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2010/2011

"Our real teacher has been and still is the embryo,
who is, incidentally, the only teacher
who is always right."

Viktor Hamburger (1900-2001)

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Abstract

Primordial germ cells (PGCs) are the founders of the gametes. They are one of the first discernible embryonic cell types which are set aside very early in the developing embryo. As it happens in other organisms, in chicken embryos, PGCs migrate from their extraembryonic location to combine with the somatic component of the gonad. An ingression of PGCs from the hypoblast into the vascular system, around stage HH8-10, was previously addressed using SSEA-1 as a marker for PGCs. Nonetheless, the isolation of the chicken *vasa* homolog (c*vh*), which is known to play a role in chicken germline, and the production of an antibody against VASA protein, permitted its use as a reliable marker to analyze the migration process of PGCs in chicken embryos. In this work we compared the specificity of VASA antibody with the specificity of SSEA-1 antibody for PGCs at different developmental stages. We concluded that SSEA-1 is not a specific marker for PGCs. Therefore we decided to characterize the position of PGCs, in different developmental stages, using VASA antibody. We identified the presence of PGCs in one structure that was not related with their migration in chicken embryos, or even in other animal model: the amnion/chorion. Taking advantage of the easiness of chicken embryos manipulation, we preformed a functional study to understand if amnion/chorion is having a role on PGCs migration. Our experiments demonstrated that embryos cultured with a modified "cornish pasty" method have a normal development, regarding the body structures and also the extra and intraembryonic vascular system. However the development of somatopleure is affected, and there is no separation between chorion and amnion or development of the amnion/chorion fold. The absence of PGCs in the genital ridges at stage HH17 and their presence in the somatopleure, allowed us to conclude that the developing amnion/chorion is involved in the migration process of PGCs.

Keywords: Primordial Germ Cells, chicken embryo, amnion, chorion, migration, VASA, SSEA-1, somatopleure, splanchnopleure.

Resumo

A formação de órgãos durante o desenvolvimento embrionário baseia-se na cooperação de diferentes tipos celulares. Algumas células são definidas a alguma distância do local onde o futuro órgão se formará. A migração celular é um fenómeno central em vários momentos do desenvolvimento embrionário permitindo que estas células se desloquem até à estrutura onde virão, mais tarde, a desempenhar o seu papel biológico correctamente.

As células germinais primordiais (CGPs) são um dos primeiros tipos celulares a distinguirem-se no embrião. As CGPs constitutem as progenitoras dos gâmetas desempenhando, por isso, uma função fundamental nos organismos sexuados. Depois de especificadas as CGPs são mantidas longe da gónada, até ao momento em que os movimentos celulares embrionários estabilizam, o que permite que mantenham a sua identidade. Mais tarde, as células iniciam a sua migração em direcção às gónadas, onde encontrarão as células somáticas ali presentes que lhes servirão de suporte nutritivo, o que permitirá seu correcto desenvolvimento. Ao atingirem a gónada esta fica apta a produzir gâmetas femininos ou masculinos, processo que se completará na puberdade. Os gâmetas são responsáveis por uma parte da variabilidade dos organismos sexuados, sendo por isso células com um papel muito importante tanto ao nível biológico individual como ao nível evolutivo. No caso da ave, a migração das CGPs ocorre da parte anterior extraembrionária para uma parte posterior do embrião, as cristas genitais, local onde se formará a futura gónada. Inicialmente, no estádio X (Eyal-Giladi and Kochav), as CGPs encontram-se no epiblasto, mais precisamente no centro da zona pelúcida. As células são deslocadas até ao hipoblasto. Durante a formação da linha primitiva as CGPs são transferidas para uma região extraembrionária, anterior à linha primitiva em desenvolvimento, denominada crescente germinal. É na zona do crescente germinal que estas células se incorporam na rede vascular aí formada, durante os estádios HH8-10, migrando pelo sistema vascular do embrião, até atingirem as futuras gónadas entre os estádios HH15-19. No entanto alguns destes estudos foram realizados usando marcadores não específicos para esta linha celular, nomeadamente o stage specific embryonic antigen 1 (SSEA-1). Neste trabalho avaliámos a especificidade do marcador SSEA-1 nestas células. Usámos como marcador mais fidedigno o anticorpo contra VASA, proteína que sabemos estar envolvida na identificação destas células em diferentes grupos taxonómicos ao longo da evolução (incluíndo o grupo *Gallus gallus*). Ao longo nosso trabalho, o marcador SSEA-1 não se revelou específico desta linha celular, sobretudo em estádios mais precoces. Esta observação levou-nos à necessidade de caracterizar a posição destas células no embrião de galinha, em diferentes estruturas embrionárias ao longo de vários

estádios de desenvolvimento, a fim de perceber melhor o seu processo de migração.

Ao analisar imunohistoquimicamente embriões entre os estádios de desenvolvimento HH5-19, deparámos com a presença destas células, em estádios HH8-13, numa estrutura nunca antes referida no seu processo de migração: o âmnio/córion.

Para compreender se o âmnio e o córion têm um papel na migração das CGPs, cultivámos embriões de galinha pelo método de cultura "cornish pasty". Este método de cultura *in vitro,* permite o correcto desenvolvimento do embrião com excepção do desenvolvimento da somatopleura e da prega amniótica, que devido à ausência de pressão da cabeça sobre os tecidos adjacentes, não consegue elevar-se sobre o embrião. Desta forma, a somatopleura continua o seu desenvolvimento à volta da área extraembrionária. A análise imunohistoquímica destes embriões, permitiu concluir que as CGPs no estádio HH10 se encontram no local onde seriam encontradas num embrião com desenvolvimento *in ovo* (localizadas numa posição anterior à cabeça, e na qual, num embrião com desenvolvimento *in ovo* se elevaria a prega amniótica). No entanto em estádios intermédios (HH13) estas células encontram-se na somatopleura. Verificámos também que a presença destas células nesta estrutura se mantêm em estádios mais avançados (HH17), nos quais é de esperar a presença das CGPs nas futuras gónadas. A partir destas observações concluímos que estas células estão a usar esta estrutura numa parte inicial da sua migração.

Ao longo deste trabalho deparámo-nos com lacunas na literatura à cerca do âmnio que, desta forma, encontrámos algumas lacunas na literatura relativamente aos mecanismos inerentes ao seu desenvolvimento. Assim, usando técnicas histológicas tradicionais aliadas a técnicas de live imaging, procedemos à análise do desenvolvimento desta estrutura que pensamos ter um papel importante na migração das CGPs. Concluímos dessa forma que esta estrutura extraembrionária parece ter um desenvolvimento dependente da formação da cabeça embrionária e do proamnio. O proamnion, estrutura diblástica anterior à cabeça, estará a servir de "clip", possibilitanto que a cabeça "mergulhe" sobre os tecidos subjacentes à mesma, ajudando ao correcto desenvolvimento da prega amniótica mediana pre-axial sobre a cabeça.

A integração dos dados obtidos neste trabalho, levou-nos a propor um novo mecanismo de migração para estas células com uma fase transitória de migração através do âmnio/córion. As CGPs, inicialmente presentes no epiblasto e mais tarde deslocadas para o hipoblasto, são depois incorporadas na mesoderme. A mesoderme, que ingressa pela linha primitiva, dará origem à espancnopleura e somatopleura. Estas duas estruturas estão envolvidas na formação do sistema vascular extraembrionário e do âmnio/córion, respectivamente. Assim o nosso

modelo considera que as CGPs, presentes na mesoderme, durante a formação da somatopleura e da esplancnopleura, são incorporadas na somatopleura. O nosso modelo não exclui o facto de que algumas destas células poderão ficar na mesoderme que, em conjunto com a endoderme, formará a esplancnopleura e que por essa razão tenham sido identificadas nas ilhas sanguíneas, em estádios precoces do desenvolvimento (HH1O). O facto de as CGPs usarem o âmnio/córion como estrutura transitória na sua migração poderá estar relacionado com o facto de as CGPs, apesar de mais tarde usarem o sistema vascular para se deslocarem até à futura gónada, estarem a evitar os processos envolvidos na vasculogénese e angiogénese que estão a ocorrer ao nível das ilhas sanguíneas. Durante o processo de vasculogénese e angiogénese são recrutados inúmeros sinais químicos e parácrinos que poderiam conduzir a uma alteração da linhagem das CGPs.

O âmnio e o córion foram uma das novidades evolutivas mais importantes no decurso da evolução, permitindo a independência de ambientes aquáticos para a reprodução. Permitiram a exploração de uma enorme variabilidade de nichos terrestres. No entanto, parece-nos que além da sua função como protector da dissecação em amniotas, esta estrutura poderá estar a ser usada em outros processos embrionários.

Este trabalho veio assim redefinir o papel do âmnio/córion na migração das CGPs. Esta estrutura extra-embrionária, constitui um suporte com características que permitem a manutenção da estaminidade celular, como tem sido descrito recentemente. Este trabalho demonstra assim a necessidade de um estudo mais aprofundado deste anexo embrionário que poderá ter mais aplicações, na área das células estaminais, do que inicialmente se esperava, sendo o facto de as CGPs estarem a usá-lo no seu processo de migração um indicador revelador dessa capacidade de manutenção da estaminidade celular.

Palavras-chave: Células Primordias Germinais, embrião de galinha, âmnio, córion, somatopleura, esplancnopleura, migração celular, VASA, SSEA-1.

Table of contents

I. Introduction

I.1. Primordial Germ Cells

When August Weismann developed the germ-plasm theory of heredity, he was far from knowing how important it was for the understanding of many different biological processes that are inherent to this commitment. Early in development a **commitment**, in cells from embryos of all sexually reproducing animals, happens between two type of cells: the cells that constitute the **germ line** (**primordial germ cells** (PGCs)), that are segregated from the **somatic cell lineages** committed to become tissues, organs and systems of the adult. PGCs are the precursor cells of the **eggs** and **sperm**, carrying on them the genetic information throughout generations. PGCs are therefore the engine of evolution, contributing for genetic variability in sexually reproducing animals *(1)*.

Besides the importance of **PGCs** in evolution, the mechanisms governing PGC specification are not conserved. Two general mechanisms were defined for PGCs specification. **Preformation** describes a mechanism in which PGCs are specified cell-autonomously by molecules inherited from the egg, known as germ plasm. And **epigenesis**, a mechanism which involves extracellular signals that trigger PGC specification from pluripotent precursors. Both mechanisms, preformation and epigenesist, contribute to define the germ plasm, which gives a greater evolvability to the species that contain it, allowing the soma to evolve more rapidly due to weak linkage with germ line development *(2)*.

There are some characteristics and mechanisms inherent to the developmental process of PGCs that are maintained in species where the formation of these cells is regulated from both, epigenesis and preformation. Germ cells are formed **early in development**, they have distinct **morphology** from somatic cells and they **migrate** actively from their extraembryonic place of origin to meet with the somatic gonad. At their destination, the future gonad, the somatic cells are responsible for maintenance and nourishment of PGCs through their developmental process *(3)*. Nonetheless many differences can be found between the migratory process used by PGCs at different animal model systems, that have been used to study the genetics of germ cell migration, each one with their own advantages *(1)*. In this work we focused on the migration of PGCs in ***Gallus gallus*** embryos.

Understanding the details surrounding the specification and migration of PGCs had occupied scientists for more than a century. The study of PGCs is not only important for the **scientific knowledge** but also for some direct **applications** in medicine, taking into account that aberrant migratory pathways could cause developmental defects including cancer and infertility *(4)*.

I.1.1. On the origin and development of PGC in chicken embryos

In contrast to other organisms, such as *Drosophila melanogaster*, *Xenopus laevis* or *Mus musculus*, the developmental dynamics of early segregation of the germline in birds is largely unclear. It is known that the precursors of PGCs are localized in the central zone of the **zona pellucida**, on the ventral surface of **epiblast**, at stage X (the roman numerals refer to the staging system used in *(5)*). Around stage XIV, the PGCs, are gradually translocated from the epiblast to an extra-embryonic structure, the **hypoblast**. Thereafter PGCs are carried anteriorly by the hypoblast to an extraembryonic region located anterior to the developing embryo, the **germinal crescent**, away from the primitive streak that starts to move forward from the posterior area of the **blastoderm** *(6)*. The germinal crescent became localized in the border region of the **zona pellucida** and **zona opaca**, anterior to the developing embryo *(7)*.

Previous studies, using periodic acid-Schiff staining (PAS), described these subsequent active displacements of PGC into four phases. First, PGCs are segregated from the endodermal layer and are accumulated in the **mesoderm** localized between the **ectoderm** and **endoderm** (Fig. 1b) during stages HH4 to HH8 (this staging system was developed by *(8)* and it is based in different morphological landmarks). Subsequently the PGCs, in a second phase of their migratory process, start ingressing in the **vascular system** and this process occurs at the same time when the formation of the **blood islands** starts, around stage HH10 (Fig. 1c). In the third phase of this process, around stage HH12, PGCs start to appear in the **extra-embryonic blood vessels** (Fig. 1d). In the last phase the PGCs start leaving the visceral branches of the aorta and begin to penetrate the **gonadal epithelium** two day and a half after the beginning of incubation, around stage HH15 (Fig. 1e). At stage HH 17 the majority of the PGCs have settled in the gonadal epithelium *(9)* (Fig. 1f).

Dubois et al. *(10)* gave some highlights about a possible **chemotactic** attraction exerted by the gonadal epithelium in the absence of a vascular system: the PGCs *in vitro* were capable of leaving the germinal crescent from one embryo cultured in the same petri dish with the gonads of other embryos, colonizing the gonadal epithelium without a vascular system. Therefore they demonstrated that if in one hand the vessels are allowing the PGC's migration, on the other hand the chemotatic signals are enough to allow PGCs to reach the genital ridges, demonstrating that the **vascular system is not necessary** for their correct migration process. Moreover is not proved when and how the PGCs, at stages HH8-10, are able to leave the germinal crescent to ingress into the developing **blood vessels**.

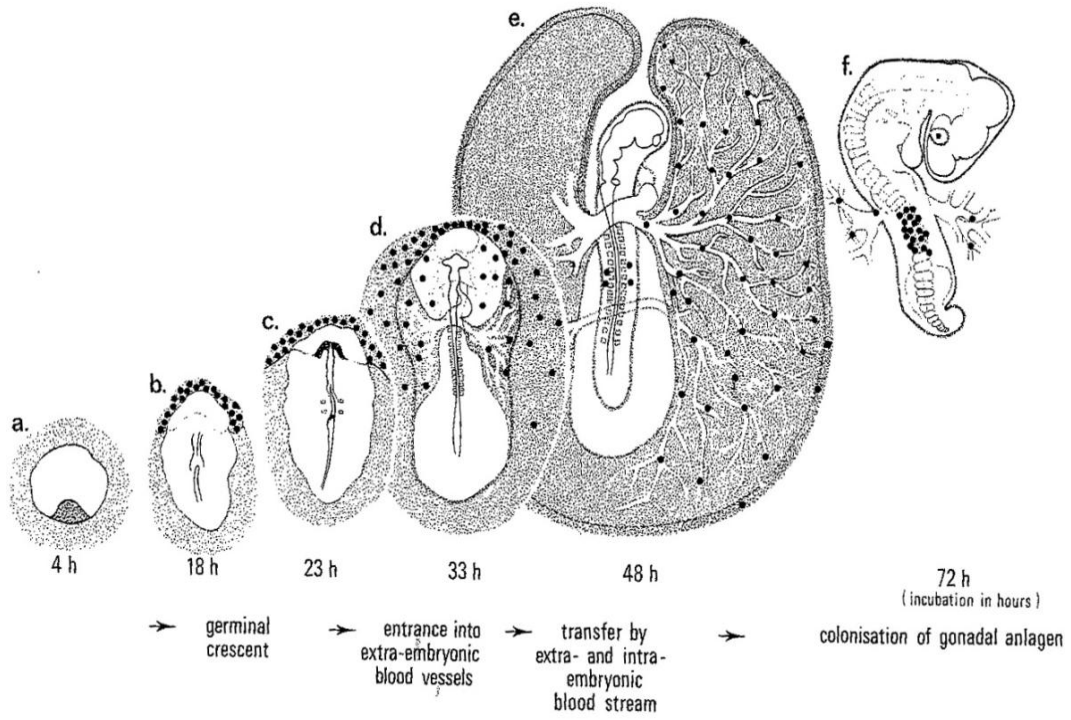


Figure 1 - Schematic representation of the PGC's migration on chick embryo along different developmental stages *(adapted from P. D. Niewkoop et al., 1979) (66)*. a) PGCs were not identifiable prior to primitive streak formation using PAS and ultrastructural criteria, b) and c) demonstrate an accumulation of PGCs in the germinal crescent anterior to the head-process and head-fold stages. d) There is an active penetration of the PGCs into the developing blood islands, around stage HH10, and beginning of their transfer by blood vascular system. d) PGCs are circulating through the entire extraembryonic vascular system. e) Colonization of the genital ridge by actively migrating PGCs starts around stage HH15. e) 72 hours after the beginning of incubation, the majority of the cells are already settled in the genital ridges.

I.1.2. Markers for PGCs in chicken embryos

In birds many approaches have been used to investigate the origin and development of the germline (*(11),(12),(13),(14),(15),(16),(17)*). PGCs in early developing embryos, stages X-HH5 have been distinguished by their **morphological characteristics**, classical stainings and immunocytochemical stainings for the stage specific embryonic antigen 1 (SSEA-1) and for the epithelial membrane antigen 1 (EMA-1) *(18)*. The **alkaline phosphatase** reaction has long been used to identify PGCs in mammalian species. Due to a generalized reaction in many tissues of the chicken embryo, alkaline phosphatase cannot be use as a marker for PGCs in *Gallus gallus* before stage HH10. Moreover alkaline phosphatase staining have shown that the specificity of this enzyme for PGCs in older embryos, stage HH15-17, is different between PGCs migrating actively in the

dorsal mesentery and PGCs passively migrating through the blood vessels, demonstrating that is not specific for labeling of all the PGCs *(19)*. Therefore the most common classical stain used for differentiating PGC from somatic cells is **periodic acid-Schiff** (PAS) *(20)*. PAS is a generally accepted method to identify PGC. PGCs have high glycoprotein content, when compared with surrounding somatic cells, which make germ cells PAS positive and somatic cells PAS negative *(9)*.

There are also immunological markers against cell-surface glycoproteins that are found in PGC but not in somatic cells. **SSEA-1** is likely one of the best-characterized antigens of PGCs in mammals. Anti-SSEA-1 was directed against a carbohydrate epitope with a galactose (β1-4) N-acetylglucosamine (α1-3) fucose linkage similar to the Lewis × antigen *(21)*. Undifferentiated multipotential mouse cells, chicken embryonic stem cells, and murine embryonic germ cells express the SSEA-1 epitope (*(22)*; *(23)*) suggesting that the antigen is not germline-specific. Moreover, upon differentiation, these cells are no longer SSEA-1 positive. Nevertheless previous studies defined SSEA-1 as an appropriate marker for germ cells in chicken embryos older than stage HH10 because the only SSEA-1–reactive cells in avian embryos beyond stage HH10 are PGCs (*(24)*; *(25)*).

The *vasa* gene in *Drosophila melanogaster* is one of the genes involved in maternal-effect mutations that are responsible for defect in the formation of germline precursor cells *(26)*. The **VASA** protein, coded by this gene, is specifically localized in the pole granules, *Drosophila*'s germ plasm, and the zygotic *vasa* expression is initiated in pole cells soon after fertilization *(27)* and during subsequent stages of development (*(28)*; *(29)*; *(30)*). The molecular function of proteins encoded by *Drosophila*'s *vasa* and its homologs is not fully understood. In *Drosophila* a possible function of the VASA protein is to bind target mRNAs involved in **germline determination** controlling the onset of the translation *(31)*. In *Caenorhabditis elegans* the injection of antisense RNA for *vasa* homolog (*Glh*) demonstrated that this gene plays an essential role for germline segregation *(32)*. Likewise in *Xenopus*, microinjection of antibodies against *Xenopus* VASA homolog protein (XVLG) into blastomeres at the 32-cell stage caused a reduction in the number of PGCs in the tadpole stage *(33)*. These previous findings indicate that even if the function of *vasa* family genes is not well understood it is indispensable for germ cell development and for that reason is **conserved** throughout evolution of animal species.

Tsunekawa et al. *(34)* isolated the chicken *vasa* homolog (*Cvh*) gene and have shown its germline-specific expression. Immunohistochemical analyses, using specific antibodies against CVH protein, demonstrated that CVH-expressing cells were detectable during early embryogenesis, starting from the first cleavage of fertilized eggs, and being expressed throughout all stages of development.

I.2. Blood vessels development in chicken embryos

The **first functional organ system** developing in vertebrate embryos is the cardiovascular system. The first step of blood vessel formation is the differentiation of vascular endothelial cells, which later cover the entire inner surface of all blood vessels. The mesodermal layer residing on the endoderm, known as splanchnopleuric mesoderm, is the only extraembryonic tissue capable of blood islands formation *(35)*. The activation of **FGF-receptor**, for signal transduction, leads to the activation of genes that are involved in the aggregation of mesodermal cells adjacent to the endoderm (splanchnopleura). The mesodermal cells from splanchnopleura start the differentiation of **angioblasts** in the **zona pellucida**, or angioblast and **hematopoietic** cells in the **zona opaca** and paraaortic clusters. The further growth and migration of endhotelial cells connect the blood islands and lead to the formation of a plexus of capillaries remodeled into a branched network *(36)*.

This **vascular network** expands from a thin band of endothelial and hematopoietic cells to cover the entire **yolk sac** forming the **capillary vascular plexus** (Fig. 2A, B) *(37)*. The **capillary vascular plexus** matures by a process termed angiogenesis, which involves capillary sprouting, splitting and remodeling leading to the reorganization of the primary vessels into large and small vessels (Fig. 2C, D). The **blood flow** has a role in the differentiation and patterning of endothelial cells. The expression and release of growth factors implicated in vascular remodeling, is altered by the blood flow and the hemodynamics is responsible for controlling the vessel **plasticity** *(38)*.

After the onset of cardiac activity, and during the subsequent stages, the **arterial network** expands and some small capillary side branches are selectively disconnected from the arterial network reconnecting again to the venous plexus. *(37)*. The relatively high pressure in the arteries repels the expanding disconnected segments, which avoid the arteries and can only reconnect to lower **pressure** veins. The hemodynamics plays a determinant role in the **remodeling** of the arterial growth and branching. The remodeling of the branches leads to different shapes that optimize the relation between regulation of peripheral oxygen diffusion and cardiac activity in the final **vascular structure** *(39)*.

The development of the vascular system starts early in development but is a very dynamic process. On embryos at stages HH8-13, are undergoing deep rearrangements and the vascular system is just defined around stage HH18 *(40)*.

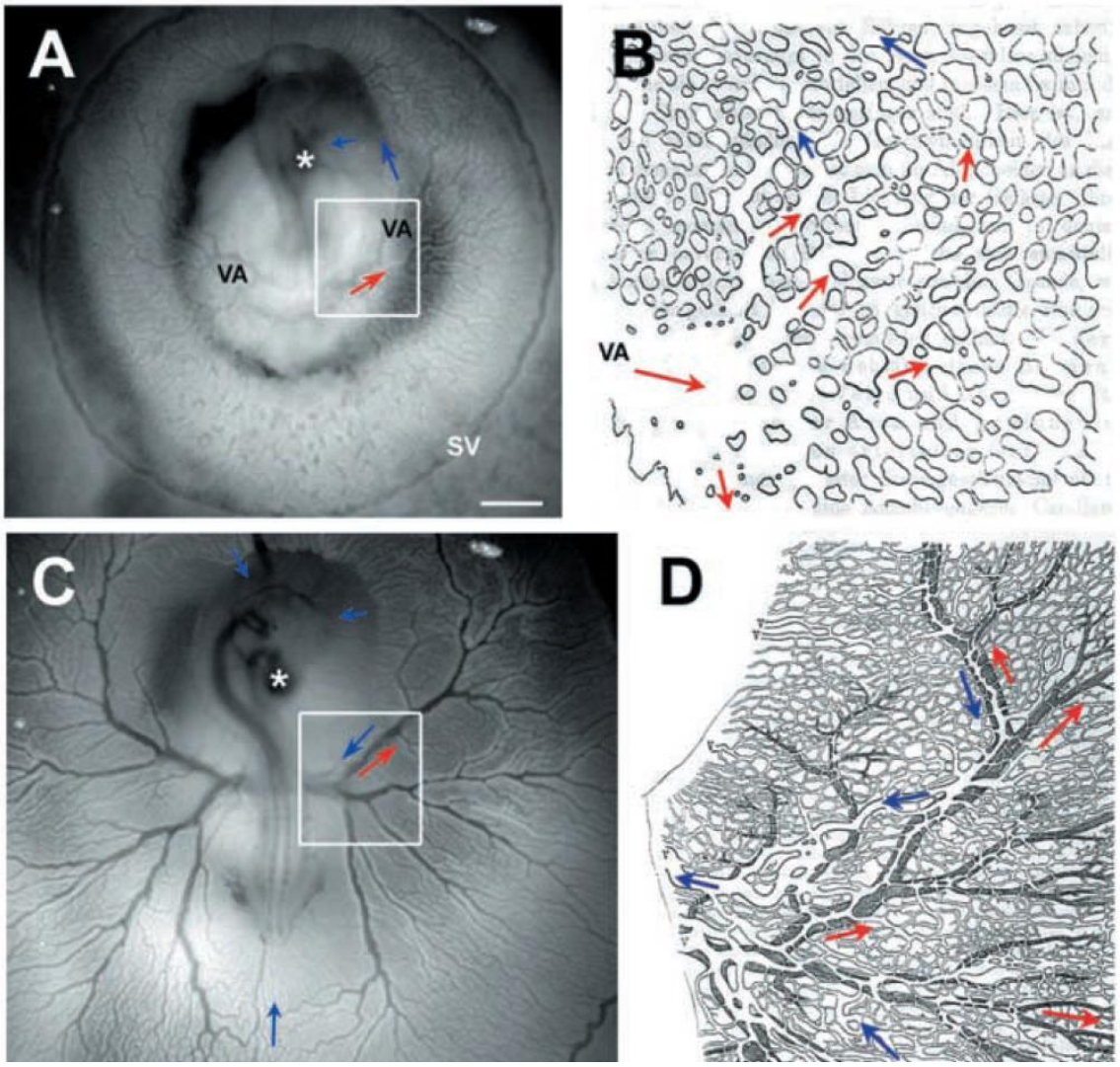


Figure 2- Overview of the extraembryonic blood system development in chicken embryos *(adapted from F. Le Noble et al., 2005)(39)*. **(A)** Extraembryonic vessels from the yolk sac vessels at stage HH13. The vitelline artery (VA) is just beginning to form. Red and blue arrows are showing the direction of arterial and venous blood flow respectively. The (*) is indicating the position of the heart. **(B)** Schematic representation of vascular system as seen in the boxed area in A. **(C)** Embryo at stage HH18 and the **(D)** schematic representation of the vascular system as seen in the boxed area in C. There was a rearrangement of the vascular system, and the veins have come to lie parallel to arteries, a venous network is covering the arterial network dorsally. Scale bar: 1100 mm.

I.3. The amnion development in chicken embryos

The **amniotic egg** had such profound effects on the evolutionary trajectory of the vertebrates that all groups after the moment of its appearance in evolution (reptiles, mammals and birds) adopted this developmental architecture. The major novelties of the amniotic egg were the four **extraembryonic membranes**.

The extraembryonic membranes allowed the embryo to carrier its own water and food supplies, necessary for the embryo development. This provided the independence to reproduction from the aquatic environment, allowing animals to explore the **terrestrial habitats** with different niches *(41)*.

In the amniotes, the embryo develops in a **blastodisc** over a large yolk mass. Initially there are no distinctions between embryonic and extraembryonic domains. As the body of the embryo takes shape, the combination of ectoderm and mesoderm (**somatopleure**) forms an inner layer, the **amnion**, and an outer layer, the **chorion**. The combination of endoderm and mesoderm (**splanchnopleure**) forms the **yolk sac** and **allantois**. Between the two layers, somatopleure and splanchnopleure, there is an cavity - the **extraembryonic coelom** *(41)*.

In chicken embryos the separation between amnion and chorion results from a **fold** of the somatopleure which begins in the head region and progresses posteriorly on either side of the embryonic axis (Fig.2A). The fusion of the folds over the dorsal side of the embryo begins anteriorly. **Fusion** is generally completed by 72 h of incubation, around stage HH18. As the fold progresses posteriorly, the ectodermal thickening of opposite sides fuse progressively, beginning in the center of the posterior edge of the head-fold and proceeding posteriorly. Presumably, the tension caused by this fusion is sufficient to lift the somatopleure up in the form of **lateral folds** (Fig.2B,C) around the embryo *(42)*. The **amniotic fluid** accumulates in the amniotic cavity, constituting an aqueous environment around the embryo, protecting it from desiccation. The amnion is neither innervated nor vascularized but possess a **smooth muscle** which acts as an autonomous biological stirrer, mechanically ensuring the relative homogeneity of the amniotic fluid. For animals that develop *in uterus*, it is thought that the movement of the mother provides enough mixing of the amniotic contents to make such smooth muscle unnecessary *(43)* .

On the other hand, the **chorion** allows the gas exchanges, also important for land-dwelling eggs. In birds and reptiles, this membrane adheres to the shell allowing the exchange of gases between the embryo and the environment. To nourish the developing embryo, a complex system of blood vessels develops from the splanchnopleure of the **yolk sac** and spreads into and out of the embryo. These vessels supply yolk and egg white materials to the embryo. The **allantois** also has an important role in the amniotic egg, and is an evagination of the primitive gut

(archenteron or gastrocoel) connected to the **extra-embryonic coelom** which stores the toxic by-products of the embryo *(44)*.

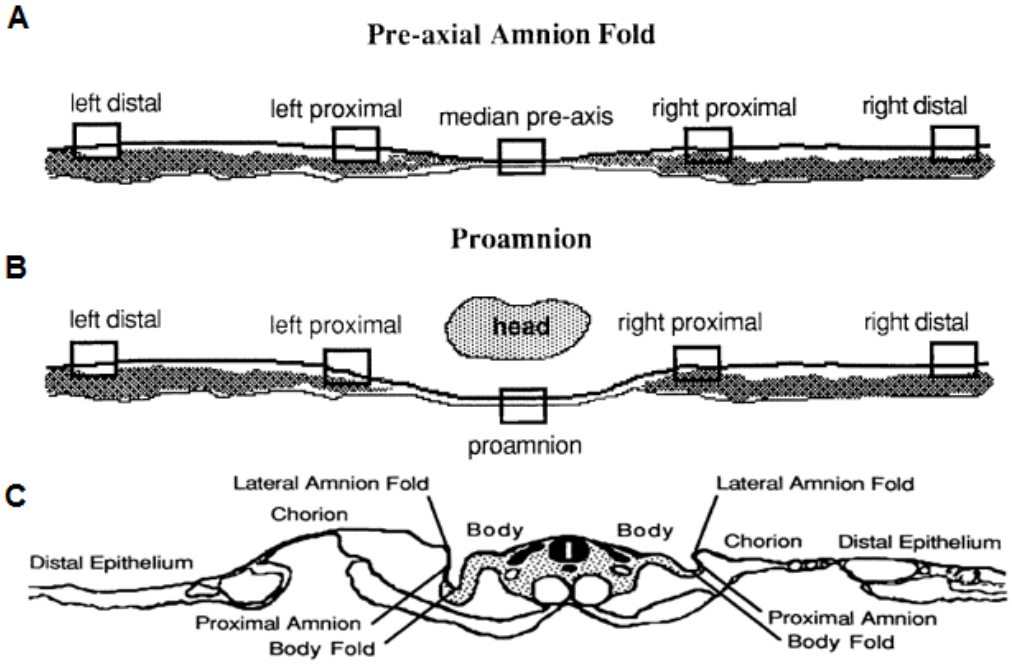


Figure 3- Overview of the amnion and chorion development in chicken embryos *(adapted from Miller et al., 1994)(67)*. **A)** The avian amnion developmental process starts with a median pre-axial amnion fold anterior to the head. **B)** The right and left proximal sides of the embryo elevate as two lateral amnion folds. **C)** Differentials in cellular proliferation within the somatopleure contribute to formation of lateral amnion folds and also for chorion development.

I.4. Live imaging techniques for the study of migratory cells and developing structures

The first report describing the use of live imaging microscopy was 50 years ago, when David Rogers made a movie in 16-mm of a neutrophil chasing a bacteria (David Rogers, Vanderbilt University, http://www.biochemweb.org/neutrophil.shtml). With new technical advances including sensor sensitivity, computing power, brighter and more-stable fluorescent proteins (FPs), and new **fluorescent probes** for cellular compartments *(45)* this approach has become commonly used in many fields of life and physical sciences, revealing aspects of **cellular dynamics** and function that were not accessible before, changing old dogmas and giving rise to new hypothesis and theories.

To perform such experiments it is crucial that cell viability is not altered ensuring that the physiological and biological processes that are under investigation are not perturbed in any way. Live tissues must be kept in an environment that does not induce stress responses which can alter the cellular processes of interest which easily could lead us to misleading conclusions. Some of the key factors to considerer are the type of **culture medium**, **temperature** and **pH**. Beyond biological parameters, bioimaging techniques introduce new variants that the researcher needs to be aware of. Most tissues and cells are never exposed to light or atmospheric **O_2 conditions** in their normal life, moreover we know that focused infrared (IR) light cause localized **heating**, ultraviolet light **damages DNA** and fluorescence excitation causes **phototoxicity** to tissues and cells. The oxygen-dependent reaction of free radical species, generated during the excitation of fluorescent proteins or dye molecules, is the main cause of phototoxicity in living cells. Thus, for live-cell imaging, it is best to use minimal amounts of excitation light by optimizing the efficiency of the light path through the microscope, and by using detectors that are optimized to detect most of the fluorescence emission *(46)*.

For this it is important to optimize each system independently, developing ways to minimize the introduction of variables and thus to obtained results the more faithful as possible to *in vivo*. This is a slow and hard process, which includes several control experiments and the development of new techniques that could improve the efficiency of our system. In this work an inverted culture system for live imaging of chicken embryos was developed.

II. Objectives

Gallus gallus is the animal with the longest and most continuous history as an experimental model for the understanding of multiple biological processes such asvertebrate limb development, somitogenesis and gastrulation. In the last 50 years it has contributed to some of the most important general concepts in vertebrate developmental biology.

The availability of fertilized eggs that can be easily maintained and incubated to any stage of interest, the easiness of experimental manipulation and the fact that chicken embryos mimic many biological processes in human, constitute chicken embryo as a powerful system for developmental biology. This system has now been complemented by new discoveries, related with the chicken genome, giving us new tools that could help to clarify our knowledge in many fields *(47)*.

Regarding the understanding of the emergence and fate of PGCs in chicken embryos, previous studies were developed in *in vitro* systems, or in fixed animals using cytological and enzymatic staining as markers and these approaches could be hiding the real dynamic of PGCs. Using reliable markers and live imaging techniques, the aim of this thesis was to characterize the migration of primordial germ cells during different developmental stages in chicken embryos, and understand how the developing amnion/chorion could be involved in PGCs migration process.

III. Material and methods

III.1. Embryos collection and manipulation

Fertilized chicken (*Gallus gallus*) eggs were incubated in a humidified atmosphere at 37.5°C until different HH stages *(8)* .

Embryos were washed and manipulated on a 2% agar-coated petri dish containing phosphate buffer solution (PBS) (see appendix I). The viteline membrane (the membrane that surrounds the embryo and the yolk) was removed and the embryos were isolated keeping the zona opaca and zona pellucida.

The fixation was performed overnight at 4˚C in a solution containing 4 % of paraformaldehyde in PBS (PFA) (see appendix I).

III.2. Classical histological stainings in paraffin sections

Hematoxyline and eosine staining was used in paraffin sections in order to understand the development of the amnion/chorion in chicken embryos.

III.2.1. Paraffin sections

For paraffin inclusion, embryos from each stage were dehydrated in a glass vial following a graded series of ethanol (70%, 80%, 90% and 100%) and cleared in xylene.

The embryos were individually placed in a plastic box containing liquid paraffin at 70˚C, which was replaced by new liquid paraffin after 30 minutes, and the process of embedding occurred overnight at 70˚C. The embryos were carefully oriented anterior-posteriorly in the same plastic box, and stored at 4˚C until further use.

Afterwards the embryos were sectioned, 5µm of thickness, transversally or sagittally using a Leica/Reichert 2055 Autocut Microtome. The sections were placed sequentially in glass slides (Superfrost Plus) containing a drop of milli-Q water which allowed their stretching over preheated plates at 50˚C. Thereafter the slides containing the sections were left to dry overnight at 37˚C and stored at 4ºC until further use.

III.2.2. Hematoxyline and Eosine (H&E) staining

The protocol followed for the H&E staining was Mayer's protocol (Lillie & Fullmer, 1976). The paraffin sections, previously placed in slides, were rehydratated starting with xylene followed by a decreasing series of ethanol (100%, 90%, 80%, 70%) and finishing with milli-Q water. The solution of hematoxylin for the counterstaining was previously prepared and reutilized from

the common stock (see appendix I). The staining was performed during 1 minute in a plastic box and the sections were then washed in flowing tap water during 10 minutes. Thereafter, for the cytoplasm staining eosin (see appendix I) was used during one minute.

The sections placed in the slides were washed in milli-Q water, dehydrated in a increasing series of ethanol and finally cleared with xylene. The samples were mounted in Entellan (Merck, 107961**)** and covered with a cover glass.

III.3. Antibodies and immunohistochemistry

The first antibodies used, in this work, were rabbit IgG anti-CVH *(34)* in a concentration 1:500 and mouse IgM anti-SSEA-1 (Santa Cruz, SC21702) in a concentration 1:10. The secondary antibodies were, respectively, Alexa Fluor 488 donkey anti-rabitt IgG (Invitrogen, A-21206) in a concentration 1:1000 and Alexa Fluor 568 goat anti-mouse IgM (Invitrogen, A-21043) in a concentration 1:500.

III.3.1 Whole mount Immunostaining:

For the whole amount immunochemistry embryos from the same stage were placed, using a glass Pasteur pipette, in a Falcon™ round-bottom tube and washed in PBS with 0.5% Triton (PBT) (see appendix I) rotating overnight at 4˚C.

After permeabilization, they were kept in the same vials and washed in PBS during 1 hour at room temperature (RT). Groups of four embryos each were placed in 1,5mL eppendorfs and incubated with the first antibodies.

The first antibodies were diluted with 1% of bovine serum albumin (BSA) (Invitrogen, 15260-037) in PBS (see appendix I), and the incubation occurred during 24 hours at 4˚C with rotation. The embryos were placed again in a Falcon™ round-bottom tube and washed in PBS during 1 hour with rotation at RT.

The incubation with the respectively secondary antibodies diluted in 1% BSA/PBS was also performed in 1,5 mL eppendorfs during 24 hours at 4˚C with rotation.

The embryos were washed in PBS and placed in a glass slide (Superfrost Plus) using a glass Pasteur pipette. The embryos were oriented, with the dorsal side of the embryo facing “upward”, the excess water was removed and they were mounted with VECTASHIELD mounting medium with Dapi (Vector, H-1200) and covered with a cover glass.

III.4 Image acquisition and analysis

Several optical systems were used for image acquisition.

For lower magnifications of whole amount immunostained embryos was used a Leica DFC90 digital camera (5 megapixel, 12bit) attached to a Stereoscope

MZ FIII equipped with epifluorescence optics and a Nikon E4500 Coolpix (4 megapixel, 12 bit) attached to a simple stereoscope Leica M420.

For higher magnifications of the embryos' sections and whole mount stainings, a Spot RT3 Slider Digital Camera from Diagnostic instruments (2 megapixel, 12bit) attached to a microscope Olympus AX20 equipped with epifluorescence and Nomarski optics was used.

For confocal images, a Leica TCS SP5 confocal microscope operating under the Leica Application Suite Advanced Fluorescence software was used. During the confocal image acquisition, the detection parameters were adjusted to avoid under- or overexposed pixels.

Leica Application Suite, Adobe Photoshop CS3 and Adobe Illustrator CS3 were used for image analysis and processing.

III.5 Statistical analysis

The positive VASA and SSEA-1 cells on embryos processed for whole mount immunochemistry were counted. The data was processed to calculate averages, percentages and medians using Microsoft Excel.

III.6 Chicken embryo culture and live-imaging

To understand the role of the amnion in PGCs migration and to study its development we used two different culture techniques.

III.6.1 *Ex ovo* culture I: modified "cornish pasty" culture

Preparation of the embryos for the culture was similar to what was described for the original "cornish pasty" culture for *ex ovo* culture (*(49)*;*(50)*). The name for this culture system was inspired by the british pasty. This culture system allows the growth of chicken embryos without the vitelline membrane in a fish embryo-like topology on top of a ''yolk sphere" resembling the shape of those traditional appetizers (Fig. 4).

Figure 4 – Cornish pasty. Chicken embryos cultured in the "cornish pasty" culture system resemble a british cornish pasty.

Embryos from the stages HH4/5 were removed from the egg, cleared of excessive yolk with PBS (Fig. 11A), and folded parallel to the primitive streak into a half moon. Forceps were used to gently press two sides together along the intended cut line (Fig. 11B).

A narrow margin of the area opaca was cut off with a pair of micro dissection scissors and the embryo was left to heal undisturbed for about one hour in Pannett-Compton solution at room temperature (Fig. 11C). A blunt end of a

glass Pasteur pipette was used to transfer the embryos to the culture media (2:1 ratio of thin albumen and the Pannett-Compton solution with Pen/Strep (Invitrogen, 15070-063) in a final concentration of 1:300).

The embryos were placed in a 94x16mm cell culture dish (Cellstar) containing 35mL of culture medium, and incubated at 37˚C with an atmosphere of 5% CO_2 during 24, 48 and 72 hours *(51)*.

III.6.2 *Ex ovo* culture II: Inverted chicken embryo culture system

Fertilized chicken eggs (*Gallus gallus*) were placed in an incubator at 38°C. Eggs were windowed and embryos staged according to Hamburger and Hamilton *(8)*. The stages used for our experiments were HH10-13.

The eggshells were opened and the content was deposited directly into a petri dish (Fig. 5A). The blastoderm was positioned uppermost and centered on the yolk. The thick albumen was removed and a piece of filter paper with a central aperture was placed onto the albumen at the edge of the blastoderm (Fig. 5B) and gently drawed away with the forceps and pulled from the yolk in an oblique direction.

The excess of yolk was washed with PBS and the embryo attached to the filter paper, and with its ventral side facing upward, was placed in a petri dish with a glass bottom (MatTek) and containing a drop of PBS (Fig. 5C/D). The borders of the cover of the petri dish were coated with humid paper and the box was sealed with parafilm to avoid evaporation. (Fig. 5E).

II.7. Image acquisition

With the ventral side of the embryo facing “upwards” embryos were time-lapsed using a fully automated Leica AF6000 LX microscope system equipped with an inverted DMI 6000B microscope, a DFC350 FX monochrome digital camera (1.4 Megapixel, 12 bit) and a climate chamber (Leica Microsystems).

The system was set to record an image each 8 minutes over the course of 24-48h. This interval between time-points was chosen because it is an interval that allows the capture of different biological movements, such as heart beating, increase in the number of somites, and amnion development.

To ensure a proper development of chicken embryos the climate chamber was kept at 5% CO_2 atmosphere at 37,5˚C. To avoid oscillations in temperature, the climate chamber was preheated 1 hour before the start of the experiment.

To maintain a humid environment we placed a 1L goblet filled with water inside the climate chamber.

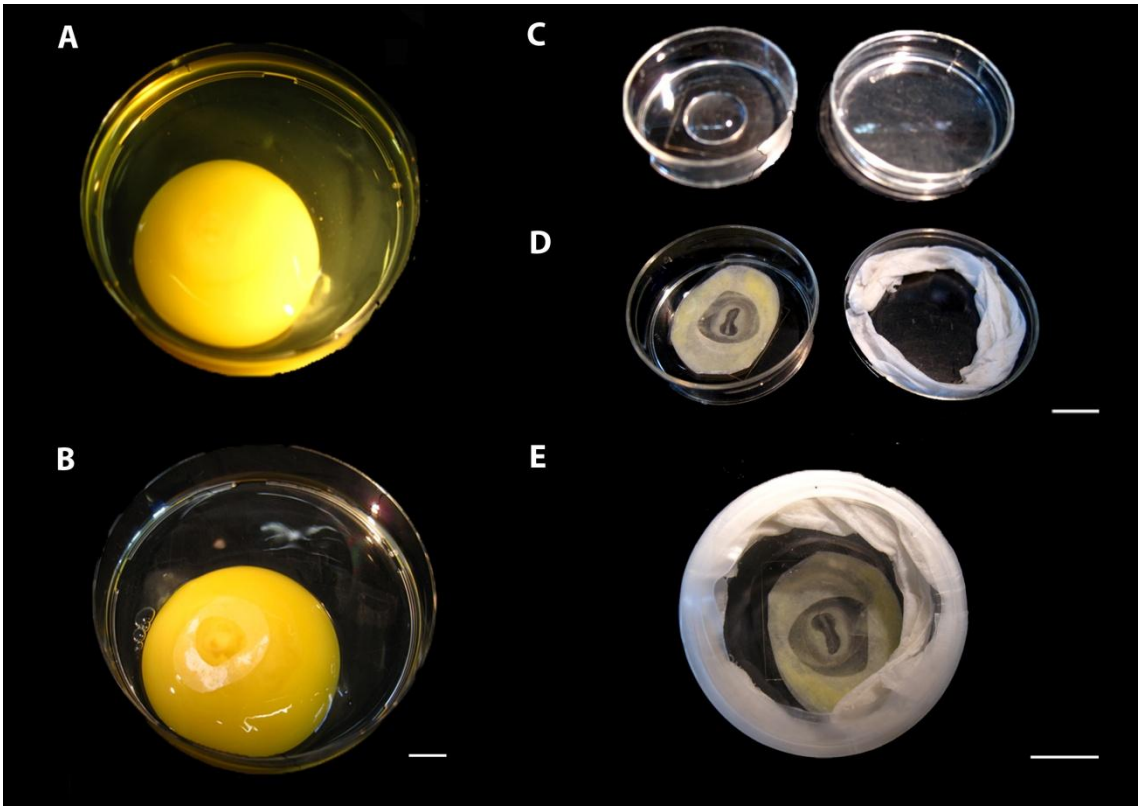


Figure 5 – Inverted cultured system for live imaging study of chicken embryos. A) The content of the egg was deposited into a petri dish and the blastoderm was positioned uppermost. **B)** A piece of filter paper, with a central aperture, was placed at the edge of the blastoderm. **C)** A drop of PBS was placed in a petri dish with a glass bottom, and the cover of the petri dish was coated with humidified paper. **D)** The petri dish containing the embryo was sealed with parafilm. Scale bar: 1 cm.

IV. Results

IV.1. The expression of SSEA-1 in PGCs present in defined embryonic structures is variable along different developmental stages of chicken embryos

To address the specificity of SSEA-1 as a marker for PGCs in *Gallus gallus* we preformed a whole mount immunostaining for VASA and SSEA-1 in chicken embryos from different developmental stages between HH5-19.

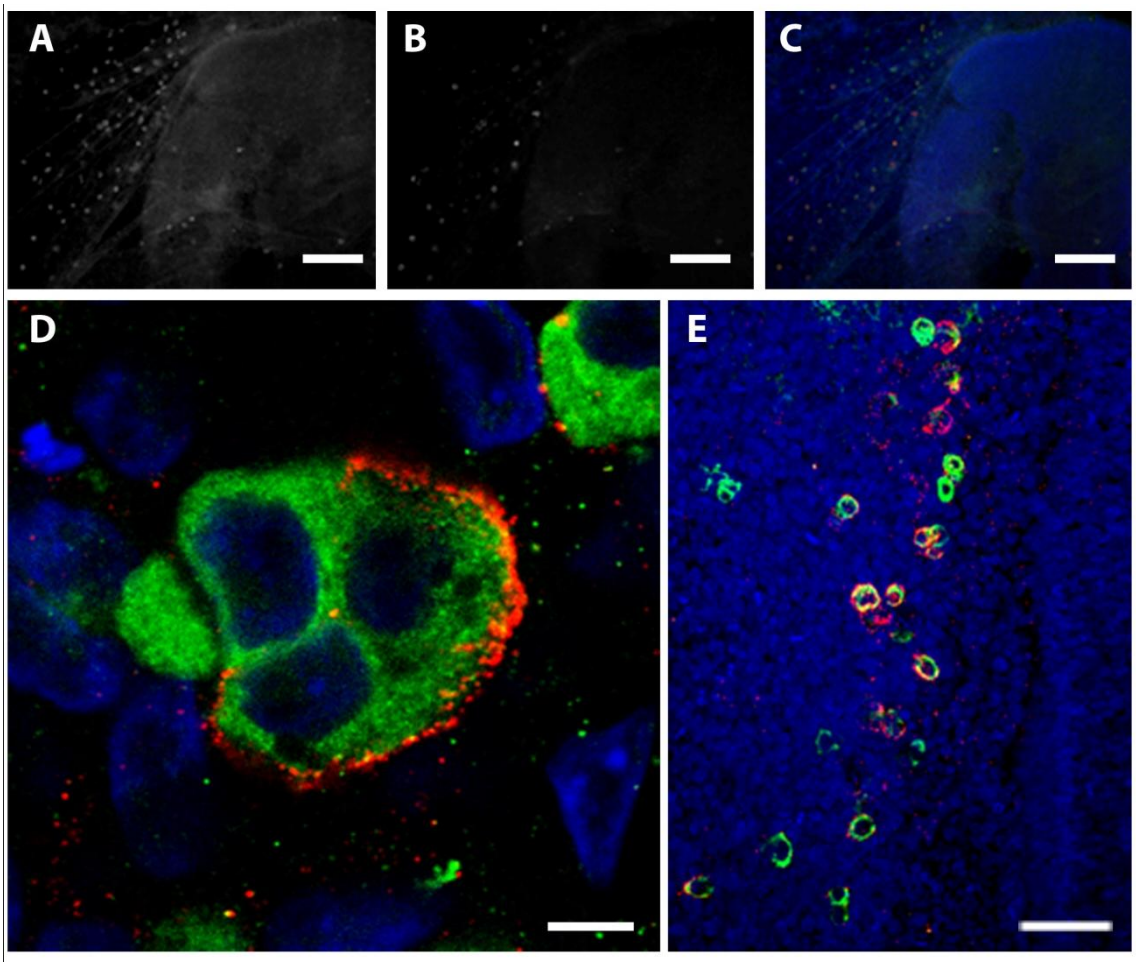
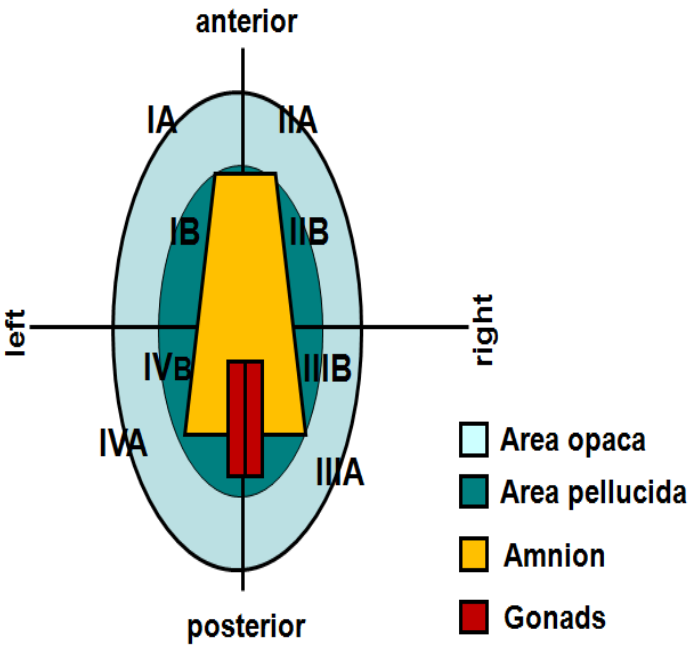


Figure 6- Expression of VASA and SSEA-1 in different embryonic structures along different developmental stages. A) VASA positive cells were found in the amnion fold of embryos from stage HH12. **B)** Expression of SSEA-1 in the amnion fold at stage HH12. **C)** Some of VASA positive cells in the amnion fold at stage HH12 are expressing SSEA-1. **D)** Cells from the same cluster, in the anterior part of zona pellucida, at stage HH10, have different expression of SSEA-1. VASA (green) is expressed in the cytoplasm while SSEA-1 (red) expression is restricted to the cell surface. **E)** At stage HH16 the majority of VASA positive cells present in the genital ridges are expressing SSEA-1. Scale bars: (A-C) 100µm, D) 5µm and E) 50 µm.

Analyzing chicken embryos at stages HH5-19, we verified that VASA is expressed in the cytoplasm of cells with a big and spherical nuclei, while SSEA-1 is visible in the cell's surface (Fig.6D).

The expression of SSEA-1 seems variable during different developmental stages and also between embryonic structures. The expression of SSEA-1 in different structures, such as the developing amnion at stages HH12 (Fig.6C), and the gonads at stage HH16 (Fig.6E) is different from the expression of VASA. Moreover, in some clusters we found variable expression of SSEA-1 in VASA positive cells present in the same cluster (Fig.6D).

To analyze the expression pattern of SSEA-1 we defined a regional map for the chicken embryo. In our map we included different embryonic structures, and their relative position in the embryo: zona opaca, zona pellucida, amnion and genital ridges (gonads). In order to understand more precisely the SSEA-1 expression in each structure, we built a dynamic map adapted for each stage. The embryonic structures are developing through the analyzed stages, from HH5 and HH19. For a better positional understanding of these cells we identified the four quarters of zona opaca and zona pellucida with roman numbers (I, II, III, IV).



I and IV label the structure in the left and II and III to label the structure in the right, the roman numbers I and II are also identifying the anterior regions of zona opaca and zona pellucida, while III and IV are identifying the posterior regions of zona opaca and zona pellucida in the chicken embryo.

Figure 7 - Dynamic map for the analysis of PGCs position through chicken embryonic development. In this map are included four distinguishable embryonic structures: zona opaca, zona pellucida, amnion and gonads. The numbers are indicated the position of zona opaca (blue) and zona pellucida (green) in each quarter of the embryo (I-IV). The structure that we defined as "amnion" (yellow) includes the median pre-axial and the lateral amnion folds in stages between HH8-HH12 and the proper amnion from stages HH13-19. The structure gonads (red), genital ridges, is defined for embryos from the stage HH12.

The VASA and SSEA-1 positive cells that we found in the median pre-axial, the proximal lateral amnion folds from stages HH8-HH12 and the proper amnion from stages HH13-18 were included in the "amnion" of our dynamic map. The gonads, or genital ridges, were considered as a present structure on embryos at stages HH12-19 (Fig.7).

We counted the VASA and SSEA-1 positive cells in the previously defined regions in each embryo. We compared the number of VASA positive cells (as the most reliable marker for PGCs) and SSEA-1 positive cells. The average of the percentage of SSEA-1 positive cells is variable during the development of chicken embryos, and also between the analyzed embryonic structures (see table in appendix 2). No correlation was found between the specificity of the expression of SSEA-1, embryonic structure or developmental stage. The higher values of SSEA-1 specificity for PGCs were found in the PGCs already settled in the genital ridges at stages HH14-19. SSEA-1 is not a reliable marker for PGCs on chicken embryos at stages HH5-19.

IV.2. The analysis of VASA expression demonstrate that PGCs migrate from the anterior region of zona opaca at stage HH8 to the amnion at stage HH8-14 and start reaching the gonads at stage HH15

To understand the migratory process of PGCs in chicken embryos we used the VASA antibody to label the cells on chicken embryos at stages HH5-19. The percentage of embryos without PGCs is shown in Figure 8A. At stages HH10, HH12 and HH16, we found 25%, 50% and 40% of embryos without any VASA positive cells (Fig. 8 A).

The total number of VASA positive cells was counted for each embryo. The average of VASA positive cells per embryo, during stages HH5-19, is shown in Figure 8B. We observed that the average of PGCs at stage HH5 is 372 and at stage HH19 is 289. The average of PGCs in the intermediate stages is variable reaching its maximum at stage HH9 (580 VASA positive cells) and its minimum at stage HH14 (220 VASA positive cells) (Fig. 8 C).

The dynamic map previously defined, was also used in the analysis of the position of PGCs during the embryonic development. In order to understand the relative anterior-posterior position of PGCs in the chicken embryos we analyzed the anterior region of zona opaca and zona pellucida (IA+IIA and IB+IIB), their respectively posterior region (IIIA+IVA and IIIB+IVB), the amnion (including the median pre-axial and proximal lateral amnion folds) and the gonads. The median of VASA positive cells in each structure per stage was used to analyze the position of PGCs in each structure per stage and its represented in Figure 9 A,B.

A

Stage HH	Nº of embryos	Average of VASA +	Standard deviation
5	4	372	195
6	2	316	216
7	1	391	
8	3	328	51
9	3	481	471
10	2	290	95
11	3	462	300
12	7	340	137
13	2	332	199
14	5	220	74
15	1	500	
16	3	226	92
17	1	278	
18	2	235	8
19	1	289	

B

Stage HH	Nº of embryos with PGCs	Nº of embryos without PGCs	% Embryos without PGCs
5	4	0	0%
6	2	0	0%
7	2	1	50%
8	4	1	25%
9	3	0	0%
10	4	2	50%
11	4	1	50%
12	9	2	22%
13	5	3	60%
14	6	1	17%
15	2	1	50%
16	5	2	40%
17	1	0	0%
18	2	0	0%
19	1	0	0%

C

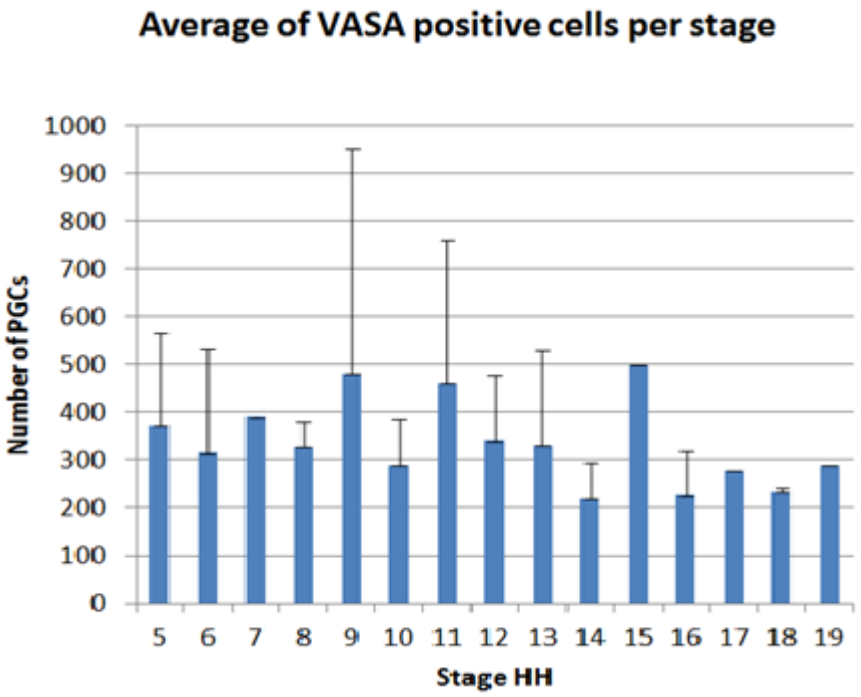


Figure 8 - Analysis of the average of VASA positive cells (PGCs) in stages HH5-19. A) Number of embryos analyzed with and without PGCs. **B)** Table with average of VASA positive cells (PGCs) in stages HH5-19. **C)** Dynamic of the average of VASA positive cells (PGCs) on embryos between stages HH5-19.

A

Stage HH	IA+IIA	IIIA+IVA	IB+IIB	IIIB+IVB	Amnion	Gonads
5	0	0	323	0	-	-
6	62	2,5	195	57	-	-
7	0	0	381	10	-	-
8	132	6	135	2	62	-
9	125	19	114	6	29	-
10	77	23	56	3	132	-
11	256	12	0	0	219	-
12	145	32	0	3	145	0
13	92	28	0	1	211	0
14	0	0	69	4	36	0
15	91	63	170	0	16	160
16	3	0	0	0	0	218
17	0	0	0	0	0	278
18	0	0	0	0	0	235
19	0	0	0	0	0	289

B

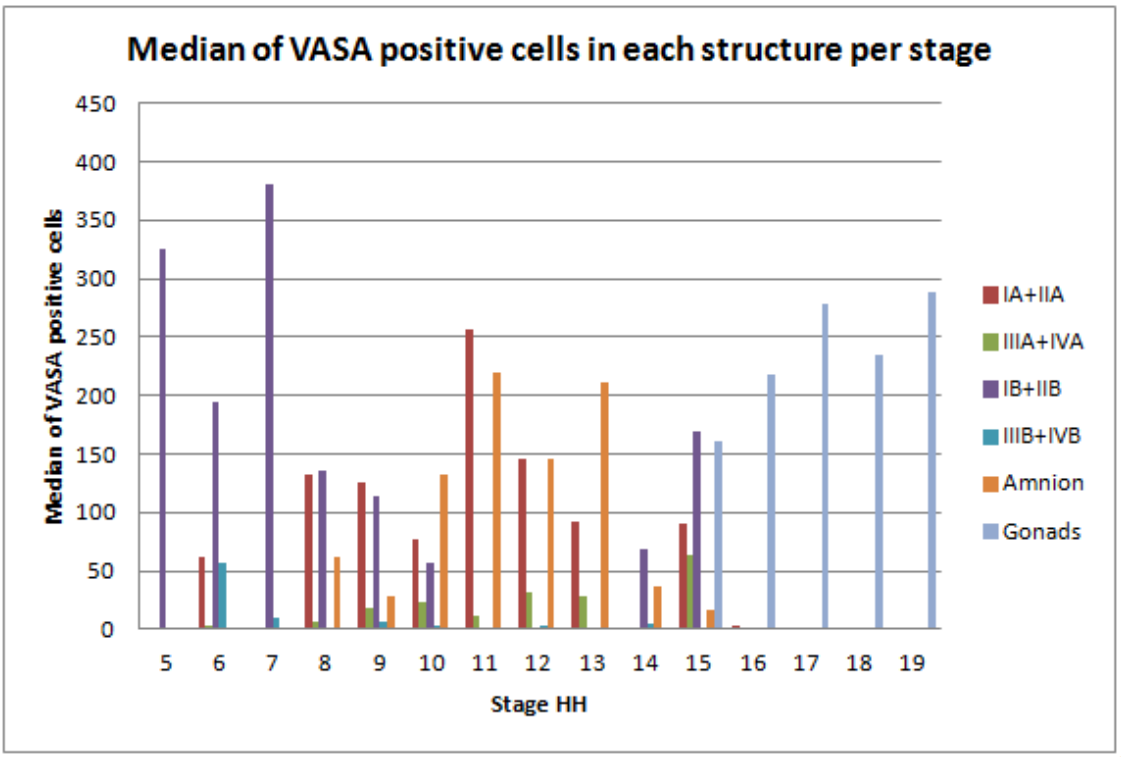


Figure 9 - Analysis of the position of VASA positive cells (PGCs) along different developmental stages between HH5-19. A) Table with median values of VASA positive cells (PGCs) present in each embryonic structure per stage. **B)** Dynamic of the position of VASA positive cells (PGCs) in each embryonic structure through developmental stages HH5-19.

Anti-VASA staining of stage HH5 embryos showed a median of 323 PGCs, in the zona pellucida. Around stages HH6 the majority of VASA positive cells are in the anterior region of the zona pellucida (median of 195 PGCs).

PGCs seem to be translocated from the anterior region of zona pellucida at stage HH5-7 to the anterior region of zona opaca at stage HH8.

Between stages HH8-10 the number of PGCs in the anterior region of zona opaca decreases from a median of 132 PGCs at stage HH8 to a median of 77 PGCs at stage HH10. At the same stages, HH8-10, the number of PGCs present in the median pre-axial amnion fold increases from a median of 62 PGCs at stage HH8 to a median of 132 PGCs at stage HH10.

PGCs seem to be translocated from the anterior region of zona opaca at stage HH8 to the median pre-axial fold at stage HH8-10.

The number of PGCs in the median pre-axial amnion fold reaches its maximum at stage HH11 (median of 219 PGCs). At stage HH13 the majority of PGCs (median of 211 PGCs) are in the amnion. The median number of PGCs in the anterior region of zona opaca decreases from stage HH11 to HH14. At stage HH14 the median of PGCs in the anterior region of zona opaca is 0.

PGCs seem to be translocated from the anterior region of zona opaca to the amnion at stages HH11-14.

Around stage HH15 the PGCs showed a widespread distribution pattern through the embryo. A median of 170 PGCs was found in the vessels localized in the posterior region of the zona pellucida. At stage HH15 a median of 160 PGCs were found in the genital ridges. PGCs seem to be translocated from the amnion at stage HH14 to the vessels in the zona pellucida at stage HH15. At stage HH15 PGCs are migrating through the vessels of zona pellucida and start reaching the gonads at stage HH15.

Between stages HH16-19 there is an increase of the number of PGCs in the genital ridges. The number of PGCs in the genital ridges increases from a median of 160 PGCs at stage HH16 to a median of 289 PGCs at stage HH19.

PGCs seem to be translocated from the vessels in the zona opaca at stage HH15 to the genital ridges at stages HH15-19.

IV.3. At stage HH13 the PGCs are present in the amnion/chorion

The whole mount immunostaining revealed the presence of PGCs in the amnion/chorion at stage HH13 (Fig. 10A-G). The amnion started its folding and at stage HH13 the median pre-axial fold and the two lateral amnion folds are not distinguishable. At stage HH13 the amnion is covering the anterior region of the head. In the present image (Fig. 10A) the amnion fold is bellow the embryo, showing that PGCs are in the amnion and not in the head of the embryo (Fig. 9D). Some VASA positive cells are also expressing SSEA-1 (Fig. 9F). There is a strong SSEA-1 positive border in the basis of the amnion fold. (Fig. 9G).

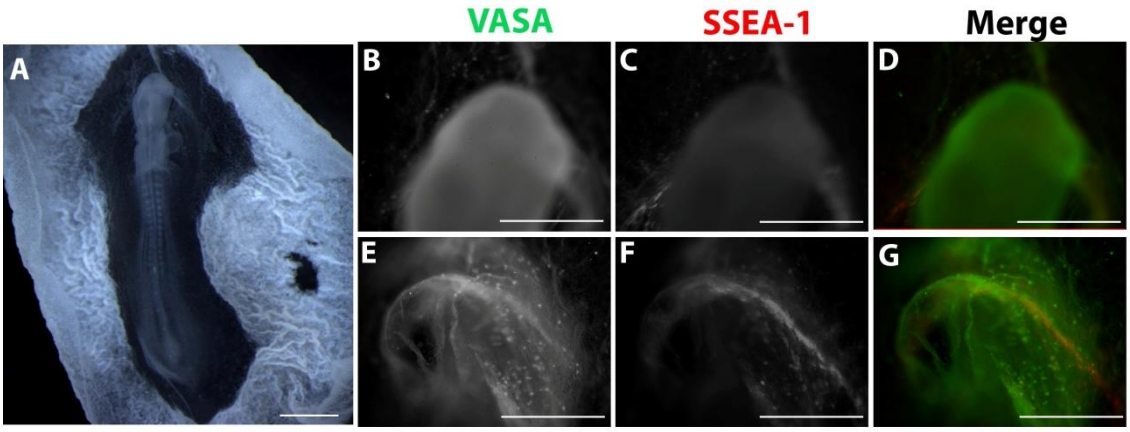


Figure 10- Expression of VASA and SSEA-1 in whole mount chicken embryos from stage HH13. A) Chicken embryo from stage HH13, the amnion is already covering the anterior region of the head. **B,C,D)** Expression pattern of VASA (B) and SSEA-1 (C) in the anterior dorsal side of the embryos, PGCs are localized in the amnion. **E,F,G)** Expression of VASA (E) and SSEA-1 (F) in the ventral side of the embryos. VASA positive cells are localized in the amnion fold and some are expressing SSEA-1. A SSEA-1 positive border is visible in the basis of the amnion fold. Scale bars: (A) 100μm (B-G) 50μm.

IV.4. Embryos cultured with the modified "cornish pasty" method have a normal development except for the somatopleure development

Embryos from stage HH10-17 cultured in the "cornish pasty" method (Fig.11 A) showed a fish embryo-like topology, with the embryo developing in the top of a "yolk sphere". The majority of operated embryos took the shape of an inflated balloon after 24 hours of culture, and was floating in the media.

Two in every ten cultured embryos were sunk to the bottom of the dish, due to a failure in proper heal after the initial cutting which resulted in their death, and were removed from the culture.

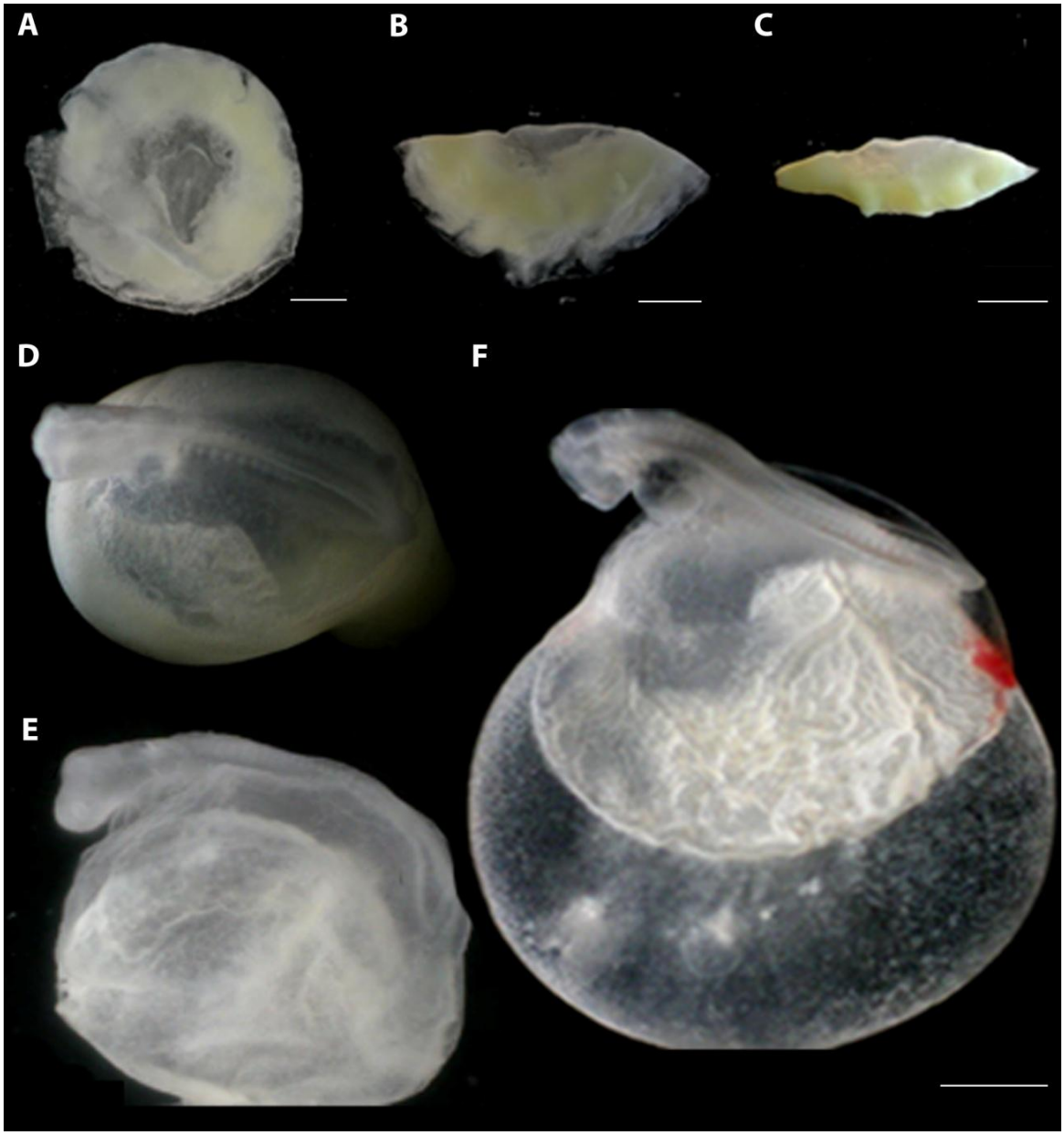


Figure 11 – Embryos cultured in the modified "cornish pasty" method. (A) Embryos from stages HH4/5 were removed from the egg and cleared of excessive yolk **(B)** and folded parallel to the primitive streak into a half moon. **(C)** A narrow margin of the area opaca was cut off and the embryo was left to heal undisturbed for about an hour in Pannet-Compton solution at room temperature. **D)** After 24 hours of culture embryos from HH10 were collected, showing typical characteristics of stage HH17 embryos growing *in ovo*. Embryos from stages HH13 **E)** and HH17 **F)** were collected after 36 hours and 48 hours of culture, respectively. The somatopleure in cultured embryos at stage HH13 and HH17 did not developed correctly and the amnion/chorion is not covering the embryos. Scale bar: 100 μm (A-C) and 250 μm (D-F).

After 24 hours in culture, embryos from stage HH10 were collected. The head was properly formed, with the five brain vesicles formed, and 10 somites were already defined. In the "yolk sphere" the vascular plexus in the zona opaca, composed by several blood islands, was visible (Fig. 11D).

After 36 hours we collected embryos from stage HH13. The head showed a normal development and the embryos had 17 somites. The heart was already beating and the circulation well-established in approximately two thirds of the embryos, which corresponds to stage HH13 *in ovo*. The amnion was not covering the head in embryos from stage HH13 cultured in the "cornish pasty" method as it happens in the embryos from stage HH13 growing *in ovo* (Fig. 11E).

After 48hours we collected embryos from stage HH17 showing a normal vascular system development, with a beating heart and a visible blood flow (movie 1 in the CD). The embryos developed normally above the "yolk sphere".

Cultured embryos at stage HH17 showed a normal growth with 29 somites defined, which correspond to stage HH17 *in ovo*. In embryos from stage HH17 growing *in ovo* the amnion/chorion is already covering the embryo. In cultured embryos at stages HH13-17 the somatopleura did not have split in amnion and chorion and had grown around the "yolk sphere".

The amnion/chorion did not develop correctly in cultured embryos at stage HH17 (Fig. 11F). The amnion and the chorion seem to fail their split in somatopleure, and they are not able to cover the embryo.

The growth rate in embryos, at the analyzed stages, cultured in the "cornish pasty" culture was the same to the one that occurs *in ovo*. The embryos showed a normal morphology but with some developmental defects in what concerns to somatopleure (Fig.11). Therefore embryos cultured in a modified "cornish pasty" method are a good system to study if the amnion/chorion has a role in PGCs migration.

IV.5. In cultured embryos from stage HH17 PGCS are in the ectopic somatopleure but not in the genital ridges

We analyzed the expression of VASA and SSEA-1 in embryos cultured with the modified "cornish pasty" method. The analysis of the expression pattern of VASA and SSEA-1 in cultured embryos with development defects in somatopleure, allowed us to address if the amnion and the chorion, derived from the somatopleure, are playing a role in the migration of PGCs

Cultured embryos from stages HH10, HH13 and HH17 were processed for whole mount immunostaining.

We analyzed the expression pattern of VASA and SSEA-1 in 3 embryos from stage HH10, and 5 embryos from stages HH13-17. The following results were concomitant between all the embryos analyzed per each stage.

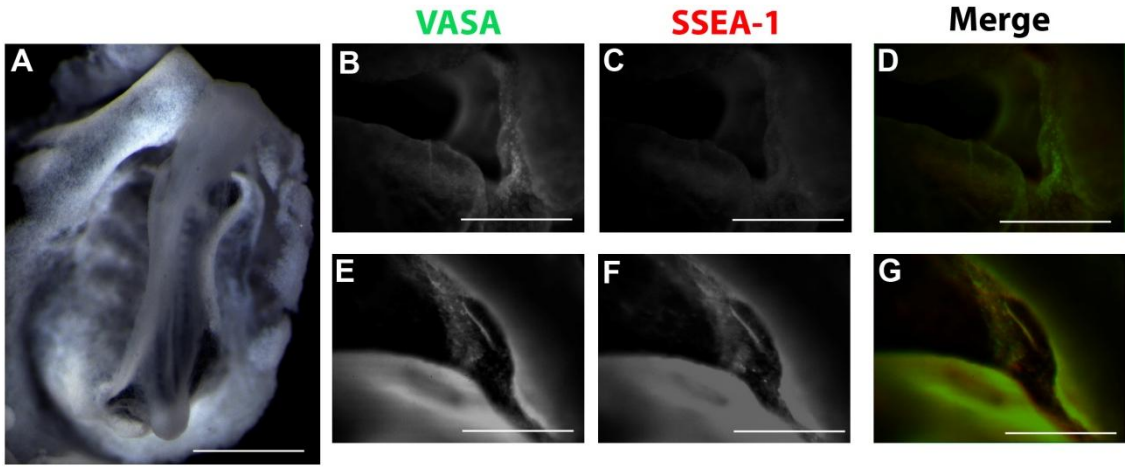


Figure 12 – Whole mount imunnostained chicken embryos, at stage HH10, cultured in the modified "cornish pasty" method. (A) Embryos cultured during 24hours shown a typical morphology of embryos growing *in ovo* from stage HH10. **B)** VASA positive cells are present in the ventral side of the median pre-axial fold of the amnion, no cells expressing SSEA-1 **(C)** were found. **E)** Dorsal view of the left zone adjacent to the head where VASA positive cells can be seen. Scale bars: (A) 100µm, (B-G) 50 µm.

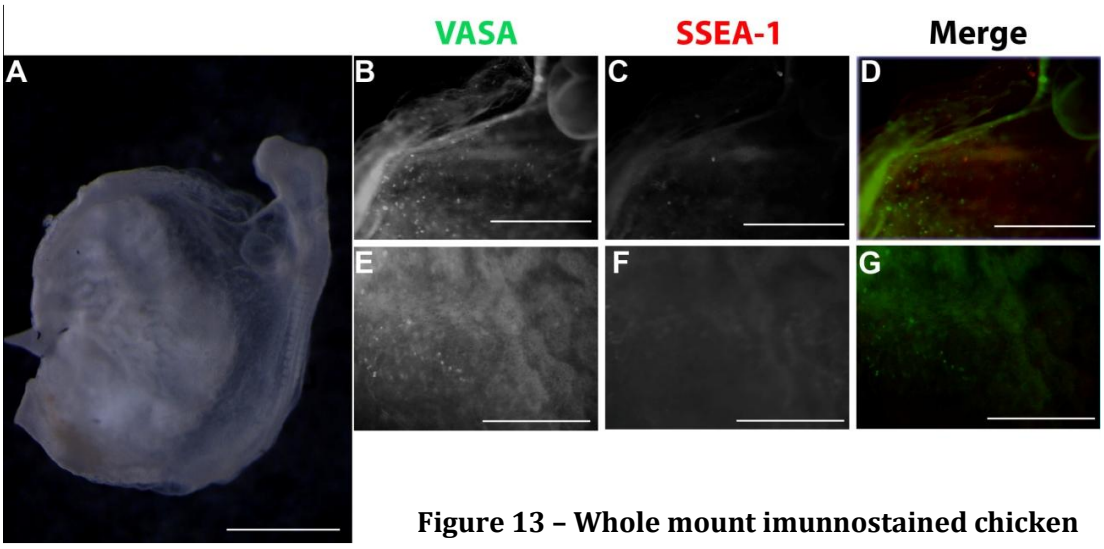


Figure 13 – Whole mount imunnostained chicken embryos, at stage HH13, cultured in the modified "cornish pasty" method. A) Embryos cultured during 36 hours shown a typical morphology of embryos growing *in ovo* from stage HH13. **B)** VASA positive cells are present in the somatopleure adjacent to the heart. **C)** Some VASA positive cells are expressing SSEA-1. **D)** No VASA or SSEA-1 positive cells were found in the vessels. Scale bars: (A) 100µm, (B-G) 50 µm.

The distribution pattern of PGCs in cultured embryos from stage HH10 (Fig. 12A) was similar with the pattern of PGCs previously described in the embryos from stage HH10 that had a normal development *in ovo*. PGCs are in the median pre-axial amnion fold, anterior to the head, and could be seen in the ventral side of the median pre-axial amnion fold (Fig. 12B) and in the lateral proximal left amnion fold (Fig. 12F). Some VASA positive cells are expressing SSEA-1 (Fig. 12C and F).

In cultured embryos from stage HH13 (Fig. 13A), PGCs are localized in the ectopic somatopleure, anterior to the head and above to the developing heart. Some PGCs are expressing SSEA-1 (Fig. 13B). No PGCs were found in the vessels of cultured embryos from stage HH13 (Fig. 13E).

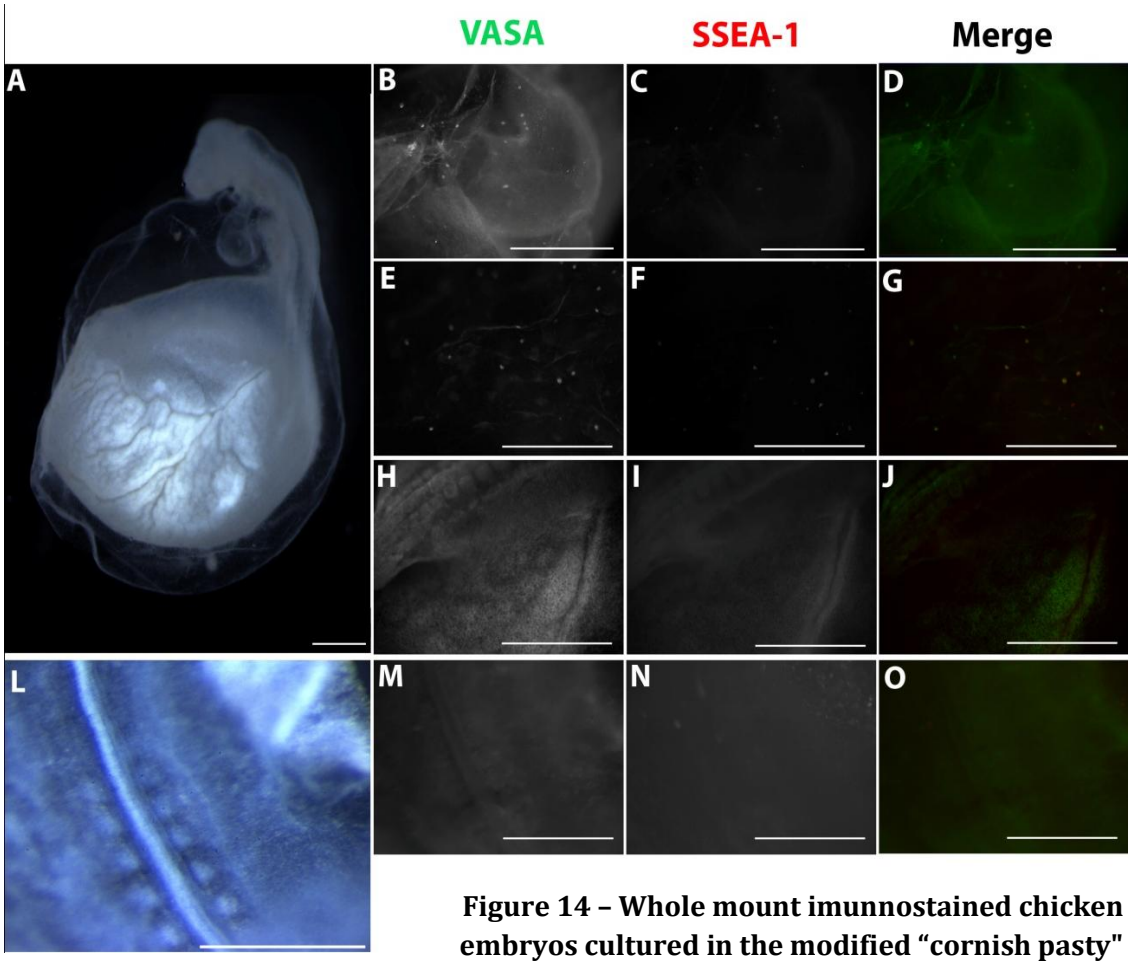


Figure 14 – Whole mount imunnostained chicken embryos cultured in the modified "cornish pasty" method at stage HH17. A) Embryos cultured during 48 hours showed a typical morphology of embryos growing *in ovo* from stage HH17. The heart was beating and the blood flow was present. **A)** VASA positive cells are present in the somatopleure adjacent to the heart **E)** and also distributed along the somatopleure involving the "yolk sphere". **C,F)** Some of VASA positive cells are also expressing SSEA-1. **H)** No VASA or **I)** SSEA-1 positive cells were found in the vessels. **L)** In the isolated genital ridges no positive **M)** VASA or **N)** SSEA-1 were found. Scale bars: (A) 100µm, (B-G) 50 µm.

In cultured embryos from stage HH17 (Fig. 14A), PGCs showed a wide distribution pattern in the ectopic somatopleure. PGCs are present in the somatopleure above the heart (Fig. 14B) and in the somatopleure above the vascular system (Fig. 14E). Some VASA positive cells in somatopleura have also shown expression of SSEA-1 (Fig. 14 C,F). No PGCs were found in the vessels from the vascular system that is connecting to the posterior region of the embryo (Fig. 14G).

To analyze the expression of VASA and SSEA-1 in the gonads, cultured embryos at stage HH17 were removed from the "yolk" sphere. We verified that there are no VASA or SSEA-1 positive cells in the genital ridges in cultured embryos at stage HH17 (Fig. 14O).

On embryos at stage HH17 cultured in the modified "cornish pasty" method, PGCs have not reached the gonads as it happens on embryos from stage HH17 developing *in ovo*.

IV.6. The anterior fold of the amnion in chicken embryos is formed by the fusion of the median pre-axial amnion fold with two lateral amnion folds

To understand the development of the amnion in chicken embryos we performed the classical H&E staining in sagittal and transverse paraffin sections of embryos at different developing stages. H&E is a general histological staining in which the nuclei acquire a purple color while the cytoplasm becomes red. Our analysis is focused on chicken embryos from stage HH 10-13.

We defined the structures present at each stage of development, considering their position and level of development on embryos from stages HH10-13. For a better understanding of the localization of each section, besides the indication of the section in the whole mount figure of the embryo from each stage, we are going to use the brain vesicles as landmarks.

The prosencephalon is the rostral portion of the brain, being visible in the most anterior sections. The diencephalon, visible at the level of the two optical visicles, is followed posteriorly by the mesencephalon and rhombencephalon. The rhombencephalon at stage HH13 is divided in two other vesicles: the metencephalon and the myelencephalon. Moreover at stage HH13 the developing brain is bent ventrally and the vesicles are not linear to each other. Therefore the myelenchephalon, at stage HH13, is sectioned at the level of the diencephalon.

At stage HH10 the extraembryonic coelom development already started (Fig. 15B). The coelomic cavity resulted from the delamination of the lateral plate mesoderm into the somatic and splanchnic mesoderm, and is visible between these two structures in both sides of embryo's body (Fig.15 A-H).

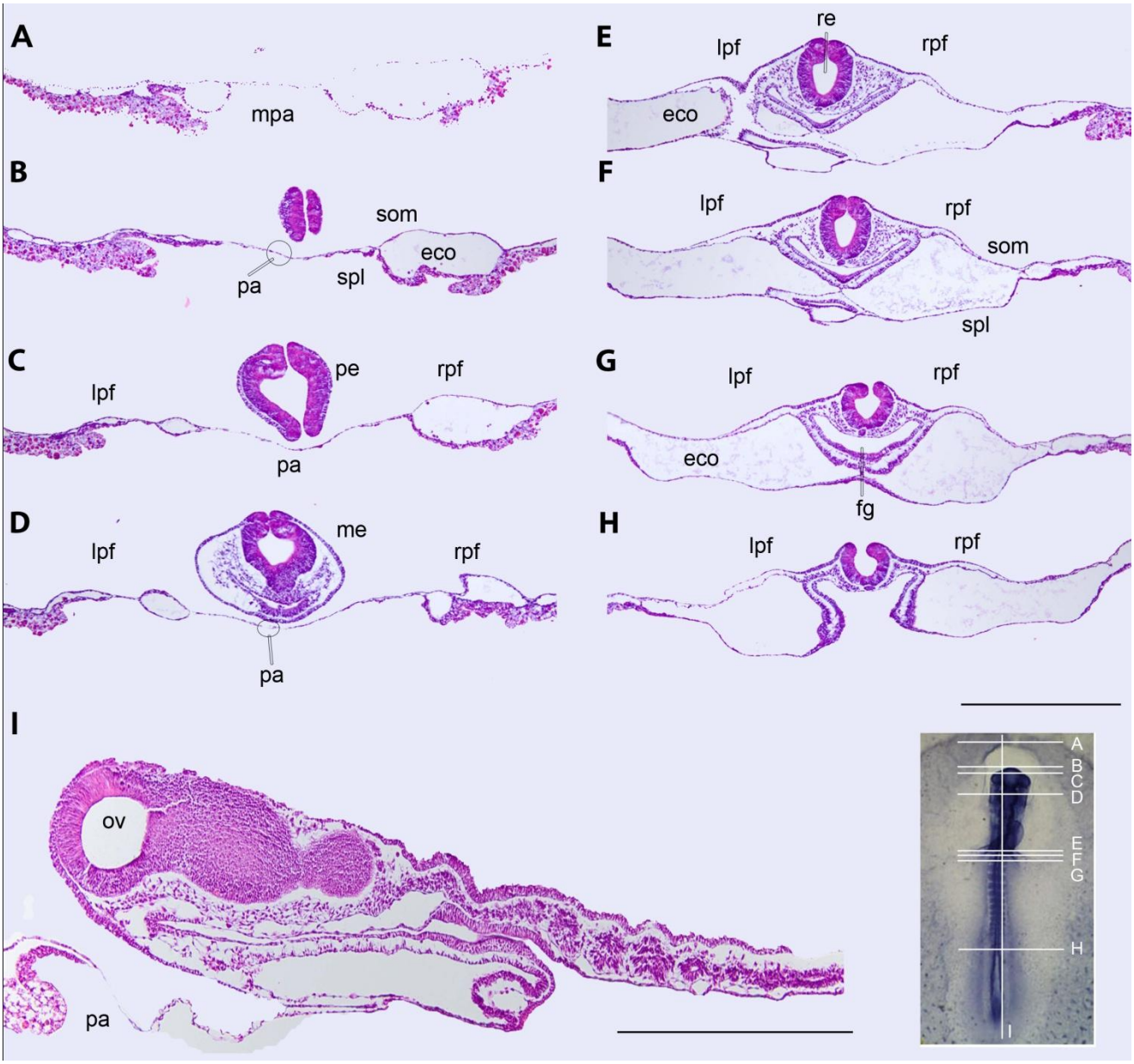


Figure 15 – Anatomical study of chicken embryos from stage HH10 using H&E staining in paraffin sections. **A)** Anterior to the head, there is the (mpa) median pre-axial amnion. At stage HH10 the median pre-axial amnion fold did not start its folding yet. The left proximal and right proximal folds (lpf) (rpf), are both surrounding the lateral sides of the embryo and are going to constitute the lateral folds of the amnion. The lateral amnion folds are constituted by the somatopleure (som), over the extraembryonic coelom (eco). The extraembryonic coelom is itself splitting the somatopleure from the splanchopleure (spl). **B, C)** Under the developing prosencephalon (pe) there is a diblastic region composed only by ectoderm and endoderm: the proamnion (pa). **D)** The proamnion is visible under the ventral side of the mesencephalon (me). **E,F)** At rombencephalon's (re) level the lateral amnion folds are visible. **G,H)** At the level of the neural groove, the lateral amnion folds are smaller when compared with anterior sections. **I)** The sagittal section from an HH10 embryo shows the position of the proamnion in the ventral side of the head, and the optical vesicle already formed (ey). Scale bar: 100 µm.

The top membrane constitutes the somatopleure and the bottom membrane the splanchnopleure (Fig. 15B). Anterior to the head there is the median pre-axial amnion fold, which will constitute the initial folding of the future amnion (Fig. 15 A). At stage HH10 the median pre-axial fold did not start its folding yet.

More posterior and bellow to the ventral side of the developing prosencephalon, there is a diblastic region composed only by ectoderm and endoderm, the proamnion (Fig. 15 B-D).

In both sides of the developing head, at the prosencephalon and mesencephalon's level, there are the left and the right lateral proximal amnion folds (Fig. 15 B-D). The sections showed that the lateral folds, at prosencephalon and mesencephalon's levels, are closer to the embryo's body when compared with posterior sections at the level of the rombencephalon (Fig. 15 B-D). The lateral amnion folds are closer to the embryo in the posterior regions of the embryo's head.

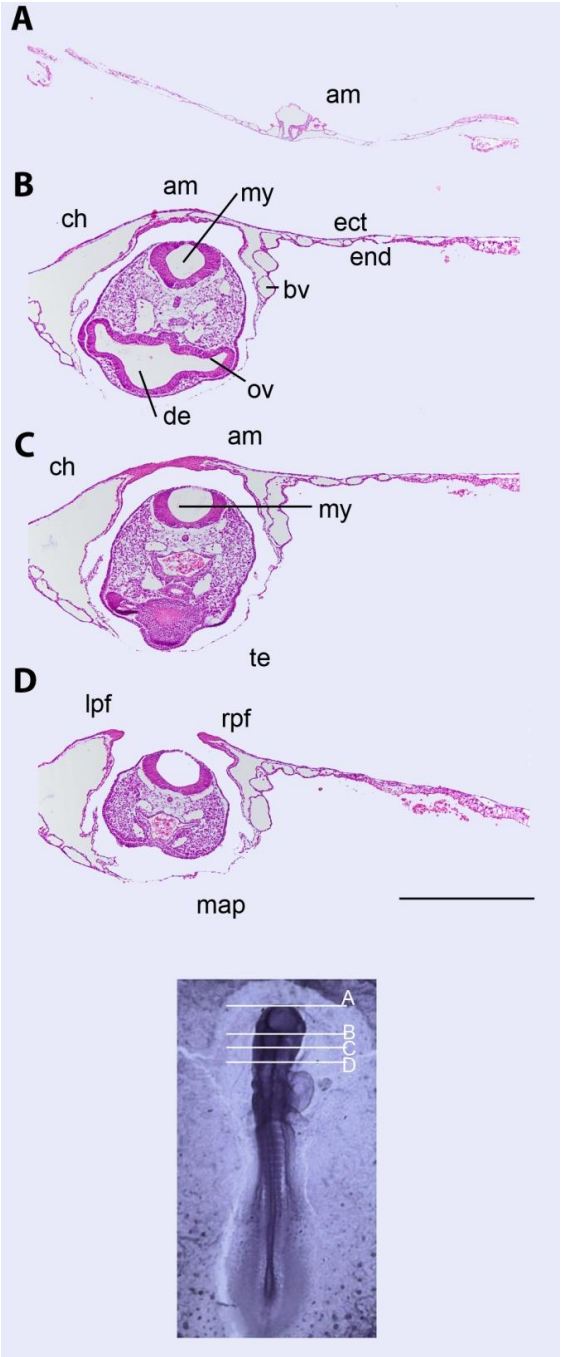


Figure 16 – Anatomical study of chicken embryos from stage HH13 using H&E staining in paraffin sections. A) The head is already enclosure in the amniotic cavity at stage HH13. The amnion (am) is visible anterior to the head. **B)** Besides the amnion that derived from the medial pre-axial amnion, the head in enclosure in the two lateral proximal body folds that are closing the myelencephalon (my) in the amniotic cavity. **C)** The telencephalon (te) is also visible in this section, due the flexure of the at stage HH13. The chorion (ch) is in contact with the amnion. The chorion is derivated from the somatopleure composed by mesoderm and the ectoderm (ect). Close to the body wall and to the amnion are some blood vessels (bv). Blood vessels derived from the splanchnopleure, composed by endoderm (end) and mesoderm.. **D)** At the level of mandibular arch pharynx the two lateral proximal amnion folds are visible again (rpf and lpf). Scale bar: 100 μm.

On embryos at stage HH13, the anterior head (at the level of telencephalon) is already enclosed in the amniotic cavity. The enclosure of the head in the amniotic cavity results by the fusion of the median pre-axial fold (Fig. 15A) with the lateral folds at rombencephalon's level (Fig. 15D) at stage HH10. The median pre-axial amnion fold ceased to exist at stage HH13.

At the top of the diencephalon, at stage HH13, the lateral amnion folds are enclosing the embryo (Fig. 16 B). At myelencephalon's level the closure of the two lateral amnion folds over the myelencephalon is visible (Fig. 16 C). At the level of the myelencephalon, at stage HH13, the amnion/chorion is enclosing the embryo (Fig. 16 C).

In posterior sections, at the level of the mandibular arch at stage HH13, the two lateral amnion folds are already distinguishable, but the embryo is not enclosed inside the amniotic cavity (Fig. 16 D).

The closure of the amnion started with the fusion of the median pre-axial amnion fold with the lateral amnion folds at the mesencephalon's level (Fig. 15 A-B). The fusion of the median pre-axial fold occurred with the lateral amnion folds that are more remote to the head at mesencephalon's level (Fig. 15 B). There is a "zipper" closuring, at rombencephalon's level, of the lateral amnion folds that are more closed to the embryo (Fig. 15 D) and the closing develops posteriorly. The "zipper closure" of the lateral amnion folds advance progressivelly from the anterior region of the brain (at the myelencephalon's level at stage HH13 (Fig. 16 B) to the posterior region of the myelencephalon (Fig. 16 C). The embryo is progressively becoming enclosed inside the amniotic cavity from anterior to posterior. At a posterior position (at the level of the mandibular arches (Fig. 16 D)) the two lateral amnion folds did not closed yet.

IV.7. The median pre-axial amnion fold does not fuse with the lateral amnion folds on embryos from stages HH11-12 cultured in the inverted culture system.

Embryos at different developmental stages were used to start the inverted system culture: HH10, HH111, HH12 and HH13 (Fig. 17). The movies (movies 2 ,3, 4, 5) are in the CD that is accompanying this thesis.

In order to better understand our analysis, figure 19 shows the frames at the beginning, then after 12 and 22 hours each. The lateral amnion folds, proamnion, median pre-axial fold and the amnion are indentified with different colors: yellow, red, blue and green, respectively.

Chicken embryos from stages HH10-11 (Fig. 17 A-F) seem to have a normal development. The vascular plexus is visible around the embryo and the heart is

showing a normal development. The developing heart starts the cultures as a simple heart tube, start beating after 12 hours in culture, and is completely formed in the end of the filming frames (movie 2) (Fig. 17 C). On embryos from stage HH11 the heart showed a defective development in the last hours of culture: its size increase abnormally in the end of time lapse (movie 3) (Fig. 18F). The head is showing a normal development: in the end of the culture the five brain vesicles are defined at stages HH10-11 (Fig. 17 C, F). The process of the flexure of the body is visible at stages HH10-11, around 22 hours of culture for stages HH10 (Fig. 17 C) and 12 hours for stages HH11 (Fig. 17 E). On embryos from stage HH12 the heart is beating during the entire filming frame, and the body's flexure become more prominent during the culture progress (movie 4). At stage HH13 the heart is beating and the head is growing until the end of culture (movie 5).

The amnion/chorion is not having a normal development on embryos from stages HH10-12. At stages HH10-11 the median pre-axial amnion fold is not folding yet (Fig. 17 C,F), and we did not indicate this structure in the color map of our analysis at stage HH10-11.

At stages HH10-11 the lateral folds of the amnion are already present in both sides of the embryo's body. At stage HH10 the lateral amnion folds are present from the mesencephalon's level till the heart's level at stage HH10 (Fig. 17A, yellow strips). The two lateral folds at stage HH11 are developed from the optical vesicle's level until the heart's level (Fig. 17 D, yellow strips). During culture time, on embryos from stage HH10 the lateral folds are growing anteriorly achieving the proamnion after 22 hours of culture (Fig. 17 B, yellow strips). On embryos from stage HH11 the lateral amnion folds reach the proamnion very soon (2 hours after the start of the culture) (movie 2). The proamnion is visible since the beginning of time lapse anterior to the head (Fig. 17 A-F, red). This structure seems to be limiting the progression of the lateral amnion folds, that are converging anteriorly but without further progression after reaching the proamnion (Fig.17 C , F). On embryos from stages HH10-11 the lateral amnion folds did not show any anterior progression after reaching the proamnion, and the median pre-axial fold did not developed over the head of embryos at stage HH10-11 (Fig. 17 E, yellow strips).

In the beginning of culture in embryos from stage HH12 the median pre-axial amnion fold had already started its folding (Fig. 17 G, blue). The lateral amnion folds are visible in both, right and left, sides of the embryo (Fig. 17 G, yellow).

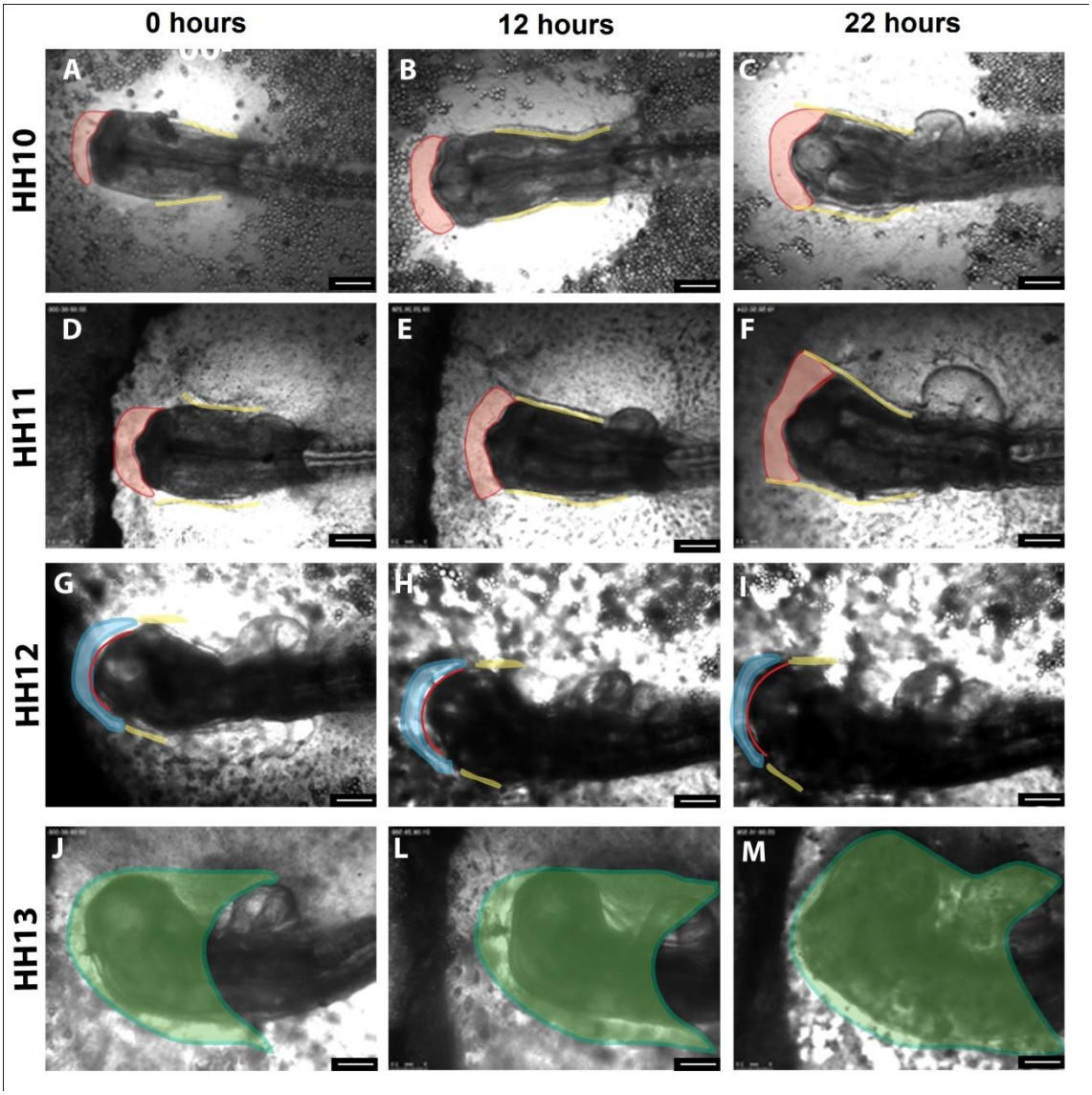


Figure 17 – Frames (from stages HH10, 11, 12 and 13) taken in the first moment of the culture, then 12 hours and 22 hours after the beginning of culture. The proamnion is defined as the red structure anterior to the head, the two lateral amnion fold as the two yellow stripes laterally to the body, the median pre-axial fold anterior to the head as the blue structure, while the amnion is represented with the green colour. **A-C)** Frames from the culture started with the embryo from stage HH10, the two lateral folds during the filming frames are converging to the proamnion. The median pre-axial fold is not folding at stage HH10-11 and is not identified at these stages. **D-F)** At stage HH11, the converging growing of the lateral amnion folds to the proamnion is also visible, and the heart is showing an abnormal size in the end of the culture. **G-I)** At stage HH12, the median pre-axial fold is visible anterior to the head, and underlined ventrally by the proamnion. The embryo at stage HH12 is growing properly in the inverted cultured system but there is no fusion of the median pre-axial fold with the lateral amnion folds. **J-M)** In the beginning of the culture of embryos at stage HH13, the amnion is already formed and is covering the head and the anterior region of the heart. In the end of the filming frame the amnion had progressed posteriorly and is covering the heart. Scale bars: 250 μm.

The proamnion at stage HH12 is localized immediately posterior, and slightly ventrally, to the median pre-axial amnion fold (Fig. 17 G, red). During culture of embryos from stage HH12, the median pre-axial fold did not fused with the lateral amnion folds and remains in the anterior region of the embryo after 22 hours of culture (Fig. 17 I).

The fusion of the pre-axial amnion fold and the lateral folds already had occurred on embryos from stage HH13. The amnion is enclosing the head and the anterior portion of the heart (Fig. 17 J). During culture, the amnion is progressively enveloping the embryo from anterior to posterior. At the end of the culture the heart is almost totally enclosed in the amnion (Fig. 17 M). At stage HH13 the embryo growth seems to help the closure of the amnion, due to the dislocation of the head to a more anterior position (movie 5). At stage HH13 the development of the amnion is not affected in embryos cultured in the inverted culture system.

We developed the inverted culture system to study the migration process of PGCs in chicken embryos. Our system showed that the amnion formation is affected depending on the developing stage used in the beginning of the culture.

The median pre-axial amnion fold on embryos from stage HH10-11 did not fold on embryos cultured in the inverted method, and is not able to fuse with the lateral amnion folds present in stages HH10-11 (Fig. 18 A-F). At stage HH12 the median pre-axial fold started its folding but on embryos at stage HH12 cultured in the inverted method is not able to fuse with the lateral amnion folds (Fig. 18 G-I). At stage HH13, the fusion of median pre-axial fold with the lateral folds already occurred. On embryos at stage HH13 cultured in the inverted system the amnion develops normally from anterior to posterior (Fig. J-M).

Our system did not revealed to be suitable for the study of the migration process of PGCs. Embryos at stage HH13 are showing a correct development of the amnion. However Stage HH13 is too advanced to study how PGCs are ingressing in the amnion/chorion at stages HH8-10. Nevertheless, we used the inverted system to help us to clarify the development of the amnion in chicken embryos.

V. Discussion

V.1. SSEA-1 is not a reliable marker for PGCs in chicken embryos

SSEA-1 is being used as a label for live cells in many approaches *(52)(53)*. The initial goal of this work was the understanding of the migration process of PGCs in live chicken embryos. Therefore, we analyzed the specificity of SSEA-1 as a marker for PGCs in chicken embryos. However our data showed that SSEA-1 is not specific for PGCs. Its expression seems to increase in the PGCs already settled in the gonads on embryos at stages HH14-19.

Some of the previous studies that revealed SSEA-1 as a marker for PGCs were performed in cultured PGCs *(54)(55)*. Our analysis was performed *on embryo*, and the expression of SSEA-1 at cell's surface of PGCs seems to be dynamic. The dynamic expression of SSEA-1 in PGCs is largely variable through different embryonic structures at different developmental stages. Moreover we found VASA positive cells in the same cluster with different expression of SSEA-1, reporting that PGCs from the same linage have differences in the expression of SSEA-1.

The specificity of SSEA-1 in the PGCs settled in the genital ridges at stages HH14-HH19 is relatively high but we did not find more relations between the expression of SSEA-1 and the position of the PGCs in the embryo. Nevertheless we are able to conclude that due to all the differences that we found in the expression of SSEA-1 in PGCs, this marker is not reliable to study PGCs in chicken embryos.

V.2. PGCs are migrating through the amnion/chorion at stage HH13

The variable expression of SSEA-1 on PGCs in chicken embryos led us to reevaluate the migratory process of PGCs in chicken embryos using anti-chVASA.

The analysis of the expression of VASA through different developmental stages allowed us to redefine the migratory route for PGCs in chicken embryos. Concomitant with previous studies *(56)*, demonstrated that PGCs are settled in the anterior region of the zona pellucida at stage HH5. Moreover our data demonstrated that PGCs seem to migrate from the anterior region of zona pellucida, at stage HH5, to the anterior region of zona opaca around stage HH8.

It was not shown before that, at stages HH8-11, PGCs are also in the median pre-axial amnion fold and that around HH13 the majority of the PGCs are present in the amnion/chorion. PGCs seem to be translocated from the anterior region of zona pellucida at stage HH8 to the amnion at stages HH8-14.

At stage HH15 PGCs were found in the vessels at the anterior region of zona pellucida. Therefore there seems to be a translocation of PGCs, present in the

amnion at stage HH14, to the extra-embryonic vessels in zona opaca and zona pellucida at stage HH15.

At stage HH15 PGCs start reaching the genital ridges. Our observations indicate that PGCs are migrating from the amnion to the vessels and from the vessels to the genital ridges at stage HH15.

Our observations led us to propose that PGCs, present in the anterior region of the zona pellucida at stage HH5 are dislocated to the anterior region of zona opaca at stage HH8. PGCs migrate from the anterior region of zona opaca at stage HH8 to the developing amnion at stages HH8-14. At stage HH15 PGCs are migrating in the blood vessels and start reaching the genital ridges at stage HH15. At stages HH16-19 PGCs are already settled in the genital ridges.

V.3. The correct development of the amnion/chorion is necessary for the PGCs to reach the genital ridges.

The presence of PGCs in the amnion/chorion of embryos from stage HH13 led us to questioning if these structures have a role in the migration of PGCs. To address this question we performed a functional essay which consisted in analyzing embryos cultured in a modified "cornish pasty" method. Embryos cultured with "cornish pasty" method have a normal development but failed in the correct development of somatopleure, from which derives the amnion and the chorion *(57)*.

In cultured embryos from stage HH17, PGCs showed widespread distribution in the ectopic somatopleure formed around the "yolk sphere". The PGCs in HH17 embryos failed to reach the gonads, and no VASA or SSEA-1 positive cells were found in the gonads from cultured embryos at stage HH17.

We conclude that the amnion/chorion is having a role in PGCs migratory process. It remains unclear if the PGCs are localized in the chorion or in the amnion. The analysis of sectioned chicken embryos, stained with anti-VASA, will help us to address this question.

V.4.The development of the embryonic head, around stages HH10-12, is necessary for the fusion of the median pre-axial with proximal lateral amnion folds

Live imaging techniques had a huge impact in biological sciences, allowing a better understand of many processes *in vivo (58)*. PGCs are migratory cells and for that reason, in order to understand the dynamic of this process, we wanted to study PGCs *in vivo*. We built a live imaging system that revealed not to be adequate for the study of the migration process of PGCs *in vivo*. The inverted cultured system prevents the correct development of the amnion of stages HH10-12 chicken

embryos, and cannot be used to the study of the migration process of PGCs in the amnion.

Nevertheless the inverted culture systems allowed us to better understand the developmental process of the amnion in chicken embryos. We hypothesize that the sinking of the head in the yolk, at stages HH10-12, is necessary for the fusion of the median pre-axial amnion fold with the lateral amnion folds. The fusion of the median pre-axial fold with the lateral amnion folds is necessary for the correct folding of the amnion over the developing head.

On embryos that started to be cultured at stage HH10-11, the lateral proximal folds of the amnion developed normally. The inverted culture system does not allow the head to sink correctly on the above tissues. The absence of the sinking of the head in the above tissues does not allow the fusion of the median pre-axial fold with the lateral amnion folds. Therefore there is no folding of the amnion over the developing head.

We propose that gravity is having a role the amnion formation, allowing the sinking of the head in the anterior region of the embryo, which led to the fusion of the median pre-axial amnion fold with the lateral folds allowing the correct folding of the amnion.

V.5. The proamnion allows the correct folding of the amnion over the developing head

Our live imaging data allowed us to propose that the proamnion is probably having a role in the amnion's folding process, functioning as a "clip". The proamnion is a "clip" localized posterior to the median pre-axial fold of the amnion. The head needs the proamnion to sink in a pouch of somatopleure, becoming progressively enclosed in it and therefore allowing the fusion of the median pre-axial fold with the lateral amnion folds.

At stage HH12, the proamnion is visible posterior to the median pre-axial fold that already started its folding. The two lateral proximal amnion folds are converging anteriorly to the proamnion along the culture and are not able to fuse with the median pre-axial fold.

We observed that the head failed to sink, due to the inverted position of the embryo. Both lateral proximal amnion folds are converging to the proamnion, without any progression after reach the proamnion. Therefore in embryos cultured in the inverted culture system the lateral amnion folds are not able to fuse with the median pre-axial fold anterior to the proamnion.

In order to confirm if the proamnion is acting as a "clip" that allows the correct development of the amnion, we need to make a comparative live imaging analysis of embryos cultured in the inverted culture system with embryos with a

normal amnion developmental process. Embryos developed in the "New" system culture *(59)* can be placed in a normal position which allows the correct development of the amnion. Therefore, the "New" system, commonly used in *ex ovo* culture of chicken embryos for the study of different processes, could help us to address this question.

V.6. Hitchhiking through the amnion/chorion: proposing a new model for the mechanism of PGC's migration in chicken embryos

Our experiments allowed a better understanding of the position of the PGCs through different developmental stages and also the mechanisms underlying the amnion formation in chicken embryos. Integrating our data we want to propose a new model for PGCs migration in chicken embryos regarding a transitory migration of PGCs through the developing amnion/chorion.

Previous studies have shown that initially (stage X) the precursors of PGCs are localized in the central area of the zona pellucida *(60)*, on the ventral side surface of the epiblast, being gradually translocated to the hypoblast around stage XI-XIV *(54)*. During the formation of the primitive streak the PGCs are carried anteriorly to the germinal crescent region. This aspect of their migration allow the PGCs to be a part of all the morphogenetic movements that are underlying the formation of the primitive streak, which otherwise could disturb their commitment *(61)*.

It has been reported that, around stage HH10, PGCs begin to concentrate at the region anterior to the head *(13)*. Nakamura et al. also showed that VASA positive cells are in the head region between stages HH10-14, without addressing any hypothesis. The presence of VASA positive cells in the head region is concomitant with our results. We found PGCs in the head region, more precisely in the amnion/chorion. Nakamura et al. *(13)* have shown that at stages HH10-12 the PGCs began to appear in the blood islands. They suggested that PGCs enter in the blood vessels from the anterior region of the head around stages HH10-12. At stage HH10-12 PGCs enter in the embryonic circulation at the anterior region of the head, migrating through the vascular system, occupying their position at the genital ridges around stage HH15 *(13)*.

Our results suggested that PGCs are not only entering in the vascular system at the anterior region of the heat at stages HH10-12. We are showing that PGCs are also migrating through the amnion/chorion around stages HH8-13. An explanation to this intermediate step could be the fact that at stages HH8-13 the circulatory system is not completely developed. There are undergoing many rearrangements *(62)* in its morphology and paracrine factors, such as growth factors from FGFs' family *(39)*, are being released in the developing vessels. For that reason, the

circulatory system, at stages HH8-13, do not constitute a good environment for PGCs to maintain their identity. On the other hand previous studies have shown that the amnion is a good substrate for stem cells culture *(63)*. Therefore we hypothesize that the amnion could be a good environment for PGCs that are escaping from the deep rearrangements that are occurring in the vascular system.

Therefore in our model we propose that PGCs, localized in the hypoblast, move to the germinal crescent where they are incorporated in the anterior extraembryonic mesoderm (Fig. 18 A). The formation of the extraembryonic coelom around stage HH7 *(57)*, allow the separation of the extraembryonic mesoderm which together with ectoderm and endoderm will develop in somatopleure and splanchnopleure, respectively (Fig. 18 B).

We propose that at stage HH7-8, the PGCs that are present in the mesoderm are being carried with the

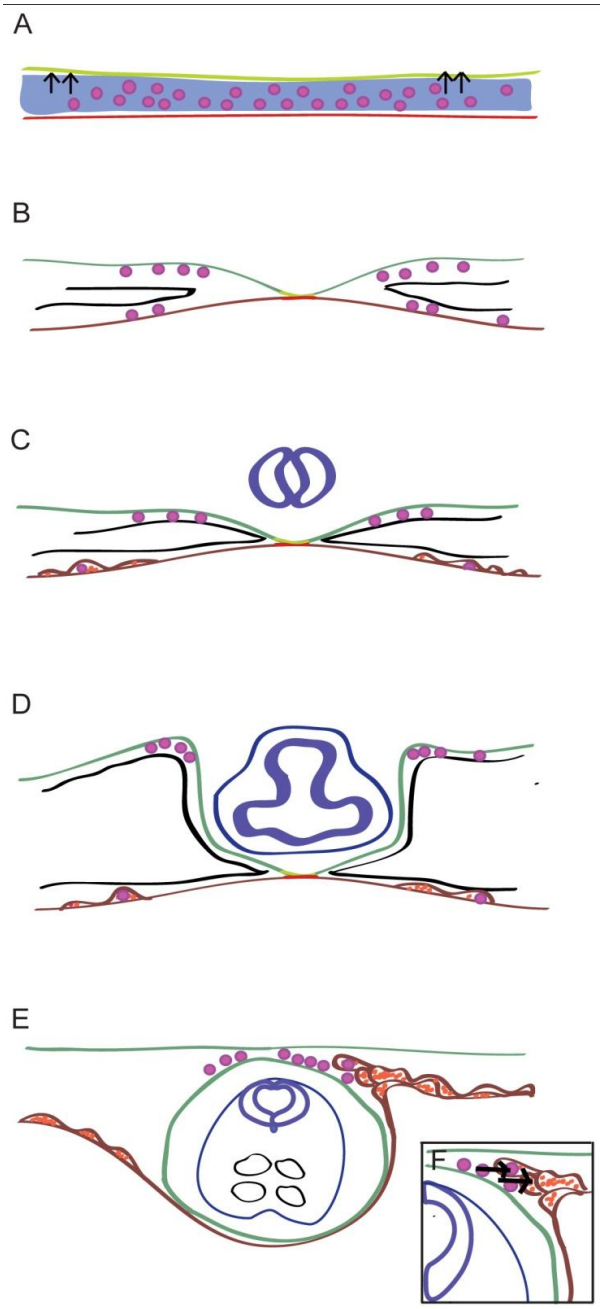


Figure 18 – Hitchhiking through the amnion/chorion: a new model for the migration process of PGCs. A) PGCs at stage HH6 are present in the mesoderm, between the ectoderm and endoderm. The black arrows represent signals that are probably being send to the PGCs to ingressing the future somatopleura. **B)** Around stage HH7, the mesoderm starts splitting in two layers: the upper layer that together with the ectoderm will form the somatopleura, and a lower layer that together with the endoderm will form the splanchnopleure**. C)** At stage HH10, the majority of the cells ingressed the developing somatopleura, that has already formed the median pre-axial fold of the amnion. However some of PGCs become enclosure in the blood islands at this level. **D)** The amnion progresses its development around the head at stage HH11, and **E)** at stage HH14 are visible some vessels close to the amnion that could be sending some **F)** signals (black arrows) to PGCs, attracting them to the vessels.

mesoderm that is forming the somatopleure. Some PGCs stay in the mesoderm that will form the splanchnopleure and therefore will become included in the blood islands that are going to develop from this structure (Fig. 18 C). PGCs are therefore "hitchhiking" trough the amnion/chorion, avoiding all the morphogenetic movements that are occurring in the developing circulatory system at stages HH8-12.

We do not have data to confirm it, but maybe the ectoderm is secreting some factors that could be involved in the attraction of these PGCs to ingress the mesoderm that forms the somatopleure (Fig. 18A, black arrows).

From the somatopleure will derive the amnion and the chorion, which progressively will cover the developing embryo *(64)*, and the PGCs will become enclosed in the amnion/chorion at stages HH13-14. Besides to the fact that no contact between the circulatory system and the amnion had been observed in chicken embryos, our anatomical studies showed that there are some vessels close to the amnion.

Moreover it was previously report that SDF-1α plays a role in the proper migration of PGCs in chicken embryos at the time they migrate through the endothelium but is not playing a role in PGC migration before stage HH 11 *(65)*. Molyneaux et al. *(65)* observed that SDF-1α chemokine is expressed at the level of the head and posterior to the heart in the ectoderm of somatopleure and in the dorsal region of the extraembryonic mesoderm. This expression pattern was observed on embryos from stages between HH10-14. They also found a correlation between the position of the PGCs during later stages of migration and the expression pattern of SDF-1, raising the possibility that SDF-1 provides the PGCs with directional cues by activating its receptor, CXCR4, expressed by the migrating PGCs.

The function of SDF-1α as a PGC attractant in the chick appears to be temporally restricted to the second phase of PGC migration, the stage at which germ cells leave the vascular network and migrate towards the genital ridge (HH 15–29). Molyneaux et al. *(65)* addressed the presence of SDF-1α in the vessels. The presence of SDF-1α in the vessels, makes SDF-1α a good candidate for be responsible of the attraction the PGCs, present in the amnion, to ingress in the vascular system (Fig. 18 E, black arrows).

The presence of SDF-1 α in the vessels on embryos at stage HH14-19 should be addressed in future experiment in a way to understand if SDF-1α is signaling to the PGCs, present in the amnion, to ingress the vascular system (Fig. 18 F). Moreover we cannot discard the possibility that SDF-1α is having a role in the attraction of PGCs by the ectoderm, when the somatopleure is being formed. The presence of SDF-1 α in the dorsal region of the mesoderm that forms the

somatopleure around stage HH10 *(65)*, is concomitant with the possibility of SDF-1α being have a role in the attraction of PGCs from ectoderm.

Thus we should investigate the expression of SDF-1α through different developmental stages in the ectoderm at stage HH8-9 where the splitting of the mesoderm is happening, the point when we considered that the PGCs are integrating the somatopleure.

VI.Conclusion

SSEA-1 immunostaing in whole mount chicken embryos revealed that SSEA-1 is not specific for PGCs between HH5 and HH19. We redefined the migration process of PGCs in chicken embryos using an antibody against chVASA. We conclude that between stages HH8-12 PGCs are in the median pre-axial and proximal lateral amnion folds and at stage HH13-14 the PGCs are in the amnion/chorion.

We used embryos cultured *ex ovo* with a modified "cornish pasty" method to understand if the deficient development of the somatopleure, observed in cultured embryos, has an effect in the migration of PGCs. In cultured embryos at stage HH17, PGCs remain immobilized in the abnormal somatopleure and do not reach the genital ridges as it happens on embryos growing *in ovo*. We concluded that the abnormal development of somatopleura in cultured embryos prevents the PGCs to reach the genital ridges.

Our results led us to propose a new model for the migration of PGCs in chicken embryos which involves a hitchhiking of PGCs through the extraembryonic amnion/chorion. This intermediate migration step is probably keeping the PGCs apart from all morphogenetic changes that are involved on the development of the circulatory system in early chicken embryos at stage HH8-12. The amnion/chorion is allowing the PGCs to keep their identity throughout PGCs' migration in chicken embryos.

Our work is redefining the migratory route of PGCs in chicken embryos and opening new questions in this field. We are proposing several hypotheses to address this question and further experiments, regarding the analysis of sectioned embryos and a live imaging approach would need to be preformed to define a accurate model for the migration of PGCs in chicken embryos.

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APPENDIX I – BUFFERS, FIXATIVES AND OTHER SOLUTIONS

BUFFERS FOR MULTIPLE USES

PBS 1X	
NaCl	137 mM
KCL	2,7 mM
KCL	2,7 mM
Na2HPO4	10 mM
KH2PO4	2 mM
	Adjust pH to 7.4 with HCl

PBT 0,5%	
Triton	0,5%
PBS	to final volume

TPB 0,2%	
Tween	0,2%
PBS	to final volume

Blocking Buffer 1%	
BSA	1%
PBS	to final volume

FIXATIVE SOLUTION

Paraformaldehyde 8%	
Paraformaldehyde	40g
Milli-Q water	400 mL
NaOH (1 M)	10 drops
Milli-Q water	to 500mL

Phosphate buffer stock solution (0.2 M)	
$NaH_2PO_4.2H_2O$	2,76 g
$Na_2HPO_4.2H_2O$	7,6 g
milli-Q water	to 100 mL

4% Paraformaldehyde in 0,1 M phosphate buffer	
8% Paraformaldehyde (stock)	1:2
0,2 M Phosphate buffer (stock)	1:2

SOLUTIONS FOR STAININGS

Hematoxylin according to Mayer	
Haematoxiline (Merck1.04302)	1,5g
Potasium-aluminium sulfate	51g
Chloral hydrate	51g
Sodium iodate	0,2 g
Citric accid	1g
Milli-Q water	to 1000 mL

Eosine	
Eosine G (Merck 1.15935)	20g
Ethanol 96%	710 mL
Saturated picric acid in milli-Q water	100mL
Milli-Q water	192mL
	Stir till everything is dissolved.

SOLUTIONS FOR CRYO EMBEDDING

Phosphate buffer stock solution (0.24M pH 7.2)	
$NaH_2PO_4.2H_2O$	7.2 g
$Na_2HPO_4.2H_2O$	33.85g
milli-Q water	to 1000 mL

Soltution 1	
Sucrose	4g
$CaCl_2$ (1M)	12 µL
Phosphate buffer	50mL
milli-Q water	to 100 mL

Soltution 2	
Sucrose	15g
Phosphate buffer	50mL
milli-Q water	to 100 mL

SOLUTIONS FOR MODIFIED "CORNISH PASTY" CULTURE METHOD

Pannett-Compton saline°

Soltution A	
NaCl	121 g
KCL	15.5 g
$CaCl_2.2H_2O$	10,42 g
$MgCl_2.6H_2O.H_2O$	12,7g
H_2O	to 1000 mL

Soltution B	
$Na_2HPO_4.2H_2O$	2.365 g
$NaH_2PO_4.2H_2O$	0.188 g
H_2O	to 1000 mL

°For **final solution**, before use, mix in order: 120 ml solution A, 2700 ml H_2O and 180 ml solution B.

APPENDIX II –ANALYSIS OF SPECIFICITY OF SSEA-1 IN DIFFERENT EMBRYONIC STRUCTURES PER STAGE

Table 1 – Average of the specificity of SSEA-1 for VASA positive cells in different embryonic structures per stage. To address the specificity of SSEA-1 for PGCs in each structure we counted the VASA and SSEA-1 positive cells in the previously defined regions per embryo. We compared the number of VASA positive cells (as the most reliable marker for PGC) and SSEA-1 positive cells. We calculated the values for percentage of SSEA-1 positive cells relative to VASA positive cells and we calculated the average of the percentage of SSEA-1.

		IA+IIA+IIIA+IVA				IB+IIB+IIIB+IVB				Amnion				Gonads			
Stage HH	Nº of embryos	Average VASA	Std VASA	Average %SSEA-1	Std %SSEA-1	Average VASA	Std VASA	Average %SSEA-1	Std %SSEA-1	Average VASA	Std VASA	Average %SSEA-1	Std %SSEA-1	Average VASA	Std VASA	Average %SSEA-1	Std %SSEA-1
5	4	0	-			372	195	0%				0%				0%	
6	2	3	91	0%		252	125	0%				0%				0%	
7	1	0				391		0%		0		0%				0%	
8	3	174	122	5%	6	133	107	0%		21	36	2%	4			0%	
9	3	217	252	1%	10	176	125	2%	2	88	103	0%				0%	
10	2	100	141	8%	11	59	83	3%	5	132	130	28%	4			0%	
11	3	245	195	6%	10	20	30	19%	32	198	98	5%	9			0%	
12	7	184	152	26%	19	6	6	33%	21	150	37	7%	11	0	0	0%	
13	2	120	4	28%	14	1	-	21%	17	211	194	10%	14	0	-	0%	
14	5	31	42	18%	25	95	60	18%	19	37	33	0%		58	112	23%	9
15	1	154		24%		170		10%		16		0%		160		0%	
16	3	20	33	22%	38	0		0%		0		9%	16	206	120	42%	11
17	1	0		0%		0		0%		0		0%		278		62%	
18	2	0		0%		0		0%		0		0%		235	8	64%	1
19	1	0		0%		0		0%		0		0%		289		57%	