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The handle http://hdl.handle.net/1887/37294 holds various files of this Leiden University dissertation

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Title: Primordial germ cells and amnion development in the avian embryo
Issue Date: 2016-01-26

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General discussion and future perspectives

AVIAN PRIMORDIAL GERM CELLS

Molecular markers. Chicken PGCs have been identified using different molecular markers: periodic acid-Schiff (PAS)[1], stage-specific embryonic antigen-1 (SSEA1), embryonic mouse antigen-1 (EMA1)[2] and chicken vasa homolog (CVH)[3]. In Chapter 2 we studied the expression of SSEA1 and CVH in chicken PGCs between stages HH5-19. SSEA1 is a marker of pluripotent stem cells in mouse that is also expressed by multiple cells in the beginning of chicken development. Our findings show that SSEA1 is downregulated at stage HH8 and peaks in germ cells localized in the gonads (60%).

In mouse there is also dynamic expression of pluripotency genes, and their up regulation in the germ cells localized in the mouse gonads has been observed [4-6]. Regarding the chicken, one study has recently analyzed the expression of pluripotent PGCs in the chicken at HH14, HH18 and HH28 [7]. Mohsen Naeemipour and colleagues have shown that the expression of pluripotency markers such as *NANOG*, *OCT4* and *SOX2* is down regulated in germ cells localized in the genital ridges [7]. It would be interesting to analyze the expression of germline-specific genes, such as *TUDOR*, *DAZZL*, *NANOG*, *DEAD END*, *PRDM14* and *SOX17* in greater depth in chicken PGCs. Recently, analysis of *BLIMP1* expression in the chicken germline has shown that *BLIMP1* is expressed in presumptive PGCs at stage X and it remains expressed in germ cells in the adult. The role of *BLIMP1* in chicken germ cells, however, remains unclear [8]. Although this remains to be confirmed, preformation is most likely the underlying mechanism of PGC development in the chicken. Detailed analysis of gene expression in PGCs will be very useful to elucidate on this. Moreover, the dynamics of PGCs in mammals, like mice and humans, is better known and constitutes a good point of comparison with birds.

In Chapter 2 we show that two different populations of germ cells can be identified in the gonads: germ cells double positive for CVH and SSEA1 and germ cells positive only for CVH. In mammals, different population of germ cells have been described in the gonads and are dependent on the maturation state of the oogonia or spermatogonia [9]. In chicken, as we show in Chapter 3, the location of germ cells in the gonads affects the expression of the meiotic markers, SYCP3 and H2AFX. We show that germ cells in the right gonad only express H2AFX between stages HH38-45, while left gonad germ cells can express both, H2AFX and SYCP3, at stage HH45. We report, for the first time, the influence of cell position on the expression of meiotic markers, which is associated with different maturation stages of chicken germ cells (Chapter 3). However, we still do not know if other features of germ cells, such as the expression of different genes related to pluripotency or germline identity, are also affected by the position of the cells in the gonads. Analyzing the relationship between position and gene expression will shed some light on the mechanisms underlying germ cell maturation in the chicken.

Furthermore, epigenetics in chicken PGCs is a process that is still not completely understood. [10]. The study of PGC epigenetics in mammals has elucidated how DNA methylation, histone modifications, imprinting and X reactivation occur in PGCs. A good example of these studies in mammals, is the recent work by Azim Surani's group, that analyses the transcriptional network and epigenetic reprogramming of human germ cells in different developmental time points [11]. A temporal analysis of gene expression and DNA methylation in chicken would further elucidate the epigenetic mechanisms regulating PGC (pre)formation in a non-mammal organism.

Moreover, this will allow a better understanding of how epigenetics is regulated in sexual system of birds, which is different from that in mammals. While in mammals the females have two homogametic chromosomes (XX) and the male has two heterogametic chromosomes (XY), in the chicken the opposite occurs: the male has two homogametic chromosomes (ZZ) and the female has two heterogametic chromosomes (ZW) [12]. Mammalian X and bird Z chromosomes are significantly larger than the respective Y and W chromosomes. However, while one of the X chromosomes is widely inactivated in homogametic mammal females, promoting dosage compensation of mammal's sexual chromosomes, in homogametic bird males some genes seem to be silenced in the Z chromosome, not only in one but in both Z chromosomes [13, 14]. The male hypermethylated (MHM) region constitutes the locus on the Z chromosome where most of transcriptionally silenced genes are localized. [14, 15]. On the contrary, in chicken females, the MHM region, in the single Z chromosome, is transcribed as a long non-coding RNA. Interestingly, it has been suggested a role for these long non-coding RNA are responsible to control differential expression of the Z chromosome and regulate dosage compensation, but this is still not clear [14, 16]. These differences between mammals and birds, in regard to sexual chromosomes and dosage compensation, are due to evolutionary divergences that are still not completely understood [17]. While the XY sexual system has been widely studied, the ZW is less understood. Therefore, in order to better analyze the two systems and determine their relative evolutionary significance, more attention should be given to studying epigenetics in the chicken.

Isolation of avian PGCs. In order to address molecular, genetic and epigenetic features of the chicken germline, it is necessary to develop protocols that would allow isolation of chicken PGCs. On the one hand, the availability of embryos and the fact that PGCs in the chicken circulate in the blood, which can easily be collected, constitutes an advantage when compared with species such as mice or humans. On the other hand, our limited knowledge of chicken PGC (surface) markers makes the development of effective isolation protocols difficult.

For this reason, two main techniques have been used to isolate PGCs in chicken embryos to date: Ficoll density-gradient centrifugation [7, 18] and FACS [2, 19, 20]. Ficoll density-gradient centrifugation consists of isolating PGCs from chicken blood distributed over a sucrose gradient. The presence of PGCs in the isolated fraction is confirmed by analyzing the expression of germline markers, such as CVH. FACS is based on the use of specific antibodies for germ cells, of which the most commonly used are SSEA1 and EMA1. However, as we showed in chapter 2 it is not clear whether even these antibodies used together would identify all PGCs or just a subfraction: it is expressed by other cells at early stages of development, and not by all germ cells localized in the gonads.

Nevertheless, isolation protocols for chicken PGCs rely on the identification of PGC markers that can be used in live cells. In contrast to CVH (cytoplasmic protein), SSEA1 is cell surface protein and therefore fixation and permeabilization of cells is not needed to allow the antibody to bind. EMA1 [2] has also been used as a tool to access gonadal germ cells. Recently it has been shown through immunohistochemistry that expression of EMA1 does not completely overlap with CVH, and therefore EMA1 seems to also not be a specific marker for PGCs in the chicken. Finding new surface markers for chicken germ cells is still a challenge in the field, and a bottleneck for defining PGC isolation protocols.

Migration. Unlike mammals, chicken PGCs use blood vessels to migrate to the gonads. Several studies have addressed the question of how chicken germ cells migrate into the gonads [21-23]. Due to the use of non-specific markers in previous studies, some features of PGCs remain unclear, such as molecular mechanisms guiding migration or the migratory route followed by PGCs.

In Chapter 2 we provide a detailed study on the migration of PGCs from the extraembryonic circulation into the embryo. We counted the number of PGCs in different areas of the developing embryo from HH5-19, and observed that from stage HH14, germ cells started to appear in the genital ridges. Our results on the number of PGCs are in accordance with other groups that also analyzed whole-mount chicken embryos from different stages stained for CVH [22]. However, our study goes beyond what has been established regarding PGC migration, since we focus on understanding the mechanisms used by PGCs to migrate into the embryonic vasculature. A closer look at substage HH13circ showed that PGCs had accumulated in the medial part of area pellucida and sinus terminalis. Therefore we decided to investigate the anatomical position of PGCs at this stage, through histological sections and 3D reconstruction. Curiously, our results showed that the majority of germ cells were actually localized in the anterior vitelline veins. We blocked the anterior vitelline veins at HH13 by clamp, and verified a decrease in the number of germ cells localized in the gonads at HH15 (Chapter 2). In order to

observe if PGCs were able to find alternative ways to colonize the gonads, it would be interesting to leave the embryos until later stages of development.

Regarding the role of chemotaxis in directing PGCs to the gonads, it has been suggested that SDF1/CXCR2 plays a role in the process [23]. Manipulation of chicken embryos *in ovo*, can be used to test the relative importance of blood circulation and signalling clues sent by the gonads in PGC homing. A possible experiment would be placing the genital ridges of HH12 embryos in specific regions of the extraembryonic vasculature, in order to test the effect on the migration of germ cells. If chemoattraction plays a role at this stage, we would expect the cells to redirect their migration to the new place of the gonads.

From our analysis of whole-mount chicken embryos stained with CVH we also observed that PGCs migrate preferentially to the left gonads, in both females and males (Chapter 3). We showed that between HH15-19 there are more germ cells localized in the left than in the right genital ridge. Our results are in accordance with others. It has been suggested that the difference in the number of PGCs, at these stages, in both the right and the left side, is due to secretion by the presumptive gonads of molecules that attract PGCs preferentially to the left side [23] or secretion of molecules that trigger mitotic activity of PGCs in the left [24]. Moreover, it has also been suggested that the asymmetrical expression of *BMP7* and *PITX2* in the gonads is involved in asymmetrical migration (Chapter 3). Despite these leads, this process is still poorly understood and deserves more attention.

Gonadal asymmetry and meiosis. Contrary to what happens in most animals, where the two embryonic gonads develop into two functional organs, in the female chicken, as in most birds, only the left gonad will develop into a functional ovary [25]. Interestingly, gonadal asymmetry already starts to be evident during migration in both sexes: germ cells migrate preferentially to the left gonad (Chapter 2). However, after sex differentiation, differences between right and left gonads with regard to the number of PGCs and their morphology become more pronounced in the female chicken [26].

In Chapter 3 we analyzed in detail the differences in the expression of different meiotic markers in germ cells localized in different regions of the right and left gonad. Our study is the first study to report, simultaneously, the dynamic expression of two different meiotic markers (H2AFX and SYCP3) in chicken gonadogenesis (Chapter 3). Moreover, we looked for massive apoptosis in the right gonad, as had previously been suggested [27]. We have observed expression of H2AFX from stage HH38 in both female and males, which we show not to be correlated with massive germ cell apoptosis. On the contrary, H2AFX expression seems to indicate that germ cells are in the pre-leptotene

stage until hatching. It is not clear whether the expression of FASLG-FAS in germ cells localized in the extremities of the cortex and medulla in HH45 indicates future apoptosis (Chapter 3). However, our study does not analyze the expression of apoptotic markers in the gonads after hatching. This analysis is necessary for understanding if the expression of FASLG-FAS in germ cells at stage HH45 dictates future apoptosis.

Simultaneous analysis of the expression of two different meiotic markers shows that meiosis in chicken germ cells depends on their position in the gonad. Moreover, we are the first to report a meiotic wave in chicken gonads: we showed that germ cells localized in the center of the left cortex are more mature (late zygotene/early pachytene and late pachytene/early zygotene) while germ cells in the extremities of the left gonad are less mature (early leptotene or pre-leptotene). Germ cells localized in the right gonad remain in pre-leptotene until before hatching. The existence of different meiotic stages in the left gonad, has been shown by the analysis of meiotic spreads [28]. Moreover, the results from other studies regarding immunohistochemical analysis of the expression of meiotic markers, suggest that they are not expressed uniformly in the gonads. However, conclusions regarding differences in the maturation state of germ cells have not been made, since in these studies only one meiotic marker was used [29-31]. Our study is, however, mostly descriptive and so there are several aspects that remain unclear: Are the mesonephros, in close connection with the gonad, responsible for secreting signals involved in this meiotic wave or is the gonad itself? Are the molecules involved in chicken asymmetric gonadogenesis affecting meiosis? We (data not shown) and others [29, 32] have cultured chicken gonads *in vitro*. In this system, gonads can be cultured with or without the presence of the mesonephros, which can further help elucidate whether the presence of the mesonephros affects meiosis in the gonad. Moreover, this system offers a model to culture gonads in the presence of a variety of signalling inhibitors or activators. Blocking signaling pathways involved in gonadal asymmetry, such as Retinoic Acid, PITX2 or BMP 7, and verifying their effects on the expression of meiotic markers, can shed further light on which genes involved in asymmetry affect germ cell meiosis. The availability of embryos is an advantage, since it allows the optimization of the culture system, with regard to concentrations of inhibitors/activators and culturing times.

Production of transgenic birds. Studying the germline in the avian model also contributes to our understanding of pluripotency in a non-mammalian system: the chicken. Several research groups have succeeded in producing chicken embryonic germ cells (cEGs) by culturing circulating PGCs (cPGCs) [33, 34] and gonadal PGCs (gPGCs) [35, 36]. In these studies, blood or chicken gonads are cultured in the presence of leukemia inhibitor factor, stem cell factor and basic fibroblast growth factor [33, 34, 36]. Contrary to chicken embryonic stem cells (cESC), isolated from the blastoderm [37], cEGs derived by cPGCs and gPGCs can contribute to the germline and have been used in

the production of germline chimeras[33]. cEGs obtained from gPGCs revealed to be less efficient regarding germline chimeras[35]. Marie-Cécile van de Lavoir and colleagues were the first to induce transgenesis by electroporation of a non-viral expression vector into cEGs in culture, cEGs were subsequently introduced in host embryos in order to produce transgenic birds [33]. Since then, the protocols for transgenesis in birds have diversified and efficiency has improved [38], but research on chicken germline and pluripotency is still needed for further optimization. In turn, transgenic birds will become a valuable tool to understand PGC mechanisms but also in the conservation of endangered species and poultry production for agriculture (reviewed in [39]). Moreover, the generation of transgenic birds for production of recombinant human proteins is also a major application in the pharmaceutical industry [38].

AMNIOGENESIS IN CHICKEN

While studying the migration of PGCs in the chicken we made a curious observation: we found PGCs, which are normally localized in the splanchnopleure and in the vasculature of the yolk sac, present dorsally over the head (Chapter 2, Chapter 5). This observation led us to investigate the formation of the anterior amnion fold in chickens. We studied transversal sections and 3D reconstructions of chicken embryos and provide functional evidence, using *ex ovo* cultures of chicken embryos, to explain the role of the proamnion in the development of the anterior amnion fold in the chicken (Chapter 5). The proamnion is a diblastic structure composed of ectoderm and endoderm. The proamnion is present in different species, and it seems to have different roles in amnion development, as we discuss in Chapter 5.

In relation to chicken amniogenesis, Thomas Shore and J.W. Pickering were the first to anatomically describe the proamnion as a structure underlying the developing head at stage HH10 and to suggest its involvement in amnion formation [40]. In Chapter 5 we revisited their anatomical model using two different functional assays: the inverted culture system and the suspension culture system. In the former, the chicken embryos were grown with their ventral side facing upwards, a condition which impairs the formation of the anterior amnion fold. In the latter culture system, the embryo grows in a “Cornish pasty” shape and as a result the anterior amnion fold is not formed and does not cover the head of the embryo. Our results showed that sinking of the head in the proamnion between HH10-14 is of paramount importance to the formation of the anterior amnion fold in the chicken. This sinking of the head in the proamnion is responsible for the replacement of the splanchnopleure on top of the head, explaining why the PGCs can be found dorsally at this point of embryonic development (Chapter 5). With regard

to amnion development, it will be interesting to analyze if culturing the chicken embryo in an inverted position also effects the formation of the posterior amnion fold. The anterior amnion fold is the first to develop, and therefore the sinkage of the embryo in the proamnion can also have implications for the correct development of the posterior amnion fold.

In Chapter 2 and Chapter 4 we present two different *ex ovo* culture systems: the inverted/non inverted culture system and suspension culture system. The inverted culture system is an adaptation of a protocol published by Chapman and colleagues [41] and embryos were maintained for maximum of 2 days in culture. The suspension culture system is an adaption of Cornish pasty method [42] and the embryos were maintained in culture for 72 hours. Both systems improved the survival rate of the embryos and the reproducibility of results, and therefore constitute a useful system for further exploration through embryonic manipulation and live imaging. However, they also present some limitations,with respect to morphological defects observed in the cultured embryos such as the absence of anterior vitelline vessels in the suspension system or absence of the anterior amnion fold development in both, inverted and suspension systems. Therefore the applicability of each culture system to other research questions, should always take into account the morphological defects caused by the culture systems themselves.

AVIAN MODELS IN DISEASE

Epithelial ovarian cancer (EOC) is one of the leading causes of cancer-related deaths among women [43]. This is due to the fact that EOC is often asymptomatic, and its location in the peritoneal cavity makes early detection difficult and facilitates the spread of the disease (reviewed in Chapter 4). Moreover, we still lack efficient treatment due to poor information regarding the origin and development of the disease. Different models have been used in EOC research: fruit fly, mice, *in vitro* systems and the adult hen. In Chapter 4 we reviewed different models used in EOC research with a focus on the advantages of using the avian model in the study of this disease. The adult hen offers a particularly valuable model, since, as in humans and unlike in any other species, the disease develops spontaneously. Moreover, the metastatic processes are similar to those in humans, and the use of the avian model allows manipulation of environmental variables with regard to nutrition, hormones and drugs [44].

Besides the advantages of studying EOC in the adult hen, in Chapter 4, we discussed the advantages of using the asymmetric development of chicken gonads to model cancer pathways in EOC. We highlighted the fact that PITX2, overexpressed in EOC [45], is also an important player in gonadal asymmetric development in the chicken

[30]. In gonadogenesis, PITX2 is expressed in the left gonad and has been associated with higher proliferation, but its role in cancer development is still not clearly understood [45]. Silvana Guioli and colleagues have studied the role of PITX2 in chicken gonadogenesis using RCAS virus to induce expression of PITX2 in the chicken gonad at different time points [30]. The versatility of the chicken embryo as an experimental model that allows physical manipulation together with gene silencing and activation assays makes the chicken an interesting model to study the role of PITX2 in EOC. Inhibiting or activating this pathway in cancer would be interesting in order to understand its role in disease.

FUTURE PERSPECTIVES

The chicken embryo is the experimental model with the longest history in developmental biology and disease [46]. The similarities with humans, and the ease of experimental manipulation have made the chicken a good system in basic scientific research. However, compared to other models, such as mice and flies, the availability of genetic and molecular tools for research is still a limitation in birds. Only lately have chicken laboratory tools for genomics, proteomics and metabolomics become available and this has contributed to transforming the chicken from "a great model system" to "become even greater" [47].

The future of the chicken in scientific research is, however, dependent on the continuation of the development of dedicated tools that can improve methods of experimental manipulation and analysis. In Chapter 4 we provide an extensive discussion on the advantages and disadvantages of using the chicken in modelling EOC. There are however many other applications for chicken laboratory technologies. Another good example is that of the gene deletion or mutation technologies. While in mice and flies, gene knockout is nowadays established in relating cause and effect, in the chicken they are still not established although RNA interference is indeed "up-and-running" in the chicken; this has allowed scientists to understand gene function but only by post-transcriptional gene silencing [48]. The new tool of Crispr/Cas9 gene editing still has to be explored. The availability of optimized protocols for the production of transgenic birds, still dependent on efficient establishment of cSC lines and transgenic techniques for cEGs, will offer a great improvement regarding the development of avian gene knockouts [39]. Avian gene knockouts are fundamental tools to address many questions that remain unanswered due to the impossibility to perform functional assays on gene silencing. Understanding of the mechanism of preformation in avian germ cells or the role of genes expressed asymmetrically in the chicken gonad in the process of meiosis, the subjects of this thesis, are only some examples. The production of transgenic birds will have a direct impact in fundamental research, applied biomedical research and medicine but also in regard to agriculture and pharmaceutical industry.

REFERENCES

1. D. M. Application of the Periodic Acid-Schiff Technique to Whole Chick Embryos. Biotechnic & Histochemistry. 1960;35(2):83-9.
2. Mozdziak PE, Angerman-Stewart J, Rushton B, Pardue SL, Petitte JN. Isolation of chicken primordial germ cells using fluorescence-activated cell sorting. Poultry science. 2005;84(4):594-600.
3. Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development. 2000;127(12):2741-50.
4. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes & development. 2003;17(1):126-40.
5. Yamaguchi S, Kimura H, Tada M, Nakatsuji N, Tada T. Nanog expression in mouse germ cell development. Gene expression patterns : GEP. 2005;5(5):639-46.
6. Payer B, Chuva de Sousa Lopes SM, Barton SC, Lee C, Saitou M, Surani MA. Generation of stella-GFP transgenic mice: a novel tool to study germ cell development. Genesis. 2006;44(2):75-83.
7. Naeemipour M, Dehghani H, Bassami M, Bahrami A. Expression dynamics of pluripotency genes in chicken primordial germ cells before and after colonization of the genital ridges. Molecular reproduction and development. 2013;80(10):849-61.
8. Wan Z, Rui L, Li Z. Expression patterns of prdm1 during chicken embryonic and germline development. Cell and tissue research. 2014;356(2):341-56.
9. Anderson RA, Fulton N, Cowan G, Coutts S, Saunders PT. Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. BMC developmental biology. 2007;7:136.
10. Jang HJ, Seo HW, Lee BR, Yoo M, Womack JE, Han JY. Gene expression and DNA methylation status of chicken primordial germ cells. Molecular biotechnology. 2013;54(2):177-86.
11. Tang WW, Dietmann S, Irie N, Leitch HG, Floros VI, Bradshaw CR, et al. A Unique Gene Regulatory Network Resets the Human Germline Epigenome for Development. Cell. 2015;161(6):1453-67.
12. Arnold AP, Itoh Y, Melamed E. A bird's-eye view of sex chromosome dosage compensation. Annual review of genomics and human genetics. 2008;9:109-27.
13. Kuroiwa A, Yokomine T, Sasaki H, Tsudzuki M, Tanaka K, Namikawa T, et al. Biallelic expression of Z-linked genes in male chickens. Cytogenetic and genome research. 2002;99(1-4):310-4.
14. Melamed E, Arnold AP. Regional differences in dosage compensation on the chicken Z chromosome. Genome biology. 2007;8(9):R202.
15. Caetano LC, Gennaro FG, Coelho K, Araujo FM, Vila RA, Araujo A, et al. Differential expression of the MHM region and of sex-determining-related genes during gonadal development in chicken embryos. Genetics and molecular research : GMR. 2014;13(1):838-49.
16. Teranishi M, Shimada Y, Hori T, Nakabayashi O, Kikuchi T, Macleod T, et al. Transcripts of the MHM region on the chicken Z chromosome accumulate as non-coding RNA in the nucleus of female cells adjacent to the DMRT1 locus. Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology. 2001;9(2):147-65.
17. Hen G, Friedman-Einat M, Sela-Donenfeld D. Primordial germ cells in the dorsal mesentery of the chicken embryo demonstrate left-right asymmetry and polarized distribution of the EMA1 epitope. Journal of anatomy. 2014;224(5):556-63.

18. Chang IK, Tajima A, Yasuda Y, Chikamune T, Ohno T. Simple method for isolation of primordial germ cells from chick embryos. Cell biology international reports. 1992;16(9):853-7.

19. Motono M, Ohashi T, Nishijima K, Iijima S. Analysis of chicken primordial germ cells. Cytotechnology. 2008;57(2):199-205.

20. Mozdziak PE, Wysocki R, Angerman-Stewart J, Pardue SL, Petitte JN. Production of chick germline chimeras from fluorescence-activated cell-sorted gonocytes. Poultry science. 2006;85(10):1764-8.

21. Kang KS, Lee HC, Kim HJ, Lee HG, Kim YM, Lee HJ, et al. Spatial and temporal action of chicken primordial germ cells during initial migration. Reproduction. 2015;149(2):179-87.

22. Nakamura Y, Yamamoto Y, Usui F, Mushika T, Ono T, Setioko AR, et al. Migration and proliferation of primordial germ cells in the early chicken embryo. Poult Sci. 2007;86(10):2182-93..

23. Stebler J, Spieler D, Slanchev K, Molyneaux KA, Richter U, Cojocaru V, et al. Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. Dev Biol. 2004;272(2):351-61.

24. Swartz WJ, Domm LV. A study on division of primordial germ cells in the early chick embryo. The American journal of anatomy. 1972;135(1):51-70.

25. AL R. The avian embryo: structural and functional development. : New York: Macmillan; 1960.

26. Smith CA, Sinclair AH. Sex determination: insights from the chicken. Bioessays. 2004;26(2):120-32.

27. Ukeshima A. Germ cell death in the degenerating right ovary of the chick embryo. Zoolog Sci. 1996;13(4):559-63.

28. del Priore L, Pigozzi MI. Chromosomal axis formation and meiotic progression in chicken oocytes: a quantitative analysis. Cytogenetic and genome research. 2012;137(1):15-21.

29. Smith CA, Roeszler KN, Bowles J, Koopman P, Sinclair AH. Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. BMC developmental biology. 2008;8:85.

30. Guioli S, Lovell-Badge R. PITX2 controls asymmetric gonadal development in both sexes of the chick and can rescue the degeneration of the right ovary. Development. 2007;134(23):4199-208.

31. Guioli S, Nandi S, Zhao D, Burgess-Shannon J, Lovell-Badge R, Clinton M. Gonadal asymmetry and sex determination in birds. Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation. 2014;8(5):227-42.

32. Yu M, Yu P, Leghari IH, Ge C, Mi Y, Zhang C. RALDH2, the enzyme for retinoic acid synthesis, mediates meiosis initiation in germ cells of the female embryonic chickens. Amino acids. 2013;44(2):405-12.

33. van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, et al. Germline transmission of genetically modified primordial germ cells. Nature. 2006;441(7094):766-9.

34. Yamamoto Y, Usui F, Nakamura Y, Ito Y, Tagami T, Nirasawa K, et al. A novel method to isolate primordial germ cells and its use for the generation of germline chimeras in chicken. Biology of reproduction. 2007;77(1):115-9.

35. Han JY, Park TS, Hong YH, Jeong DK, Kim JN, Kim KD, et al. Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained *in vitro* for an extended period. Theriogenology. 2002;58(8):1531-9.

36. Park TS, Hong YH, Kwon SC, Lim JM, Han JY. Birth of germline chimeras by transfer

of chicken embryonic germ (EG) cells into recipient embryos. Molecular reproduction and development. 2003;65(4):389-95.

37. Lavial F, Pain B. Chicken embryonic stem cells as a non-mammalian embryonic stem cell model. Development, growth & differentiation. 2010;52(1):101-14.

38. Park TS, Han JY. Genetic modification of chicken germ cells. Annals of the New York Academy of Sciences. 2012;1271:104-9.

39. Intarapat S, Stern CD. Chick stem cells: current progress and future prospects. Stem cell research. 2013;11(3):1378-92.

40. Shore TW, Pickering JW. The Proamnion and Amnion in the Chick. J Anat Physiol. 1889;24(Pt 1):1-21.

41. Chapman SC, Collignon J, Schoenwolf GC, Lumsden A. Improved method for chick whole-embryo culture using a filter paper carrier. Developmental dynamics : an official publication of the American Association of Anatomists. 2001;220(3):284-9.

42. Nagai H, Lin MC, Sheng G. A modified cornish pasty method for *ex ovo* culture of the chick embryo. Genesis. 2011;49(1):46-52.

43. Lowe KA, Chia VM, Taylor A, O'Malley C, Kelsh M, Mohamed M, et al. An international assessment of ovarian cancer incidence and mortality. Gynecol Oncol. 2013;130(1):107-14.

44. Johnson PA, Giles JR. The hen as a model of ovarian cancer. Nat Rev Cancer. 2013;13(6):432-6.

45. Fung FK, Chan DW, Liu VW, Leung TH, Cheung AN, Ngan HY. Increased expression of PITX2 transcription factor contributes to ovarian cancer progression. PloS one. 2012;7(5):e37076.

46. Kain KH, Miller JW, Jones-Paris CR, Thomason RT, Lewis JD, Bader DM, et al. The chick embryo as an expanding experimental model for cancer and cardiovascular research. Developmental dynamics : an official publication of the American Association of Anatomists. 2014;243(2):216-28.

47. Stern CD. The chick; a great model system becomes even greater. Developmental cell. 2005;8(1):9-17.

48. Sifuentes-Romero I, Milton SL, Garcia-Gasca A. Post-transcriptional gene silencing by RNA interference in non-mammalian vertebrate systems: where do we stand? Mutation research. 2011;728(3):158-71.