiss, Goettingen, Germany) equipped with the Axio Vision software release 4.6.3 SP1 (11-2007) was used for investigation of sections.

Results

In the genetically female control group (*n* = 330) reared at a constant temperature of 28°C, no males were observed. The temperature-treated group (36°C from 10 to 20 dpf), consisting of 330 fish in total, exhibited a share of 20.3% males. Overall, the survival rate in both groups was 100%. Histological analysis revealed that male individuals from the treatment group showed complete sex reversal; no intersex fish were observed.

Generally, ovarian structures were more consistently conserved in fixated samples than in those without fixation. Samples of ovaries for the PolyFreeze-tissue-freezing-medium-embedded approach showed a high degree of shrinkage (online suppl. Fig. 1, see www.karger.com/doi/10.1159/000479396). Therefore, analysis of the germline was based on evaluation of paraffin-embedded sections only. Germline stem cells in the ovaries were identified based on the following criteria: detection of a strong Vasa signal in early germ cells; a typical morphology of early germ cells as single cells or within nests [Nakamura et al., 2011]; typical localization of germline stem cells at the germinal epithelium of the ovigerous lamellae; a characteristic PCNA signal in the nuclei of pro-

Fig. 2. Vasa-PCNA immunostaining of ovary cross-sections in control and temperature-treated female Nile tilapia at 90 dpf displays germline stem cells at the germinal epithelium of the ovigerous lamellae. Vasa-positive staining is in red, PCNA-positive staining in green, and DAPI-stained DNA in blue. Arrows indicate the germinal epithelium of the ovigerous lamellae. OC, ovarian cavity. Scale bars, 50 μ m.

liferating germ cells. Ovarian germline stem cells were identified at all sampling times (60–210 dpf) in both control and treatment group. Expression of PCNA and Vasa was detected in all ovary sections irrespective of age and treatment. Ovarian germline stem cells were found exclusively in the germinal epithelium of the ovigerous lamellae, both in the control and in the treatment group (Fig. 2). Moreover, ovarian germline stem cells of 2 distinct types were identified: single, isolated germline stem cells and nested germline stem cells in interconnected clutches (Fig. 3), both of which were located in niches. Niches resemble the germinal cradle, a structure containing germline cells, advanced cells, and supporting cells. Nakamura

et al. [2010] defined the germinal cradle as a cluster of early-stage germ cells supported by interwoven thread-like cords of *sox9b*-expressing somatic cells. The niches were exclusively located in the germinal epithelium of the ovigerous lamellae. Nested germline stem cells as well as single cells were in active proliferation, indicated by PCNA-positive staining. Moreover, single isolated germline stem cells were long and of irregular shape, whereas nested germline stem cells had a round shape.

At an age above 90 dpf, a tendency for decreased germline stem cell numbers in the germinal epithelium of the ovary was observed. Although differences could not be quantified, the decrease in germline stem cell numbers occurred to equal extents in the control and treatment group. Nevertheless, at advanced age, both nested germline stem cell clutches and single isolated germ cells were detected around the germinal epithelium of the ovigerous lamellae without discernible differences between treatment groups (Fig. 4). Whereas PCNA signals were comparable at 60, 120, 180, and 210 dpf, at 90 dpf, ovaries possessed distinct and strong PCNA immunostaining in germline stem cells irrespective of treatment (Fig. 5). Moreover, differences in PCNA signal strength between single isolated germinal stem cells and the nest-like clutches were observed. Single isolated germinal stem cells expressed strong signals, whereas amongst cells within nest-like structures both strong and weak Vasa-PCNA signals were detected. Overall, the comparison of ovaries derived from temperature-treated and control females revealed only subtle, if any, differences in immunostaining.

Discussion

Little is known about the effect of temperature treatments during the thermo-sensitive period on subsequent gonadal development in the non-responsive and non-masculinized females and its implications for the non-determinate proliferation of germ cells. The present study clearly indicates that the temperature treatment applied during the critical phase of sex differentiation from 10 to 20 dpf leads to normal ovaries in the temperature-treated but non-masculinized females. Intersex structures in the gonads were not observed. Ovaries of temperature-treated genetically female (XX) individuals showed no abnormalities or deviations from normally developed germline stem cells. The immunohistological analysis provided evidence for the identification of germline stem cells in ovarian tissue of control and temperature-treated indi-

Fig. 3. Vasa-PCNA immunostaining of female ovary cross-sections in control and temperature-treated Nile tilapia at 90 dpf reveals single (arrows) and nest-like clutches (asterisks) of ovarian germline stem cells. Ovaries display primary growth phase oocytes (po) and germline stem cells. DAPI stained DNA is in blue; Vasa-positive staining in red; PCNA-positive staining in green. *nu*, nucleus. Scale bars, 50 μ m for control females and 10 μ m for treatment group females.

viduals. In the present study, both, temperature-treated as well as control females showed germline stem cells, based upon positive PCNA-Vasa and DNA staining.

In contrast to female mammals which have only a finite number of germ cells, teleost germ cells exhibit a lifelong ability to multiply [Hanna and Hennebold, 2014]. On the male side, teleosts show similar characteristics as mammals, featuring germline stem cells. Grier [2000] described germline stem cells in common snook and also in rainbow trout [Grier et al., 2007]. In the latter, they identified the germinal epithelium in spent females and found that it contained oogonia, mitotic oogonia, early diplotene oocytes, and prefollicle cells sheathed within nests. Grier et al. [2007] stated that the germinal epithelium is a source of germline stem cells both in male and female individuals. In the present study, germline stem cells were only identified in the germinal epithelium. Moreover, there were no obvious differences between the temperature-treated and control group, at least based on the immunostaining. The temperature treatment applied from 10 to 20 dpf induced a complete female-to-male sex reversal in a certain proportion of the population. In temperature-treated but unaffected females the development of the ovary and function of germline stem cells seemed to remain unaffected. Nevertheless, Vasa and PCNA immunostaining alone are not able to fully address this question; additional comparative reproduction trials would allow a more comprehensive understanding. Pandit et al. [2015] demonstrated in their experiments that elevated temperature of 37°C applied at 9, 50, 100, or 160 dph for a period of 45 days induced sterility in female Nile tilapia. The resulting ovaries had a thread-like appear-

ance and were devoid of germ cells. The authors proved an absence of germ cells in temperature-treated individuals using Vasa immunostaining. A temperature treatment of 30 days at 37°C showed a lower efficiency in sterility induction [Pandit et al., 2015]. Further, the authors applied an experimental temperature of 35°C in their experiments, which did not result in sterile gonads. In the present study, an intermediate temperature of 36°C combined with a 10-day duration was chosen for the temperature treatment. Thus, the higher temperature of 37°C used by Pandit et al. [2015] in combination with the drastically extended treatment time seem to be responsible for the irreversible and complete loss of germ cells. The present study did not aim to produce sterile fish; the intention was to elucidate the impact of high-temperature treatments (36°C for 10 days), with the purpose of phenotypic sex reversal, on development of the ovarian germline and the non-determinate lifetime proliferation of the female germline. In the present study, the temperature as well as the treatment duration were sufficient to induce female-to-male sex reversal in a share of 20.3% of individuals, but failed to provoke persistent adverse or irreversible effects on the female ovary and germline, at least based on immunostaining using Vasa and PCNA. Ba-roiller et al. [2009] outlined that only a portion of germ cells might be negatively affected by the temperature treatment, or that “neo”-germ cells could recolonize the gonads. This hypothesis was partially confirmed by Pandit et al. [2015] and could explain the results of the present study, too.

Immunostaining of ovarian tissue in Nile tilapia indicated that germline stem cells were localized in the ger-

Fig. 4. Development of ovarian germline stem cells from juvenile (60 dpf) to adult (210 dpf) control (28°C) and temperature-treated (36°C) Nile tilapia females. Germline stem cells (arrows) were identified by Vasa (red)–PCNA (green) immunostaining. DAPI stained DNA blue. Scale bars, 20 µm for 60 dpf, 10 µm for 90 dpf, 20 µm for 120 dpf, and 13 µm for 210 dpf tilapia from the control group and 50 µm for 210 dpf tilapia from the treatment group.

minal epithelium of the ovigerous lamellae. Moreover, 2 types of ovarian germline stem cells were identified: single isolated germline stem cells as well as nest-like clusters of germline stem cells, resembling germinal cradle-like structures. Nakamura et al. [2010] first described the “germinal cradle” as a germline stem cell-supporting structure in medaka. Germline stem cells need a micro-environment or niche supported by somatic cells for their existence [Spradling et al., 2011]. This micro-environ-

ment provides essential factors for cell identity, proliferation, and survival. Further, Wildner et al. [2013] investigated early germ cells in ovaries of ostariophysian fish, *Pimelodus maculatus* and *Serrasalmus maculatus*, identifying 2 kinds of oogonia, single oogonia and proliferating oogonia that build nests. Oogonia of both types were also found in ovaries of Nile tilapia, irrespective of the treatment.

Fig. 5. Strong (arrows) and faint Vasa-PCNA expressing cells (asterisks) revealed by immunostaining of female ovary cross-sections in control and temperature-treated Nile tilapia at 90 dpf. DAPI stained DNA blue. Scale bars, 50 µm.

Spradling et al. [2011] stated that the mechanisms of germline stem cell renewal are probably conserved amongst species. They assumed that stem cell renewal occurs to balance the number of daughter cells and induce stem cells. Likewise, in the present study germline stem cells were observed at all developmental stages, indicating a continuous renewal and proliferation.

In summary, germline stem cells were identified in the germinal epithelium of ovigerous lamellae in control and temperature-treated genetically female Nile tilapia at age 90 to 210 dpf. Immunostaining, using PCNA and Vasa, revealed the presence of both single isolated as well as nest-like clutches of germline stem cells, the latter resembling the germinal cradle of the ovarian cord. Furthermore, between females that had not responded to temperature treatment and untreated control females no differences were found with regard to proportions of germline stem cells or immune staining. It may thus be assumed that in non-responding females the production of gametes and, thus, maintenance of reproductive fitness, had remained unimpaired; an assumption that needs to be confirmed by appropriate fertilization trials. Further studies are needed to analyze the effect of temperature treatment on germline stem cells and their relation to reproductive fitness of females.

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Statement of Ethics

All procedures were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German Animal Welfare Act.

Disclosure Statement

The authors have no conflicts of interest to declare.

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