Development of *Xenopus laevis* bipotential gonads into testis or ovary is driven by sex-specific cell-cell interactions, proliferation rate, cell migration and deposition of extracellular matrix

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ABSTRACT

Information on the mechanisms orchestrating sexual differentiation of the bipotential gonads into testes or ovaries in amphibians is limited. The aim of this study was to investigate the development of *Xenopus laevis* gonad, to identify the earliest signs of sexual differentiation, and to describe mechanisms driving these processes. We used light and electron microscopy, immunofluorescence and cell tracing. In order to identify the earliest signs of sexual differentiation the sex of each tadpole was determined using genotyping with the sex markers. Our analysis revealed a series of events participating in the gonadal development, including cell proliferation, migration, cell adhesion, stroma penetration, and basal lamina formation. We found that during the period of sexual differentiation the sites of intensive cell proliferation and migration differ between male and female gonads. In the differentiating ovaries the germ cells remain associated with the gonadal surface epithelium (cortex) and a sterile medulla forms in the ovarian center, whereas in the differentiating testes the germ cells detach from the surface epithelium, disperse, and the cortex and medulla fuse. Cell junctions that are more abundant in the ovarian cortex possibly can favor the persistence of germ cells in the cortex. Also the stroma penetrates the female and male gonads differently. These finding indicate that the crosstalk between the stroma and the coelomic epithelium-derived cells is crucial for development of male and female gonad.

1. Introduction

The sexual differentiation of the gonadal anlagen into testis or ovary requires a binary developmental decision. During this process a distinct testis- or ovary-specific structures emerge and the bipotential gonad acquires features enabling it to produce sperm or eggs, and male or female steroid hormones (Piprek et al., 2016). The gonad development, the formation of sex cords and the molecular and cellular mechanisms driving these processes have been described in details in mammals (Hummitzsch et al., 2013; Nel-Themaat et al., 2009; reviewed in Piprek, 2016), chicken (Smith and Sinclair, 2004; Smith et al., 2007), and red-eared turtle *Trachemys scripta* (Yao et al., 2004). These studies indicated that among various developmental processes, the cell proliferation and migration are crucial for establishment of the gonadal fate (Schmahl et al., 2000; Schmahl and Capel, 2003; Tilmann and

Capel, 1999). In amphibians, the development of gonads has been extensively studied at the light microscopy level (Falconi et al., 2004; Piprek et al., 2010; Saotome et al., 2010; Tanimura and Iwasawa, 1988, 1989; Witschi, 1929). The structure of developing amphibian gonads deviates from that of amniotes, and it is unknown whether the mechanisms responsible for testis and ovary development in amphibians and amniotes are similar. *Xenopus laevis* is a model anuran species, and many laboratories studied the role of steroid hormones and xenobiotics (Piprek et al., 2012 and citations there; Piprek et al., 2013a) and gene expression (Osawa et al., 2005; Piprek et al., 2013a; Yoshimoto et al., 2008) in developing gonads of *X. laevis*. However, developmental mechanisms driving sexual differentiation of *Xenopus* gonads are still poorly understood. The studies of the structural aspects of gonad development in *X. laevis* are limited to primordial germ cell migration and their settlement in the earliest genital ridges (Wylie and

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Heasman, 1976), the organization of germ cells during ovarian cyst formation (Kloc et al., 2004), and the induction of meiosis (Piprek et al., 2013b). Previously, we studied the external structure of developing gonads in *Xenopus* and other anurans (Piprek et al., 2014). Witschi (1929) described the early structural changes during gonad development in *Lithobates sylvaticus*. The ultrastructure of developing gonads was studied in *Pelophylax nigromaculatus*, *Rhacophorus arboreus*, *Bufo bufo*, *Bombina variegata* (Falconi et al., 2004; Piprek et al., 2010; Tanimura and Iwasawa, 1988, 1989). Cell proliferation was studied in *P. nigromaculuarus* and *Glandirana rugosa* (Saotome et al., 2010; Tanimura and Iwasawa, 1991). Many studies were focused on the gene expression in developing gonads in *G. rugosa* because of its unusual mode of sex determination (Kato et al., 2004; Nakamura, 2009; Oike et al., 2016, 2017; Oshima et al., 2005; Yamamura et al., 2005).

The aim of present study was to investigate the morphology of the gonads at the earliest stages of gonads formation in *Xenopus*, and to shed a light on cellular and molecular mechanisms of gonad development and sexual differentiation in *Xenopus*. We used genotyping to establish the sex of analyzed animals before they were morphologically distinguishable, and analyzed morphology of the gonads using light and transmission electron microscopy. These analyses indicated that the early testes and ovaries differ in cell adhesion and extracellular matrix formation. In order to get further insight into mechanisms driving early gonad development we studied cell junctions and the extracellular matrix (ECM) formation, as well as cell proliferation and migration. We discuss *Xenopus* gonad development in the context of other vertebrates, and address the question if the vertebrate gonadogenesis is driven by an universal mechanism.

2. Materials and methods

2.1. Animals

Larvae of the African clawed frog (*Xenopus laevis* Daudin, 1802; Pipidae) were obtained in the laboratory. The tadpoles were reared in 10-L aquaria (30 tadpoles per 10 L) at 22 °C and fed with powder food Sera Micron (Sera) daily. They were staged according to Nieuwkoop and Faber (1956). After staging tadpoles from the stage NF45 until stage NF66 (completion of metamorphosis) were anesthetized with 0.1% MS222 solution. Numbers of analyzed specimens are shown in Supplementary Table 1. All the specimens used in experiments were acquired according to Polish legal regulations concerning the scientific procedures on animals (Dz. U. nr 33, poz. 289, 2005) and the permission from the First Local Commission for Ethics in Experiments on Animals.

2.2. Gender determination by PCR

The genetic sex of tadpoles was determined using PCR detection of female-specific *DMW* gene. DNA was isolated from tadpole tails using NucleoSpin Tissue Kit (Macherey-Nagel, 740952.240 C). *DMW* gene (W-linked female-specific marker) and *Dmrt1* gene (positive control) were used to determine ZZ or ZW status of tested animals. PCR was performed as previously described (Yoshimoto et al., 2008). The mixture of the following pairs of primers were used: for *DMW*: 5′-CCACACCCAGCTCATGTAAAG-3′ and 5′-GGGCAGAGTCACATATACTG-3′, and 5′-AACAGGAGCCCAATTCTGAG-3′ and 5′-AACTGCTTGACCTCTAATGC-3′ for *Dmrt1*.

2.3. Light microscopy

The tadpoles were dissected and the whole urogenital ridges (*meso*nephroi and gonads) were fixed in Bouin's solution overnight, dehydrated and embedded in paraffin (Paraplast, Sigma, P3683). Samples were serially sectioned at 6 μm, and stained with hematoxylin and picroaniline according to Debreuill's procedure (Kiernan, 1990). This trichromatic staining allowed for visualization of extracellular

matrix, stroma, and thus gonadal interior structure. Images were taken with Nikon Eclipse E600 light microscope.

2.4. Transmisison electron microscopy

Urogenital ridges were dissected from tadpoles and fixed in Karnovsky's fixative. After rinsing in cacodylate buffer and postfixtion in 1% osmium tetroxide solution (Ito and Karnowsky, 1968), samples were dehydrated and embedded in Epon812. Ultra-thin sections were stained with uranyl acetate and lead citrate. The sections were analyzed using JEOL JEM2100 transmission electron microscope. The germ cells were easily distinguishable due to their big size, and large, pale, euchromatic nuclei.

2.5. Immunohistochemistry

Bouin's solution-fixed and paraffin-embedded samples were serially sectioned at 4 μm. Sections were deparaffinated, rehydrated and heat-induced epitope retrieval was performed using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6) at 95 °C for 20 min. Subsequently, the sections were blocked with 3% H_2O_2 followed by 6% Bovine Serum Albumin (BSA, Sigma) and incubated with primary antibodies (rabbit polyclonal anti-laminin #L9393, Sigma; anti-E-cadherin #ab152102, Abcam; both 1:200) for 15 min at room temperature (RT), and with UltraVision Quanto Detection System (ThermoFisher, TL-125-QHD). Mayer's hematoxylin was used as a counterstain. Sections were viewed under Nikon Eclipse E600 microscope.

2.6. Proliferation assay

In order to investigate cell proliferation we used BrdU labeling and PCNA immunodetection. Bromodeoxyuridine (BrdU, Sigma, B5002) was diluted in PBS (10 mM; pH 7.4) and 50 μL of solution was injected intraperitoneally to the tadpoles. After 24, 48, and 72 h tadpoles were anesthetized and gonads were dissected and fixed in Bouin's solution overnight.

Bouin's solution-fixed, deparaffinised and rehydrated samples were rinsed in PBS. Samples were boiled in citrate buffer (10 mM tri-sodium citrate, 0.05% Tween-20, pH 6.0) to retrieve epitopes. Then samples were incubated with blocking solution (6% Bovine Serum Albumin (BSA, Sigma)) for 30 min followed by overnight incubation with primary antibodies in PBS with 1% BSA. The primary antibodies against BrdU (1:1000, mouse monoclonal antibody, Sigma, B8434) and against human PCNA (1:3000, rabbit polyclonal antibody, Sigma, HPA030521) were used. Incubation with secondary antibodies was performed at RT for 1 h with Cy3-conjugated goat anti-mouse secondary antibodies (Sigma, C2821) at 1:100 dilution or with Alexa Fluor 488-conjugated goat-anti rabbit secondary antibodies (ThermoFisher, A20181) at 1:200 dilution. DAPI (10 μM; Sigma, D9542) was used to stain nuclei. Samples were mounted with SlowFade Gold Antifade Reagent (Molecular Probes). Immunostained samples were viewed under confocal microscopy LSM 510 META.

2.7. Cell counting

For total and proliferating cell counting we used 4 μm-thick sections immunostained for PCNA as described in 2.6. Analysis was performed with Nikon Eclipse E600 fluorescence microscope. Cell counting was performed on five sections through the gonomere of the medial part of the gonad, and averaged for each gonad. The cell number was counted from three gonads from the same sex and NF stage. Analyses were performed with the use of statistical software package Statistica (version 12.0 StatSoft PL).