Primordial germ cells and amnion development in the avian embryo
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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.
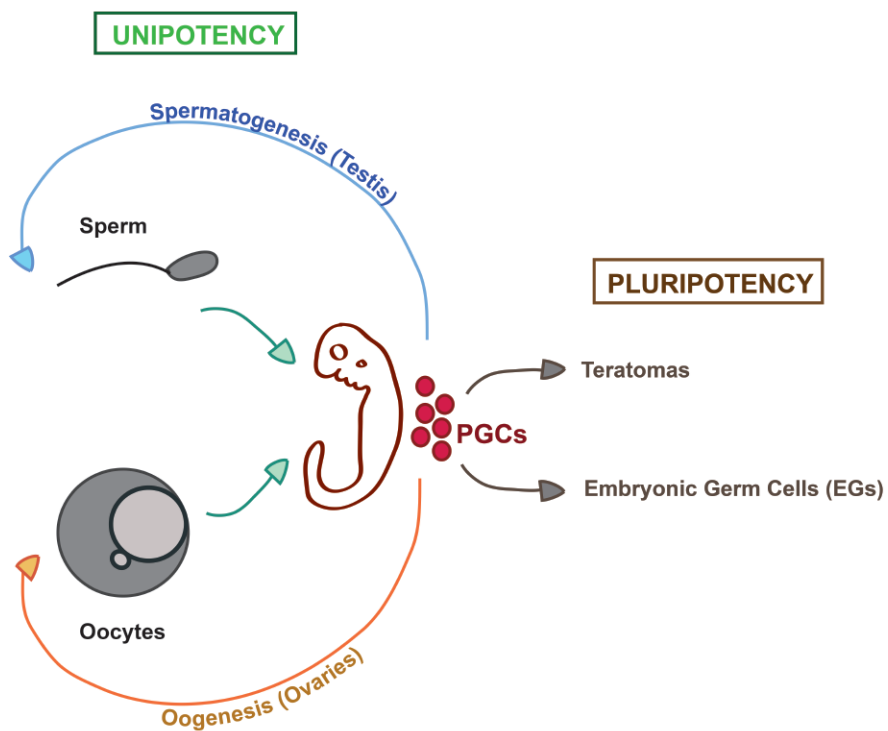

Figure 1. The immortal germline. Primoridal germ cells (PGCs) are unipotent cells that are the progenitors of the oocytes and sperm. When fused, oocyte and sperm give rise to the zygote, a totipotent cell that will develop into a complete organism. During abnormal development, PGCs give rise to teratomas, 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.
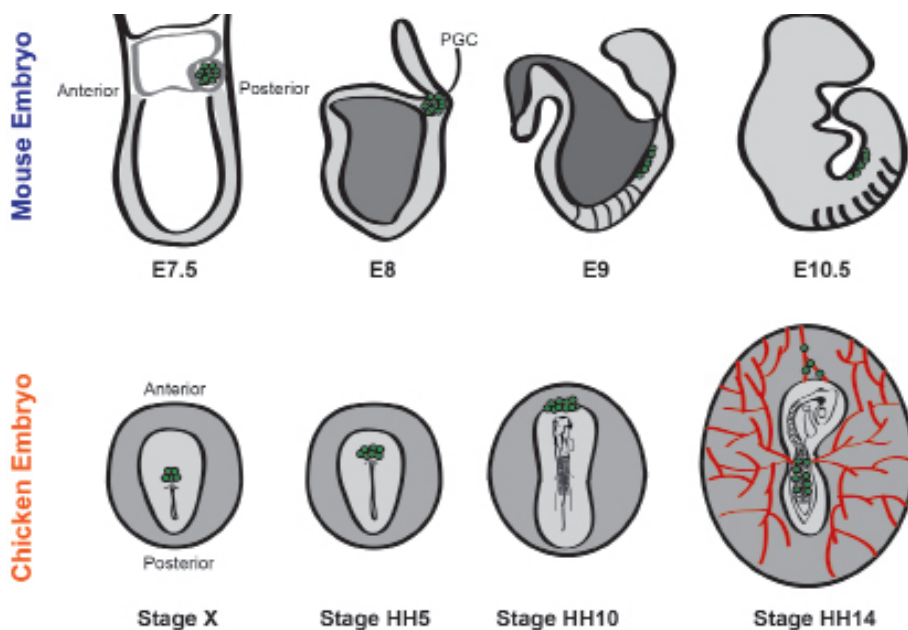

Figure 2. Migration of PGCs in the mouse and chicken. In the mouse, PGCs are specified in the proximal epiblast. At E8 PGCs are localized in the base of the allantois. From there, they migrate to the future gonads along the hindgut (Lateral view of the mouse embryo). In the chicken, at the beginning 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.
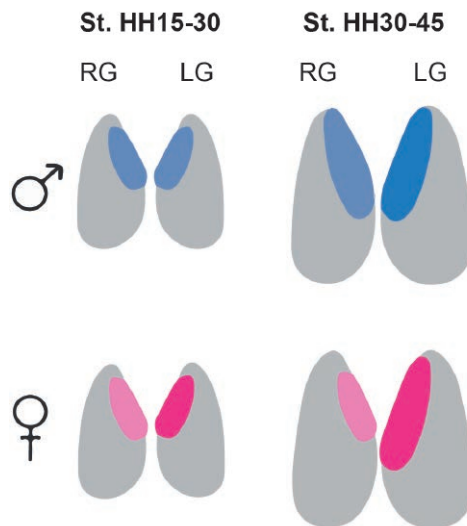

Figure 3. Assymetric gonadogenesis in the chicken. Before sexual differentiation (Stages HH15-30), the differences between the right and left gonad are not visible macroscopically. Only with the beginning of sexual differentiation at stage HH30, the differences between the right and left gonad start 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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