Primordial Germ Cells and Amnion Development in the Avian Embryo

Ana de Melo Bernardo

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Principles for the Development of a Complete Mind:
Study the science of art. Study the art of science.
Develop your senses - especially learn how to see.
Realize that everything connects to everything else.

Leonardo Da Vinci (1452 - 1519)

CONTENTS

1

General Introduction and Outline of this Thesis

THE "IMMORTAL" CYCLE OF THE GERMLINE

The question of how humans and other organisms originate, has fascinated scientists and society throughout history. In 1651, the English physician William Harvey claimed "*ex ovo* Omnia" - all life originates from an egg – suggesting the importance of the egg in the origin of all living animals, and refuting the idea of spontaneous generation [1]. A few years later, Antoni van Leeuwenhoek was able to build a microscope that allowed him to observe spermatozoa [2]. Van Leeuwenhoek would become the first scientist to suggest that the spermatozoid penetrates the egg in a process that we now call fertilization. His ideas contradicted Harvey's and the then common belief that fertilization occurred due to "vapors" originating in the seminal fluid. Nevertheless, Van Leeuwenhoek was never able to observe the spermatozoid penetrating the egg under his microscopes [3]. Only towards the end of the next century, in 1891, did Oscar Hertwig observe the process of fertilization in the sea urchin [4]. Even now, after almost 400 years of research, we continue to unravel the most fascinating mechanisms underlying the gametes and their progenitors: the primordial germ cells (PGCs).

PGCs are specialized cells that are formed outside the developing embryo, from where they migrate into the gonad, and give rise to the gametes (reviewed in [5]). PGCs, and subsequently the derivative gametes, form the so-called germline, which is at the same time an "immortal cell line", responsible for transmitting genetic information through generations; this constitutes the raw material for evolution [6, 7]. Evolution has brought about a number of interesting characteristics that allow the "immortality" of the germline: PGCs for example give rise to haploid gametes, possess highly regulated transcriptional genetic programming, and have specialized epigenetic regulation (reviewed in [8]). Once in the gonads, germ cells can develop into oocytes or sperm through a sex-dependent maturation process – oogenesis or spermatogenesis, respectively – that includes the transition from a diploid to a haploid state through meiosis [9]. The fusion of the haploid gametes results in a diploid totipotent cell – the zygote – which is the origin of a complete organism and its extraembryonic structures [10]. The unipotent state of PGCs is therefore very tightly controlled and disruption of the control mechanisms can lead to developmental abnormalities. For example, the migration of PGCs into ectopic sites or abnormal signalling in the gonad can initiate inappropriate pluripotency in germ cells and lead to the development of malignant or benign tumors (called teratocarcinomas or teratomas, respectively)[11]. Moreover, when cultured *in vitro*, PGCs can give rise to embryonic germ cells, which behave as pluripotent cell line, capable of self-renewal, differentiation and contribution to chimaeras when introduced into an embryo, similar characteristics as embryonic stem cells (ESCs) [12] (Figure 1).

Different animal models have been used to study different aspects of germline biology, ranging from flies, frogs and fish to birds and mammals. This dissertation focuses on PGC migration as they move to the gonads (Chapter 2) and meiosis (Chapter 3) in the chicken embryo.

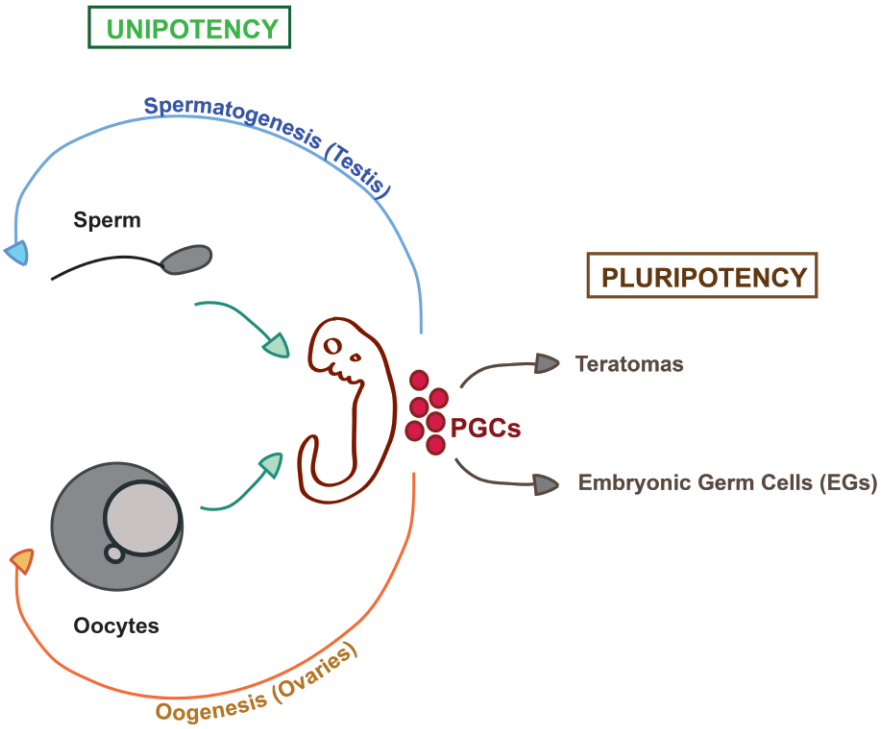

Figure 1. The immortal germline. Primoridal germ cells (PGCs) are unipotent cells that are the progenitors of the oocytes and sperm. When fused, oocyte and sperm give rise to the zygote, a totipotent cell that will develop into a complete organism. During abnormal development, PGCs give rise to teratomas, that demonstrate their pluripotent characteristics. When cultured *in vitro*, PGCs can also acquire pluripotency (EGs).

ON THE ORIGIN OF PGCs: EPIGENESIS AND PREFORMATION

Due to the importance of PGCs in development and evolution, the mechanisms regulating germline specification are strictly regulated during development. PGCs are specified early in development, a processed defined by the segregation of the germline from the somatic line [8]. Although the germline represents a major evolutionary step that enabled sexual reproduction in the metazoan species, PGC specification mechanisms are not conserved. Two main mechanisms are responsible for the development of PGCs in vertebrates: epigenesis and preformation (reviewed in [13]).

Preformation. In the late nineteenth century August Weissman introduced the concepts of germline and soma. He suggested that the oocyte contained germ plasm from the mother and that this plasm was responsible for the specification of germ cells after fertilization [14]. Robert Hegner was the first to identify germ granules, in which the germ plasm is transported, in the germ cells of beetles. They introduced the first concepts of "preformation", the process by which germ cell precursors are defined by maternal factors contained in the egg [8]. This was later shown to be responsible for the development of germ cells in *C. elegans*, *D. melanogaster*, *D. renio*, *X. laevis* and *G. gallus* [15].

The most well documented organism with respect to germline development is the fly. In the fruitfly *Drosophila*, PGC segregation depends on the asymmetrical deposition of germ plasm in the posterior pole region of the oocyte, where the germline forms [16, 17]. The development of the embryo consists of the formation of a syncytium, meaning that the embryo goes through nuclear divisions without cell division, from the center to the periphery of the oval-shaped *Drosophila* embryo. The nuclear division in the pole leads to the formation of PGC precursors, and the cells are individualized (i.e. not only do the nuclei divide, but cells form earlier than in the rest of the embryo) and called pole cells [18]. During gastrulation around 40 pole cells containing germ plasm, composed of maternal proteins and mRNA, are carried into the embryo [15]. The germ plasm is responsible for the activation of a germline genetic program that promotes the specification of PGCs [19]. The molecular content of the germ plasm has been determined largely through the analysis of mutant flies [19, 20]. The fact that female mutants for certain genes, such as vasa, valois or tudor, do not develop germ cells, revealed the importance of these factors in PGC development in flies [21]. In the chicken, it seems that preformation is also the mechanism underlying PGC specification. In 2000, the isolation of a chicken homolog of VASA (CVH) by Naoki Tsunekawa and colleagues allowed the identification of cells expressing CVH from the first cleavage in the chicken embryo: 1) in granulofibrilar structures around the mitochondrial cloud and spectrin protein-enriched structure, and 2) in a germ plasm-like structured localized in

the basal part of the first embryonic cleavage [22].

In chicken embryos, two systems are used to define developmental stages: Eyal-Giladi & Kochav′s staging is commonly used from the first cleavage (stage I) until pre-primitive streak stage (stage X) [23]; and Hamburger and Hamilton staging that considers chicken development from early primitive streak (stage HH2) until just before hatching (stage HH45) [24]. Both systems consider morphological landmarks of the chicken embryo. The first embryonic cleavage is therefore defined by Eyal-Giladi & Kochav system as stage I [23]. Following the expression of CVH, after the first embryonic cleavages, it was shown that CVH remained in 6-8 cells located in the center of the blastocyst at stage V. Moreover, *in vitro* experiments have shown that inducing vasa overexpression in chicken stem cells (cSC) upregulates the expression of other germline markers, such as TUDOR, SDF1, CXCR4 and DAZZL [25]. Interestingly, these same cells, induced *in vitro*, were able to migrate to the embryonic gonad [25]. Although this seems to be evidence for the origin of PGCs by preformation in the chicken, functional studies are still necessary to prove this. However, this is presently not possible to do due to the lack of molecular and genetic tools for the chicken.

Epigenesis. In 1947, almost one hundred years after Weissman, Pieter Nieuwkoop, showed that 'unspecialized' cells, localized in the primitive ectoderm of axolotl embryos, could be induced into PGCs [26]. Nieuwkoop's observation led to the suggestion that PGCs can also be induced without the presence of the germ plasm, in a process that is now called epigenesis [26]. Subsequent analysis of gene knockout and transgenic mice revealed part of the mechanism of epigenesis. In mice, it is now known that a population of presumptive PGCs is founded in the posterior part of the embryo at E6.25. This population was first characterized by the expression of Blimp 1, a transcriptional repressor of somatic genes in PGCs [27]. Nowadays it is known that the interaction between three transcription factors, Blimp1, Prdm14 and Ap2γ, controls specification of PGCs in mice. These factors are responsible for the repression of the somatic programme in these cells [28]. At E7.25, a population of 40 PGCs, derived from the presumptive PGCs and localized in the same position, can be identified by Alkaline Phosphatase activity, and expression of Stella [28]. Concerning signaling pathways involved in PGC specification in mice, it has been shown that Bone morphogenetic protein (BMP) 4 plays a crucial role, since *BMP4* knockout embryos lack PGC precursors [29]. Other growth factors in the same pathway, such as Bmp2 [30] and Bmp8b [31], also produced by the extraembryonic tissues and acting via Smad1 [32, 33] , Smad5 [34] and Alk2 [35], have been shown to have important roles in the specification of PGCs. In contrast to what happens in preformation, in principle all epiblast cells can differentiate into PGCs in the presence of the above signaling cues, since this does not depend on the presence of germ plasm. Recently, Katsuhiko Hayashi and colleagues were able to recapitulate *in vitro* the

signaling cues necessary to produce eggs from mouse embryonic stem cells (mESC) and mouse-induced pluripotent stem cells (mIPSC) [36, 37]. Although there are differences between mouse and human PGCs, studies in mice have been fundamental in bringing us closer to producing human gametes *in vitro* [38].

ON THE ROAD: THE MIGRATORY ROUTE OF CHICKEN PGCs

After specification, PGCs are maintained outside the developing embryo, most likely to avoid signals that could compromise their strictly-controlled developmental program. When the basic pattern of the organism is formed, PGCs migrate from their place of origin to meet the somatic cells of the gonads [39]. In the gonads, PGCs continue their development into oogonia or spermatogonia in the female or male gonads respectively [40]. PGC migration mechanisms are also not conserved between organisms.

In the mouse, at E7.25 PGCs are localized in the base of the allantois from where they start to migrate to where the genital ridges will form [41]. From the base of the allantois, PGCs migrate to the adjacent endoderm, which will develop into the hindgut [42]. At E8.5 PGCs are found migrating along the midline of the embryo, from the hindgut through the dorsal mesentery. At around E10.5, PGCs reach the genital ridges [42] (Figure 2). The interaction of Sdf-1 and its receptor Cxcl12 is fundamental to chemotaxis of PGCs toward the genital ridges. In mice it was shown that the use of Sdf-1-coated beads in slice cultures of mouse embryos caused defective movements of PGCs and decreased survival [43, 44]. Moreover, homozygous knockout mice lacking Cdx2 show a dramatic decrease in the number of germ cells colonizing the genital ridges. This interaction seems to be important for the behavior of germ cells once they leave the hindgut [44].

In contrast to what happens in the mouse, in the chicken embryo the migration of PGCs occurs through the vascular system and starts from the anterior part of the embryo. Tsunekawa et al. [22]. From the germinal crescent, PGCs start to accumulate in the extraembryonic mesoderm between stages HH4-8. At stage HH10 blood islands start to form in the splanchnopleure, and since PGCs are localized in the mesoderm, from there they ingress into the vascular system. At around stage HH12, PGCs begin to appear in the extra-embryonic blood, and at stage HH14 they start to colonize the gonads (reviewed in [45]) (Figure 2).

The vascular system guides them to the posterior region of the embryo, where the genital ridges are localized. At stage HH17 the majority of PGCs is localized in the gonads [46]. Regarding chemotaxis, SDF-1/CXCL2 interaction seems to be conserved

between mice and chicken. In the chicken a role for SDF-1/CXCL2 has been shown in directing the migration of chicken PGCs toward the genital ridges, at stages HH11-16, when the cells are already migrating through the vasculature [47]. In Chapter 2 we describe the migration of chicken PGCs from the extraembryonic circulation into the embryo using CVH as a PGC marker. We show for the first time the role of the anterior vitelline veins in this process.

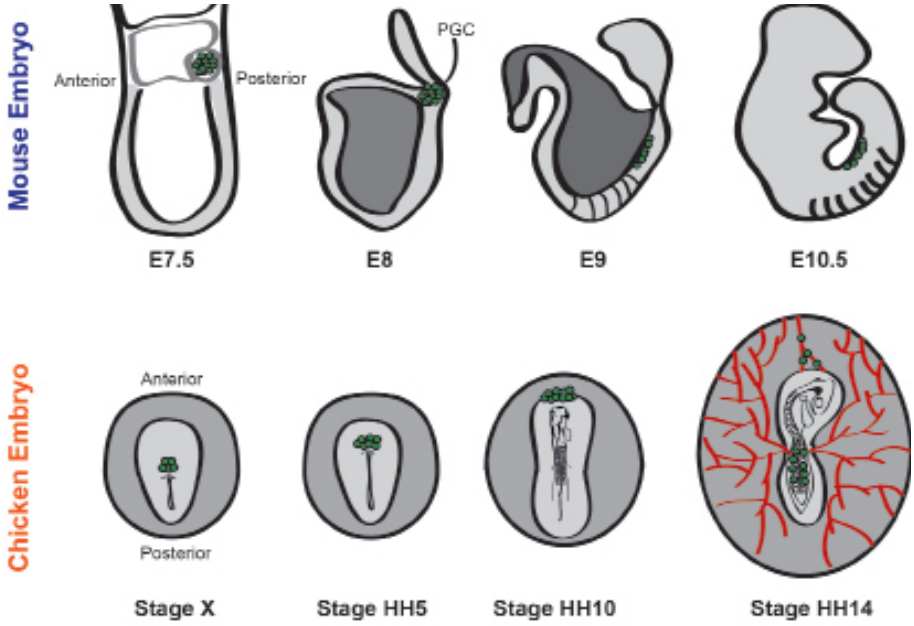

Figure 2. Migration of PGCs in the mouse and chicken. In the mouse, PGCs are specified in the proximal epiblast. At E8 PGCs are localized in the base of the allantois. From there, they migrate to the future gonads along the hindgut (Lateral view of the mouse embryo). In the chicken, at the beginning of gastrulation the primitive streak develops and localizes PGCs in the anterior region, where the germinal crescent is formed. At HH10, germ cells are localized at the anterior region of the head. At HH14 the cells migrate into the embryo through the anterior vitelline veins. (Top view of the chicken embryo).

THE CURIOUS CASE OF CHICKEN GONADOGENESIS: ASYMMETRY, MEIOSIS AND CANCER DEVELOPMENT

The genital ridges, precursors of the gonads, arise at stage HH20 in the ventromedial surface of the mesonephros, and constitute two macrosymmetrical structures, right and left, before sexual differentiation [48] (Figure 3). Undifferentiated female and male gonads are composed of the inner cortex and outer medulla [49]. The migration of PGCs toward the genital ridges occurs before sexual differentiation in an asymmetric way, since the right gonad generally presents more cells than the left gonad, in males and females, in early stages of development stages HH15-30 [50]. Although there are differences in terms of germ cell colonization, before the sexual differentiation there is almost no detectable morphological asymmetry between right and left. The development of the cortex, which is thicker in the left gonad in both sexes, is an exception to this [51] .

Only during sexual differentiation, which starts at around stages HH29-30, do differences in morphology and size between right and left gonads start to be more pronounced in females compared to males (Figure. 3) [49]. As a result, in males both gonads develop into a functional testis while in females only the left gonad develops into a functional ovary [49, 51].

Differential genetic expression in males and females leads to differences in the sexual morphology of the gonads. On the one hand, embryological testes have a greater medullary development, and the testicular cords containing germ cells will develop in that layer. On the other hand, the ovary presents a pronounced development of the cortex that will host the female germ cells [52]. DMRT1 [53] and SOX9 [54] seem to contribute to the male phenotype, while HINTW [55], FET1 [56] and FOXL2 [57] contribute to the female phenotype.

Regarding the pronounced asymmetry between the right and left gonad in the females, PITX2 [58], BMP7 [59] and estrogen receptor α (ERα) [60] seem to have a determinant role, but little detail is known on their signalling pathways in chicken. PITX2, known to determine asymmetry in other model animals (reviewed in [61]), seems to contribute to cell proliferation in the left cortex of the chicken ovary [58]. *BMP 7* is also expressed asymmetrically in the gonads, and seems to act early in gonadogenesis [59]. *ERα* is also expressed asymmetrically but its role in gonadogenesis is still unclear [62]. Moreover, it has not been studied whether this asymmetric development of chicken ovaries also affects meiosis. Indeed, the right ovary has often been neglected in germ cell studies, since it has been thought that germ cells in the right gonad are degenerating [63]. In order to understand the effect of asymmetry in the chicken ovary, we have analyzed the dynamics of the expression of meiotic markers – synaptonemal complex protein 3 (SYCP3)

and phosphorylated histone H2A (H2AFX) - in both, right and left gonads, in both, male and female. Our results are presented in Chapter 4. We suggest that the localization of germ cells with respect to the left versus right gonad, cortex versus medulla of the left gonad, and central part versus the extremities versus the left cortex, influences meiotic maturation of the germ cells.

The adult hen is a model for epithelial ovarian cancer (EOC) in humans [64]. EOC is the most lethal gynaecological cancer, and the fifth cause of death in cancer-related mortality in women [65]. This is due to lack of treatments that specifically target EOC, difficulty in recognizing the symptoms and the wide spread of the disease in the peritoneal cavity [66]. Various models have been used to understand the mechanisms underlying the disease, including fruit flies, mice, *in vitro* models and the hen. In Chapter 5 we discuss the advantages of the avian model compared with other models in EOC. Moreover, we suggest that studying genes that are differentially expressed in chickens during asymmetric gonadogenesis and expressed in human EOC, such as *PITX2*, could offer a model to study the molecular basis of EOC in humans.

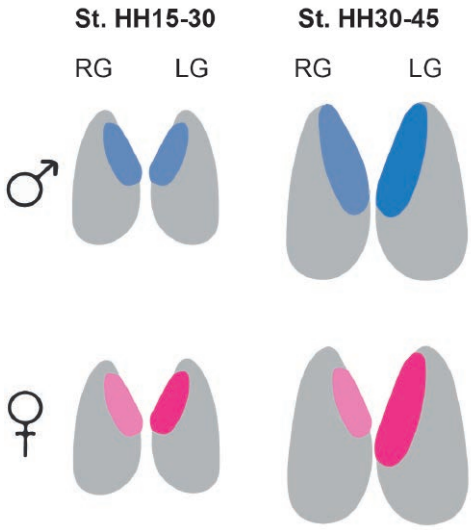

Figure 3. Assymetric gonadogenesis in the chicken. Before sexual differentiation (Stages HH15-30), the differences between the right and left gonad are not visible macroscopically. Only with the beginning of sexual differentiation at stage HH30, the differences between the right and left gonad start to be more pronounced between left and right gonad in the female chicken. This differences allow to distinguish female and male embryos, since the left gonad is distinticvely bigger when compared with the rigth gonad. In males the both gonads are the same size. gonad. In males the both gonads are the same size.

THE DEVELOPMENT OF EXTRAEMBRYONIC MEMBRANES IN AMNIOTES

As discussed previously, in the mouse embryo the PGCs are localized in the base of the allantois before they start to migrate, while avian PGCs use the vessels of the yolk sac to migrate into the embryo. Allantois and yolk sac are part of the extraembryonic membranes of amniotes and there is a tight relationship between their development and the initial developmental dynamics of PGCs. How does extraembryonic development occur? In amniotes, the embryo develops in a blastodisc on top of the yolk mass, and during gastrulation, the borders between embryo and extraembryonic membranes are not clearly defined [64]. Only after gastrulation is complete, when the embryo is already taking shape, do the endoderm and mesoderm (splanchnopleure) form the yolk sac and allantois, while the ectoderm and mesoderm (somatopleure) start to form the amnion and the chorion. In between the somatopleure and splanchnopleure, a cavity is formed – the extraembryonic coelom [67].

The splanchnopleure of the yolk sac contributes to the development of a vascular network, that is part of the first functional organ system in the embryo – the cardiovascular system. The activation of FGF-receptor in the splanchnopleure activates the differentiation of blood islands: angioblasts in the area pellucida and angioblasts and hematopoietic cells in the area opaca and paraaortic clusters [68]. The endothelial cells are then responsible for connecting the blood islands and remodeling blood vessels into a branched network that will cover the entire yolk sac. The process of vasculogenesis, the formation of the blood vessels, is followed by angiogenesis – capillary sprouting, splitting and remodeling – that leads to a reorganization of the vascular network. Moreover, after the heart starts beating, the resulting hemodynamics will have an important role in remodeling its branching and growth [68]. The development of the vascular network is a complex process, which starts at stages HH8-13 and is only defined at stage HH18 [69]. At the same time, the allantois, also derived from the splanchnopleure, connects to the extraembryonic coelom, and stores toxic by-products [70].

On the other hand somatopleure will give rise to the amnion and the chorion, and does not have angiogenic properties. Amniogenesis, the development of the amnion, starts with the formation of an anterior amnion fold that will involve the head from anterior to posterior [70]. As the anterior amnion fold develops, it will fuse with its posterior counterpart in the middle of the embryo after 72 hours of incubation, at stage HH18 [70]. When the two amnion folds fuse, the chorion and the amnion are separated from each other: the amnion surrounds the embryo in the amniotic cavity filled with amniotic fluid, and protects the embryo from desiccation, while the chorion underlies the inner surface of the shell, and allows gas exchange. The initial steps of amniogenesis in

the chicken are described in detail in Chapter 5. Our results revisit an old model [71] for amnion formation, where the proamnion plays an important role.

AIM AND OUTLINE OF THIS THESIS

The mechanisms underlying PGC biology have been extensively studied in different organisms over the last several centuries. Nonetheless, many underlying mechanisms governing PGC behaviour are still unclear and deserve a more detailed analysis. The aim of this thesis is to study the migration of PGCs and meiosis in the chicken embryo. We also discuss the advantages and applicability of the avian model for ovarian cancer research in humans. Furthermore, we provide experimental evidence for the role of the proamnion in amnion development in the chicken, a structure that is often neglected in the literature.

In **Chapter 2** the migration route of germ cells from the extraembryonic circulation into the chicken embryo is described in detail. We show that SSEA1 is not a good marker for chicken PGCs at this time of development, since not all CVH-positive cells stain with an anti-SSEA1 antibody. Focusing on CVH as a marker for PGCs, we analyze the position of PGCs in the chicken embryo between stages HH10-19. We redefine the migration route of PGCs, providing evidence that the anterior vitelline veins are the main vehicles of transportation of germ cells from the anterior region of the extraembryonic vasculature into the genital ridges.

In **Chapter 3** we show a detailed analysis of the expression of two different markers SYCP3 and H2AFX. We conclude that there is no evidence for apoptosis of germ cells localized in the right ovary as had been suggested by other authors. Moreover we demonstrate that differences in the expression of meiotic markers reveal three different aspects influencing the meiotic maturation of germ cells localized in female chicken gonads: their localization in the left or right gonad, cortex or medulla in the right gonad, and their position in the left cortex.

Chapter 4 provides a review of the use of different models in the study of epithelial ovarian cancer. We focus on the advantages of using the avian model, such as the similarities with the disease in humans, in improving the outcome of clinical research.

Chapter 5 describes chicken amniogenesis. We revisit an old model in amnion formation proposed in 1888 by Shore and Pickering. We provide a detailed anatomical study, revealing the importance of the proamnion in the correct formation of the amnion. The role of the proamnion in chicken amniogenesis is often ignored in the literature. For

the first time, we show, through functional assays, the importance of sinking of the head in the proamnion for the development of the anterior amnion fold.

Finally, **Chapter 6** provides a general discussion of the results obtained in the previous chapters. Moreover, we discuss the study of germ cell development in chickens and also its importance in pluripotency studies in a non-mammalian model.

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2

Chicken primordial germ cells use the anterior vitelline veins to enter the embryonic circulation

Ana de Melo Bernardo[1], Kaylee Sprenkels[1], Gabriela Rodrigues[2], Toshiaki Noce[3] and Susana M. Chuva De Sousa Lopes[1]

[1]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands;
[2]Centro de Biologia Ambiental e Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal;
[3]Department of Physiology, Keio University School of Medicine, Tokyo, Japan

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ABSTRACT

During gastrulation, chicken primordial germ cells (PGCs) are present in an extraembryonic region of the embryo from where they migrate towards the genital ridges. This is also observed in mammals, but in chicken the vehicle used by the migratory PGCs is the vascular system. We have analysed the migratory pathway of chicken PGCs, focusing on the period of transition from the extraembryonic region to the intraembryonic vascular system.

Our findings show that at Hamburger and Hamilton developmental stage HH12–HH14 the majority of PGCs concentrate axially in the sinus terminalis and favour transport axially via the anterior vitelline veins into the embryonic circulation. Moreover, directly blocking the blood flow through the anterior vitelline veins resulted in an accumulation of PGCs in the anterior region and a decreased number of PGCs in the genital ridges. We further confirmed the key role for the anterior vitelline veins in the correct migration of PGCs using an *ex ovo* culture method that resulted in defective morphogenetic development of the anterior vitelline veins.

We propose a novel model for the migratory pathway of chicken PGCs whereby the anterior vitelline veins play a central role at the extraembryonic and embryonic interface. The chicken model of PGC migration through the vasculature may be a powerful tool to study the process of homing (inflammation and metastasis) due to the striking similarities in regulatory signalling pathways (SDF1–CXCR4) and the transient role of the vasculature.

INTRODUCTION

Early during the development of amniotes, the germline is segregated from the somatic cell lineages. This is an important event because the primordial germ cells (PGCs), the precursors of the oocytes and sperm, carry the genetic information throughout generations and are therefore the engine of evolution, contributing to genetic variability in sexually reproducing animals [1]. Even though PGCs can be formed by two distinct mechanisms, epigenesis and preformation, they show some common characteristics, including early segregation, similar morphology cross-species [2] and a distinct migratory period from a peripheral or extraembryonic location to the place where the somatic gonad compartments are formed. Understanding the details surrounding the migration of PGCs is important because an aberrant migration can cause cancer and infertility (reviewed by [3]). Interestingly, in *Gallus gallus* the PGCs migrate from an anterior location towards the genital ridge compartment, whereas in *Mus musculus* the PGCs migrate from a posterior/caudal location towards the genital ridges [4].

In chicken, the staining method classically used to distinguish PGCs from the somatic cells was the periodic acid-Schiff (PAS) staining [5]. There are also immunological markers against cell-surface glycoproteins present in PGCs, like SSEA1, which is commonly used to identify mammalian and chicken PGCs. However, SSEA1 is not restricted to chicken or mammalian PGCs, but is found in several types of undifferentiated multipotent mouse and chicken cells [6, 7]. More recently, Tsunekawa and colleagues identified the chicken vasa homolog (*CVH*) gene and have shown its germline-specific expression [8]. The function of *VASA* is not well understood, but it has been shown that vasa is indispensable for germ cell development and it is present in the germline of many animal species, suggesting a conserved role throughout evolution (reviewed by [3]). Immunohistochemical analyses, using specific antibodies against CVH protein, demonstrated that CVH-expressing cells were detectable during early embryogenesis of chicken embryos, starting from the first cleavage of fertilized eggs,[8], suggesting that a preformation mode of germline specification was adopted in chicken.

At stage X [the roman numerals refer to the staging system used by Eyal-Giladi and Kochav, [9] the PGCs are localized in the central zone of the area pellucida, on the ventral surface of the epiblast [10]. At this stage, the PGCs are gradually translocated from the epiblast to an extra-embryonic structure, the hypoblast and carried anteriorly by the hypoblast to the so-called germinal crescent region, away from the primitive streak that starts to move forward from the posterior area of the blastodisc [10]. At HH4–5 [referring to the staging system used by Hamburger and Hamilton in 1951, and reprinted in 1992 [11], the germinal crescent containing the PGCs is localized at the border region between the area pellucida and area opaca, anterior to the developing embryonic disk [12, 13].

The PGCs move from the hypoblast layer to accumulate in the extraembryonic mesoderm localized between the ectoderm and hypoblast. Subsequently, the PGCs become lodged in the vascular system as the blood islands are formed in the yolk sac around HH10 and by HH12 use those extraembryonic blood vessels as a vehicle to reach the embryo [5, 12, 13]. By HH15, the PGCs start leaving the vascular system close to the genital ridges, just caudally from the vitelline arteries and by HH17 the majority of the PGCs have settled in the genital ridges [5, 13-16]. The mechanism by which the PGCs enter the vascular system is less well understood than the mechanism by which the PGCs exit the vascular system (SDF1–CXCR4) to colonize the gonads [17] that has clear similarities with the process of homing of lymphocytes during inflammation and tumor metastasis [18, 19].

Here, we have investigated the vasculatory route used by the PGCs from the extraembryonic germinal crescent to the intraembryonic vascular system as this has also not been well described to date. We observed that PGCs concentrate and make effective use of the two large calibre blood vessels that flow into the embryo from left and right: the anterior part of the sinus terminalis and the anterior vitelline veins. A defective development of the anterior vitelline veins or the direct blocking of the blood flow through the vitelline veins resulted in an accumulation of PGCs anteriorly and a concomitant decrease in the number of PGCs that reached the genital ridges. We propose a novel model of PGC migration in chicken embryos.

MATERIAL AND METHODS

Embryo collection and manipulation

Fertilized White Leghorn chicken (*Gallus gallus*) eggs were incubated in a humidified atmosphere at 37.0°C until the desired HH stage [11]. Embryos were washed and manipulated on 2% agar-coated petri dishes containing phosphate buffer solution (PBS). The vitelline membrane was removed, the embryos were isolated with intact area opaca and pellucida and fixed overnight (o/n) at 4°C either in 4% paraformaldehyde (PFA) for whole mount immunofluorescence or in Bouin's solution (Sigma) for immunohistochemistry and stored in PBS at 4°C until further use.

Whole mount immunofluorescence

Fixed embryos were permeabilized with 0.5% Triton (Sigma) in PBS (PBT) o/n at 4°C with rotation. Thereafter, they were washed in PBS and incubated 24 hours at 4°C with the first antibodies diluted in 1% bovine serum albumin (BSA, Fraction V) (Gibco) in PBS. The first antibodies used were rabbit anti-CVH IgG at 1:500 and mouse anti-SSEA1 IgM (TG1) at 1:10. Next, the embryos were washed in PBS for 1 hour and incubated with the respective secondary antibodies diluted in 1% BSA/PBS for 24 hours at 4°C. The secondary antibodies used were Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes) and Alexa Fluor 568 goat anti-mouse IgM (Molecular Probes), both used at 1:1000. The embryos had a final rinse in PBS and were enclosed with Vectashield with Dapi (Vector), covered with a cover glass and sealed with nail polish. For this analysis, we considered only embryos that showed normal morphology, including the presence of PGCs. The total number of PGCs per embryo was counted and plotted.

Immunohistochemistry

For paraffin inclusion, embryos from HH13 were dehydrated following a graded series of ethanol (70%, 80%, 90% and 100%) and cleared in xylene. The embryos were individually embedded in paraffin (2× 30 minutes) at 70°C and stored at 4°C. The embryos were sectioned (transverse sections, 5 μm) using a rotatory microtome RM2255 (Leica, Nussloch, Germany). The sections were rehydrated starting with xylene and followed by a decreasing series of ethanol (100%, 90%, 80%, 70%) followed by milli-Q water and PBS. The inhibition of endogenous peroxidase activity was performed by treatment with a freshly prepared 0.3% H2O2 in PBS for 20 minutes at room temperature (RT). Next, the

sections were blocked for 1 hour at RT in fresh 1% BSA/PBS. The slides were incubated with rabbit anti-CVH IgG at 1:500 diluted in blocking o/n at 4°C, washed in PBS and incubated with BrightVision Poly-HRP anti-rabbit (Immunologic) for 30 minutes at RT. Thereafter, the slides were washed first with PBS, than with 0.05M Tris-maleate buffer (pH 7.6), revealed with a solution of 0.4 mg/ml 3,3-diaminobenzidine (DAB) and finally counterstained with Mayer's Hematoxylin. The sections were washed in water, dehydrated in an increasing series of ethanol and finally xylene. Thereafter, the samples were mounted in Entellan (Merck). For this analysis, we considered only embryos that showed normal morphology, including the presence of PGCs. The total number of PGCs per embryo was counted and plotted.

In ovo clamp experiments

At HH14, an opening was made in the shell of eggs and part of the vitelline membrane was removed to expose the embryo and some drops of PBS were added to avoid embryo drought. To block blood flow though the anterior vitelline veins a knot was tied using a small semicircular multipass needle attached to a prolene monofilament (Ethicon). The opening made in the eggs was closed and the eggs incubated for 6 hours (until HH15). The control embryos were treated similarly, but the vitelline veins were not clamped. After the incubation time, the embryos were isolated, fixed in 4% PFA o/n and processed for whole mount immunofluorescence. The total number of PGCs per embryo was counted and statistical analysis to compare the distribution of PGCs in the two groups of embryos was performed using the non-parametric Mann–Whitney test.

Ex ovo culture of chicken embryos

Preparation of the embryos for *ex ovo* culture was performed as described [20]. This culture system allows the growth of chicken embryos without the vitelline membrane in a fish embryo-like topology on top of a "mini yolk sac-like". HH5 embryos were removed from the egg, cleared of excessive yolk with PBS and folded by the anterior–posterior axis into a half circle. Forceps were used to gently press the edges of the area opaca together to create a "sealed" half circle. Outside the sealed area, the rest of the area opaca was cut off with micro scissors and the embryo was left to heal undisturbed for 30 minutes in Pannett–Compton solution [21] at RT. Thereafter, the embryos were cultured for 30 hours (HH13) or 48 hours (HH17) on a petri dish in suspension in medium consisting of a mix 2:1 of thin albumen and Pannett–Compton solution containing 1:300 Penicillin/Streptomycin (Gibco) at 37°C with humidity on air.

After the incubation time, the embryos were isolated, fixed in 4% PFA o/n and processed for whole mount immunofluorescence.

Image acquisition and analysis

Whole mount embryos were imaged on a Leica M420 stereoscope (Leica, Rijswijk, the Netherlands) equipped with a Nikon E4500 coolpix camera (Nikon, Tokyo, Japan), fluorescence images were made on a Leica MZFIII stereoscope (Leica, Rijswijk, the Netherlands) equipped with a Leica DFC90 camera (Leica, Heerbrugg, Switzerland) and confocal images were made on a Leica TCS SP5 confocal inverted microscope (Leica, Mannheim, Germany) operating under the Leica Application Suite Advanced Fluorescence software (Leica, Mannheim, Germany). Sections were imaged on an Olympus AX70 microscope (Olympus, Zoeterwoude, Netherlands) equipped with either an Olympus XC50 camera (Olympus, Tokyo, Japan) or a Spot RT3 camera (Diagnostic Instruments, Sterling Heights, MI, USA). For 3D reconstruction, serial paraffin sections immunostained for CVH followed by Hematoxylin staining were digitalized using a Pannoramic MIDI scanner (3D Histech, Budapest, Hungary) and reconstructed with Amira 4.1 software (Visage Imaging, Carlsbad, CA, USA).

RESULTS

The number of chicken PGCs remained constant, but increasing numbers of PGCs expressed SSEA1 between HH8–HH19

We analysed the number of PGCs in White Leghorn chicken embryos between HH5–HH19 (n=42) by whole mount double immunofluorescence for CVH and SSEA1, counting the total number of PGCs present in the embryo, area pellucida and area opaca. We observed a high variation in the total number of PGCs between embryos of the same developmental stage; however, the average number of PGCs present in the germinal crescent at HH5 was similar to the average number of PGCs present in the genital ridges between HH16–HH19. The majority of the embryos exhibited between 200 and 450 PGCs (Fig. 1A). Between HH5–HH7, all the CVH-positive cells were positive for SSEA1 (Fig. 1B), but SSEA1 was observed in many other cells and tissues in the embryo, making SSEA1 an inadequate marker of the germline at those stages. At HH8, SSEA1 was drastically downregulated in the CVH-positive PGCs and was then slowly upregulated in a fraction of the germ cells (Fig. 1B,C) until it stabilized at 60% of the cells by the time the PGCs colonized the genital ridges at HH16–HH19 (Fig. 1B,D). Interestingly, in the typically 3–4 cell clusters of germ cells, already described in 1914 by Swift [13], we often observed both SSEA1-positive and SSEA1-negative cells (Fig. 1E). In agreement, Swift noticed pronounced differences in the yolk content and yolk coloration among PGCs [13] and this may be directly linked to the heterogeneity observed in SSEA1 staining.

At HH13, the PGCs localized to the sinus terminalis and anterior vitelline veins

We analysed the distribution of the CVH-positive PGCs in detail between HH5–HH19 in whole mount chicken embryos and observed PGCs in three different structures: the area opaca, the area pellucida and the genital ridges (Fig. 2A,B). Between HH5–HH8, the great majority of the PGCs were located at the anterior region of area pellucida, bordering with the area opaca, the germinal crescent (Fig. 2B). However, at HH8–HH10, the PGCs were displaced to the area opaca adjacent to the germinal crescent, where they were predominantly found between HH11–HH12 (Fig. 2B). From there, the PGCs migrated transiently through the anterior area pellucida, towards the embryo, during a period of 12 hours between HH13–HH15. By HH16, the majority of the PGCs had reached the genital ridges (Fig. 2B). The number of PGCs present in the posterior part of the embryo, both in the area opaca and area pellucida, was consistently low during the period of development analysed (Fig. 2B).

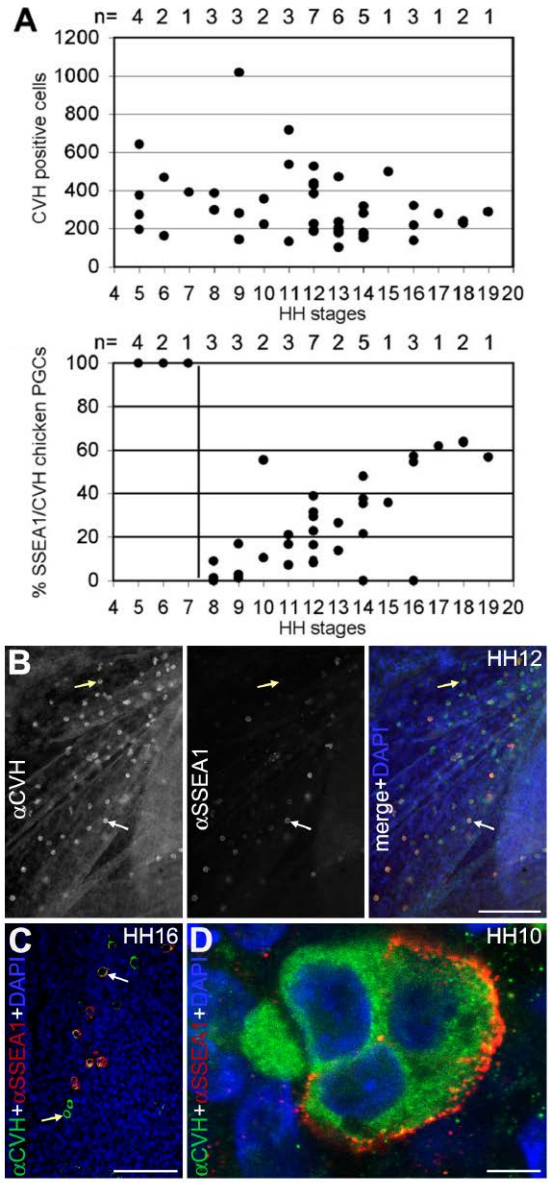

Figure 1. Chicken PGCs between HH5–HH19. (A) Total number of CVH positive cells present between HH5–HH19. n is the total number of embryos analyzed. (B) Percentage of SSEA1-positive cells in the CVH-positive population of PGCs between HH5–HH19. n is the total number of embryos analyzed. (C) Expression of CVH and SSEA1 in area pellucida, lateral to the head region at HH12. The PGCs (CVH positive) were SSEA1-positive (white arrows) or SSEA1-negative (yellow arrows). (D) In the genital ridges at HH16 the PGCs (CVH-positive) were SSEA1-positive (white arrows) or SSEA1-negative (yellow arrows). (E) PGCs from the same cluster showed different expression of SSEA1. CVH (green) is expressed in the cytoplasm while SSEA1 (red) expression is restricted to the cell surface. Scale bars: 100 mm in C,D and 5 mm in E.

At HH13, the PGCs start to transit between the anterior region of area opaca, area pellucida and the genital ridges and this coincides with the period of initiation of the (vitelline) blood circulation. Therefore, we zoomed in at HH13 and defined two developmental sub-stages, HH13 and HH13circ. At HH13, the PGCs were relatively dispersed in the anterior central part of the area opaca and anterior central area pellucida (Fig. 2C). However, in some HH13 embryos, the PGCs concentrated in a narrower continuous axial region between the area opaca and the area pellucida (Fig. 2D). Both HH13 and HH13circ embryos contained about 17–19 somites and approximately the same number of PGCs (with similar medians) in the yolk sac in the area opaca and in the area pellucida, the amnion and ectopically, in particular in the head vasculature (Fig. 3A).

The histological analysis and 3D reconstruction of the vasculature and the position of the PGCs confirmed that at HH13circ the majority of PGCs are concentrated in specific blood vessels in the yolk sac, namely in the sinus terminalis (in the area opaca) and continuous to the developing anterior vitelline veins (in the area pelucida) (Fig. 3B). Our observations contrast with the current model where the PGCs were thought to be scattered broadly throughout the yolk sac vasculature and enter the embryo through the omphalomesenteric veins. Also of note was the fact that, at HH13circ the PGCs were clearly present both inside (in the lumen of the vessel) and outside the blood vessels in the yolk sac (Fig. 3C–F), suggesting that they are not simply engulfed by the blood vessels as they form. In addition, PGCs were also observed frequently in the amnion (somatopleura) (Fig. 3G) and ectopically in the vasculature of the embryo head (Fig. 3H). The PGCs in the amnion probably mislocated when the somatopleura (amnion/chorion) and splanchnopleura (yolk sac) separated.

Finally, we also report that the head (at the level of the prosencephalon) at stage HH13circ, while extending anteriorly, becomes transiently enveloped in the proamnion, a bilaminar tissue consisting of hypoblast and epiblast (and no mesoderm); and, as a result, both the somatopleura (amnion/chorion) and splanchnopleura (yolk sac) are transiently localized above the developing head (Fig. 3E).

The PGCs migrated towards the embryo primarily using the anterior vitelline veins

To experimentally test whether the anterior vitelline veins play a key role in the migration of PGCs from the extraembryonic to the intraembryonic vasculature, we blocked the anterior vitelline veins (by clamping the veins) in embryos at stage HH14 (Fig. 4A) and allowed the embryos to develop *in ovo* for 6 hours (from HH14 to HH15) to check whether that impacted on PGC migration.

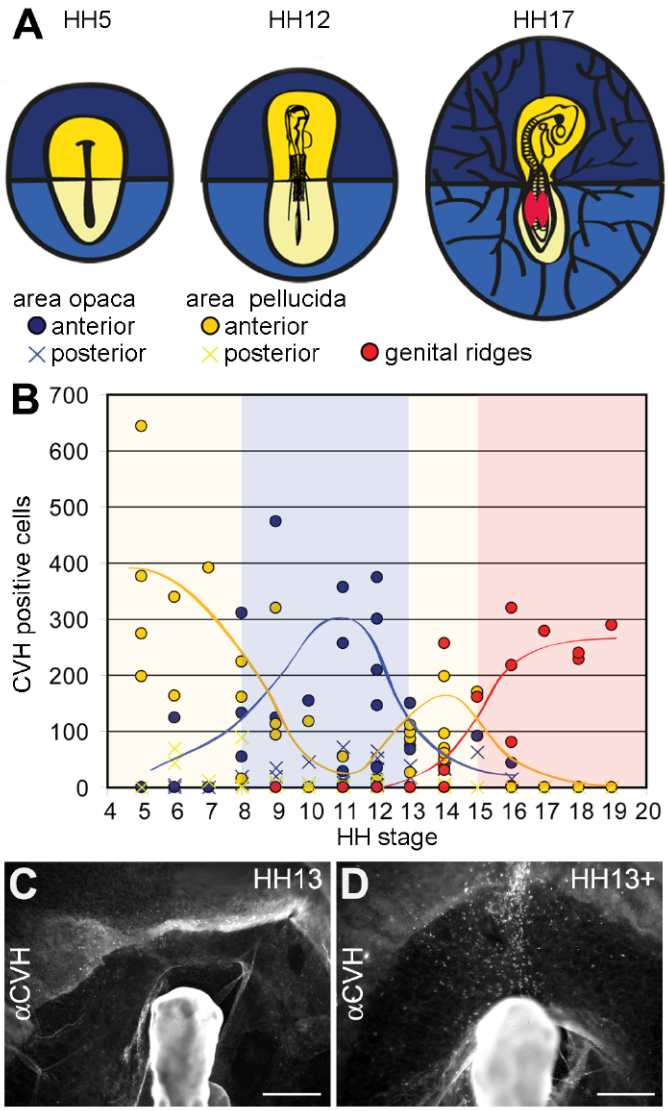

Figure 2. Tracking of the migration of PGCs using CVH as a marker. (A) Cartoon defining the different regions analysed during chicken development in several stages (HH5, HH12, HH17): anterior (dark blue/circle) and posterior (light blue/cross) regions of the area opaca; anterior (dark yellow/ circle) and posterior (light yellow/cross) regions of area pellucida; and genital ridges (red/circle). (B) Total number of PGCs in the areas defined (A) showed predominant localization in 4 different structures during migration: at HH5–8 PGCs localized in the anterior region of area pellucida (dark yellow circle), at HH8–12 there was a displacement of the PGCs to the anterior region of area opaca (dark blue circle), from there the PGCs are migrating back to the anterior region of area pellucida (dark yellow circle) at HH13–15, and from HH14 on, the PGCs started to settle on the genital ridges (red circle). (C,D) At HH13, PGCs are either sparsely localized between area opaca and area pellucida (white arrows) (C) or they have aligned axially in the area pellucida (white arrows). We define this novel stage as HH13circ. Scale bars: 500 mm in C,D.

After 6 hours of culture, in control embryos (n=7) the majority of PGCs found their way to the embryo and colonized the genital ridges (Fig. 4B–D). However, in experimental embryos (n=6), the PGCs remained clustered in the region of the clamped anterior vitelline veins in the axial anterior area pellucida (Fig. 4B,E) and showed a reduced number of PGCs transiting through the embryo and in the genital ridges when compared to the controls (Fig. 4B,F). Our results indicate that blocking the blood flow from the anterior vitelline veins at HH14 has a significant effect in the correct migration of PGCs towards the genital ridges. We concluded that the anterior vitelline veins are the main vehicle used by the PGCs during their migration from the extraembryonic vasculature into the intraembryonic vasculature.

To further confirm the role of the anterior vitelline veins in the migration of PGCs from the extraembryonic to the intraembryonic circulation, we analyzed embryos cultured *ex ovo* using a modified Cornish pasty method that results in primary defects in the morphogenesis of the anterior extraembryonic structures [20]. Using this method, the somatopleura (amnion/chorion) and the splanchnopleura (yolk sac) separate, but the amniotic folds from the head, lateral and tail do not form leaving the embryo exposed. Moreover, the anterior axial conversion of the sinus terminalis and the anterior vitteline veins does not occur and therefore we investigated whether the PGCs were able to find their way into the genital ridges from the germinal crescent. For the modified Cornish pasty method, embryos at HH5 were removed from the egg, folded in two by their anterior–posterior axis and the edges of the semi-circle were pressed together to create a "mini yolk sac-like" (Fig. 5A) and cultured in suspension.

After 30 hours *ex ovo*, embryos at stage HH13 were collected and immunostained for CVH (Fig. 5B,C). In general, the head showed a normal development, the embryos had 17–19 somites (n=10), the heart was beating and the extraembryonic circulation well-established in the posterior region of the embryo. As expected, the headfold of the amnion did not form leaving the head exposed and tilting upwards. We observed many PGCs (± 200) dispersed in the splanchnopleura, anterior to the head at the border between the area opaca and pellucida and some PGCs mislocated in the somatopleura (Fig. 5C).

Ater 48 hours *ex ovo*, we could collect embryos corresponding to stage HH17 (Fig. 5D,E) showing a beating heart with visible blood flow and about 29–32 defined somites (n=10), which corresponds to stage HH17 *in ovo*. HH17 *ex ovo* embryos showed a well-established circulation in the "mini yolk sac" and well-developed posterior vitelline arteries (Fig. 5D). However, there were clear defects in the morphogenesis and positioning of the large calibre anterior vitelline veins. We observed a general defect in the axial movement and fusion of the left and right side of the sinus terminalis and the two anterior vitelline veins. We counted the total number of (CVH-positive) PGCs in

several HH17 *ex ovo* embryos (n=5) and the majority of the PGCs (± 150) were present in the region of the splanchnopleura between the left and right vitelline veins and anterior to the heart. In those embryos, only a very low number of PGCs (< 10) was observed in the region of the genital ridges. We concluded that due to the developmental defects in the position of the sinus terminalis and the anterior vitelline veins in the HH13 and HH17 *ex ovo* embryos, the PGCs fail to find and ingress these blood vessels and therefore remained ectopically in the "germinal crescent" region. We propose a novel model for the migration of PGCs in the chicken with a key role for the sinus terminalis and the anterior vitelline veins (Fig. 5E) as part of a defined or preferred vascular pathway used by the PGCs to travel from their position in the germinal crescent into the heart via the omphalomesenteric veins.

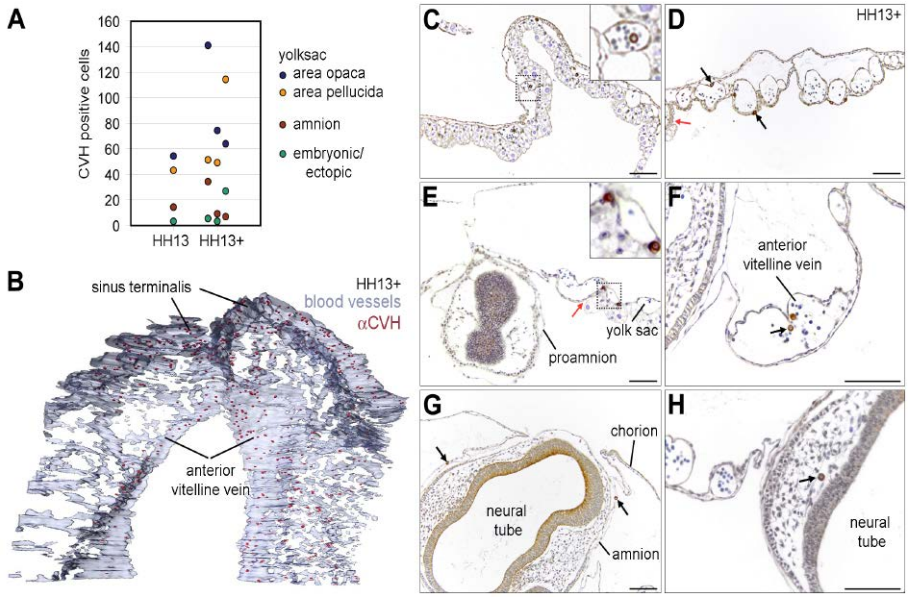

Figure 3. At HH13circ the majority of PGCs is localized in the sinus terminalis and anterior vitelline veins. (A) Analysis of position of PGCs in sectioned embryos at stage HH13circ. PGCs at HH13 and HH13circ are present in similar numbers in the yolk sac in the anterior area opaca and pellucida; the amnion and ectopically in the embryo head. (B) 3D reconstruction of the extraembryonic vasculature of embryos at HH13circ has shown that the PGCs were mainly localized in the anterior vitelline veins and the sinus terminalis. (C–E) Transverse sections of HH13circ embryos immunostained for CVH. PGCs were dispersed in the area opaca (C) and area pellucida (D) anterior from the head and at the level of the head (E). PGCs were observed inside and outside the blood vessels (black arrows). The junction between the area opaca and pellucida is marked by a red arrow. Note in E, that the head at the level of the prosencephalon is completely surrounded by proamnion. (F) PGCs (black arrow) in the anterior anterior vitelline

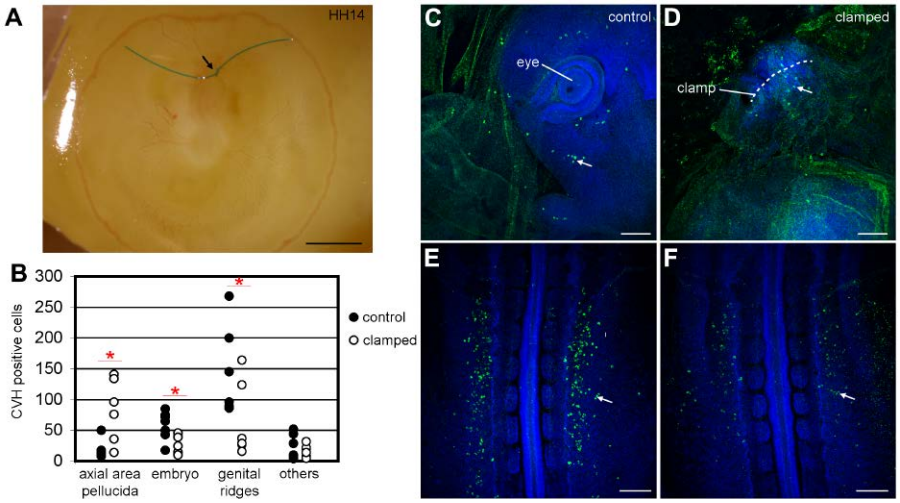

Figure 4. Blocking the anterior vitelline veins prevented the correct migration of PGCs towards the genital ridges. (A) The anterior vitelline veins were clamped in HH14 embryos growing in ovo and the embryos were allowed to develop for 6 hours. (B) Analysis of the total number of PGCs in control (n57, black dots) and experimental embryos (n56, white dots) in different regions. The differences in distribution of the PGCs in the axial area pellucida, the embryo and genital ridges were statistically significant (P,0.05) using the non-parametric Mann–Whitney test [(*) P,0.05]. (C–F) The number of PGCs (white arrows) present ectopically in the embryo head (C) and genital ridges (D) was consistently higher in control embryos than in experimental embryos, where the PGCs concentrated surrounding the clamped vitelline veins (E) and the number of PGCs settled in the genital ridges was reduced (F). Scale bars: 500 mm in A, 100 mm in C,E and 200 mm in D,F.

DISCUSSION

SSEA1 has been used as an appropriate marker to identify and isolate PGCs from chicken embryos [22-27]. However, by performing double immunofluorescence analyses, using antibodies against CVH and SSEA1, we have now demonstrated that although SSEA1 marked PGCs, it is only expressed by a fraction, albeit increasing, of CVH-positive PGCs between HH8–HH19, but not in the entire population of PGCs. This perhaps explains why the fraction of circulating PGCs at HH13–HH15 isolated by fluorescence-activated cell sorting (FACS) on the basis of SSEA1 expression by Mozdziak and colleagues was smaller than the fraction of circulating PGCs isolated using a Nycodenz density gradient [28] or found in chicken blood by PAS staining [29]. Interesting, pluripotent mouse embryonic stem cells, which are closely related to PGCs, also show pronounced heterogeneity for SSEA1 staining [30].

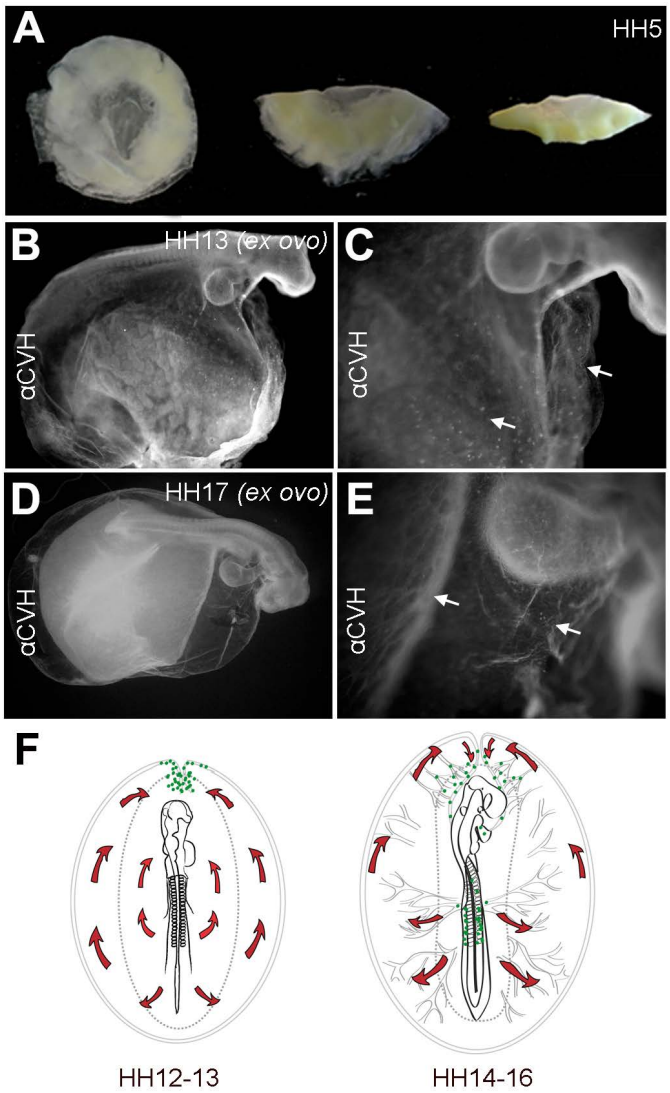

Figure 5. A new model for PGCs migration in chicken embryos. (A) Embryos at HH5 prepared to be cultured using the Cornish pasty method. (B,C) Ex ovo embryos at HH13 showed a relatively normal embryonic morphology (B) and the PGCs were observed in the germinal crescent area in both the somatopleura and splanchnopleura (white arrows). (D,E) Ex ovo embryos at HH17 showed a relatively normal embryonic morphology and the formation of well-developed posterior vitelline arteries (black arrow) (D) and the PGCs were still observed in the germinal crescent area in both the somatopleura and splanchnopleura (white arrows) (E). (F) A new model for PGCs migration in chicken embryos. At HH12–13, the yolk sac circulation courses in loop (red arrows) to enter the embryo via the heart. At this stage, the majority of PGCs (green dots) localized axially at the border between the area opaca and pellucida, where the sinus terminalis converged in the anterior vitelline veins. At HH14–16, the PGCs (green dots) circulated effectively towards the embryo via the sinus terminalis and the anterior vitelline veins towards the heart. Thereafter, the PGCs traffic via the aorta to the caudal part of the embryo and become lodged in the genital ridges. Scale bars: 100 mm in A–E.

It will be important to identify additional lineage-specific markers, like *NANOG* and *DEAD END* [31-33] and in particular novel cell surface markers to study chicken PGCs. This will be important to investigate both embryonic pluripotency and PGC development at very early developmental stages, where the CVH antibody is the only available to identify PGCs.

We and others [29] have observed a large individual variation in the number of PGCs between HH5 and HH19. This results from genetic variation as demonstrated by Tajima and colleagues that showed that specific hens layed eggs with embryos containing consistently high or low number or circulating PGCs [29]. Maybe due to this high variability in PGC numbers, we were unable to observe an increasing number of PGCs between HH5 and HH19 as reported [12]. In our hands, the number of PGCs remained approximately constant between HH5 and HH19. In agreement, Fujimoto and colleagues described 312 PGCs at HH10 [5], Motono and colleagues referred to about 300 PGCs at HH13–HH16 [24] and Nakamura described embryos containing on average 300 PGCs from HH14 to HH20 [34]. Together, we concluded that from HH5, when the PGCs are present in the germinal crescent, until HH19, when the PGCs have reached the genital ridges, the number of PGCs remained constant and range from 200–400.

Two studies have mapped the position of PGCs during their migration from the germinal crescent (HH4) to the genital ridges (HH17) by analysis of whole amount embryos [5, 12]. None of them mentioned the vitelline veins, even though there is a clear concentration of PGCs visible at the junction between the sinus terminalis and the vitelline veins at HH10–11. Curiously, neither Fujimoto nor Nakamura analysed embryos at stage HH13 and this could be the reason why the concentration of PGCs in the anterior vitelline veins has remained unnoticed.

Different groups have collected circulating PGCs at HH13–HH14 using blood from the sinus terminalis, the vitelline vessels, the heart and the dorsal aorta [12, 15, 25, 26, 28, 29, 35, 36]. Although none referred to a specific vascular route used by the PGCs, their methods to collect circulating PGCs support the idea that the majority of the PGCs indeed concentrate and use the sinus terminalis and anterior vitelline veins as an effective way to reach the embryo via the omphalomesenteric veins that enter the heart. Circumstantial evidence was also provided by Nakamura and colleagues by injecting quail PGCs in chick vitelline vessels at HH15 and later observing those quail PGCs in the recipient chicks' genital ridges [37]. We now provide functional data indicating a key role for the anterior vitelline veins transporting PGCs towards the embryo.

The PGCs leave the heart between HH13–HH15 and use the aorta to transit towards the genital ridges. At these stages, the ventral aorta develops first cranially

before it turns caudally, via the first aortic arch, into the dorsal aorta. It is therefore not surprising that we and others [34, 38] observed that HH13–HH15 PGCs had the tendency to become trapped in the cephalic capillary network when being pumped out of the heart, instead of performing the U-turn towards the dorsal aorta. In embryos in which the posterior part, including the gonads, have been excised, the PGCs still accumulate in the head capillaries [34]. Interestingly, stromal cell-derived factor-1 (SDF1/CXCL12), a chemokine involved in the extravasation of the PGCs from the vascular system to the mesenchyme of the genital ridges [17], is expressed at HH12–HH15 specifically both in the area of the genital ridges and the head region [39] and could promote migration of PGCs into both areas.

The signaling pathway involved in attracting the PGCs into the vascular system is less understood. Having a better understanding of the vascular route taken by the PGCs and the markers that can be used to follow the population of PGCs will greatly facilitate the investigation of the mechanisms used by PGCs to enter the vascular system. In turn this may prove an important model to understand how metastatic cells behave on their way to form secondary tumors and how leukocytes behave during processes like infection and inflammation.

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3

Meiotic wave adds extra asymmetry to the development of female chicken gonads

Ana de Melo Bernardo[§1], A. Marijne Heeren[§1,2], Liesbeth van Iperen[1], Maria Fernandes[1], Nannan He[1], Stanford Anjie[1], Toshiaki Noce[3], Ester S. Ramos[4] and Susana M. Chuva de Sousa Lopes[1,5]

[1]Dept. of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands; [2]Current address: Dept. of Obstetrics and Gynaecology, VU University Medical Center, Amsterdam, The Netherlands; [3]Dept. of Physiology, Keio University School of Medicine, Tokyo, Japan [4]Department of Genetics, Ribeirao Preto Medical School, University of Sao Paulo, Sao Paulo, Brazil; [5]Dept. for Reproductive Medicine, Ghent University Hospital, Ghent, Belgium; [§] Equal first authorship contribution

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ABSTRACT

Development of female gonads in chicken is asymmetric. This asymmetry affects gene expression, morphology and germ cell development. As a result only the left ovary develops into a functional organ, whereas the right ovary remains vestigial. In males, both gonads develop into functional testes.

Here, we revisited the development of asymmetric traits in female (and male) chicken gonads between Hamburger Hamilton stage 16 (HH16) and hatching. At HH16, primordial germ cells (PGCs) migrated preferentially to the left gonad independently of gender, accumulating in the left coelomic hinge between the gut mesentery and developing gonad. Using the meiotic markers SYCP3 and (phosphorylated) H2AFX, we were able to identify a pronounced asymmetry regarding meiotic progression in the germ cells located in the central, lateral and extremity part of the cortex of the left female gonad from HH38 until hatching. This has not been previously described. Moreover, in contrast to the current view that medullary germ cells enter apoptosis during development in the right female gonad, we show that medullary germ cells are not apoptotic, but arrested in pre-leptotene until hatching.

We provide a systematic analysis of the asymmetric distribution of germ cells in female chicken gonads until hatching and propose an updated model suggesting that the localization of germ cells [(1) in the left or right gonad, (2) in the cortex or medulla of the left gonad and (3) in the central part or the extremities of the left cortex] has direct consequences for their development and functionality in reproduction.

INTRODUCTION

In chicken, primordial germ cells (PGCs), the progenitors of the gametes, are of extraembryonic origin and, at Hamburger and Hamilton stage (HH)10-12 [1], they are found in the developing blood islands in a region of the yolk sac anterior to the head. From there, they migrate axially through the bloodstream, concentrating in the sinus terminalis, and enter the embryo mainly through the anterior vitelline veins [2]. The PGCs then travel through the embryonic vasculature to reach the gonadal ridges. After the PGCs colonize both left and right gonadal ridges, those undergo sex differentiation, to become morphologically different according to their gender developing as ovaries or testes. In males, both gonads develop into functional testes but in females, as in most birds, it is only the left gonad that develops into a functional ovary, while the right gonad remains rudimentary [3,4]. Before any signs of sex differentiation, the number of germ cells present in the gonadal ridges shows a sex-independent asymmetrical distribution with a preference for the left side in both males and females at HH15-HH17 [5] and at HH22-26 [6]. This sex-independent left-right asymmetry in the number of germ cells is still present at HH35 [7]. In addition to the asymmetry in the number of germ cells, the thickness of surface epithelium of the gonadal ridges also has pronounced sex-independent left-right asymmetry, being consistently thicker in the left gonad until HH36 [8,9].

During sex differentiation, the differences between the female left and right gonads are enhanced [4]. The left female gonad develops a strong spatial asymmetry by forming a germ cell-rich "cortex" and germ cell-poor "medulla" from HH32 onwards [10,11]. By contrast, the right female gonad does not develop a "cortex" and seems to be formed only by germ cell-poor "medulla". The left "cortex" harbors the great majority of the PGCs that cluster in compact cords, whereas the lacunar medulla of both left and right female gonads contains single or small clusters of dispersed germ cells [10,11]. Here, we have examined the sequential steps in gonadogenesis from HH16 until hatching that lead to the asymmetric development of the female chicken gonads. We were particularly interested in the events leading to the "regression" of the right female gonad and wanted to determine the timing of apoptosis assumed to occur in the germ cells present in the germ cell-poor "medulla" in both left and right female gonads [12]. However, until HH45 and using immunostaining for both early and late markers of apoptosis we were unable to confirm the massive wave of apoptosis in medullary germ cells reported previously in the left or right female gonad. Instead, using immunostaining for (phosphorylated) H2AFX (also known as γH2A.X), considered a marker of both apoptosis and meiosis, and the meiotic marker SYCP3, we detected a pronounced spatial wave of meiosis progression in the cortex of the left chicken gonad from the central part of the cortex to its extremities. This previously overlooked wave in meiosis progression adds a novel layer of asymmetric development to germ cell development in chicken.

MATERIAL AND METHODS

Embryo collection and sexing

Fertilized White Leghorn chicken (*Gallus gallus*) eggs were incubated in a humidified atmosphere at 37°C. Eggs were windowed and embryos staged [1]. The sex of HH35 embryos until prior to hatching was determined by eye, whereas the sex of HH16-30 embryos was determined by genomic polymerase chain reaction (PCR) as described [37]. Embryos were isolated and used whole from HH16-HH30, whereas from HH35 until prior to hatching, the paired gonads were further dissected out of the embryo.

Immunofluorescence on whole mount and paraffin section

Embryos and gonads were fixed in 4% paraformaldehyde (PFA) (MERCK, Germany) in phosphate-buffered saline (PBS) overnight (o/n) at 4˚C. Immunofluorescence on HH16-HH19 embryos (n=7) was performed as whole amount and on HH25-HH42 embryos (n=14) on paraffin sections was performed essentially as described [2,38]. Primary antibodies used were rabbit anti-DDX4 (1:500, gift from T. Noce), mouse anti-PCNA (1:500, sc-56, Santa Cruz Biotechnology, USA), rabbit anti-cleaved CASP3 (Asp 175) (1:300, 9661S, Cell Signaling Technology, USA), mouse anti-H2AFX (Ser139) (1:500, 05-636, Millipore, USA) and rabbit anti-FASLG (1:100, sc-6237, Santa Cruz Biotechnology, USA). Secondary antibodies used were Alexa Fluor 568 goat anti-mouse (1:500, A-21124, Life Technologies, UK) and Alexa Fluor 488 donkey anti-rabbit (1:500, A11008, Life Technologies, UK). DNA fragmentation was determined by TUNEL assay with TMR red In Situ Cell Death Detection Kit (Roche Applied Science, Germany) for 1 hour at 37°C following manufacturer's instructions. Slides were counterstained with 1:1000 dilution of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies, UK) in PBS for 1 minute and mounted with ProLongGold antifade reagent (Life Technologies, UK).

Germ cell counting and FACS-analysis

Germ cells were counted in the right and left gonad in whole mount HH16-HH19 embryos (n=3 females; n=4 males) and sequential paraffin sections of HH25-HH30 embryos (n=3 females; n=3 males) immunostained for DDX4.

The left and right gonads were collected individually from HH35 females (n=3), HH35 males (n=2), HH42 females (n=3) and HH42 males (n=3) and isolated in DPBS0 (Life Technologies, UK). To obtain single-cells, gonads were first cut into small pieces

and then incubated in TRIPLE 5x (Life Technologies, UK) at 37°C for 30 minutes, with pipetting from time to time. The single cell suspension was washed in DPBS0 and cells were fixed in 4% PFA for 10 minutes on ice. Thereafter, cells were permeabilized using 0.1% Triton/DPBS0 for 30 minutes on ice, incubated with 1:1000 rabbit anti-DDX4 in blocking solution [1% bovine serum albumin (BSA, Life Technologies, UK) in 0.05% Tween/DPBS0] for 1 hour on ice, washed with DPBS0 and incubated with 1:500 Alexa fluor goat anti-rabbit 488 in blocking solution for 1 hour on ice. After a final wash in DPBS0 cells were resuspended in FACS buffer (1%BSA, 10mM EDTA in PBS) and FACS analysis was performed using BD FACSAriaTM III (BD BioSciences, the Netherlands). Results were processed using the software BD FACSDivaTM version 6.0 (BD BioScience, the Netherlands).

The percentage of PGCs in the left and right gonad was calculated in relation to the total number of germ cells counted per embryo. The Student's T-test (two-tailed distribution, two-sample unequal variance) was used to compare the percentage of DDX4 positive germ cells between the right and left gonads. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Quantitative reverse transcription-PCR (qPCR)

The left and right gonads were harvested individually from HH35 females (n=3), HH35 males (n=3), HH42 females (n=5) and HH42 males (n=5). RNA was isolated using RNeasy Micro Kit (Qiagen, Netherlands) and cDNA made using the iScript™ cDNA Synthesis Kit (Biorad, USA). QPCR was performed using iQ™ SYBR® Green Supermix (Biorad, USA) on a CFX96TM Real-time system, C1000TM Thermal Cycler (Biorad, USA) with the condition 1x (95°C, 3 minutes), 40x (95°C, 15 seconds; 60°C, 30 seconds; 72°C, 45 seconds) and 1x (95°C, 10 seconds; 65°C, 5 seconds; 95°C, 50 seconds). The primers used here for DDX4, DAZL and the housekeeping genes RPS17 and ACTB were described elsewhere [39,40]. All reactions were performed in triplicate. Data was normalized using the DDCt method. Data from HH35 and HH42 is relative to the right male gonads.

Immunofluorescence on cryosections

Paired gonads from HH35 to prior to hatching (n=19) were fixed in 4% PFA/PBS for 10 minutes at room temperature (RT), washed three times in PBS, cryoprotected in 30% sucrose in PBS o/n at 4˚C, included in optimal cutting temperature (OCT) compound (Tissue-Tek, The Netherlands) and frozen at -80˚C. The gonads were sectioned (10µm) using a CM3050S cryotome (Leica Instruments GmbH, Germany) and mounted on Superfrost plus slides (Thermo Scientific, Germany). Cryosections were washed with PBS, blocked 1 hour at RT in 1% BSA/ PBS. The rest of the procedure was as described

above for paraffin sections. Primary antibodies used were rabbit anti-SYCP3 (1:500, NB300-232, Novus Biologicals, USA), rabbit anti-FASLG (1:100, sc-6237, Santa Cruz Biotechnology, USA) and mouse anti-H2AFX (Ser139) (1:500, 05-636, Millipore, USA) and secondary antibodies used were as above.

Imaging

Slides were either analysed on a Leica DMRA fluorescence microscope (Leica, Germany) and pictures taken with a CoolSnap HQ2 camera (Photometrics, USA); or scanned using a Panoramic MIDI digital scanner (3DHISTECH, Hungary) and representative areas for images selected using the software program 'Panoramic viewer' (3DHISTECH, Hungary). Confocal images were made on a Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany) operating under the Leica Application Suite Advanced Fluorescence software (Leica, Mannheim, Germany).

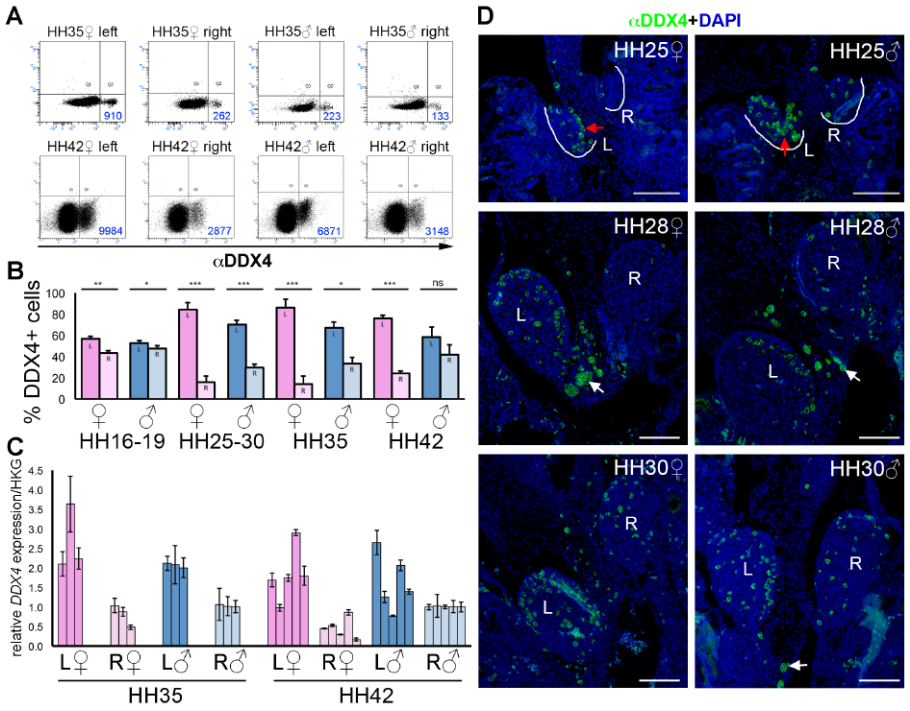

Figure 1. Difference in germ cells localization between the left and right gonads of female and male chicken at HH16-HH42. (A) Flowcytometric analysis showed less DDX4-positive cells in right gonad of each gonadal pair in both HH35 and HH42 females and males. (B) The percentage of DDX4-positive germ cells present in the in the left gonad (L) was significantly higher than in the right gonad (R) at HH16-HH19, HH25-HH30, HH35 and HH42 in both sexes, except for HH42 male gonads where this difference was not significant (ns). *, P<0.05; **, P<0.01; ***, P<0.001. (C) Relative DDX4 expression in left and right gonads of HH35 and HH42 embryos, both females and males, compared to the male right gonad. Each bar represents one gonad and data are expressed as the mean ± standard deviation of technical triplicates. Expression of DDX4 was normalized to the housekeeping genes (HKG) RPS17 and ACTB. (D) Immunostaining for DDX4 (green) showed more germ cells localized to the left gonad (L) than to the right gonad (R) in both females and males at HH25 (upper panels), HH28 (middle panels) and HH30 (lower panels). The white lines delimitate the place of the left and right gonads. Red arrows indicate germ cells accumulating just underneath the coelomic epithelium at the hinge between the gut mesentery and the developing left gonad. White arrows indicate ectopic PGC clusters. Scale bar (same for all images) is 100 µm.

RESULTS

Preferential asymmetric distribution and localization of germ cells between HH16-42

It has been reported that until HH35 both male and female chicken embryos contained a larger number of germ cells in their left gonads [5,7]. This led us to investigate the precise distribution of the germ cells in the developing gonadal ridges from the time of their arrival in the gonads at HH16 until HH42. Using immunostaining for the germ cell-marker DDX4 (also known as CVH), we either counted or FACS-analysed (DDX4)-positive germ cells in embryos from HH16 to HH42 and confirmed a consistently higher number in the left gonad independent of the gender until HH42, even though this differences were less pronounced in males (Figure 1A, 1B). At HH42, the left female gonad contains about 80% of the gonadal germ cells, whereas the male left gonad contains about 60%. This asymmetry was also observed by quantitative reverse transcriptase-PCR for DDX4 (Figure 1C) and similarly for DAZL, another germ cell marker using dissected whole gonads (Supplementary Figure S1). As expected [2,5,7], the number of germ cells in each gonad shows some variability. Between HH35-42, left-right differences in germ cell numbers persisted in the female gonads, but got less pronounced in the male gonads.

In terms of distribution, at HH25, germ cells strikingly accumulated underneath the coelomic epithelium at the hinge between the gut mesentery and the developing left gonad in both males and females (red arrows Figure 1D); this preferential localization just underneath the thick epithelial layer of the left gonad remained at HH28-30 (Figure 1D). Interestingly, outside the gonads, ectopic tight clusters of germ cells were observed both in females and males (white arrows in Figure 1D), where many remained localized in the gut mesentery. At HH35-42, the localization of germ cells to the “cortex” developing in the left female gonad became very pronounced, even though many germ cells were scattered in the “medulla” of the left and right female gonad (Figure 2A, 2B). Interestingly, between HH45 and just before hatching (BH), the germ cells in the medulla of both the right and left female gonad remained numerous (Figure 2C-2F). In the right medulla, germ cells also localized to the surface, however those did not become enclosed in germ cells cords as in the cortex of the left gonad (Figure 2C-2F).

Germ cells undergo a meiotic wave in the left cortex between HH38 and hatching

To explore further the developmental capacity of the asymmetrically distributed germ cells in the cortex and medulla of the left female gonad, we investigated

the timing of meiosis entry and progression until hatching. For this, we analysed the expression pattern of the meiotic-specific marker SYCP3 together with that of (phosphorylated) H2AFX, a marker of DNA double-strand breaks not only associated with DNA damage in apoptosis during mitosis [13], but also those DNA double-strand breaks that occur early during meiotic recombination [14,15].

At HH38, germ cells along the whole length of the left cortex as well as in the medulla of both the left and right female gonads expressed punctated H2AFX, but showed overall no/low levels of (nuclear) SYCP3 (Figure 3A, 3B; Supplementary Figure S2). However, in the cortex center of the left female gonad between HH38 and HH42, H2AFX is downregulated and at HH42, in the cortex center germ cells showed either only (punctated nuclear) SYCP3 or both SYCP3 and H2AFX (Figure 3C, 3Ci, 3Cii). In the extremities of the left cortex and in the medulla of both the left and right female gonads, germ cells maintained the characteristic pattern of H2AFX (Figure 3C, 3Ciii, 3Civ, 3D, 3Di) as observed at HH38. Combining our localization data using the intact gonads with previous data from chromosome spreads from dispersed germ cells [16-18], we conclude that the expression pattern of H2AFX and SYCP3 at HH38 corresponded to that of germ cells in pre-leptotene and, at HH42, germ cells located in the central and lateral part of the left cortex either in early leptotene (punctated SYCP3 and low/no H2AFX) or are in late leptotene/early zygotene (punctated SYCP3 and H2AFX), and those at the extremities of the left cortex and all medullary germ cells are still in pre-leptotene (no SYCP3 and high H2AFX). At HH45, the expression pattern of H2AFX and SYCP3 along the left cortex showed that germ cells in late leptotene/early zygotene (punctated SYCP3 and H2AFX) not only occupied the central part of the cortex, but now also occupied most of the lateral parts of the cortex, whereas germ cells in early leptotene (punctated SYCP3 and low/no H2AFX) became fewer and restricted to a narrow band, adjacent to the pre-leptotene germ cells (no SYCP3 and high H2AFX) in the cortex extremities (Figure 4A, 4Ai-4Aiii; representative higher magnifications in Supplementary Figure S3). This was less pronounced in the long axes of the cortex of the left gonad, but we could still find pockets of H2AFX-positive SYCP3-negative germ cells at the cortex extremities (Supplementary Figure S4).

Since several studies on chromosome spreads have described germ cells in pachytene around hatching [16-18], we extended our analysis to female gonads just before hatching (BH). We observed that the central part of the cortex indeed now contained many germ cells in late zygote/early pachytene (containing clear SYCP3-positive synaptonemal complexes but still expressing H2AFX) (Figure 4B, 4Bi; representative higher magnifications in Supplementary Figure S3). The lateral part of the left cortex still contained germ cells in late leptotene/early zygotene, flanked by germ cells in the cortex extremities in early leptotene and pre-leptotene (Figure 4B, 4Bii, 4Biii).

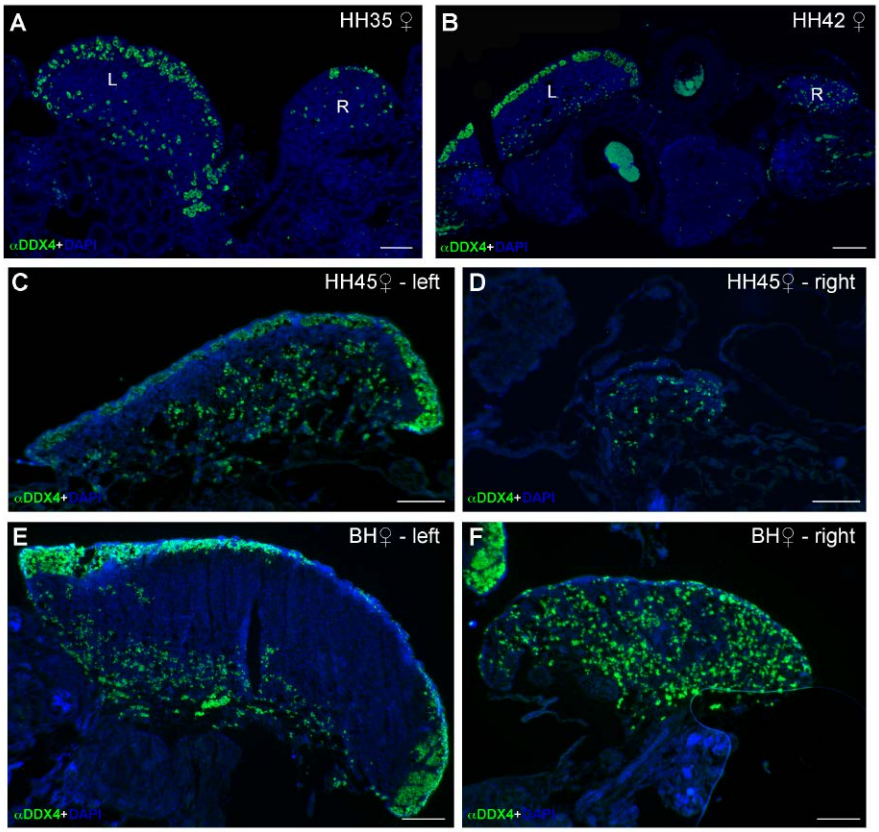

Figure 2. Distribution of (DDX4-positive) germ cells in left and right gonads in female chicken from HH35 to before hatching (BH). DDX4 (green) expression in transverse sections of female gonads showing the distribution of germ cells in left (L) and right (R) gonad at HH35 (A), HH42 (B), HH45 (C,D) and BH (E,F). Scale bars are 200 µm (A, C, D, E, F) and 500 µm (B).

Medullary germ cells in left and right female gonads are not apoptotic, but in pre-leptotene until hatching

Germ cells localized in the medulla of both left and right gonads at HH45 and before hatching (Figure 4C, 4D) showed similar nuclear features to pre-leptotene germ cells (no SYCP3 and high H2AFX) located in the extremities of the left cortex and are most probably in pre-leptotene. Interestingly, we noted that at HH45 and before hatching some small clusters of germ cells in the left and right gonadal medulla were either positive for SYCP3 (Figure 4Aiv, white arrow in Figure 4D) or expressed both SYCP3 and H2AFX (Figure 4Biv) suggesting that some medullary PGCs may in fact enter meiosis. As (phosphorylated) H2AFX is associated with DNA double-strand breaks both during apoptosis and meiosis entry, we wanted to discriminate between these two processes to understand the fate of left and right medullary germ cells. First, we investigated the expression of the proliferation cell nuclear antigen (PCNA), a marker for DNA replication in mitosis, but also involved in DNA replication during meiotic prophase [19]. At HH35-HH42, the great majority of germ cells expressed PCNA (Figure 5A; Supplementary Figure S5). The expression of both H2AFX and PCNA in medullary germ cells, suggest that these cells were in pre-leptotene, instead of apoptosis.

To further distinguish between apoptosis and meiosis, we then investigated the expression of the early apoptosis marker cleaved caspase 3 (CASP3) and assessed DNA fragmentation during late apoptosis with the TUNEL assay. In the HH35 and HH42 female gonads, we found only a few cleaved CASP3-positive cells (white arrowheads in Figure 5B) and a few TUNEL-positive germ cells and somatic cells (white arrowheads in Figure 6A) but no massive loss of germ cells in the medulla, which has been previously reported using electron microscopy [11,12]. Furthermore, the germ cells in the surface of the right gonad at HH35 and HH42 are clearly not in (early or late) apoptosis (white arrows in Figure 6A) and therefore, this cannot be the reason why a germ cell-rich cortex does not develop in the right female gonad. Until hatching, we only detected a very reduced number of either CASP-3-positive or TUNEL-positive cells in both gonads (data not shown).

We next examined the expression of FASLG (also known as FAS ligand), a marker involved in the FASLG-FAS system that plays a role in inducing apoptosis in mammalian spermatocytes undergoing meiosis [20,21]. Interestingly, HH42 germ cells in the extremities of the left cortex and some in the left and right medulla seem to upregulate FASLG (white arrowheads in Figure 6B); and at BH, many presumably germ cells in both the left and right medullas seem to become strongly positive for FASLG (white arrows in Figure 6C), suggesting that after hatching those may be eliminated by apoptosis via a FASLG-FAS dependent mechanism.

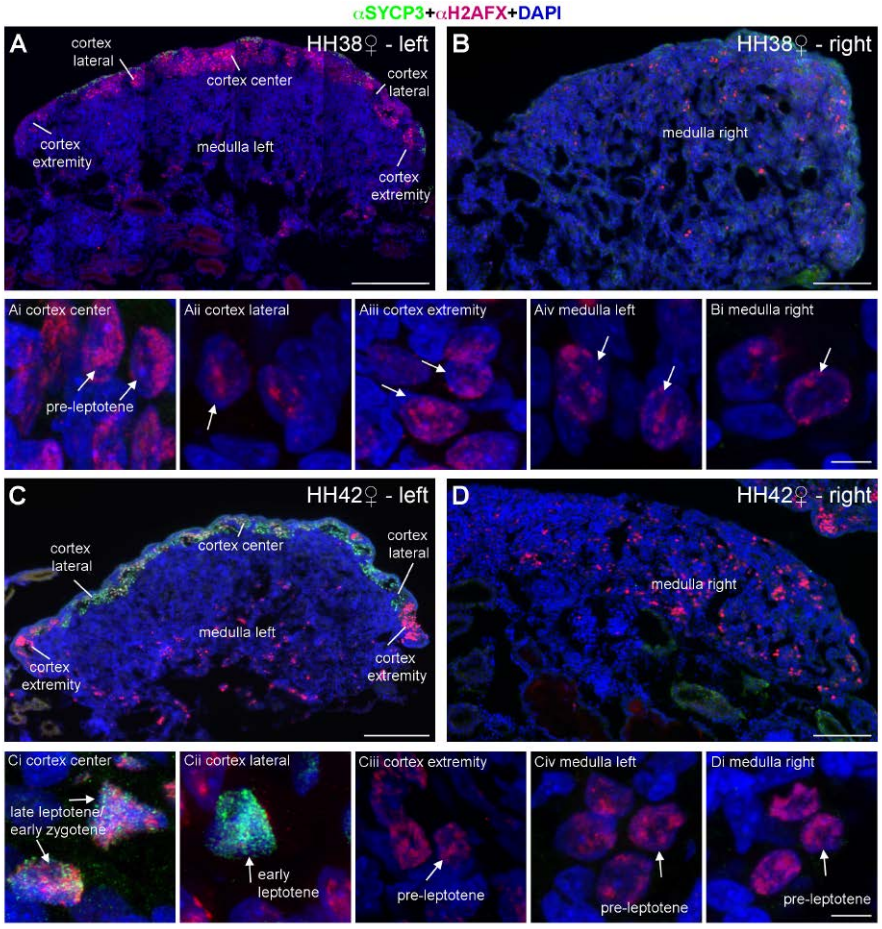

Figure 3. SYCP3 and H2AFX expression in chicken female gonads at HH38 and HH42. SYCP3 (green) and H2AFX (red) expression in HH38 female left (A) and right (B) gonads and HH42 female left (C) and right gonads (D). High magnifications are representative from three different regions of the left cortex (cortex center, cortex lateral, cortex extremity), the left medulla and the right medulla. White arrows point to germ cells illustrating specific meiotic stages (pre-leptotene, early leptotene, late leptotene/early zygotene). Scale bars are 100 μm (A, B, C, D) and 5 μm (Ai-Aiv, Bi, Ci-Civ, Di).

DISCUSSION

Sex-independent preferential migration of PGCs to the left side

Our results and those of others [5-7] show that chicken PGCs preferentially colonize the gonad on the left side of the body as they arrive at HH15, independent of their sex. Differentially expressed signaling cues could direct this asymmetric migration. Molecules that might play a role are BMP7, which is expressed in the left gonad around HH21 (3.5 days of incubation) in both sexes [22] and the transcription factor PITX2, which is expressed in the left gonad around HH18 (3 days of incubation) in both sexes [9,23-25]. Experiments by Naito and colleagues (2009) that transferred PGCs from the bloodstream of HH14-15 donor embryos to the bloodstream of age-matched recipient embryos and analysed them at 16.5 days of incubation (± HH42) suggested that female PGCs migrate preferentially to the left side of both female and male embryos but also that female embryos attracted more male or female PGCs to the left gonad [26]. It is interesting to note that also in humans, between 140-212 mm crown-rump length (about 22 weeks of gestation) the right gonad weighs more (and has a higher DNA and protein content) than the left gonad in both sexes [27], suggesting that an initial sex-independent left-right asymmetry may be conserved at least between humans and chicken.

No evidence for apoptosis in chicken medullary germ cells until before hatching

Left-right asymmetry of the chicken female gonads has been noticed and reported in morphological detail a long time ago [28]. However, since the right gonad of the chicken embryo has been considered as a degenerating structure containing germ cells in apoptosis [10,12], its study has been neglected. Ishimaru and colleagues (2008) analysed apoptosis levels at HH27 and HH29 and observed a very reduced number of TUNEL-positive germ cells in cortex and medulla in both sides [23], but we have extended this here and analysed apoptosis in both sexes between HH35 and hatching in the right and left gonad. We were unable to detect signs of robust apoptosis in the germ cells in the right gonad and our data suggest that medullary germ cells are not apoptotic, but presumable in pre-leptotene until hatching (and a few may even manage to enter meiosis). The reason for the discrepancy with previous literature is the fact that we presently use a combination of meiotic and apoptotic markers together with a robust germ cell marker, whereas previously only histological sections imaged by light microscopy of transmission electron microscopy were analyzed.

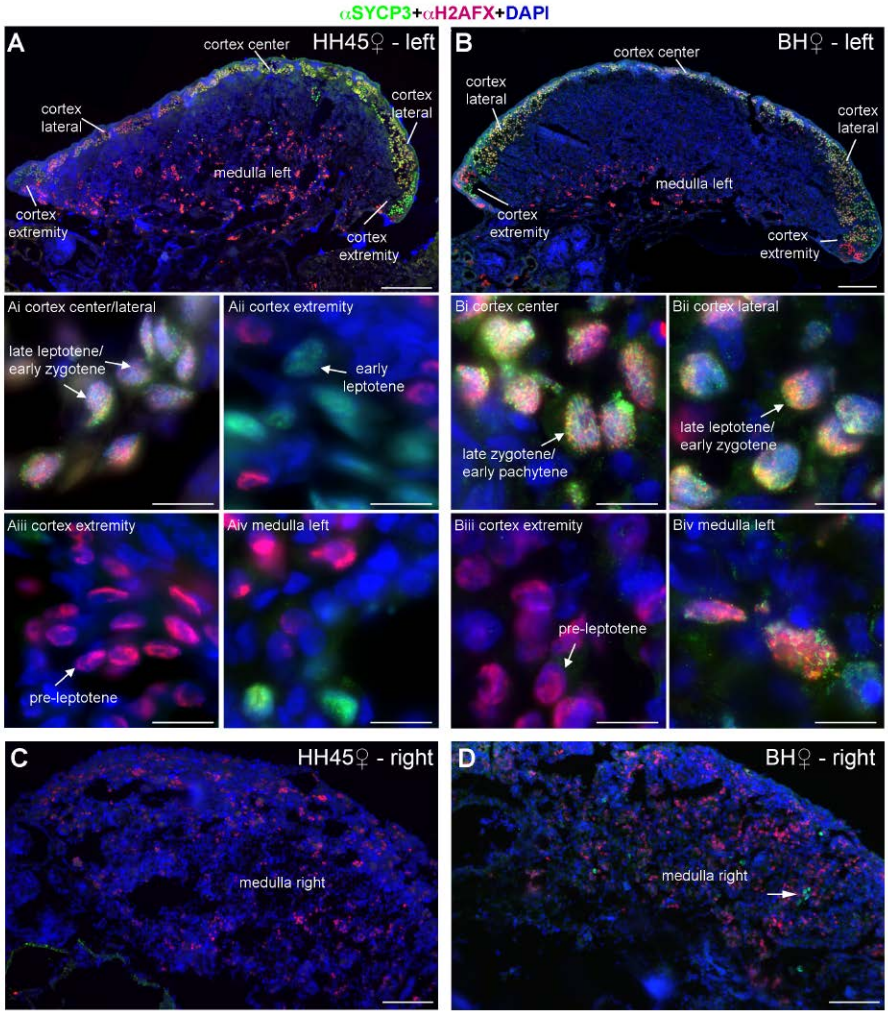

Figure 4. SYCP3 and H2AFX expression in chicken female left and right gonads at HH45 and before hatching (BH). SYCP3 (green) and H2AFX (red) expression in HH45 female left (A) and right (B) gonads and BH female left (C) and right gonads (D). High magnifications are from three different regions of the left cortex (cortex center, cortex lateral, cortex extremity), and the left medulla. White arrows point to germ cells illustrating specific meiotic stages (pre-leptotene, early leptotene, late leptotene/early zygotene, late zygotene/early pachytene). In D, the white arrow points to a cluster of germ cells that upregulated SYCP3. Scale bars are 100 µm (A, B, C, D) and 10 µm (Ai-Aiv, Bi-Biv).

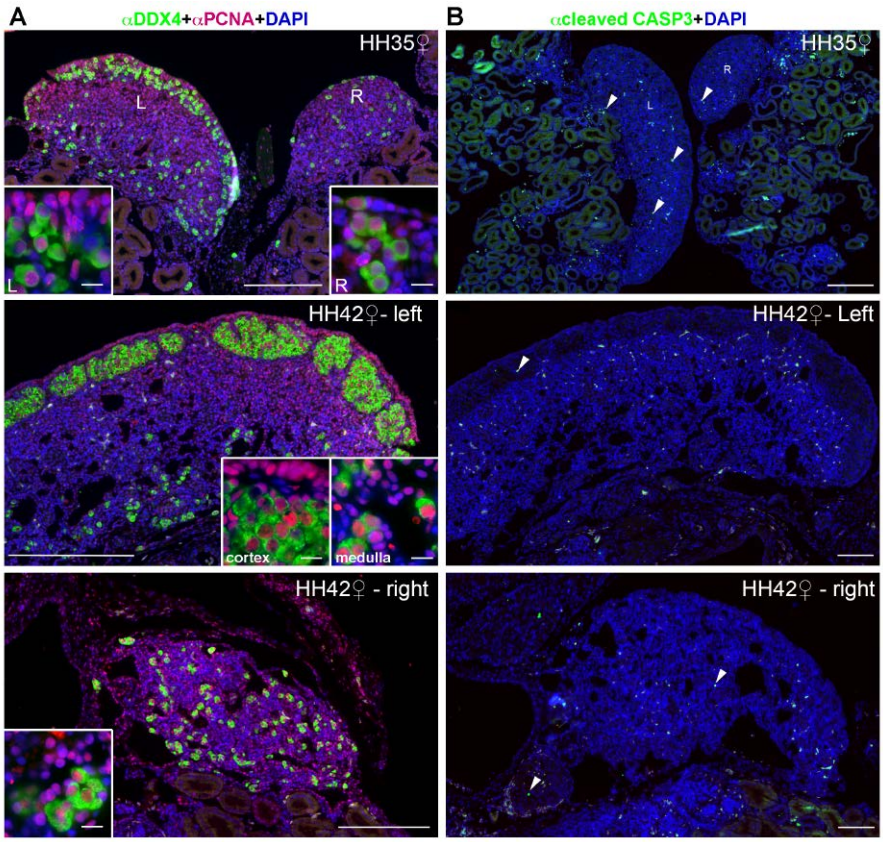

Figure 5. Expression of PCNA and cleaved CASP3 in chicken female gonads at HH35 and HH42. (A) PCNA (red) expression in female left (L) and right (R) gonads at HH35 and HH42. Most germ cells (DDX4-positive cells) at HH35 and HH42 are PCNA-positive. Inserts show a magnified detail. Ectopic PGC clusters, indicated by white arrows, also showed PCNA-positive nuclei. (B) Cleaved CASP3 expression (green) in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to cleaved CASP3-positive cells. Scale bars in (A) are 200 μm (HH35 and HH42 right gonad) and 500 μm (HH42 left gonad); in (B) are 200 μm (HH35) and 100 μm (HH42 left and right gonad); and in the inserts are 10 μm.

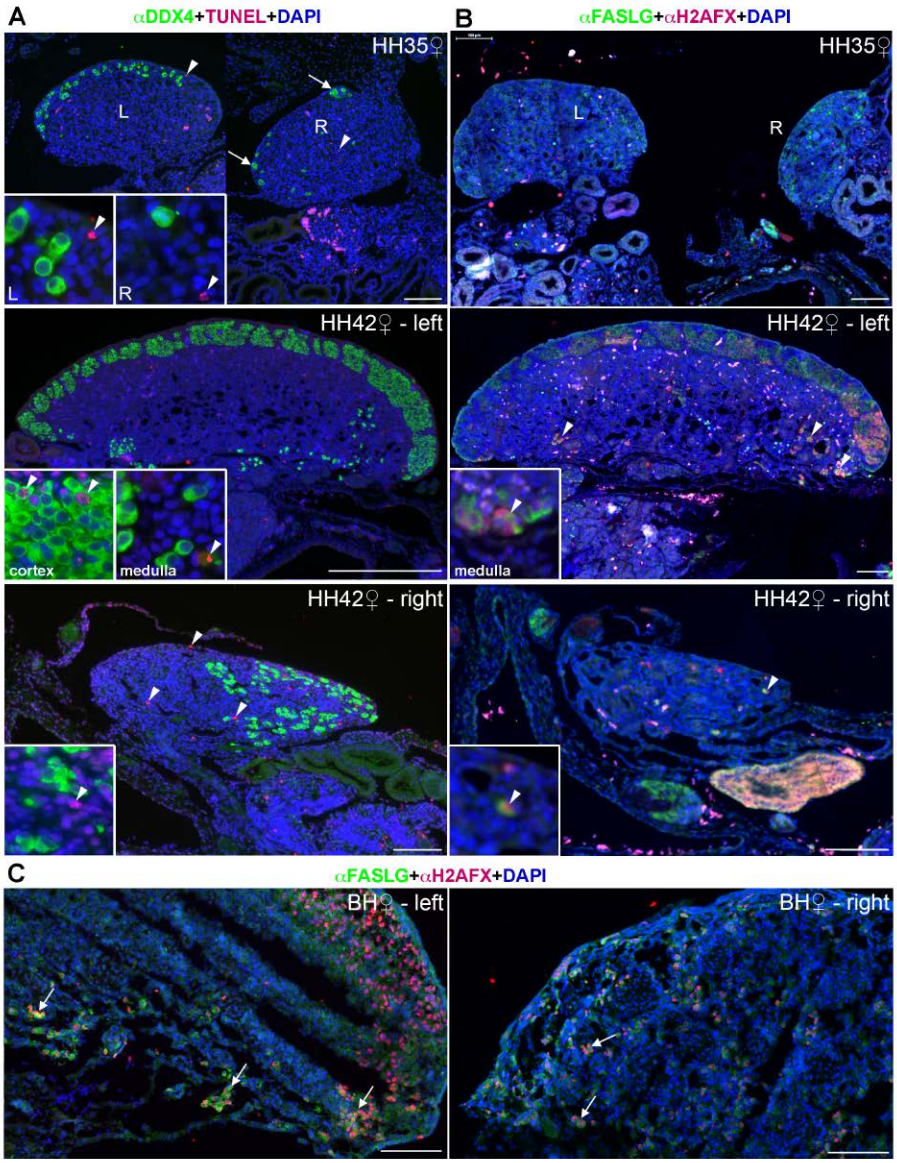

Figure 6. TUNEL and FASLG expression in chicken female gonads. (A) TUNEL assay (red) shows late apoptotic cells in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to apoptotic somatic cells and germ cells (green, DDX4-positive). Inserts show a magnified detail. White arrow point to TUNEL negative germ cells in the surface of the HH35 right gonad. (B) FASLG (green) and H2AXF (red) expression in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to double positive FASLG and H2AXF cells (C) FASLG (green) and H2AXF (red) expression in female left and right gonads before hatching (BH). White arrows point to double positive FASLG and H2AXF cells. Scale bars in A and B are 100 µm, in C are 200 µm and in the inserts are 10 µm.

In agreement, the overexpression of PITX2 *in ovo* in the right female gonad from HH8-10 has been demonstrated sufficient to induce a robust cortex formation in the right gonad similar to that of the left gonad containing H2AFX-positive germ cells at HH38-39 [9]. Those results indicate that germ cells in right female gonad have the capacity to develop into a normal cortex and enter meiosis if given the right environment. We suggest that until hatching, germ cells have equal potential to develop, as in males, in both left and right females gonads but they only receive the correct molecular signals, initiated by PITX2, to do so in the central and lateral part of the cortex of the left female gonad.

Interestingly, we detected expression of FASLG in both the H2AFX-positive germ cells in the extremities of the left cortex and medulla. FASLG-positive cells are not undergoing apoptosis, but are potential targets for destruction. The FASLG-FAS system plays a role in inducing apoptosis in mammalian spermatocytes undergoing meiosis [20,21]. Even though the role of the FASLG-FAS system early during oogenesis is less well studied, it is well known to play a role regulating atresia at different stages during folliculogenesis [29,30]. Whether the germ cells in the extremities of the left cortex and medulla are eliminated by apoptosis after birth, eventually via a FASLG-FAS dependent mechanism remains to be investigated.

Meiotic wave in the chicken left cortex from the center to the extremities

The development of the asymmetry between left and right in female gonads is primarily orchestrated by PITX2 expression [9,23,24]. Thereafter, meiosis entry in chicken seems to be directly related to the synthesis and breakdown of retinoic acid [31,32]. Several authors have described asynchrony in meiotic stages in dispersed chromosome spreads of chicken germ cells during late developmental stages and the first week post-hatching [16-18] or have reported immunostaining in intact gonads for either H2AFX or SYCP3 separately, but not in combination [9,31-34]. The existence of a meiotic wave in chicken has therefore remained elusive to date.

In his 1963 paper, Hughes did not refer to a meiotic wave in the left cortex although he noted that “germ cells in the central parts of the ovarian cortex are consistently more advanced in development than those at the extremities of the cortex” [28]. Our systematic study of the expression of H2AFX and SYCP3, from HH38 until hatching showed the existence of a meiotic wave in the left cortex (Figure 7).

A meiotic wave has been described in female mice from the anterior to the posterior part of the gonad during mid-gestation [35,36] and even in humans it seems to occur from the inside to the outside of the gonadal cortex during the second trimester of development (AMH and SMCSL, unpublished results), suggesting that a meiotic wave may be a conserved mechanism in animals.

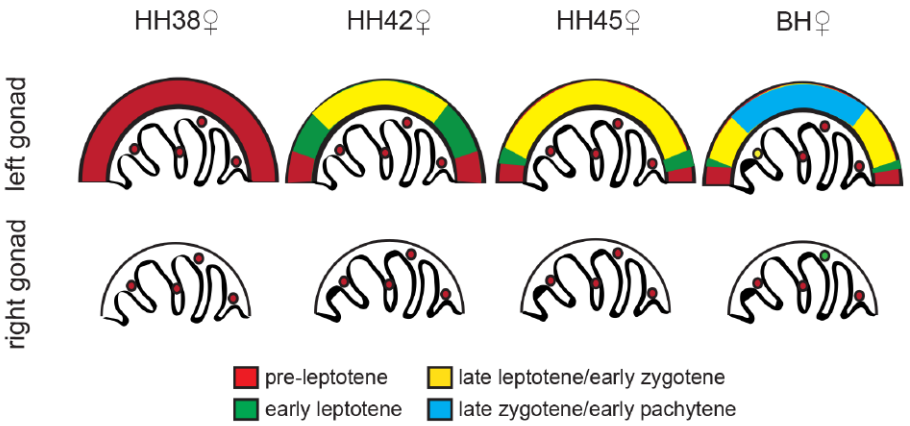

Figure 7. Proposed model of meiotic wave from HH38 until hatching in the female left and right gonads. At HH38, all germ cells independent of their localization express H2AFX and are in pre-leptotene. From HH42 until before hatching (BH), the germ cells in the left gonadal cortex exhibited a pronounced meiotic wave from the central part of the cortex to its extremities. Just BH, germ cells localized in the central and lateral part of the left cortex are in late zygote/early pachytene and late pachytene/early zygotene and germ cells in the extremities of the left gonad are in early leptotene or in pre-leptotene. The majority of the germ cells in the medulla of the left and right female gonads are in pre-leptotene

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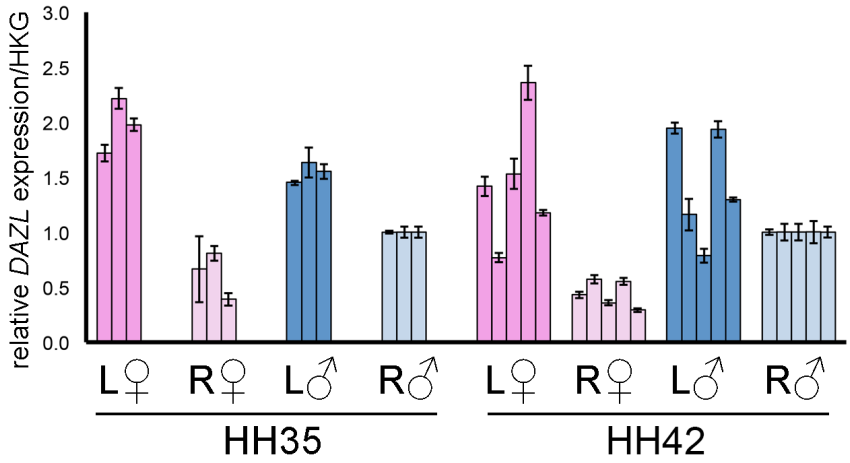
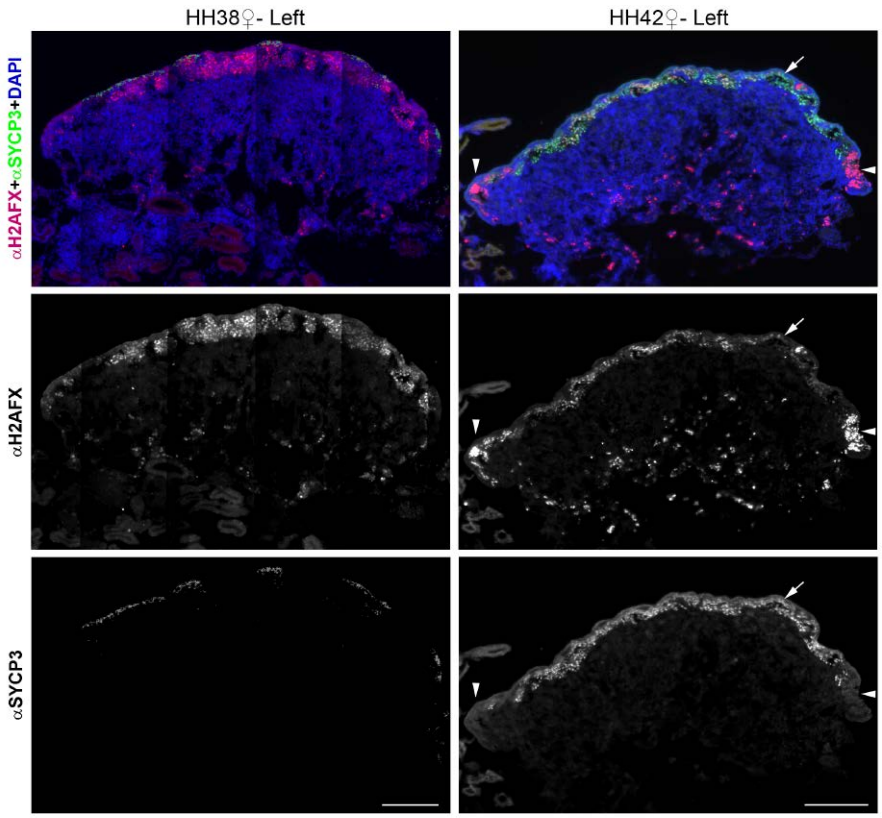
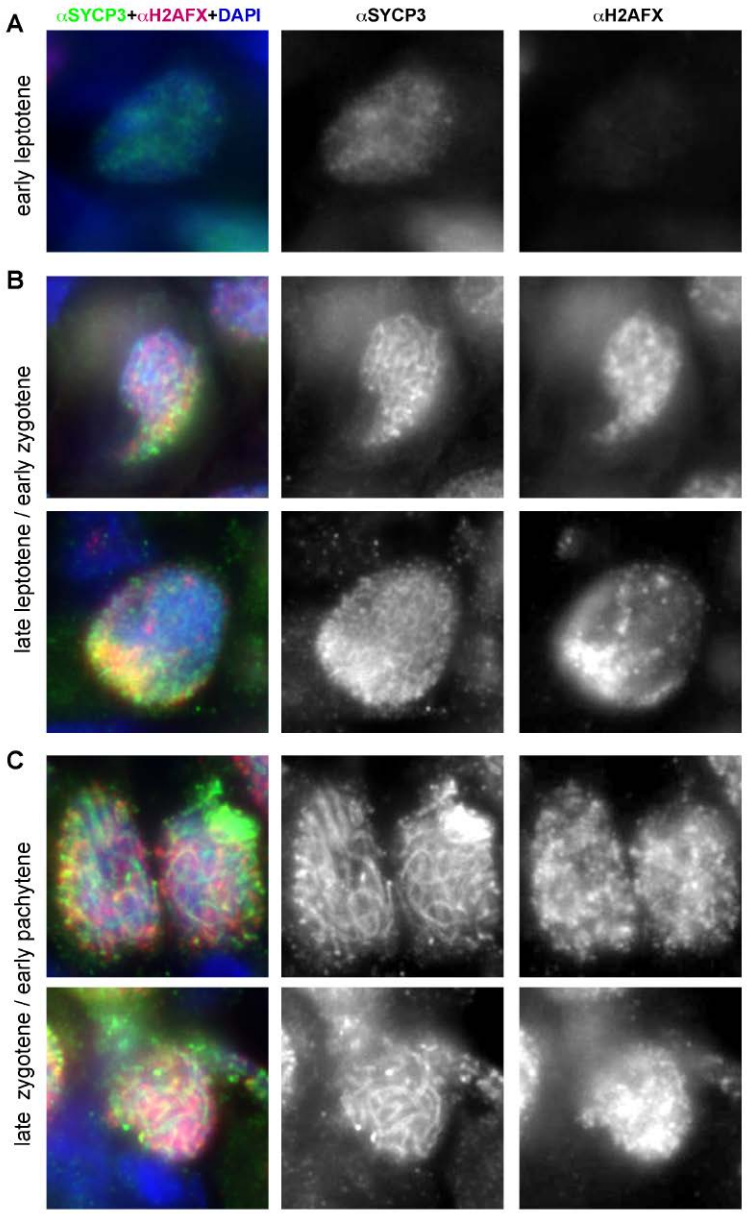
SUPPLEMENTARY FIGURES



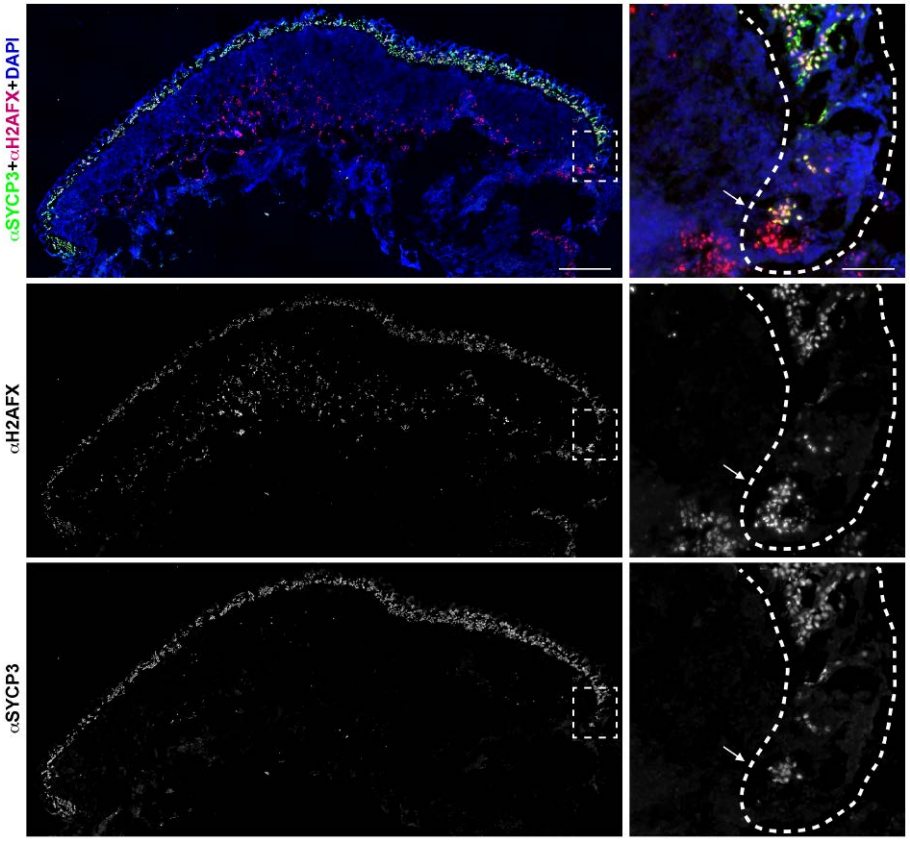
Supplementary Figure S1. DAZL expression in chicken gonads at HH35 and HH42. DAZL expression in left (L) and right (L) gonads of HH35 and HH42 chicken embryos, both females and males, compared to the male right gonad. Each bar represents one gonad and data are expressed as the mean ± standard deviation of technical triplicates. Expression of DAZL was normalized to the HKG RPS17 and ACTB.



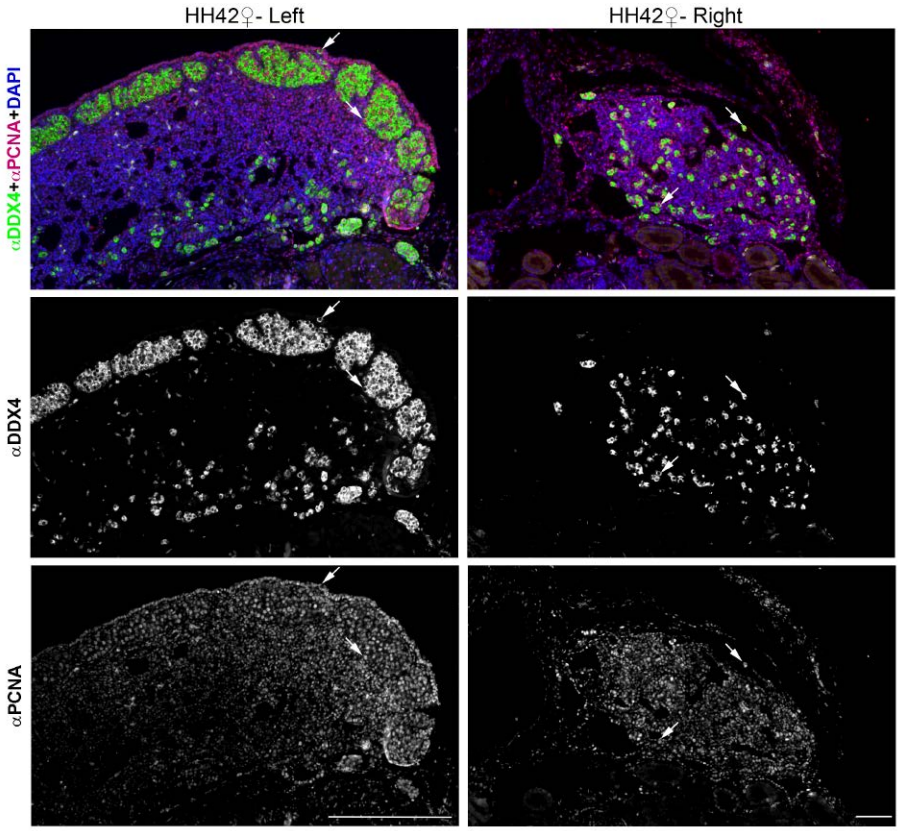
Supplementary Figure S2. Expression of H2AFX and SYCP3 in the chicken left gonad at HH38 and HH42. Sections of HH35 and HH42 female left gonads showing the merged and single channels for H2AFX (red) and SYCP3 (green). White arrows indicated germ cells with lower H2AFX and higher SYCP3 expression compared to the germ cells indicated with white arrowheads. Scale bar for HH38 is 100 μm and for HH42 is 200 μm.



Supplementary Figure S3. Different meiotic phases encountered at HH45 to before hatching. Magnified and single-channel images from Figure 4 showing different meiotic phases in female chicken gonads: (A) early leptotene stage marked by single expression of H2AFX, (B) late leptotene/early zygotene stage marked by the punctuated expression of both H2AFX and SYCP3, and (C) late zygotene/early pachytene stage marked by the expression of H2AFX and chromosomal synaptic expression of SYCP3. Scale bars are 5 μm.



Supplementary Figure S4. Expression of H2AFX and SYCP3 in the chicken left gonad at HH45. Sagittal section of HH45 female left gonad showing the merged and single channels for H2AFX (red) and SYCP3 (green). The dashed area is showed magnified in the right panel. In the left panel the dashed are depicts the cortex. White arrows indicate germ cells expressing H2AFX, but not SYCP3, in the extremity of the cortex. Scale bar is 500 μm in left panel and 75 μm in right panel.



Supplementary Figure S5. Expression of PCNA and DDX4 in chicken left and right gonad at HH42. Section of HH42 female left and right gonad showing the merged and single channels for DDX4 (green) and PCNA (red). White arrows indicate germ cells expressing PCNA. Scale bar are 500 μm for left gonad and 100 μm for right gonad.

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Avian as a model for human ovarian cancer: advantages reviewed

Ana de Melo Bernardo[1], Sólveig Thorsteinsdóttir[2] and Christine L. Mummery[1]

[1]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands; [2]Center for Ecology, Evolution and Environmental Change, Department of Animal Biology, Faculty of Sciences, University of Lisbon, Portugal

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ABSTRACT

Ovarian cancer (OC) is the most lethal gynaecological cancer. Early detection of OC is crucial for providing efficient treatment, whereas high mortality rates correlate with late detection of OC, when the tumor has already metastasized to other organs. The most prevalent type of OC is epithelial OC (EOC). Models that have been used to study EOC include the fruit fly, mouse and laying hen, in addition to human EOC cells in 3D culture *in vitro*. These models have helped in the elucidation of the genetic component of this disease and the development of drug therapies. However, the histological origin of EOC and early markers of the disease remain largely unknown. In this study, we aimed to review the relative value of each of the different models in EOC and their contributions to understanding this disease. It was concluded that the spontaneous occurrence of EOC in the adult hen, the prolific ovulation, the similarity of metastatic progression with that in humans and the advantages of using the chicken embryo for modelling the development of the reproductive system, renders the hen particularly suitable for studying the early development of EOC. Further investigation of this avian model may contribute to a better understanding of EOC, improve clinical insight and ultimately contribute to decreasing its mortality rates among humans.

INTRODUCTION

Ovarian cancer (OC) is the fifth most common cause of cancer-related mortality in women. a high incidence of OC correlates geographically with more economically developed countries [1,2]. The current treatments for OC are cytoreductive surgery and platinum/paclitaxel (Taxol®)-based chemotherapy [3]. These therapies are efficient in the treatment of 90% of patients diagnosed with OC , but only when the disease is detected at an early stage [4]. In addition, the treatments lack specificity, further contributing to the high mortality rates of OC [3,5,6]. The absence of an anatomical barrier around the ovary facilitates rapid spreading of metastases in the peritoneal cavity and late diagnosis is attributed to the minimal manifestations of early EOC [4,7]. As a consequence, the majority of OC cases are detected only when the cancer has already metastasized to other anatomical structures [8].

Epithelial OC (EOC) is the most common type of OC, constituting 90% of diagnosed OC cases [9]. One of the greatest challenges in EOC research is to understand its cellular and molecular origin(s) [10]. Different *in vivo* and *in vitro* systems have been used to model EOC. *Drosophila melanogaster* (*D. melanogaster*) and *Mus musculus*(*M. musculus*) EOC models have been helpful in elucidating the biological characteristics of EOC , such as the molecular basis of its metastatic mechanisms, which include alterations in cell adhesion or migration, or expression of genes involved in EOC development [11,12]. Unlike humans, however, neither flies nor mice spontaneously develop EOC; therefore, the translation of outcomes to humans is limited. by contrast, *in vitro* EOC models using human cells are a promising approach to testing anticancer drugs, although the absence of the tumor cell microenvironment is associated with certain limitations [13]. *Gallus gallus domesticus*, the domestic hen, is a model which appears to address some of these shortcomings and, with the recent advances in laboratory tools for chicken research, it is becoming a tractable system for the study of EOC [14]. The hen is the only animal model that, like humans, develops the disease spontaneously and exhibits similar pathology and disease progression; this appears to be associated with prolific ovulation and ageing [14].

The aim of the present review was: i) to provide an overview of the current approaches and challenges in OC research, with a focus on EOC ; ii) to provide a comparative analysis of the advantages and disadvantages of the different models used in EOC research; and iii) to investigate Gallus gallus domesticus as a model to answer fundamental questions regarding the origin of EOC that remain unanswered and to advance modalities for treatment and early diagnosis that may ultimately contribute to decreasing the mortality rate of OC.

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PATHOLOGY AND ORIGIN OF EOC IN HUMANS

Pathogenesis. More than 30 types of OC have been described, which are all derived from only three major progenitor cell types, namely stromal cells, germ cells and surface epithelial cells (Figure 1A). Stromal-cell OC (SCOC) results from the transformation of stromal cells present in the ovary and has a very low prevalence among OC s (7%); germ-cell OC (GCOC) results from germ cell abnormalities that arise during development and is the rarest histotypic origin of OC , with a prevalence of only 3%; EOC is by far the most prevalent OC histotype origin, with a prevalence of 90% (9). EOC results from the abnormal development of epithelial cells and its origin is discussed in detail below. the formation of malignant cysts from malignant epithelial cells is currently considered to herald the pathological development of EOC . Malignant epithelial ovarian cells in the cysts undergo epithelial-to-mesenchymal transition (EMT), becoming motile and capable of invading other tissues [15]. The progress of the metastatic process depends on the ability of these cells to survive and attach to other structures [8,16].

Causality. There is currently no consensus regarding the origin of EOC and it is considered to either derive from malignant alterations of the ovarian surface epithelium [17], or from the abnormal development of the fallopian tube epithelium [18]. The complexity of EOC appears to indicate that the ovarian surface epithelium as well as the fallopian tube epithelium are involved in the development of this disease [10]. The ovarian surface epithelium as the origin for EOC is the oldest hypothesis and has been associated with the high frequency of ovulation in women [19-21]. During each ovulation, this epithelium is disrupted when the mature oocyte is expelled from the ovary and inflammatory processes are then required to repair it [17,22]. During the repair process, a proportion of the cells detach and develop abnormalities, due to the DNA damage in response to inflammatory molecules, resulting in EOC [23]. A role for hormones in the damage of the ovarian surface epithelium has also been suggested [24,25].

The observation that women who use progestin-estrogen oral contraceptives have a 30-60% lower probability of developing EOC, further strengthens the hypothesis that the ovarian surface epithelium is the origin of EOC [26]. However, female mice, which ovulate approximately 4 times more than a woman during their lifespan, do not develop this disease [27]. It is possible that structural differences in the ovarian surface epithelium between mice and humans [27] allow mice to develop a form of resistance against EOC development, despite their significantly higher ovulation rates. Interestingly, it is estimated that the number of ovulations of a 2-year-old hen is similar to the number of ovulations of a woman at menopause [28]. The fact that the hen is the only animal model that develops spontaneous EOC suggests similarities in the role of ovulation in the development of the disease between hens and humans. On the other hand, EOC was

recently associated with abnormalities of the fallopian tube epithelium. The fallopian tube epithelium has been proposed as an origin of EOC , since several proteins normally expressed by the oviduct, such as paired box 8 (PAX8) and cancer antigen (CA)-125, have been found to be expressed in EOC biopsies [10,29]. Moreover, it has been suggested that a genetic predisposition in fallopian tube epithelial cells gives rise to EOC ; this includes mutations in DNA damage repair genes, such as *BRCA1* and *BRCA2* and cell cycle regulators, such as *P53* [30]. However, since the ovarian surface epithelium and the fallopian tube epithelium are contiguous, have a common early embryonic origin and are both affected by ovulation, it is difficult to distinguish whether one or both tissues are the origin of EOC [10].

CURRENT EOC TREATMENTS

There are four main factors that impede early detection of EOC. First, the location of the ovaries deep in the pelvic cavity makes it difficult to detect the initial development of EOC through pelvic probing and imaging. However, certain technological advances in this field, such as ultrasound and fluorodeoxy-glucose positron emission tomography/ computed tomography, allow for better imaging and earlier detection [6,31]. Second, EOC was until recently considered to be an asymptomatic disease. Certain attempts have been made to establish a symptom index for OC; the physical symptoms may include gastrointestinal, genitourinary and gynaecological complaints. These symptoms are, however, variable among patients, so this issue has not been resolved [4,7,32]. Third, there are currently no early tumor markers for EOC that allow early diagnosis, or population screening and later management of the disease, or monitoring of treatment effectiveness [33]. Finally, the spread of malignant carcinogenic cells in the pelvic cavity is facilitated by the absence of a physical barrier around the ovaries. This promotes the spread of EOC along other organs, such as the contralateral ovary, the uterus and the peritoneum [8].

Once EOC is diagnosed, the primary treatment is surgical removal of the tumor. The surgery is normally followed by platinum and taxol chemotherapy, which impairs cancer cell survival. Platinum-based treatments contain chemical compounds that promote DNA crosslinking, inhibiting DNA repair and synthesis [34], while Taxol promotes the assembly of microtubules in an irreversible manner, preventing cell division and promoting apoptosis of cancer cells [35]. Regrettably, the chemotherapeutic agents used against OC are very similar to those used in the 1970s, when platinum-based therapies were first used in OC treatment [3]. Alternatives to platinum-taxol chemotherapy are currently under investigation [6]. These include targeting tumor angiogenesis using inhibitors of proangiogenic proteins, such as vascular endothelial growth factor receptor

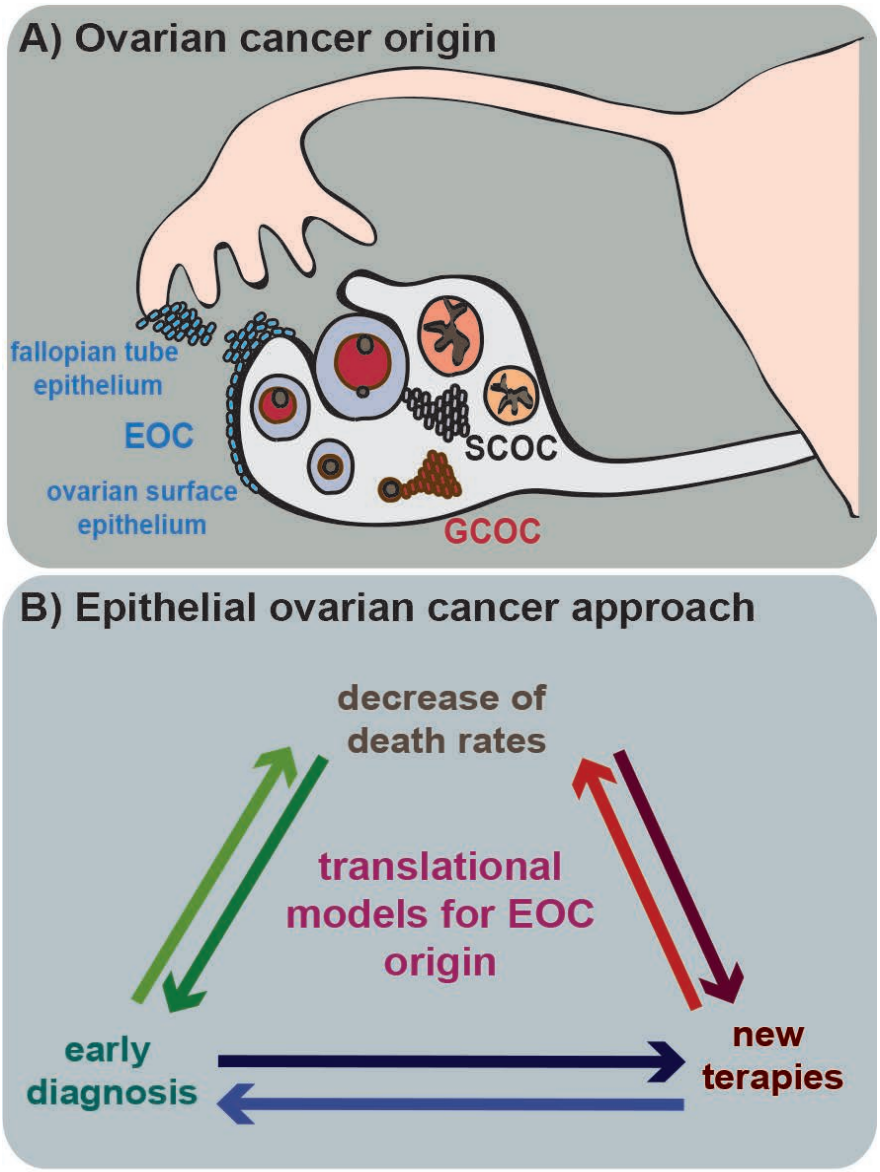

Figure 1. A) Ovarian cancer (OC) has three major types: epithelial ovarian cancer (EOC), derived from the ovarian surface epithelium and/or the fallopian tube epithelium and/or ovarian surface epithelium; germ cell ovarian cancer (GCOC), derived from abnormalities in germ cell development; and stromal cells ovarian cancer (SCOC), derived from abnormal development of stromal cells. B) Finding better translational models for EOC origin, the most prevalent OC type, is fundamental to develop early diagnostic procedures, development of new therapies and, as a consequence, decrease EOC death rates among women.

(VEGFR), platelet-derived growth factor receptor and angiopoietins; or targeting key elements in cell growth, such as epidermal growth factor receptor (EGFR), which is overexpressed in EOC cells, using tyrosine kinase inhibitors and monoclonal antibodies against the extracellular domain of EG FR [36-39]. The majority of these treatments are being developed in animal models but, unfortunately, often fail in clinical trials [33], highlighting the shortcomings of the animal models for human diseases [40]. As a consequence, the OC post-diagnosis survival rates at 1, 3 and 5 years have not changed significantly over the last 20 years [1]. Accumulating knowledge on the origin of EOC is crucial to tackling this disease in its early stages, through identifying predictive EOC biomarkers for diagnosis and improvement of therapy (Figure 1B). For this purpose, it is essential to establish a reliable experimental model capable of capturing all the characteristics of EOC pathology and origin.

ANIMAL MODELS IN EOC RESEARCH

D. melanogaster. The conserved mechanisms of molecular signalling pathways between fruit flies and humans, in combination with the ability to conduct large-scale genetic screens, makes *D. melanogaster* an excellent model for understanding the basic signalling mechanisms underlying the progression of EOC . Studies in *D. melanogaster* have helped identify tumor suppressor genes and oncogenes involved in OC development [16]. Border cells present in the fly's ovaries have been used as a model to study EMT, which is part of the cancer metastatic process [41]. These studies have identified polarity markers in the epithelium, such as E-cadherin and myosin IV, which play a role in the deregulation of proliferation and cell invasion, similar to what happens in human EOC [11]. EGFR and VEGFR are key regulators of border cell invasiveness and have been studied in the fruit fly, since they are also involved in EOC [11]. The role of the Hippo signalling pathway has also been investigated in the fruit fly as a model for EOC. Interestingly, by overexpressing the Yes-associated protein component of this pathway, which is also overexpressed in human EOC, it has been possible to induce EOC in flies, demonstrating its significance in EOC tumorigenesis and conservation of the process in humans [42]. Studying Hippo signalling in fruit flies has revealed the role of this pathway in tissue growth regulation, through programming cell death and cell fate, in flies and humans [11]. However, *D. melanogaster* remains a less than ideal clinical translational model, since it displays reduced metastatic potential and lacks the complexity of the human physiology and human immune system [41].

M. musculus. Mice are the most widely used animals for human disease modelling. In addition to a number of conserved molecular and physiological pathways, mice display

a large repertoire of genetic and laboratory tools, still unsurpassed by other laboratory species [43]. Mouse models in EOC have been extensively used to investigate disease progression in humans and to develop anti-OC drugs. Several mouse models of EOC with different characteristics have been developed. In this review, we aimed to focus on the comparison of advantages and disadvantages of three major groups of mouse models in OC research, namely xenograft, syngeneic and genetically engineered mice. Xenograft mouse models, in which human OC cells are introduced into host immunodeficient mice, enable the study of the early disease stages, as well as invasion and spreading of the cancer cells. These models have been used to evaluate therapeutic approaches, since they constitute a good representation of the disease and its heterogeneity [44,45]. The immune response, however, is completely absent in xenograft models, since the procedures are performed in immunodeficient mouse strains [43]. The development of syngeneic mouse models, in which the cancer cells are derived from the same mouse strain and are introduced into the immunocompetent host, overcome certain limitations of xenografts [46], although the EOC studied is mouse, rather than human. These models enable the study of immune response, tumor-secreting factors, epithelial-stromal interactions and tumor vascularization [43,47]. Since the development of EOC in mice is never spontaneous and must always be induced [12], this is mostly achieved using genetically engineered mice [43]. Mice have been engineered to overexpress genes associated with EOC in humans. These genes include P53, AKT, BRCA 1 and BRCA 2, which have been implicated in the progression and regression of this disease [48-51]. However, the paucity of tissue- specific promoters for ovarian surface epithelium or fallopian tube epithelium is a major limitation of this approach, since it is difficult to distinguish tissue-specific malignancy from the more general oncogenic properties of these genes [10]. Nevertheless, engineered or transgenic mice have enabled the study of the effects of different mutations in EOC and the corresponding immune interactions [12,43]. Taken together, these mouse models have overcome certain limitations of D. *melanogaster* in EOC research. However, they also present with their own biological limitations, which compromise their extrapolation to humans. For example, the heterogeneous origin of EOC requires its study in a heterogenetic background, which is not provided by inbred laboratory mouse strains. Moreover, EOC development in mice is not a spontaneous process, but rather induced as mentioned above, which, by definition, rules out the study of the origin and initial development of this disease, limiting the success of therapeutic response prediction in human patients. The development of new drugs using animal models requires a major investment from pharmaceutical companies, since only a limited number of these drugs continue to clinical trials. Failure to translate is a major obstacle towards finding cures for EOC [40].

***In vitro* models.** *In vitro* systems, based particularly on human cell lines, are in principle an attractive alternative in terms of predictive power and also have the potential to be

turned into high-throughput formats for therapeutic target identification. These *in vitro* systems may also capture patient genetic profiles, an important step in personalized medicine [13]. This promise of bench-to-clinical translation has led to various attempts of developing reliable *in vitro* models of EOC. The current challenges are determining the best source of biomaterials and improving the culture conditions of EOC in order to mimic biological environments [13,52]. Unfortunately, cells derived from untreated tumors exhibit a tendency to develop drug resistance during primary culture using the presently available methods, limiting their value [53]. Immortalized normal ovarian surface and/or fallopian tube epithelia constitute promising alternatives, since they may be genetically modified and cultured for long periods, although they do not mimic the initial stages of the disease [53]. With regard to culture conditions, cell-spreading assays, where tumor cells spread on surfaces coated with extracellular matrix (ECM) proteins, have been used to study the migration of OC cells [54,55]. However, although these ECM proteins may also be present in the tumor, they do not mimic the tumor microenvironment *in vivo*. For this reason, 3D culture systems have been developed to provide a more appropriate microenvironment for EOC cells [56]. 3D culture systems also allow other factors, such as oxygen tension, growth factor gradients and properties of the ECM, to be tightly controlled in order to test their effects on EOC development [57]. However, despite the sophistication of these 3D systems, several widely used OC cell lines and immortalized ovarian surface or fallopian tube epithelium lines have not been able to capture the biology of the tumor [13,58]. This issue has been associated with biomechanical and biophysical constraints and inappropriate ECM and, thus far, has not been resolved [59]. Several limitations, such as establishment of a proper ECM environment, absence of functional vasculature or cells that are able to mediate adaptive immune responses, remain to be overcome in order to construct truly representative EOC *in vitro* models [59]. Improving *in vitro* models for EOC may be costly, due to the need for specialized materials and expertise, but is also dependent on a better understanding of the tumor microenvironment *in vivo*, which the 3D cultures attempt to mimic. This is presently considered to be a work in progress.

THE DOMESTIC HEN: A UNIQUE MODEL TO STUDY EOC

The female hen possesses a single functional ovary, which undergoes ovulation at a high rate during its lifespan [60]. Despite anatomical differences, the laying hen is the only experimental model that develops spontaneous OC and, at the same time, offers the possibility of easy manipulation of external factors, such as nutrition or hormones and drug administration [61,62]. Moreover, the pathology and progression of the disease resembles that in humans in several respects [63,64]. Specific characteristics of the hen

also overcome several limitations of the other models already discussed in the study of OC .

Incessant ovulation hypothesis. Fathalla [17] was the first to identify a possible association between the repeated involvement of ovarian surface epithelium in the process of ovulation and the frequency of the development of the common ovarian neoplasms from this epithelium. In his "incessant ovulation hypothesis", Fathalla stresses the role of repeated repair of the ruptured ovarian surface epithelium in the induction of genetic aberrations in the tissue that culminate in the development of OC [17,65]. This hypothesis is in line with observations on the domestic hen, which ovulates daily, on average, for at least 2 years and exhibits an OC prevalence of 5-35% among adult hens, depending on the genetic strain [66,67]. Moreover, the hypothesis relates EOC incidence in humans to the fact that modern women are generally exposed to a continuous ovulatory process from puberty to menopause. A continuous ovulatory process without fertilization results from the decreased pregnancy rates in modern society, also evidenced by the geographical co-localization of high OC incidence in more economically developed countries [17]. There is strong evidence supporting an association between low prevalence of EOC and the use of oral contraceptives or/and pregnancy [65]. While wild chickens may live for 20/30 years, the domestic hen has a relatively short lifespan and is subject to intense and concentrated egg production during the first 2 years of its life, which makes it an interesting model to study the role of ovulation in EOC . Indeed, Fathalla's theory laid the foundation for different studies regarding the role of ovulation in OC [17]. The first study, using medroxyprogesterone, demonstrated decreased egg production and a 15% reduction in the incidence of EOC in 3-year-old birds [68]. More recently, using progestin as contraceptive, a 90% decrease in OC incidence was achieved in treated hens compared with the controls [69]. A short generation time, the possibility of controlling environmental factors and the availability of different genetic strains make the domestic hen a very useful model in chemoprevention experiments [61,68,69].

Biomolecular and metastatic traits. The similarities between the hen and humans with respect to EOC development are also observed in terms of pathology, with several similar histopathological subtypes identified in both species [68,70]. Moreover, the sequencing of the chicken genome 10 years ago enabled valuable molecular comparisons with human cases [71]. Different biomarkers, such as CA -125, P53 and E-cadherin, were also expressed in EOC in both species [28,72-74]. With respect to the EOC origin, the same controversies apply to human and hens. In the hen, the expression of proteins that are specifically expressed in the oviduct during the later stages of the disease, such as ovoalbumin, ovostatin 2, PAX2 protein or EG FR1, indicate involvement of the oviduct in disease development [10,72]. This finding supports the involvement of the fallopian tube epithelium in spontaneous EOC, as in humans. This trait makes the hen a particularly

useful model to better understand the origin of the OC in humans, where the oviduct also appears to play a role [63,67,75]. Since, as mentioned earlier, female mice do not develop spontaneous EOC, the involvement of the fallopian tube epithelium was only recently demonstrated: In a transgenic mouse model, in which *SV40* large T-antigen was expressed under control of a mouse Müllerian-specific *Ovgp-1* promoter, malignant progression of this epithelium was observed [76]. With respect to the pathology of EOC in the hen, this is a highly malignant cancer that metastasizes along the abdominal cavity, spreading to different organs within a short period of time [68]. Histopathological evaluation of OC metastasis reveals similar characteristics between human and hen spontaneous adenocarcinomas of the reproductive tract. Interestingly, the metastatic process of EOC, in terms of the position and location of the ascites during the later stages of hen EOC, also resembles that in humans [18]. Despite significant evidence supporting the presence of similar molecular patterns in the origin and development of EOC, the lack of commercially available antibodies for immunohistochemistry and western blot analysis remains a major limitation in the use of hen models in EOC [14,67]. In order to increase the translational power of the laying hen as a model in OC research, it is crucial to develop further chicken laboratory tools in the fields of genomics, proteomics and metabolomics. These tools will likely be useful for the study of OC, as well as that of other pathologies [77].

Anatomy and heterogenetic background. Different EOC types display remarkable diversity at the cellular and molecular levels [10,78]. There is currently a scarcity of evidence regarding the role of specific genes in the development of EOC in humans, which appears to have heterogenic causes [78]. The evidence indicating a heterogeneous background to EOC suggests that it is of paramount importance to establish an experimental model with a heterogenetic background to study this disease, rather than using inbred species [67]. The domestic hen has been extensively bred for agricultural purposes, but its genome maintains the genetic diversity of the wild chicken [71]. Studies regarding the role of ageing in the development of EOC in hens have demonstrated differences in EOC prevalence rates among different strains. Different strains appear to develop OC in parallel with ageing; however, the incidence rate of the disease differs among strains [66].

Development of the reproductive system. The fact that the hen develops *in ovo*, provides a significant advantage for *in vivo* manipulation and imaging of embryonic processes [79]. The use of chicken embryos, which are amniotes, in cell interaction studies, cell fate tracing or mechanisms of embryonic patterning, has allowed investigation of several processes that have analogies in humans [79]. The development of the urogenital system is a case in point, particularly with regard to understanding the signalling pathways underlying the development of the testes and ovaries [80]. The development of the gonads

in chickens displays one particularly striking characteristic: During gonadogenesis, the development of the gonads is asymmetric, resulting in two functional testes in males, but only one functional ovary on the left side in females [81]. This asymmetric development of the chicken gonads affects gonadal morphology and the development of germ cells, as exemplified by the asymmetric expression of meiotic markers (unpublished data). In mammals, asymmetry between the two gonads is also established during development; this does not affect their functionality, as a pair of functional testes or ovaries form. However, this asymmetry becomes evident in the development of certain sexual differentiation disorders, such as hermaphroditism [82,83]. With respect to OC , it is interesting to note that there appears to be a higher prevalence of GCOC in the right gonad compared to that in the left gonad. This asymmetric prevalence of GCOC suggests an association between this asymmetry and germ cell development [84]. The chicken provides a model for asymmetric ovarian development, a mechanism that appears to play a role in germ cell development, which is affected in GCOC . Therefore, the higher prevalence of GCOC in the right ovary may be further elucidated by understanding the asymmetrical development of the gonads in the chicken. Regarding EOC, there is no evidence supporting a role for gonadal asymmetry in the prevalence of the disease in the right or left ovary [84]; interestingly, however, paired-like homeodomain transcription factor 2 (*PITX2*), which is overexpressed in EOC , is also a key player in the asymmetric development of chicken female gonads (85,86). The expression of *PITX2* in the left gonad promotes proliferation of the left cortex, leading to the asymmetric development of the gonads [85,87]. Moreover, when induced in the left gonad, *PITX2* promotes the formation of the right cortex [87]. Interestingly, *PITX2* plays an important role during development, but is normally silenced in the adult; its role in cancer was recently demonstrated in several tumor types, such as metastatic prostate cancer and breast cancer [88-91]. The chicken embryo offers a unique experimental model to understand the role of *PITX2* in gonadal development and the effects of the inhibition or overexpression of this transcription factor during development, which may provide insight into its role in the signalling pathways involved in the development of EOC.

GENETIC TOOLS: BOOSTING AVIAN MODELS IN EOC

Since Aristotle, the first to study the avian model, the laying hen has been used extensively in experimental embryology, disease modelling and evolutionary studies [77]. The hen has contributed to our understanding of numerous processes relevant to humans, including (abnormal) cardiac development and somitogenesis, through which much of the skeletal musculature is formed [77]. The differences between birds and humans, that may complicate EOC modelling, stem from the endocrine system and relate to the

sexual hormone cycle [14,69]. However, the drawbacks of avian models in studying the origin of EOC are mostly associated with the lack of technology that provides appropriate laboratory tools [92]. In contrast to flies and mice, there are few commercial sources of antibodies for immunohistochemistry, FACS or western blot analysis and transgenic approaches are only now becoming available in the chicken laboratories [92]. Transgenesis in chicken is progressing slowly, despite the publication of the chicken genome sequence [71]. Nevertheless, small interfering RNA and morpholino oligonucleotides have already been tested successfully in the avian model, allowing gain- and loss-of-function gene studies that are controlled in space and time [93]. The development of isolation and culture methods of chicken embryonic stem cells has opened new doors in exploring chicken cell biology [94]; however, as the available protocols are far from producing the first avian knockouts, it is currently necessary to rely on data from other models. New genetic tools, associated with its extensive history as an experimental model and low costs of acquisition and maintenance compared to other models, predict remarkable advances in the use of the hen for disease modelling [77,92]. For EOC, long-term studies using the appropriate tools with regard to gene and protein expression will soon become more accessible. Together with the possibility of controlling gene expression and culturing chicken cells, these will allow researchers to investigate the spontaneous origin of EOC in a heterogenetic background and overcome certain of the limitations of other models.

THE AVIAN MODEL AS A KEY PLAYER IN EOC RESEARCH

Highlighting the advantages of the hen in studying the origin of EOC does not minimize the importance of improving other EOC models in parallel, but rather warrants the development of an integrative approach using different models, *in vivo* and *in vitro*, that may complement the discoveries made in the avian model (Figure 2). Fruit fly and mouse models in EOC research will continue to unravel the basic mechanisms in EOC development and allow the development/selection of drugs that may be screened in 3D culture systems of human EOC cells. Subsequently, the hen offers the possibility of large-scale drug screenings in heterogeneous populations, enabling the comparison of drug efficiency in a robust model in order to better select drugs for clinical trials. On the other hand, the laying domestic hen represents a unique system that mimics the disease in humans with regard to origin, development, metastatic processes and association with the ageing oviduct epithelium; in addition, the characterization of EOC in different progression stages in the adult hen may elucidate the mechanisms underlying the origin of EOC in humans. Therefore, the hen constitutes a fundamental model for the identification of candidate pathways associated with the onset, development and progression of EOC and the selection of drugs that target cancer pathways.

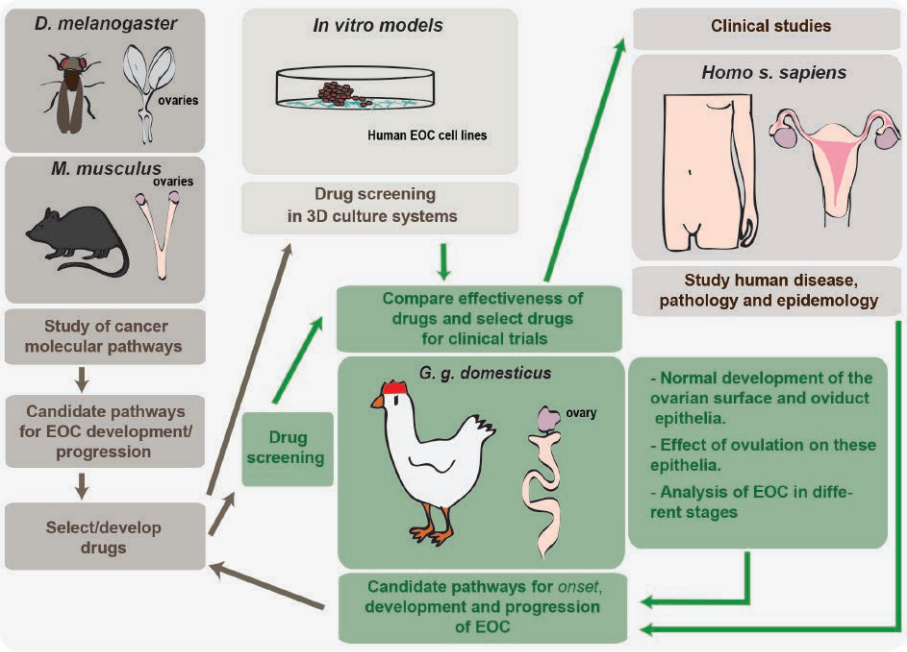

Figure 2. The role of the laying hen in an integrative epithelial ovarian cancer research effort. Drosophila melanogaster and Mus musculus are well established models to study basic molecular mechanisms in cancer. They are thus excellent models to dissect out candidate pathways involved in EOC biology. However, D. melanogaster has limited clinical translation relevance since it does not present the complexity of human physiology. M. musculus is physiologically very similar to humans, but does not provide a heterogenetic background and spontaneous development of EOC. 3D in vitro culture systems of human EOC cells can constitute an important model in drug screening, but they are still not capable of reproducing the in vivo situation. Gallus gallus domesticus is a promising model for EOC, not only by offering advantages relative to M. musculus in terms of in vivo drug validation, but it is also a unique model to

Those drugs may be screened in other models, such as fruit fly, mouse and *in vitro* systems, but also in the hen itself. The complementary study of the different models may help us elucidate the pathology and epidemiology of this disease. In conclusion, only an integrative research effort, where the avian model plays a crucial role, will enable the identification of new markers, thereby allowing the development of novel diagnostics and therapies for OC, which remains the most common cause of gynaecological cancer-related mortality in humans.

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5

The involvement of the proamnion in the development of the anterior amnion fold in chicken

Ana de Melo Bernardo[1] and Susana M. Chuva de Sousa Lopes[1]

[1]Department of Anatomy and Embryology,

Leiden University Medical Center, Leiden, The Netherlands

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ABSTRACT

The amnion was one of the most important evolutionary novelties in the animal kingdom, allowing independence of water for reproduction and subsequent exploration of terrestrial habitats, and is therefore an important structure to understand evolution. We have studied chicken amniogenesis using *ex ovo* culture systems and 3D-reconstructions of serially sectioned chicken embryos. We provide evidence for a transient depression of the head in the proamnion, forming a pouch, that positions the extraembryonic membranes dorsal to the head and that is fundamental for the correct formation of the amnion and chorion membranes. When this "sinking" process in the proamnion was blocked, the amnion/chorion did not form, even though the growth of the embryo per se seemed unaffected. Here, we give insight in the role of the proamnion in amniogenesis.

INTRODUCTION

The appearance of the amniote egg, an evolutionary novelty from the amphibian ancestors of the reptiles, opened new evolutionary paths. The amniote egg contained all the materials needed for the embryo to develop: sufficient water, nutrients and energy. Only oxygen and heat were still required from the environment. Subsequently, animals became independent of water for reproduction and took the opportunity to explore terrestrial habitats. The original pattern of the amniote egg, that comprised the formation of four extraembryonic sacs (amnion, chorion, yolk sac and allantois), was such an important novelty that it remained conserved between reptiles, birds and mammals (reviewed in [1,2]). In mammals, even in the absence of large amounts of yolk, the developing of the four extraembryonic "sacs" has been retained to a certain extent (reviewed in [1,2]).

In chicken, the generation of the extraembryonic sacs takes place after gastrulation, with the appearance of the extraembryonic coelomic cavity in the extraembryonic mesoderm (ExM) [3-6]. This cavity contributes to the separation of two major extraembryonic tissue layers: the splanchnopleure formed by endoderm and ExM; and the somatopleure formed by ectoderm and ExM. The splanchnopleure develops into a complex system of blood vessels, the yolk sac, responsible for supplying yolk and egg white materials to the embryo (nourishment); and it will architect the allantois, a structure connected to the primitive gut, which stores toxic by-products produced by the embryo. On the other hand, the somatopleure gives rise to both the chorion and the amnion. The chorion will allow gas exchanges with the external environment, while the amnion constitutes a protective membrane that surrounds the embryo and prevents its desiccation.

Interestingly, the ExM does not populate the extraembryonic area immediately anterior to the chicken foregut (and the developing heart), the so-called proamnion, but as the ExM spreads anteriorly it does so by circumventing the proamnion with two separate lateral wings that fuse axially. The proamnion remains diblastic composed only of ectoderm and endoderm and during the presomitic stages (until Hamburger and Hamilton stage (HH)7 [7]) it has been shown to express retinoic acid receptor isoform β2 (RARβ2) [8]. The proamnion, as diblastic structure, disappears gradually [9,10], however according to Rosenquist (1971), endoderm fate-mapped to the proamniotic region can become incorporated in the ventral foregut and midgut [11]. The proamnion should not be confused with the buccopharyngeal membrane, another cranial diblastic membrane, present in both human and chick embryos, that gives rise to the opening of the oral cavity [12,13].

The current model of amnion development in chicken describes the separation between the amnion and the chorion from four distinct folds of somatopleure: the anterior amnion fold, two lateral amnion folds and the posterior amnion fold [7,9,14-16]. The growth of the anterior amnion fold would create sufficient tension to elevate the somatopleure, subsequently leading to the formation of the two lateral amnion folds [17]. The posterior amnion fold surrounds the caudal region, similarly to the anterior amnion fold, but growing in opposite direction with an 18 hour delay. The embryo becomes enclosed (by amnion and chorion), after the fusion of the four different amniotic folds over the dorsal side of the embryo by 72 hours of incubation [15].

Recently, we have investigated the migratory route of the primordial germ cells (PGCs) in chicken embryos from the germinal crescent region of the yolk sac to the genital ridges and noticed that the PGCs would often be situated on an extraembryonic membrane clearly positioned above (or dorsal to) the head of the embryo [18]. As classically the PGCs are localized in the splanchnopleure and later in the vasculature of the yolk sac [19], we were surprised by their dorsal localization and we have investigated the formation of both the anterior amnion fold and the yolk sac between HH10 and HH13 by culturing embryos *ex ovo* and by performing 3D-reconstructions of serially sectioned chicken embryos. We concluded that the developing head at HH13 is submerged in a pouch formed by the diblastic proamnion. This displacement of the head positioned both the splanchnopleure (yolk sac and hence the PGCs) and the somatopleure transiently in a dorsal position to the head, explaining our observations of PGCs present dorsally to the head [18]. In 1889, an anatomical study by Shore and Pickering suggested the importance of the proamnion in the formation of the anterior amnion fold [9], but their model has been ignored in most textbooks to date, where the proamnion is not depicted. We now experimentally show that the depression of the head in the proamniotic pouch is paramount for the correct position of the anterior amnion fold, as without this depression of the head in the proamnion, the somatopleure is unable to elevate sufficiently to cover the extending embryo and to surround the embryo generating both the amnion and the chorion.

METHODS

Embryo collection and histology and 3D reconstruction

Fertilized White Leghorn chicken (Gallus gallus) eggs were incubated in a humidified atmosphere at 37.0°C until HH11-13 [7]. The embryos were isolated (HH11 n=6; HH12 n=10; HH13 n=13), fixed overnight in 4% paraformaldehyde, included in paraffin, sectioned and stained for hematoxylin and eosin (H&E) as previously described [18]. Sections were imaged on an Olympus AX70 microscope (Olympus) equipped with an Olympus XC50 camera (Olympus). For 3D reconstructions, serial paraffin transversal sections of freshly isolated embryos at stage HH12 and HH13 stained with H&E were digitalized using a Panoramic MIDI scanner (3D Histech) and reconstructed with Amira 4.1 software (Visage Imaging).

Ex ovo culture systems

The "inverted" culture system was developed combining the "filter paper" method of Chapman and colleagues [26] with that of Nicolet and Gallera [25]. In our method, embryos of HH11 were isolated using a piece of filter paper with a central hole (+/- 1 cm in diameter) placed onto the albumen with the embryo in the middle (Figure 3A-B). The border of the filter paper with the embryo attached was cut with small scissors and gently drawn away with forceps and pulled from the yolk in an oblique direction. The excess of yolk was then washed with PBS, while keeping the embryo attached to the filter paper. Thereafter, the embryos were placed with either their ventral (n=14) or dorsal (n=8) side facing upwards in a petri dish (MatTek) with glass bottom on a 500 µl drop of PBS (Figure 3C-D). The borders of the lid of the petri dish were first sealed with humid paper (with PBS) and then wrapped with parafilm to avoid evaporation (Figure 3D-E). Thereafter, the embryos ("inverted" group and "non inverted" group) were cultured for 7:30 hours (until HH12) at 37°C with humidity on air. As control, an opening was made in the shell of control eggs with embryos at HH11 (n=15), part of the vitelline membrane was removed to expose the embryo and some drops of PBS were added to avoid embryo drought. The eggs were then re-sealed with tape and incubated for 7:30 hours at 37°C with humidity on air, and the embryos were collected afterwards. Somites were counted at the onset and the end of the culture period. The resulting embryos were isolated in PBS and some embryos were processed as described above for histology. For the "suspension" culture system, embryos of HH5 (n=10) were prepared for *ex ovo* culture essentially as described [18,22]. Briefly, chicken embryos were grown on top of a mini yolk sac structure in a fish embryo-like shape. After 72 hours of incubation the embryos were analysed.

Image acquisition of whole mount embryos and statistical analysis

A Leica M420 stereoscope (Leica, Rijswijk, the Netherlands) equipped with a Nikon E4500 Coolpix camera (Nikon, Tokyo, Japan) was used to image whole mount embryos. ImageJ was used to measure the distance between the tip of the anterior intestinal portal and the border between the proamnion and the yolk sac in the embryos cultured *ex ovo* "inverted" and "non inverted" and control embryos grown *in ovo*. IBM SPSS Statistics 20 was used to perform a one-way ANOVA test to compare the average of the distance in each of the three conditions. $P<0.05$ (*) were considered statistically significant.

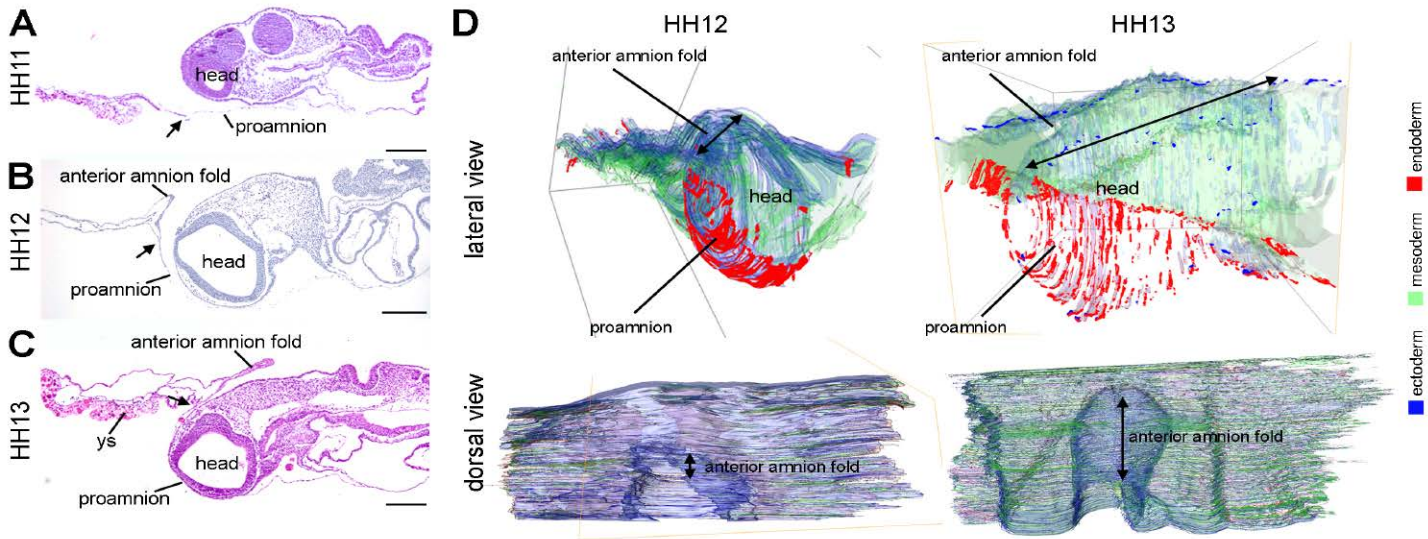

Figure 1. The head tip submerged progressively in proamnion between HH11–13. (A–C) Medial sagittal sections stained with H&E of chicken embryos at HH11 (A), HH12 (B) and HH13 (C). Black arrow points to the junction between proamnion, splanchnopleure/yolk sac and somatopleure/amnion. (D) Lateral and dorsal views of 3D reconstruction of serial transversal sections of embryos at HH12 and HH13 (sectioned plane) with the different germ layers in different colors (ectoderm in blue, mesoderm in green, endoderm in red). Double headed arrows indicate the developing anterior amnion fold. Abbreviations: ys, yolk sac. Scale bars: 200 μm.

RESULTS AND DISCUSSION

The head sinks in proamnion, positioning both the somatopleure and splanchnopleure dorsally

To understand the formation and position of the anterior amnion fold and yolk sac in chicken embryos, we isolated chicken embryos at HH11, HH12 and HH13 containing the extraembryonic membranes and serially sectioned them sagittally and transversally. At HH11, the embryo head rested on top of the proamnion (Figure 1A) and as development proceeded, the anterior amnion fold is formed starting to involve the tip of the head and the head progressively submerged in the proamnion, forming a pouch, at HH12-HH13 (Figure 1B,C). The point of separation between proamnion (endoderm/ectoderm), splanchnopleure (endoderm/mesoderm) and somatopleure (ectoderm/mesoderm) was clearly visible at HH11, HH12 and HH13 (black arrow in Figure 1A-C). At HH13, the proamnion covered the forebrain and as a consequence both the splanchnopleure and somatopleure were elevated and positioned dorsally from the forebrain, facilitating the growth of the amniotic fold to cover the midbrain (Figure 1C).

In the absence of known molecular markers for the proamnion at HH12-HH13, we performed 3D reconstructions of serial transverse-sectioned embryos to visualize the boundaries of the proamnion in more lateral positions to the head (Figure 1D). The reconstructions showed that the proamniotic domain extended laterally between HH12 and HH13 as the head submerged (Figure 1D). In addition, the dorsal view of the 3D reconstructions showed that the anterior amnion fold is growing dorsally and progressively in an anteroposterior direction between the two stages (Figure 1D, dorsal view), while the head is sinking in proamnion (Figure 1D, lateral view). A detailed analysis of whole mount embryos of HH12-HH13 (Figure 2A-B) also revealed that the head indeed submerged in a proamniotic pouch, positioning the boundary between the proamnion, the splanchnopleure/yolk sac and the developing anterior amnion fold dorsally from the head (Figure 2A and white dotted line in Figure 2B), allowing the dorsal expansion of the anterior amnion fold in a posterior direction (yellow arrows in Figure 2B). Histological analysis of HH13 embryos sectioned transversally revealed that the tip of the head was indeed completely surrounded by proamnion (Figure 2C). In 1951, Hamburger and Hamilton described the presence of the anterior amnion fold covering the forebrain's region at HH12 and extending to the midbrain and the anterior part of the hindbrain at HH13 [7], indicating a directional growth from anterior to posterior. The proamnion was not mentioned in that classical paper.

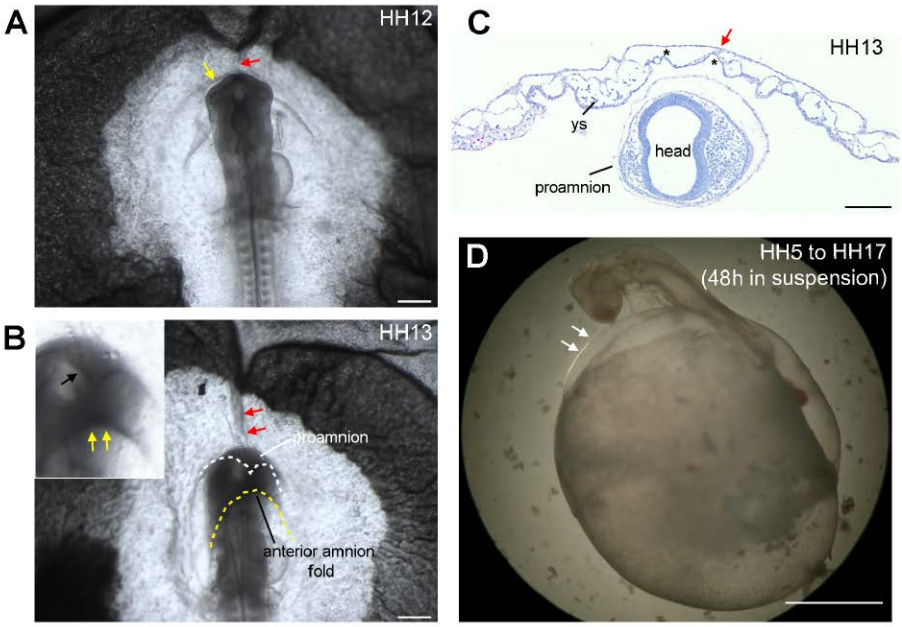

Figure 2. Passive displacement of somatopleure and splanchnopleure due to the sinking of the head in proamnion. (A) Whole mount HH12 chicken embryo with the anterior amnion fold forming (yellow arrow) and an axial strip visible from the proamnion until the area opaca (red arrow). (B) Whole amount HH13 chicken embryo showing the head sinking in the proamnion, which already covers the tip of the head (white dotted line and black arrow in the magnified view in the top left corner), and that the anterior amnion fold has developed posteriorly (yellow dotted line and yellow arrows in the magnified view in the top left corner). (C) Transversal section of a HH13 chicken embryo showing the head completely surrounded by proamnion and the avascular region of splanchnopleure/yolk sac (in between asterisks) attached to the somatopleure by a double layer of mesoderm (red arrow). (D) In embryos cultured ex ovo "in suspension", from HH5 to HH17 (for 48 hours), the head did not sink in proamnion and therefore the formation of the amnion was impaired. White arrows point to the somatopleure. Abbreviations: ys, yolk sac. Scale bars: 500 μm (A,

The splanchnopleure/yolk sac contains an avascular strip above the head at HH13

We have noticed an undescribed feature in the area pellucida at HH12-HH13: a prominent axial strip of splanchnopleure/yolk sac (red arrow in Figure 2A) as the embryo submerged under the anterior amnion fold. In HH13 whole mount embryos, this axial strip enlarged, extending from the border of the proamnion until the area opaca (red arrows in Figure 2B). Moreover, the axial strip of splanchnopleure/yolk sac that was present above the head was free of developing vasculature in contrast to the more lateral splanchnopleure/yolk sac (between asterisks in Figure 2C) and that the avascular region of the splanchnopleure/yolk sac remained consistently attached to the somatopleure by a thin double layer of mesodermal cells, known as the median mesoblastic septum (red arrow in Figure 2C).

This septum, also observed in reptiles, is the consequence of the growth of the two lateral mesodermal wings axially in front of the proamnion and the formation of the right and left extraembryonic coelom, keeping them physically separated axially [10,20].

When cultured "in suspension" the amnion/chorion did not develop properly

In 1948, in a series of experimental procedures on the development of the amnion in chicken embryos, Adamstone and colleagues showed that the cauterization (burning using a hot needle) of a limited group of cells in the proamnion, prevents the development of the anterior amnion fold [21], suggesting the involvement of the proamnion in this process. To understand whether the depression of the head in the proamnion was necessary for the development of the anterior amnion fold forming the amnion and chorion in the process, we cultured chicken embryo using a modified Cornish pasty method [18,22], a "suspension" culture system, whereby the embryos were cultured on top of a sealed mini yolk. In this method, the embryos were collected at HH5 and cultured until HH17 (for 48 hours) in suspension. Here, the head failed to submerge in the proamnion and as a consequence the somatopleure was not positioned dorsally to the head and therefore the amnion and chorion were unable to form properly (Figure 2D).

Amniogenesis occurred in chicken embryos growing in an "non inverted" position

An elegant *ex ovo* culture system, originally developed by Denis New and named after him, has been adapted and used in many studies on the development of chicken embryos [23,24]. In this system, chicken embryos are grown with their ventral side facing upwards up to 48 hours and did not form the amnion. In 1963, Nicolet and Gallera made some modifications to the "New" culture system, using two glass rings instead of only one and an agar-based substrate, and compared the development of the blastoderm for 48 hours between an "inverted" and "non inverted" position [25]. Interestingly, when the blastoderm is grown in a "non inverted" position, the amnion developed and covered the embryo properly. Even though the formation of a "capuchon céphalique" (head pouch) in the "non inverted" grown embryos was mentioned, Nicolet and Gallera did not investigate further the anatomical nature of this structure [25].

We hypothesized that the formation of the "capuchon céphalique" described by Nicolet and Gallera was in fact a consequence of the sinking of the head in proamnion. To test this we have developed a simple *ex ovo* culture system combining the use of filter

paper (instead of glass rings) previously introduced by Chapman and colleagues [26] and using PBS solution as substrate for culture (instead of agar) (Figure 3, Figure 4A). We cultured HH11 chicken embryos containing the intact extraembryonic membranes with their ventral side facing either upwards ("inverted") or downwards ("non inverted"), and compared those to embryos grown *in ovo* (Figure 3, Figure 4A). All embryos were allowed to grow for 7:30 hours (from stage HH11 to stage HH12), the timing when the head submerges in proamnion *in ovo* (Figure 1A,B).

The number of somites was counted in each embryo in each condition, before and after the experimental procedure. Embryos growing "inverted" (n=8) and "non inverted" (n=8) *ex ovo* and *in ovo* control embryos (n=9) formed similar number of somites during the experiment (5 pairs of somites) (Figure 4B). During chicken development, a new pair of somites emerges each 1:30 hours [27] and therefore the expected number of new somite pairs formed in 7:30 hours is 5, which was observed in all conditions.

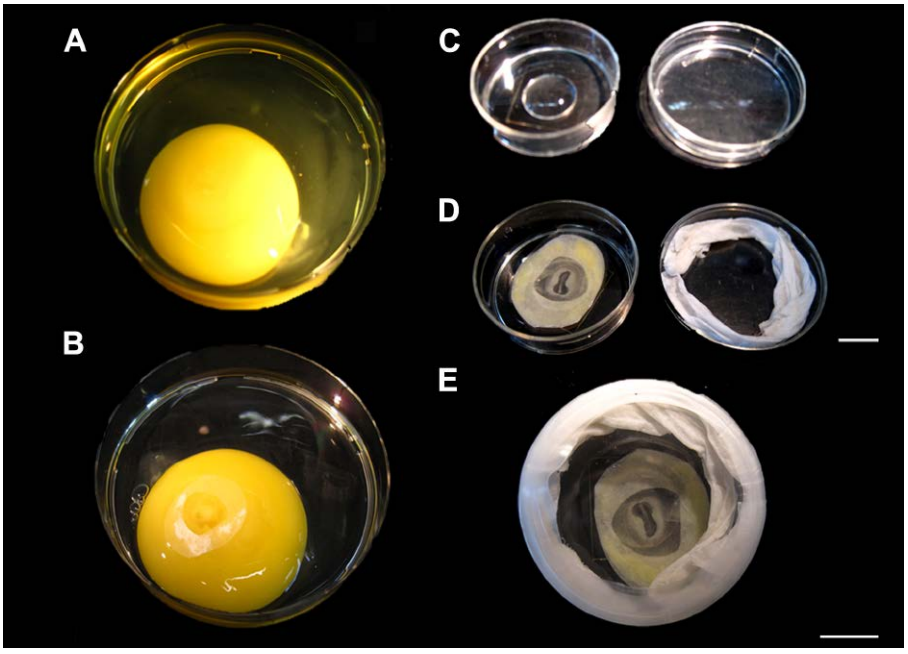

Figure 3. "Inverted and non inverted" ex ovo culture of chicken embryos. (A) The content of the egg was deposited into a petri dish, the thick albumen was removed and and the blastoderm was positioned upwards. (B) A piece of filter paper, with a central hole, was placed on the yolk, positioning the blastoderm in the central hole and the border of the filter paper with the embryo attached cut with scissors. (C) Before (B), a 500 µl drop of PBS was placed in a petri dish with a glass bottom. (D) The embryo attached to the filter paper was placed on the drop of PBS in an "inverted" or "non inverted" position. The lid of the petri dish was coated with (PBS) humidified paper before closing the petri dish. (E) The petri dish containing the embryo was sealed with parafilm. Scale bars: 1 cm in A,B,E and 1 cm in C,D.

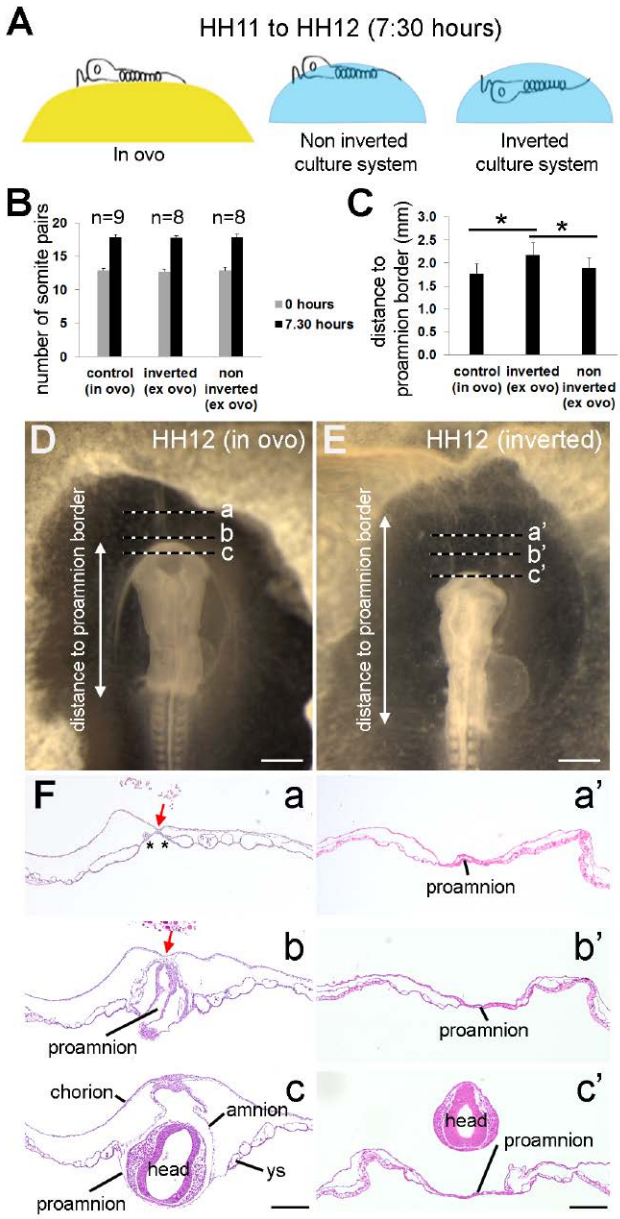

Figure 4. The anterior amnion fold did not develop in embryos growing "inverted". (A) Scheme of the "inverted" culture system where HH11 chicken embryos were cultured for 7:30 hours and compared with embryos growing in ovo. (B) Number of somite pairs at the start (0 hours) and after 7:30 hours of incubation in each group. (C) Distance between the tip of the anterior intestinal portal and the border between the proamnion and the yolk sac in each group. *, P<0.05. (D-E) HH12 embryos growing in ovo (D) and in the "inverted" culture system (E) transversally sectioned (F) at the indicated levels (a–c and a'–c'). In the control embryo note the avascular region of splanchnopleure/yolk sac (in between asterisks in a) attached to the somatopleure by a double layer of mesoderm (red arrow in a and b). Abbreviations: ys, yolk sac. Scale bars: 500 µm (D,E) and 200 µm (F).

The formation of the expected number of somites provided evidence that the *ex ovo* culture system used ("inverted" and "non inverted") did not interfere with the correct development of the embryo and therefore the abnormalities in amniogenesis observed in embryos grown "inverted" were perhaps caused by the positioning of the (normal) embryo head in relation to the proamnion.

The depression of the head in proamnion is necessary for the development of the anterior amnion fold

To quantify differences in proamniotic extension in the embryos grown "inverted" and "non inverted", we measured the distance between the tip of the anterior intestinal portal and the border between the proamnion and the yolk sac in embryos incubated in the different conditions (Figure 4C-E). Whereas both *in ovo* and "non inverted" cultured embryos showed a comparable proamnion (distance 1.77±0.21 mm and 1.86±0.22 mm respectively, P=1); the "inverted" cultured embryos showed an extended proamnion (distance 2.18±0.27 mm), significantly different from the two other groups (Figure 4C), with a P=0.005 to the control group and a P=0,043 to the "non inverted" group.

Transversal sections of control embryos showed that the splanchnopleure/yolk sac remained attached to the somatopleure by a thin double layer of mesodermal cells (septum) (red arrow in Figure 4Fa-b) and those embryos also showed the avascular axial strip in the splanchnopleure/yolk sac (asterisk in Figure 4Fa). In the inverted group, the proamnion extended anteriorly, instead of folding into a pouch as in the control and "non inverted" group (Figure 4Fa'-c' and data not shown). In the "inverted" group, both mesodermal wings formed a coelom, but they did not zipped anteriorly and, as a consequence, the anterior amnion fold was unable to form and the lateral amnion folds were underdeveloped and unable to surround the head to form either amnion or chorion (Figure 4Fa'-c'). In the control and "non inverted" group, the anterior amnion fold was visible and correctly positioned to give rise to the amnion and chorion (Figure 4Fc and data not shown). The downward movement of the head in these embryos into the proamnion is likely due to a combination of factors, including: tissue density, tissue folding induced by differential growth or tension distribution, and the specification of an ectodermal hinge point. Our anatomical analysis showed that indeed the proamnion plays a role in the development of the anterior amnion fold in chicken.

Amniogenesis in mammals

In mammals, the proamnion has been described in the rabbit [28], some species of bat [28] and in some species of marsupial [1]. It may be that depending on the type of blastocyst (existence of only mural or mural/polar trophectoderm) [29] and mode of implantation (and type of placenta) [30-32], the proamnion will be formed or not.

In mice, the amniochorionic fold is formed from the most posterior part of the primitive streak and grows (by cavitation) to fuse with the anterior part of the embryo during gastrulation [33]. There seems to be a transient diblastic region just anterior to the neuroectoderm at 7.5 days post coitum. This diblastic region may play a role as the developing head undergoes a ventral flexure repositioning the amniotic junction. However, this ventral flexure occurs after the amnion and chorion are formed.

In humans, having a similar type of blastocyst to the mouse that contains both polar and mural trophectoderm, there is no proamnion formation. In fact, early during human implantation and before gastrulation is initiated (or any mesoderm has been formed), the epiblast cavitates giving rise to amniotic cavity. The amnion will face the polar trophectoderm, while the epiblast will face the hypoblast. The human embryo completes the formation of the ectoderm part of the amnion by 9 days after conception. Later during gastrulation, the only diblastic structure is the buccopharyngeal membrane formed anteriorly (and the cloacal membrane posteriorly) [34].

The proamnion, an evo-devo perspective

In the late 1800s, it was noted that different amniote species, including different species of birds [5,9,10,35], reptiles [20,35] and mammals [28,35], share the initial steps of amniogenesis, regarding the initial sinking of the head in proamnion, positioning the somatopleure and splanchnopleure transiently above the head. In the late 1800s, some of those authors mention that “notable features are being overlooked” [20] and that “erroneous notions prevail at present day” as “classic series of diagrams constructed on this supposition being copied extensively by writers on embryology” [10] and is still the case today. We conclude that the proamniotic pouch is often being confused with the anterior amnion fold and that the position of the yolk sac, as commonly depicted in textbooks, is misplaced (Figure 5A,B).

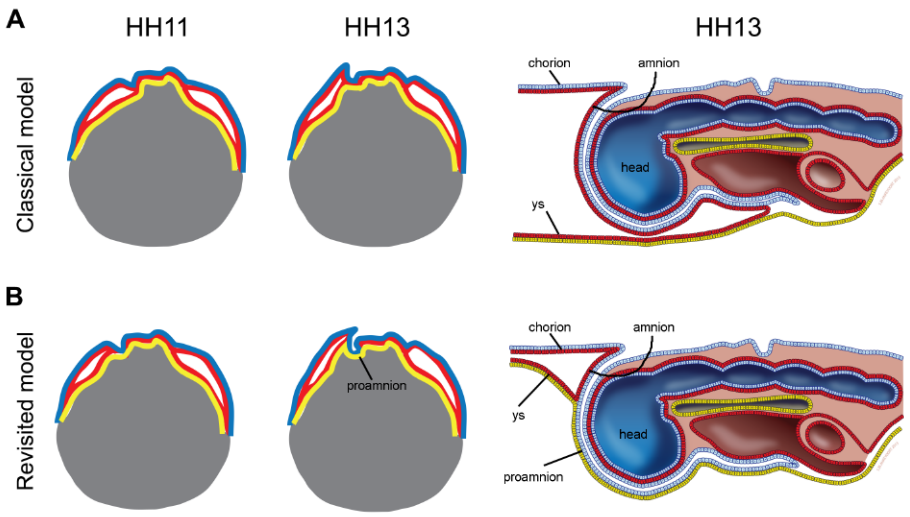

Figure 5. Two models (classical and revisited) for anterior amnion fold development in chicken embryos.(A) Sagittal scheme, representing the classical model, of HH11 and HH13 chicken embryos adapted from a Developmental Biology textbook [36] and a detail view of the head region at HH13. (B) Sagittal scheme, representing the revisited model, of HH11 and HH13 chicken embryos adapted from Shore and Pickering [9]. In both schemes, ectoderm is blue, mesoderm is red, endoderm is yellow and yolk is gray. Abbreviations: ys, yolk sac.

CONCLUSIONS

Here, we have investigated the role of the proamnion in the development of the anterior amnion fold leading to a correct amniogenesis in chicken embryos. We suggest using two different culture systems ("suspension" and "inverted/non inverted") that when the chicken (tip of the) head is not able to sink in the proamnion, the anterior amnion fold is not placed properly above the head, impairing correct amniogenesis.

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6

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.

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7

Appendix

SUMMARY

Primordial germ cells (PGCs) are the progenitors of the gametes, responsible for transmitting genetic information from generation to generation. Although there is a long history of gamete biology research, there is still a lot to be learned about many of the mechanisms underlying germ cell development. This dissertation describes and discusses the dynamics of PGCs in the chicken, with a focus on their migration to the gonads and meiosis that takes place when PGCs are already settled there. We also discussed the advantages of using the avian model in epithelial ovarian cancer research. Moreover, we revisited an often overlooked model for amniogenesis in which the proamnion plays a crucial role.

Chapter 1 presents the state of the literature on PGC development in different model organisms, with a particular focus on the avian model. It also explains the development of extraembryonic membranes.

Chapter 2 explores differences in the specificity of two different markers of germ cells used in chicken, CVH and SSEA1, through different developmental stages. This chapter presents a functional study that shows the role of the anterior vitelline veins as the main vehicles of the migration of PGCs from the extraembryonic circulation into the embryo, where PGCs become established in the gonads at stage HH15.

Chapter 3 describes in detail the asymmetric dynamic of chicken germ cells in the right and left gonads after sexual differentiation. In this chapter it is shown that there is an asymmetric migration of germ cells, which preferentially migrate to the left gonad. Moreover, it is shown that the meiotic development of chicken germ cells is affected by their position in the right or left gonad, cortex versus medulla, and their distribution in the left cortex. Moreover, we suggest that germ cells located in the right gonad are not apoptotic, contrary to what has been suggested before, but able to start their differentiation into oogonia.

Chapter 4 is a review of the use of avian models in ovarian cancer, one of the most lethal types of cancer among women. After an overview of different models used in epithelial ovarian cancer research, the review argues the value of avian models by showing its relevance to the disease compared to other models. The model captures some of the issues that have so far remained unclear in epithelial ovarian cancer, such as its cellular origin and possibilities for drug development.

Chapter 5 is an anatomical study of the role of the proamnion in the development of the anterior amnion fold in chickens. The importance of the proamnion is further demonstrated by two functional essays *ex ovo.* This chapter provides evidence

that transient sinkage of the head in the proamnion is of paramount importance to the localization of the anterior amnion fold on top of the head and that it is crucial for correct amniogenesis.

Finally, **Chapter 6** is a general discussion linking the results described in the different chapters of this thesis. It presents and discusses future perspectives on the use of avian models in stem cell research, disease and development of germ cells.

SAMENVATTING

Primordiale geslachtscellen (PGCs) zijn de voorgangers van de gameten, verantwoordelijk voor het doorgeven van genetische informatie van generatie op generatie. Hoewel biologisch onderzoek naar gameten een lange geschiedenis kent, is er nog veel te ontdekken over veel van de mechanismen die de ontwikkeling van geslachtscellen sturen. Deze dissertatie beschrijft en bespreekt de dynamiek van PGCs in de kip, met een focus op hun migratie naar de gonaden en de meiose die plaatsvindt wanneer PGCs daar zijn aangeland. We bespreken ook de voordelen van het gebruik van het vogelmodel in onderzoek naar epitheliale eierstokkanker. Tevens hebben wij een model voor amniogenese, vaak over het hoofd gezien, waarin het proamnion een cruciale rol speelt, opnieuw bestudeerd.

Hoofdstuk 1 zet de staat van de literatuur over de ontwikkeling van PGCs in verschillende modelorganismen uiteen, met een focus op het model van de vogel. Het legt tevens de ontwikkeling van extraembryonische membranen uit.

Hoofdstuk 2 verkent verschillen in de specificiteit van twee verschillende markers van kiemcellen die in de kip worden gebruikt, CHV en SSEA1, gedurende verschillende ontwikkelingsstadia. Dit hoofdstuk presenteert de resultaten van een functioneel onderzoek dat de rol van de voorste vitelline aderen als de belangrijkste vehikels voor de migratie van PGCs van de extraembryonische circulatie naar het embryo, waar PGCs zich vestigen in de gonaden tijdens stadium HH15.

Hoofdstuk 3 geeft een gedetailleerde beschrijving van de asymmetrische dynamiek van geslachtscellen van kippen in de linker- en rechtergonade na seksuele differentiatie. In dit hoofdstuk wordt aangetoond dat er een asymmetrische migratie van geslachtscellen plaatsvindt, waarbij deze bij voorkeur naar de linker gonade migreren. Bovendien wordt aangetoond dat de meiotische ontwikkeling van geslachtscellen van de kip wordt beïnvloed door hun positie in de rechter- of linkergonade, cortex of medulla , en hun verdeling in de linkercortex. Tevens suggereren wij dat geslachtscellen in de rechtergonade niet apoptotisch zijn, in tegenstelling tot wat tot nu toe gesuggereerd is, en in staat zijn hun differentiatie in oogonia te starten.

Hoofdstuk 4 is een overzicht van het gebruik van vogelmodellen in onderzoek naar eierstokkanker, een van de meest dodelijke vormen van kanker onder vrouwen. Na een overzicht van de verschillende modellen die worden gebruikt in onderzoek naar epitheliale eierstokkanker te hebben gegeven, geeft het een betoog voor het belang van vogelmodellen door hun relatieve belang ten opzichte van andere modellen te tonen, ten aanzien van sommige kwesties die tot zover onduidelijk zijn gebleven, zoals de cellulaire

oorsprong van epitheliale eierstokkanker en mogelijkheden voor de ontwikkeling van medicijnen.

Hoofdstuk 5 is een anatomisch onderzoek naar de rol van het proamnion in de ontwikkeling van de voorste plooi van het amnion in de kip. Het belang van het proamnion wordt verder gedemonstreerd door twee functionele essays *ex ovo*. De resultaten in dit hoofdstuk gepresenteerde bewijzen dat een tijdelijke daling van het hoofd in het proamnion van doorslaggevend belang is voor de lokalisatie van de voorste plooi van het amnion bovenop het hoofd en dat het cruciaal is voor correcte amniogenese.

Hoofdstuk 6, ten slotte, is een algemene bespreking die de in de verschillende hoofdstukken beschreven resultaten verbindt. Het zet toekomstige perspectieven op het gebruik van vogelmodellen in onderzoek naar stamcellen, ziektes en de ontwikkeling van geslachtscellen.

LIST OF PEER-REVIEWED PUBLICATIONS

Gomes Fernandes M., Dries R., Roost M., Semrau S., **de Melo Bernardo A.**, Davis R., Ramakrishnan R., Szuhai K., Maas E., Umans L., Albon Escalona V., Salvatori D., Deforce D., Van Criekinge W., Huylebroeck D., Mummery C., Zwijsen A. and Chuva de Sousa Lopes S.M. BMP-SMAD signaling regulates lineage priming, but is dispensable for self-renewal in mouse embryonic stem cells. Stem Cell Reports. 2015, *In press.*

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ABOUT THE AUTHOR

Ana de Melo Bernardo was born on 27 March 1988 in Covilhã, Portugal. After receiving her high school diploma in 2006, with honors, she started her Bachelor's studies in Biology at the Faculty of Sciences, University of Lisbon (FCUL). During her BSc she had the opportunity to do lab rotations in different research groups at Instituto Gulbenkian Ciência (Oeiras, Portugal) and Instituto Medicina Molecular (Lisbon, Portugal). In 2008, her last year, Ana was awarded a research grant by Fundação Amadeu Dias/University of Lisbon, allowing her to develop a project on chicken somitogenesis in Dr. Sólveig Thorsteinsdóttir's group in FCUL, under the supervision of Dr. Gabriela Rodrigues.

In 2009, Ana started her Master's degree in Evolutionary and Developmental Biology at the Faculty of Sciences, University of Lisbon. In 2010, she represented FCUL at a workshop in developmental biology at Curie Institute - University Pierre Marie Curie, Paris, France, which broadened her experience with animal models in developmental biology. In the same year, Ana was granted an ERASMUS scholarship for her Master's thesis on chicken germ cells at the Department of Anatomy and Embryology at Leiden University Medical Center (LUMC), in the group of Dr. Susana Chuva de Sousa Lopes. In 2013, she started her PhD training, continuing her work on chicken germ cells at the same group, with Prof. Dr. Christine L. Mummery as her promotor and Dr. Susana Chuva de Sousa Lopes as co-promotor.

Since January 2015, Ana has been working with Dr. Valeria Orlova at LUMC on human-induced pluripotent stem cells for vascular disease modelling.

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