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Control of Entry into Meiosis of Germ Cells Precursors in Chickens

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Control of Entry into Meiosis of Germ Cells Precursors in Chickens

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I. Introduction

Sex determination begins with colonization of the gonad (somatic nature organ) by the precursors of germ cells (PGCs) and followed by their entry into meiosis and the formation of the haploid gametes. PGCs are formed in embryonic development of birds and mammals (Murray et al, 2010). The source of the formation of gametes in birds is line of sex cells, which first appear in the early embryonic development. In the early blastula (stage X by Eyal-Giladi & Kochav, 1976) PGCs are located in the central region of the blastoderm. Then, as a result of morphogenetic movements prior to gastrulation the cells become localized in a limited region of the embryo blastodisk, so-called sexual crescent. On the third day of embryonic development PGCs begin migration via the bloodstream to the emerging gonads. The rudiments of the gonads appear on the third day of chicken embryogenesis (18-20 stages of development (Hamburger & Hamilton, 1951). From the beginning of the process of anatomical differentiation of gonads (7th day of development) reproduction rate of germ cells in the male and female gonads begin to differ. More intensive increase in the number of germ cells in the female gonad is accompanied by a significant increase in left ovary than in right ovary. In males both testes develop in similar rate. A higher rate of proliferation of germ cells is characteristic for representatives of heterogametic sex: female birds and male mammals. More intense proliferation of oogonia in the left ovary of birds leads to a considerable increase in their numbers when compared to the spermatogonia and then to an earlier entry into meiosis. Oogonia appearance characteristic of the stage of oocyte meiotic prophase leptoteny can observed at 13-14 days of incubation. In the testes the number of germ cells at similar stages of development is much smaller and at the time of hatching the population of male germ cells consists exclusively of spermatogonia (Ayers et al, 2013).

It is assumed that meiosis of female mammals is induced by retinoic acid (RA), which is inhibited in the males, by CYP26B1 (Bowles & Koopman, 2010). Unlike mammals, the molecular mechanisms of sex determination in birds remain largely unknown (Trukhina A. et al., 2013). In particular, the questions remain about the reasons why the sex cells of males and females enter meiosis during different stages of development, and what is role of sex hormones in this process.

Many epigenetic mechanisms of regulating the development of germ cells have been described in mammals. During entering into gonadal ridge genome of PGCs is undergoing methylation and chromatin remodeling (Martínez-Arroyo et al, 2014). In birds the role of epigenetic control of sex determination is less clear describe weaker for somatic gonad and PGCs (Trukhina & Smirnov, 2014). In particular, MHM locus (localized in Zp21 region) was described that consists of approximately 200 repeats of 2.2 kb and is being transcribed into noncoding heterogeneous RNA. This MHM region exhibits specific chromatin modification only in females (Itoh et al., 2011).

To confirm the possible role of retinoic acid (RA) in the induction of bird meiosis, we investigated the effect of the RA on meiosis of PGCs when it was administered to chicken embryos at different stages of development. The aim of this study was also to assay the effect of estrogen on the development of avian testicles and ovaries, as well on germ cell maturation. For this purpose, we modified the balance of sexual hormones by aromatase inhibition and altered the estrogen receptor sensibility to their hormone with corresponding modulators. Also we studied the impact of demethylation by treatment with 5-azacytidine (5-AC) embryos in the early stages of incubation.

II. Material and Methods

During the experiments, eggs of "Belaya Russkaya" breed chickens (experimental farm of All-Russia Institute of Farm Animal Genetics and Breeding, St.-Petersburg-Pushkin, Russia) or Highsex-white cross (Tosno, Lisii Nos, Russia) were used. All injections (at 100mkl into egg) were carried out into the air cells of eggs to under shell membrane in region of blastodisc. As control we carried out injections 1xPBS (at 100mkl into egg). Eggs were incubated for 17-19 days at 37,8°C

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and humidity 28%. For the first group of experiments incubated eggs were administered with RA in doses of 12.5 or 25mkg at 9th or 14th day of incubation. For the second group of experiments we have performed a number of experiments with aromatase inhibitor – letrozole (Novartis Pharma, Switzerlland) and modulator of estrogen receptors – tamoxifen (Ebeve Pharma, Austria) at concentration 1mg/ml. Injections of Tamoxifen or letrozole have been done once at the 4th day or twice at the 4th and 11th days. During one group of experiments, tamoxifen was injected first at the 4th day and then letrozole at the 11th day. At third group of experiments we carry out injections of 5-azacytidine (5-AC) at concentrations at 10, 12.5 and 15mkM at 1st or 4th day incubation.

Gonads isolated from 17 – 19 day old embryos after determination of their type (testicles or ovaries) were fixed with Clark mixture (ethanol mixed with glacial acetic acid in ratio 3:1). Gonads were then washed from the fixator, treated with alcohols of increasing concentration (ethanol–isobutanol–O-xylol), and embedded into paraffin according to the routine technique (Barikina et al., 2004) to prepare sections on the microtome. Sections were purified from paraffin, stained with Mayer's hematoxylin, dehydrated by alcohols with increasing concentration and embedded into Canadian balsam (DiaM, Russia). The meiotic marker SCP3 (Synaptonemal Complex Protein) was used for immunofluorescent staining (antibodies BDBioScience and AbCam, USA). After washing in 1xPBST (1xPBS buffer with 0.05% Tween 20), sections were incubated with secondary antibody at room temperature for 40 minutes. After that sections were stained by DAPI (12mkg/ml) (Sigma-Aldrich, USA) with adding Vectashield (Vector Laboratories, USA).

Gonadal histology was analyzed under a Leica DM6000B microscope (Leica Microsystems GmbH, Germany) equipped with a Leica DC500 CCD camera and Leica QWin v.1.2 software. We analyzed miniomum of 200 PGCs in each gonad from at least 3 embryos. Stages of meiosis prophase were determined visually. Comparison of mean values and errors at groups we calculated standardly, comparison of progressive average difference we carried out at Student t-test.

Genetic sex of each embryo was defined by polymerase chain reaction (PCR) using DNA isolated by the routine procedure (Griffiths *et al.*, 1998). Primers for the chicken *CHD1* gene were: CHD1F 5'-GTTACTGATTCGTCTACGAGA-3' and CHD1R 5'-ATTGAAATGATCCAGTGCTTG-3' (Elbrecht & Smith, 1992; Ellegren, 2002). The PCR conditions were: denaturation at 94°C for 5 min; gradual temperature decline from 60°C to 50°C, 1°C, 1 min; 35 cycles: 94°C for 30s, 50°C for 30s, 72°C for 60s. Last PCR step was performed at 72°C for 10min (Kamata et al., 2004). Amplified DNA was assayed in 2% agarose gel. Single fragment (380 bp) was identified in males and two fragments (500 and 380 bp) were observed in females.

III. Results

With the introduction of retinoic acid into eggs at 9 or 14- days of incubation in embryos on day 17 there was significlly more germ cells in the prophase stage of meiosis in left gonads of treated females than in controls. Oocytes I of control embryos were at stages of preleptotena and leptotena. At the same time most of experimentally treated embryos had oocytes I at stage of zygotena with specific arrangement of chromosomes condenseed in the characteristic form of bouquet. At a later period of incubation (19th day embryos development) control female embryos had oocytes at stage of preleptotena – zygotena. We did not find statistically significant differencesin quantities of gonocytes at the different stages of meiosis prophase between control and experimental female groups.

On 17th day of embryo's development control males had testis with only spermatogonias. At the same times experimental male had not spermatocytes I at stage of preleptotena. Quantities such cells were less than 14%. On 19th day of embryo's development control male also had testis with spermatocytes I at stage of preleptotena and their quantity was about 8.5%. In testis of experimental males number of such cells was nearly 16% (Fig.1). Number of spermatocyte I and oocytes I at various stages of meiosis prophese I (I, M, PL, L, Z) are presented in the diagram of Fig.2.

By fluorescence methods it was found that at 17th day of embryo's development germ cells of control females had meiotic SCP3 marker accumulating around their nucleus. At the same stage of development the germ cells of control males had not this marker. Gonads of experimental males had the germ cells with increasing intensive fluorescence such protein in the cytoplasm around nucleus.

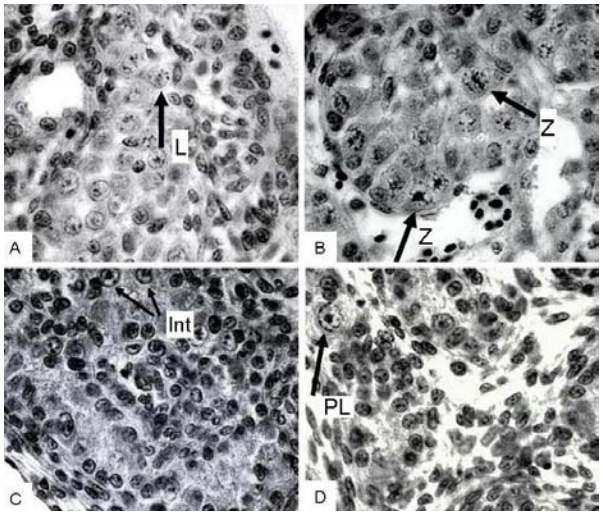

Figure 1 : Gonad sections of the experimental females (A, B) and males (C, D) at 17th day embryos development. It was injected 12.5mkg RA in egg at 9th (A, C) and 14th (B, D) day of incubation. On A and B it was showed the germ cells at stages of leptotena (L) and zygotena (Z), on C and D it was showed the germ cells at stages of interphase (Int) and preleptotena (PL)

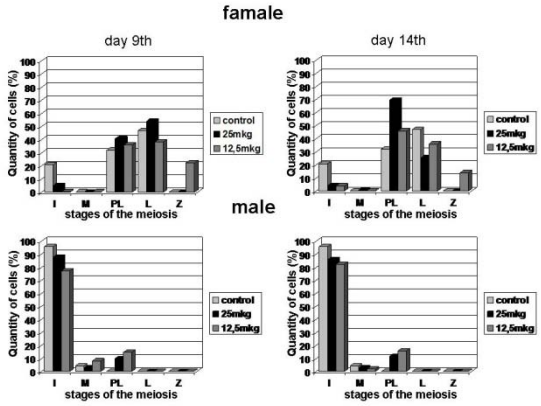

Figure 2 : The effect of RA into the entry for gonocytes I in prophase I of meiosis. At early terms of embryonic development RA in small dozes (12.5mkg) promotes to get over the first meiotic block in spermatocytes I and permit to entry into stage of zygotena for oocytes I when the synaptonemal complexes begin to form. But the effect of RA is insufficiently in order to get over the second meiotic block at the stage of diakinesis.I – interphase, M – mitosis, PL – preleptotena, L – leptotena, Z – zygotena

The effect of estrogen inhibitors (letrozole and tamoxifen) on gonad development in chicken embryos was assessed by anatomical (gonad pairs, their size and shape), histological and cytological (presence of seminiferous tubules, state of germ cells) standards. Control females have typical left gonads with medulla and cortex. Numerous germ cells at the stage of meiotic prophase were revealed in the cortex of control females. Control males of the same age had smaller testicles and a well developed system of seminiferous tubules with spermatogonia mostly not entered into the meiotic pathway. Genetic sex of control males and females fully corresponded to the phenotypic type.

The gonad morphology of experimental genetic females differed from the norm. The most apparent changes were observed after letrozole exposure: two well developed gonads, cortex thinning and structures resembling seminiferous tubules in medulla were observed. The germ cell number and their location in the cortex were unaltered. Most ovarian germ cells entered meiosis and were blocked at the meiotic prophase I. Genetic males exposed to letrozole (aromatase inhibitor) had no apparent changes in their gonads.

Single injection of tamoxifen into the egg did not produce changes in embryonic gonads of both sexes. A doubled tamoximen dose caused a slight modification: hypertrophy of the left gonad in males. Both gonads were preserved in most females; however, the right gonad was slightly smaller than the left one. Both gonads had an increased number of lacunae in medulla, thickened cortex, and rare structures resembling seminiferous tubules. The number of germ cells at the stage of meiotic prophase I in the left gonad increased. However, in the right gonad, germ cells did not enter the meiotic prophase. Simultaneous administration of tamoxifen and letrozole, but at various stages of development, increased the number of germ cells in seminiferous tubules of experimental males was observed. However, they also were blocked before the entry into meiosis. No similar changes were observed in genetic females.

In third experimental group the injections of the 5-AC at the 4th day of incubation (15mkM, V concentration) and at 1st day of incubation (10mkM, III concentration) did not change the sex of the gonads, however, it led to the entry into meiosis of males germ cells. In the developing testis the germ cells at stages of preleptotena and leptotena (Fig. 3, 4) were found and mainly in left gonad. Changes were not found in the right gonad.

IV. Discussion

Formation of the sexes begins with almost parallel derivation of the gonads of males or females and their settlement PGCs. Both of these processes are under different genetic controls (Murray et al., 2010). This paper examines the influence of the RA, aromatase and estrogen inhibitors (letrozole, tamoximen) and 5-AC on inversion of gender and entry into the meiosis in birds.

There is a view that sex of vertebrates is determined genetically and differentiation PGCs is influenced by the somatic gonad tissues (McLaren, 2003). It was suggested that the main gene responsible

for the induction of meiosis in mammals and birds is a gene STRA8, whose expression is regulated by RA (Bowles et al, 2010). We have shown that the administration of the RA in the incubated eggs at small concentration at the 9th or 14th days of development caused the emergence of the germ cells to the stage of preleptotena in the testis. Then prophase stops on this stage (Fig.2). Under the influence of RA the number of oocytes at the different stages of meiotic prophase I are increased in the female gonads. Only spermatogonia were present in the testis of control embryos. At later stages of embryo's development in the testis of 19th day embryos only a few spermatocytes appeared on stage proleptotene. In the same gonads affected by RA embryos the number of such cells reached 16% (Fig. 2). It was found that there were no meiotic gonocytes in the gonads of 20 day-old chicks,

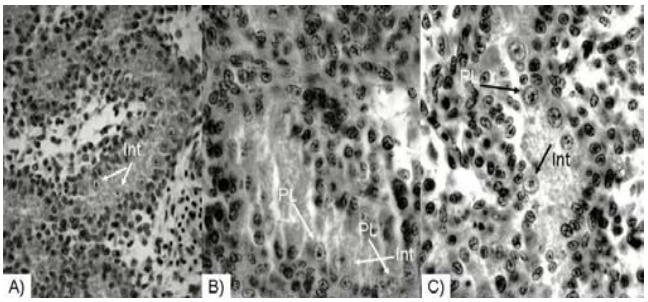

Figure 3 : The gonad sections of males at the 17th day development: A) control; B) the injection of 5-AC solution (10mkM) at the 1st day of embryos development; C) the injection of 5-AC solution (12.5mkM) at the 1st day of embryos development. It is notes spermatocytes I at the stages of interphase (Int) and preleptotene (PL).

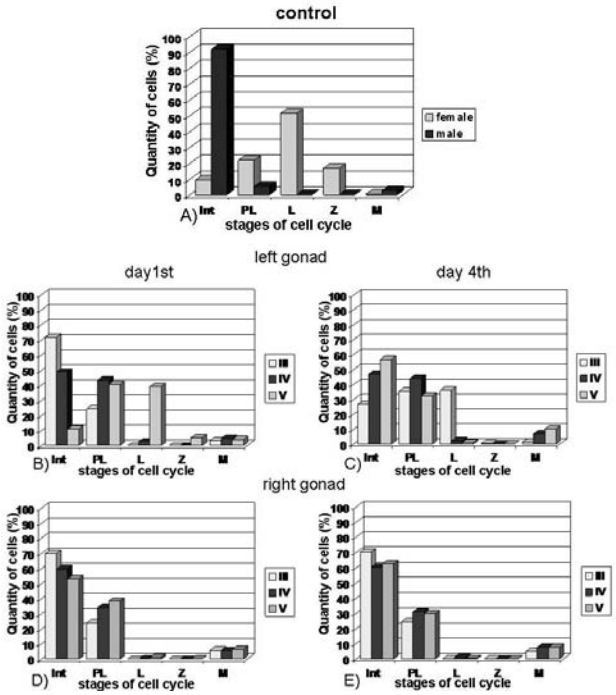

Figure 4 : The distribution of germ cells at different stages of meiotic cycle in the control female and male (A) and in the experimental male (B – E)

That were treated with RA on the 14th day of embryos development. This fact indicates that the RA is able to induce the entry into meiosis of the male cells. However, short-term exposure is not sufficient to advance the subsequent stages of meiosis and the entry into meiosis of all spermatogonia from the beginning meiotic transformations. The presence of the spermatocytes at the preleptotena stage of meiosis was confirmed by fluorescence protein SCP3 in the cytoplasm. We can assume that the RA takes part in the regulation of entry gonocytes into meiosis in birds. However, there are other factors involved. So masculinization of PGCs-supporting cells is initiated with gene *DMRT1* in some non-mammals, including chicken, *Xenopus laevis* and medaka, but in mice by induction gene *Sry* and *Sox9* expression (Yoshimoto & Ito, 2011). It is important that the injection of RA in chicken eggs during the incubation period studied is not accompanied by any sex inversion of testis.

Obtained data permits us to suggest that at early stages of embryonic development RA in small dozes (12.5mkg) allows to overcome first meiotic block in spermatocytes I and permit to entry into stage of zygotena for oocytes I when the synaptonemal complexes begin to form. During the studied terms of embryos development the germ cells of control females had not time to enter into this stage of prophase I. But the effect of RA probably is insufficient to get over the second meiotic block at the stage of diakinesis.

Earlier on the mutant of *am/am* maize it was was shown that switching over of the development program of sporogenic tissue cells from mitosis to meiosis takes place in last premeiotic mitosis. It is supposed that this mutant did not switch DNA synthesis type from mitotic type to meiotic partly delayed type of DNAsynthesis (Khvostova & Bogdanov, 1975). Very likely RA promotes this switching over. In consequence in germ cells of males first stages of meiotic prophase I begin. Moreover RA probably regulates the following events leading into meiosis: 1) delay of replication of genome DNA specific fraction (0,3%) and delay and asynchronic synthesis of all histone fractions; 2) the loss of ability to split centromeres in the next metaphase; 3) the coming prolonged meiotic prophase and the appearance spiralization of chromosomes typical for prophase type, determination of ability chromosome to the conjugation by pairs and acquisition them property to form chiasmas, forming meiotic morphology of chromosomes; 4) initiation of conjugation, synthesis of specific fraction of DNA.

According to one of hypothesis meiosis is induced by signals acting from the surrounding somatic tissues of gonad that stimulates or blocks the entry of gonocytes into meiosis in according with genetic sex of embryo (McLaren & Southee, 1997). The data about effect of RA to meiosis of mice germ cells testify in favour of the last hypothesis (Bowles & Koopman, 2010). Moreover sex hormones produced somatic cells of gonad may induce entry of germ cells into meiosis. Often it takes place at maturation time of germ cells in adult animal organism. How sex hormones influence on earlier terms of development so far is not clear.

Sex of embryos is determined chromosome set received him in the moment of fertilization. But at the stage of primary gonocytes male and female germ cells have not differences. Differences appear only after their entrance into the sex gland. In anlages of sex glands gonocytes proliferate for some time by mitosis. Oogonii cease proliferate in embryos period and turn into oocyte I. Then period of growth begins that is associate with accumulation in ovum nutritious substances from outside and with synthetic processes in ovum. The whole period previtellogenesis oocyte I gets ready for meiosis. After oocyte I entries in S-phase of reduction division, DNA amount is doubled and start prophase I of meiosis. Both oocytes and spermatocytes are in need of sex hormones those are produced by surrounding cells (Belousov, 2005).

Aromatase is a *CYP19A1* gene product revealed in both animals and plants and converse androgen to estrogen. Letrozole was used to suppress aromatase. It is less toxic to the organism (which is important for experiments with embryos) and is a nonsteroidal drug. We have shown that the sex hormone misbalance changed the female gonad differentiation into testicle formation (reduced cortex thickness, appearance of seminiferous tubules in medulla) and generation of a hermaphrodite gland, ovotestis. It should be noticed that the right gonad normally degrading during embryogenesis is preserved in genetic females with letrozole inhibited aromatase and morphologically similar to ovary. Androgen overproduction by progonadal cells abrogates the block in the development of the right gonad. The presence of the right gonad and left ovotestis in chicken genetic females creates a false impression of gonadal masculinization. This effect was also revealed by aromatase suppression by other of its inhibitors – fadrazole and vorazole (Vaillant et al., 2001; Yang et al., 2008; Li-xiu et al., 2013).

Sex hormones interact with their specific receptors (ESR1, AR), form complexes binding with regulatory regions in chromatin and initiate the transcription of particular genes (Jafarov et al., 2010). A number of chemical substances are able to limit hormone interaction with their receptors. Thus, tamoxifen is a modulator of the estrogen receptor and competes with estrogen for binding with the same receptor. As a result, estrogen penetrates into the cells of the developing female gonad in a very small, if any, amount. We suggest that the outcome should be gonadal masculinization. Indeed, we have observed this after single or double tamoxifen injection, but the effect was less apparent than after letrozole administration. A masculinization tamoxifen effect has also been observed by other researchers (Hutson et al., 1985). Also we showed that the sex hormones influence on cell differentiation of embryos gonads and formation of gonad tissues structure, physiology conditions for the future ovum and sperm. Alteration of sex hormone balance in genetic determinated embryos leads to gonad development anomalies and sterility.

In chicken meiosis of oocytes I starts in the second half of the embryonic development. At this stage in male gonads only spermatogonia are revealed. Chicken male germ cells enter meiosis only after hatching (Nakamura et al., 2013). We found that oocytes in the left gonad of ovotestis in masculinized females were at the zygotene – pachitene stage of the meiotic prophase, whereas, in the right retained gonad, germ cell differentiation was blocked. It was proposed that meiosis in females was induced by retinoic acid, the action of which may be blocked in males with cytochrome CYP26B1 (Nekrasova et al, 2011). In our experiments, the altered balance of sex hormones synthesized by gonadal cells did not influence the activity of retinoid acid and, accordingly, on the germ cell entering meiosis. The fate of germ cells in female gonads after sexual inversion requires further investigation.

An experimental approach to studying sexual inversion in birds is an important instrument for assessing the genetics of vertebrate sex determination.

It is still unclear when (on day of incubation 3, 4, 5, or 6) and where (air cavity, protein, yolk, or a "layer" of the drug on the yolk surface in the aria of the embryonic disk) it is best to carry out injections to improve drug penetration into the embryo through the blood system.

We also used an injection 5-AC as demethylating agent in the first days of incubation (1, 4 day) in order to cause the inversion of sex through the activation of MHM area of male Z chromosome and the inclusion of the two alleles of the supposed sex determining gene *DMRT1* 1. MHM area is hypermethylated in both Z-chromosomes of males and hypomethylated in the same one female region of Z. It is important that a particular methylation status is already set at 1 day of incubation (Teranishi et al., 2001). In a recent study of brazilian geneticists that used variant of RT-PCR was shown that the largest transcription of MHM for the analyzed period of incubation (4 - 14 days) is detected at 8 and 14. The males in the absence of transcription of this gene show an intense activity of DMRT1 (Caetano et al, 2014). In our experiments we used microinjection in the initial incubation period as especially important for sex determination. It turned out that the appearance of sex inversion quite rare. However, there was entry into meiosis of germ cells of males. Moreover, the cells were observed at the zygotene – pachytene stage that did not occur even with RA injection.

We want to draw attention to some interlocking control somatic and gametic sex in chickens. It is desirable to consider the role of the W chromosome in these processes (Graves, 2014).

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