Isolation and characterisation of chick embryonic primordial germ cells

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'I, Sittipon Intarapat confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Sittipon Intarapat

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in The UCL Research Department of Cell and Developmental Biology, University College London under the supervision of Professor Claudio D Stern.

Dedication

To Mr. Sopa Intarapat, my dad, you are always in my heart and you are still memorable forever.

To Mrs Noi Intarapat, my mom who gave my life, encouragement and all kind supports.

Acknowledgements

Abstract

Embryonic stem cells (cESCs) can be isolated from chick embryos, with the ability to contribute to all somatic lineages in chimaeras, but not to the germ line. However, lines of chicken embryonic germ cells (cEGCs), which are able to contribute to the germ line, can be established from chicken primordial germ cells (cPGCs). However very little is known about these cells, or about the changes that accompany the establishment of gonadal cells as self-renewing cell lines. This thesis presents a detailed study of the properties of cPGCs and the parent tissue from which they are derived. Gene expression profiles for 30 genes related to pluripotency and/or differentiation were are compared between gonads at the indifferent stage (stage 26-28HH), in primary gonocytes, established PGCs and cESCs. The results reveal great heterogeneity in the expression of various markers in culture. Several genes associated with pluripotency change dramatically upon culture. The most salient of these changes is that while *cSox3* (but not *Sox2*) is expressed in the gonads, whereas their expression becomes reversed upon culture (becoming more similar to mammalian stem cells). This suggests that these two SoxB1 class genes have swapped functions in chick. In the process of studying the expression pluripotency markers in later (stage HH35) gonads, we made an unexpected discovery: both male and female embryos show left-right asymmetric patterns of expression of some, but not all, of these markers. Expression of pluripotency (*cPouV, cNanog*, *cSox2* and *ERNI*) in the left gonad is much higher than those in the right gonad of both sexes. The expression of pluripotency markers is irrespective of its colonisation by primordial germ cells, and it appears that this left-right decision is made independently of whether the gonad will regress or be retained. These findings offer a new model system for investigating the roles of pluripotency-related markers during normal development as well as in stem cell lines.

Contents

List of Figures

List of Tables

List of Abbreviations

AP	Alkaline Phosphatase
BRL	Buffalo Rat Liver cell line
FITC	Fluorescein Isothiocyanate
FGF	Fibroblast Growth Factor
DAPI	4,6-Diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamintetraacetic Acid
EG&K	Eyal-Giladi and Kochav stage of chick embryos
EGs	Embryonic Gonads
EGCs	Embryonic Germ Cells
ESCs	Embryonic Stem Cells
H&H	Hamburger and Hamilton stage of chick embryos
IgG	Immunoglobulin G
ISH	*In situ* Hybridisation
KO-DMEM	Knock Out Dulbecco's Modified Eagle Medium
PAS	Periordic Acid Schiff
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGCs	Primordial Germ Cells
RNA	Ribo Nucleic Acid

RPM	Revolutions Per Minute
S.D.	Standard Deviation
SCF	Stem Cell Factor
STO	SIM (Sandoz Inbred Mouse) embryo-derived Thioguanine and Ouabain-resistant fibroblast cell line
WISH	Whole-Mount *In situ* Hybridisation

Chapter 1. Introduction

1.2. Germ cells

1.2.1. PGC development in chick embryos: origin and morphological characteristics of chicken primordial germ cells

From where do germ cells originate, and how do they differ from somatic cells? Since the earliest observations made by the ancient Greeks and Egyptians, and continuously until the late 18th Century, two alternative philosophical standpoints competed with each other. "Preformationists" believed that the next generation was already contained within the parent, and that the eggs or sperm contained a miniature version of the progeny. A natural extension of this (called "emboîtement") is the belief that all future generations are already preformed and contained like Russian dolls. The alternative view to preformation was "Epigenesis", which proposed that the embryo develops *de novo* by increasing complexity rather than being pre-formed (for review see (Needham, 1934)).

Although this debate has long been extinguished, there are still two theories of germ cell formation and specification, which to some extent bear a parallel to the two philosophical views of preformation and epigenesis (Extavour and Akam, 2003). In avians, it has been hypothesized that germ line formation shares some features with the preformation theory since it has been reported that it is determined by maternally inherited factors: the germ cell protein marker Vasa (Cvh) is found not only in very early embryos but even in oocytes (Tsunekawa et al., 2000).

The study of the origin of avian primordial germ cells (PGCs) started from the original observation that chick PGCs can be distinguished from somatic cells in the embryos from the late gastrula stage (Swift, 1914). Later, PGCs were identified at other stages. The identification and isolation of chick PGCs from primitive streak to somite stages has been reported (Clawson and Domm, 1969; England and Matsumura, 1993; Matsumura and England, 1993). At primitive streak stage, chicken PGCs can be found in the germinal crescent (see Figure 1.1), the anterior part of the embryo, which is devoid of mesodermal cells (Fujimoto et al., 1976). The migration of chick PGCs from epiblast to germinal crescent has also been investigated (Ginsburg and Eyal-Giladi, 1986).

However, the earliest stage at which PGCs can be found in chick embryos was the pre-primitive streak stage (Ginsburg and Eyal-Giladi, 1987; Ginsburg and Eyal-Giladi, 1989; Muniesa and Dominguez, 1990), even prior to hypoblast formation (Eyal-Giladi et al., 1976). It was suggested that chicken PGCs were located in the middle of area pellucida (Kagami et al., 1997; Naito et al., 2001). To test this more directly, an *in vitro* experiment was done by cutting central disk fragments from stage X (Eyal-Giladi and Kochav, 1976; EG&K) chick blastoderms and culturing them on coverslips; PGCs were then detected in cultures using the PAS reaction as a marker (Ginsburg and Eyal-Giladi, 1987; Ginsburg and Eyal-Giladi, 1989). Another study traced the appearance of PGCs in blastula of quail embryos using monoclonal antibody QH1 as a quail PGC marker (Pardanaud et al., 1987). The results indicated that avian PGCs are of epiblastic origin, consistent with observations using Feulgen staining (Eyal-Giladi et al., 1981) and immunocytochemistry (Pardanaud et al., 1987). Although PAS or Feulgen staining are not specific markers to identify chicken PGCs,

an objective germ cell marker, anti-CVH antibody to the chicken Vasa homologue has now been generated and can be used to trace these cells as well as the earliest expression of this protein during development. It was this approach that revealed Vasa protein in the cytoplasm of chick oocytes (Tsunekawa et al., 2000).
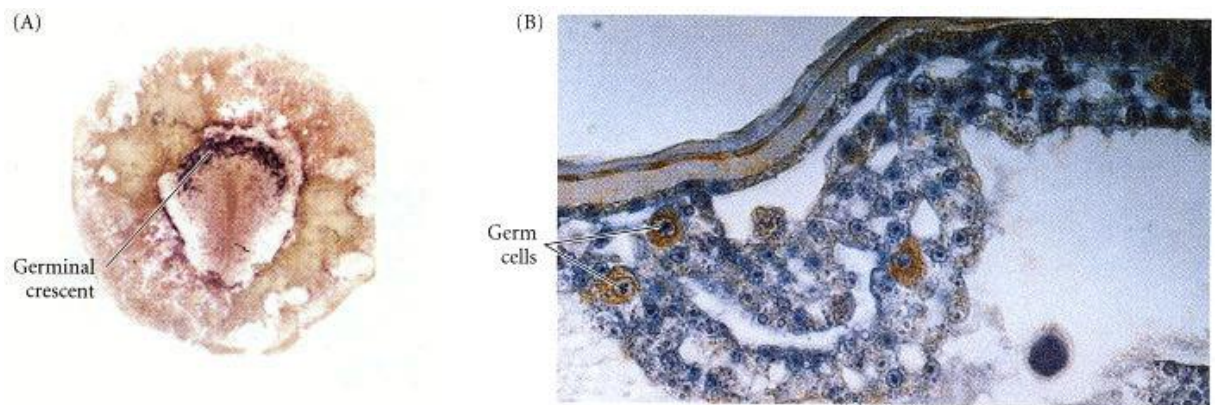
Figure 1.1: Origin of Primordial Germ Cells (PGCs) in germinal crescent region: A: The region represents germinal crescent (purple) containing PGCs in primitive streak stage (HH4) of chicken embryos. B: High magnification of PGCs (brown) in germinal crescent region. From (Tsunekawa et al., 2000).

1.1.2. Germ cell identification in chicken embryos

Before the anti-Cvh antibody was available, many studies tried to identify PGCs by other means, which involved the search for morphological features that could distinguish PGCs from other cells in birds. Electron dense and membrane bound granules were detected in the cytoplasm of quail PGCs by TEM (Yoshinaga et al., 1993). Even though avian PGCs could be easily distinguished by virtue of being

larger cells containing a larger nucleus than surrounding somatic cells (Meyer, 1964; Singh and Meyer, 1967), reliable markers needed to be developed to distinguish between PGCs. To use quail/chick chimaeras for experiments to assess the origin of PGCs, features that distinguish PGCs in chick and quail embryos were needed. Some studies reported the absence of glycogen granules in cytoplasm of quail PGCs, while chicken PGCs contain many such granules which allow chicken PGCs to be identified by PAS staining (Clawson and Domm, 1963; Fujimoto et al., 1976; Meyer, 1964). Other experiments studied the behavior of donor chicken PGs after injection into the blood stream of recipient quail embryos and vice versa (Nakamura et al., 1991b; Nakamura et al., 1992). The quail PGCs were distinguished from chicken PGCs by showing no PAS reaction in the prospective gonadal region of the recipient chick embryos, being localized among the recipient chick PGC.Quail and chick PGCs were also distinguished histochemically by double-staining with a lectin from *Wistaria floribunda* (WFA) and the PAS reaction (Nakamura et al., 1992). Since there was the difference of selective-lectin binding sites of quail an chicken PGCs, WFA and *Griffonia simplicifolia* II (GS-II) lectins were used as markers for quail and chick PGCs, respectively (Yoshinaga et al., 1992). This indicated that there are differences in sugar-binding protein among avian species.

Apart from lectin histochemistry used to identify avian PGCs, Alkaline phosphatase, a metabolic enzyme used for PGCs activity during germ cell migration (Swartz, 1982) or immunohistochemistry using monoclonal or polyclonal antibodies including QH-1 (Pardanaud et al., 1987), anti-gPGC serum (Ginsburg et al., 1989), QCR1/QB2 (Ono and Machida, 1999; Ono et al., 1996) have been reported to be useful for detecting quail PGCs, or the CVH gene in both species (Tsunekawa et al., 2000).

1.1.3. Primordial Germ Cells Migration

PGCs have the ability to migrate from the extra-embryonic region where they originate, towards the presumptive gonads. The factors involved in regulating this in avian embryos remain unknown. It has been proposed (Kuwana and Rogulska, 1999) that chemotactic factors direct PGC migration from extra-embryonic region towards the gonadal region; however, the chemotactic factor has not yet been found even though it was named "Telopheron" (Baker, 1972). Telopheron was proposed to be produced and secreted by somatic cells in presumptive gonads and to induce PGC migration towards the presumptive gonads on both sides; however, it has been known for a long time that the left side contains 70% more PGCs than the right side (Witschi, 1935). Chicken left presumptive gonads were proposed to secrete factors at higher levels than the right, which might trigger mitotic activity of PGCs (Swartz and Domm, 1972). One study in quail embryos supported this by proposing that after engrafting the number of quail PGCs differ between left and right presumptive gonads at limb bud stages (HH 18-24) (Didier and Fargeix, 1976). This phenomenon was also seen at later stages, which presumptive gonads are differentiated into the ovary since the number of oogonia in the left overy was higher than that in the right and germ cell death was also much lower (Ukeshima and Fujimoto, 1991). It has been proposed that dead oogonia are eliminated from both left and right ovary via lacunae in the medulla (Ukeshima, 1994) and that this is caused by germ cell apoptosis (Ukeshima, 1996).

What type of molecule attracts PGCs to move towards the embryonic gonads? It has been suggested that steroids might be chemotactic factor-like agents secreted by the presumptive gonads (Baillie et al., 1966; Baillie et al., 1996). One hypothesis

proposed that treatment with excess exogenous steroid hormones might disrupt endogenous steroids secreted by the presumptive gonads during PGC migration; therefore, such exogenous steroids might affect PGC migration by stimulating or inhibiting this process. This hypothesis has been tested experimentally by injecting excess testosterone cypionate (TC) into chicken embryos at 33 hours' incubation; TC inhibited PGC migration and decreased the number of PGCs localized in presumptive gonads (Swartz, 1975). Hence, this supported the hypothesis that a chemotactic-like agent secreted by the gonads, perhaps steroidal in nature, attracts PGCs towards the presumptive gonads. However, it has also been reported (Forbes and Lehmann, 1999) that the presumptive gonads secrete glycoproteins to attract migrating PGCs; it is therefore possible that more than one chemoattractant exsits, for example acting at long and short range.

In addition, it has been proposed that transforming growth factor-beta, TGF-β might be a chemotactic factor secreted by the presumptive gonads (Godin and Wylie, 1991). Furthermore, SDF-1α, the chemokine stromal cell-derived factor 1 alpha has been acted as a chemotactic factor which enhanced migrating PGCs moved toward the presumptive gonads (Stebler et al., 2004). In addition, SDF-1/CXCR4 as well as Steel factor/c-Kit has been reported that play a role for germ cells guidance (Doitsidou et al., 2002) and the gene required for such guidance was *Dnd* (*Deadend*) (Weidinger et al., 2003). Hence, these reports suggest that several chemotactic factors may be involved in guiding PGC migration.

How do PGCs migrate towards the presumptive gonads? It has been described that at the beginning of migration, PGCs migrate passively, particularly chicken PGCs,

which the germincal crescent PGCs migrate through endothelial cells of dorsal aorta via "diapedesis" (Gilbert, 2003) (see figure 1.2B). This step has been called passive migration which is a type of ameboid movement *in vivo*, occurring in blood chicken PGCs which have pseudopodia (Fujimoto et al., 1976). PGCs have been shown to use extra-embryonic blood vessels as the route to circulate throughout the entire embryo; therefore, these cells have been called "circulating-PGCs" (cPGCs) (Clawson and Domm, 1969). The morphology of circulating-PGCs has been described as round and with protruding cytoplasmic processes inserting between endothelial cells to migrate out of the embryonic blood vessels (Ukeshima et al., 1991) (see Figure 1.2A). It has been reported that circulating-PGCs that have left the blood circulation incorporate into and migrated along mesenchymal cells in the dorsal menstery at the level of the mesonephros. Hence, those PGCs have been called "tissue-PGCs (tPGCs)" (Clawson and Domm, 1969). The morphology of PGCs at this step is different from cPGCs, since they display pseudopodia, which are also characteristic of cells undergoing amoeboid movement (Fujimoto et al., 1976; Kuwana et al., 1986; Lee et al., 1978). Hence, the process that allows tissue-PGCs to migrate actively along migratory routes by themselves, has been called "active migration" (Fujimoto et al., 1976). PGCs stop active migration when they reach the genital ridges (see Figure 1.2C). It has been reported that genital ridge formation relates to the implantation of migrating PGCs. PGCs which have implanted in the genital ridges, have been called "gonadal-PGCs (gPGCs)" (Clawson and Domm, 1969). Furthermore, it has been shown that vascularization at the level of the genital ridges is crucial for PGC implantation in the genital ridges since the lack of this process has been implicated in PGC migration in chicken embryos (Perez-Aparicio et al., 1998).
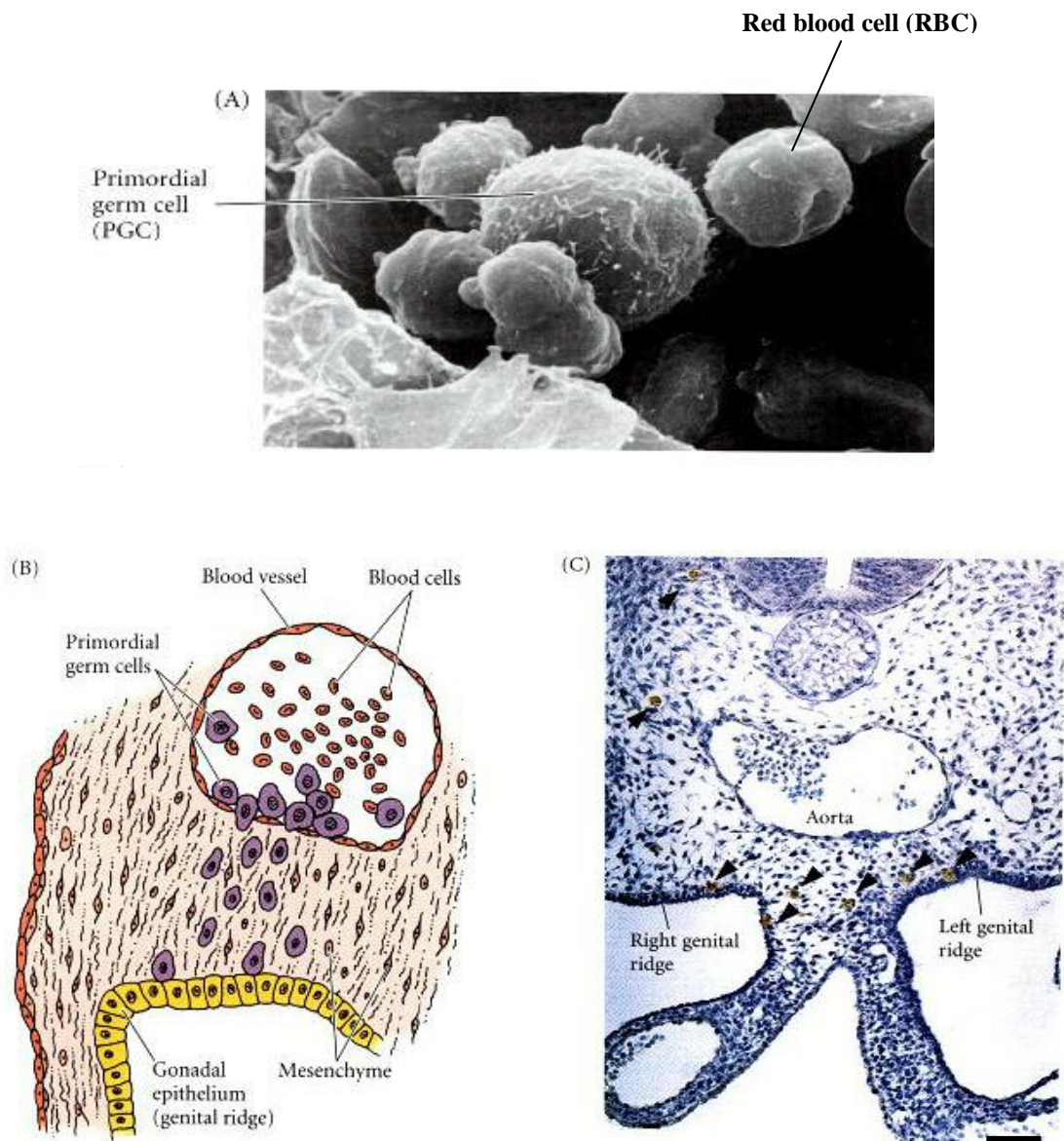
Figure 1.2: Diagram showing PGC undergoing passive and active migration:

A: Scanning electron micrograph (SEM) represents chicken primordial germ cells (PGCs) and red blood cells (RBC) in capillary at passive migration phase. B: Chicken PGCs move out from endothelial cells of dorsal aorta via diapedesis. C: Localization of chicken PGCs (black head arrows) in the genital ridges with anti-CVH immuohistochemistry at active migration phase. From (Gilbert, 2003) Bar = 100 μm.
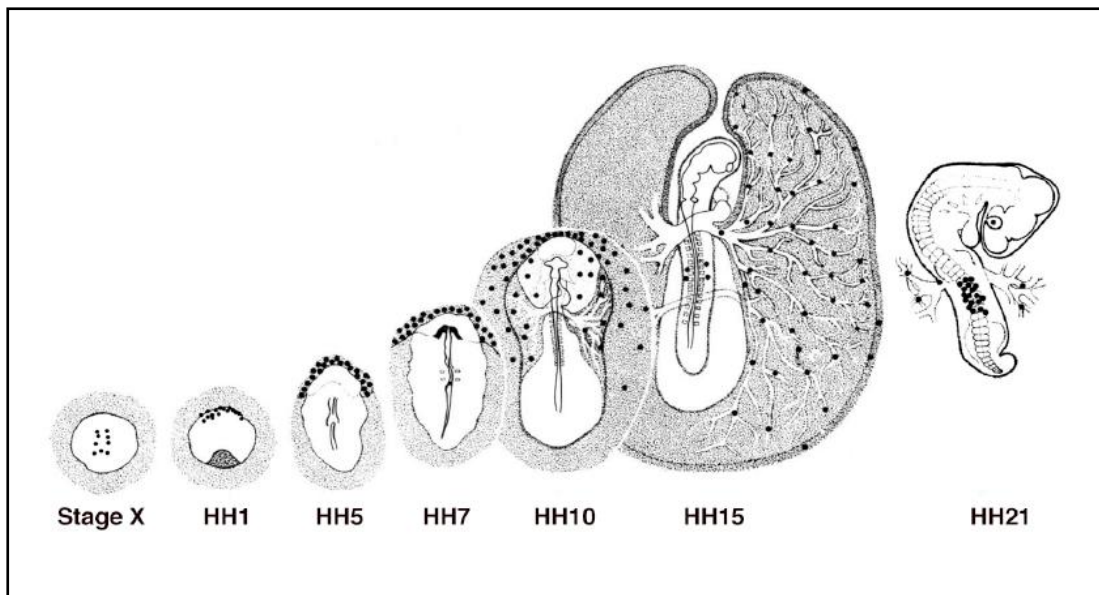
Figure 1.3: PGCs migration in different stages of chicken embryos: PGCs originate at the central zone of blatoderm at stage X (EG&K). PGCs then migrate anteriorly to the border between area opaca and area pellucid at stage HH1. PGCs could be detected at anterior part of the embryos, germinal crescent which primitive streak have been formed at stage HH5. PGCs at germinal crescent region still present since the head fold and somites have been formed at stage HH7. PGCs start to migrate from extra-embryonic region into area vasculosa (blood vessels froming region) at stage HH10. PGCs migrate passively into blood circulation (passive migration) at stage HH15. PGCs migrate actively along dorsal mesentery (active migration) inside of the embryo towards the genital or gonadal region situated at medioventral of the embryos at stage HH21). From Stebler (2005), who in turn took this from Niewkoop and Sutasurya (1979).

Unlike mammalian PGCs, chicken PGCs use the blood circulation as a migratory route during the passive migration phase (see Figure 1.3). This characteristic accounts for the ability of chicken PGCs to migrate out of the presumptive gonads and settle in the extra-gonadal regions. It has been reported that the percentage of chicken PGCs distributed in extra-gonadal regions was 20% of total chicken PGCs, while up to 90% of extra-gonadal PGCs can be found in the head region adjacent to the neural tube (Nakamura et al., 1988). These findings have been confirmed by later experiments since chicken embryos lacking presumptive gonads exhibit PGCs in the head region, migrating via endothelial cells of capillaries and cooperating with mesenchymal cells to reach such a region (Nakamura et al., 1991a). Together, these findings indicate that the presumptive gonads send signal that attract chicken PGCs towards the presumptive gonads.

In summary, the first step of chicken PGCs migration is an active but non-directional process. They originate from the blastoderm then migrate from epiblast to hypoblast of the germinal crescent and from there into the vascular system. Ultimately, circulating-PGCs actively migrate by several routes towards the genital ridges. The arguments regarding chemotactic factors involved in guiding chicken PGCs to the presumptive gonads were discussed. The specific molecules playing a role for attracting PGCs during germ cell migration and the mechanism underlying germ cell migration or displacements of PGCs inside the differentiating gonads still needed further investigation.

1.1.4. Sex determination and sex differentiation in chicken embryos

How do the sexes in avian become determined? Sex determination and differentiation in birds are controlled by genes in sex chromosomes (genetic sex determination; chromosomes Z and W). Homogametic animals (ZZ) are male and heterogametic birds (ZW) are female (Clinton and Haines, 2001; Ellegren, 2001; Smith and Sinclair, 2001; Smith and Sinclair, 2004). Although the detailed mechanism of sex chromosomes underlying sex determination is still unclear, the master gene controlling maleness in chicken embryos has been unveiled. It has been reported that *DMRT1* (doublesex-and mab-3-related transcription factor 1 gene) plays a crucial role for testicular development in male chicken embryos (Smith et al., 1999) and this gene, located in the Z chromosome, is highly expressed in male genital ridges at the gonadal differentiation stage (Raymond et al., 1999). Sex-linked genes are also present on the W chromosome and are expressed in the female genital ridges of embryonic gonads before sex differentiation occurs. Three female specific genes on the W chromosome have been identified *PKCIW* (Protein Kinase C Inhibitor W-linked gene or *Wpkci* gene) (Hori et al., 2000), ASW (Avian Sex-specific W-linked gene) (Ellegren, 2001; O'Neill et al., 2000; Pace and Brenner, 2003) and *FET-1* (Female Expressed Transcript 1 gene) (Reed and Sinclair, 2002). In addition, it has been reported that sex determination in chicken occurs on days 5-6 of embryonic development (Smith and Sinclair, 2001; Smith and Sinclair, 2004). Recently it was reported that sex determination in chick embryos is cell autonomous in tissues throughout the body, since a gynandromorphic chick, with male features on the left and female features on the right, has been created (Zhao et al., 2010). The molecular mechanism underlying

avian sex determination has been debated – this recent finding supports the idea that sex is established cell autonomously in the chick.

1.1.5. Gonadal development

Gonadal development is a sequential process which can be divided into three major events: PGC migration, sex determination and gonadal differentiation. In mammals, PGCs originate from the extra-embryonic region (in the first days of gestation) and then migrate to colonise the region where the gonads develop, the gonadal or genital ridges. PGCs differentiate from the endodermal of yolk sac, adjacent to the embryonic hindgut (see Figure 1.4A). This structure evaginates and develops into an extra-embryonic structure, the allantois (Gilbert, 2003). PGCs migrate from this extra-embryonic region to the genital ridges via the dorsal mesentery (see Figure 1.4B) by "amoeboid movement". The genital ridges are formed by bulging out of the intermediate mesoderm and lie along the medioventral aspect of the mesonephros (see Figure 1.4B). After implanting in the genital ridges (see Figure 1.4C), PGCs and surrounding somatic cells in the genital ridges develop together to form the mature gonads (see Figure 1.4D).
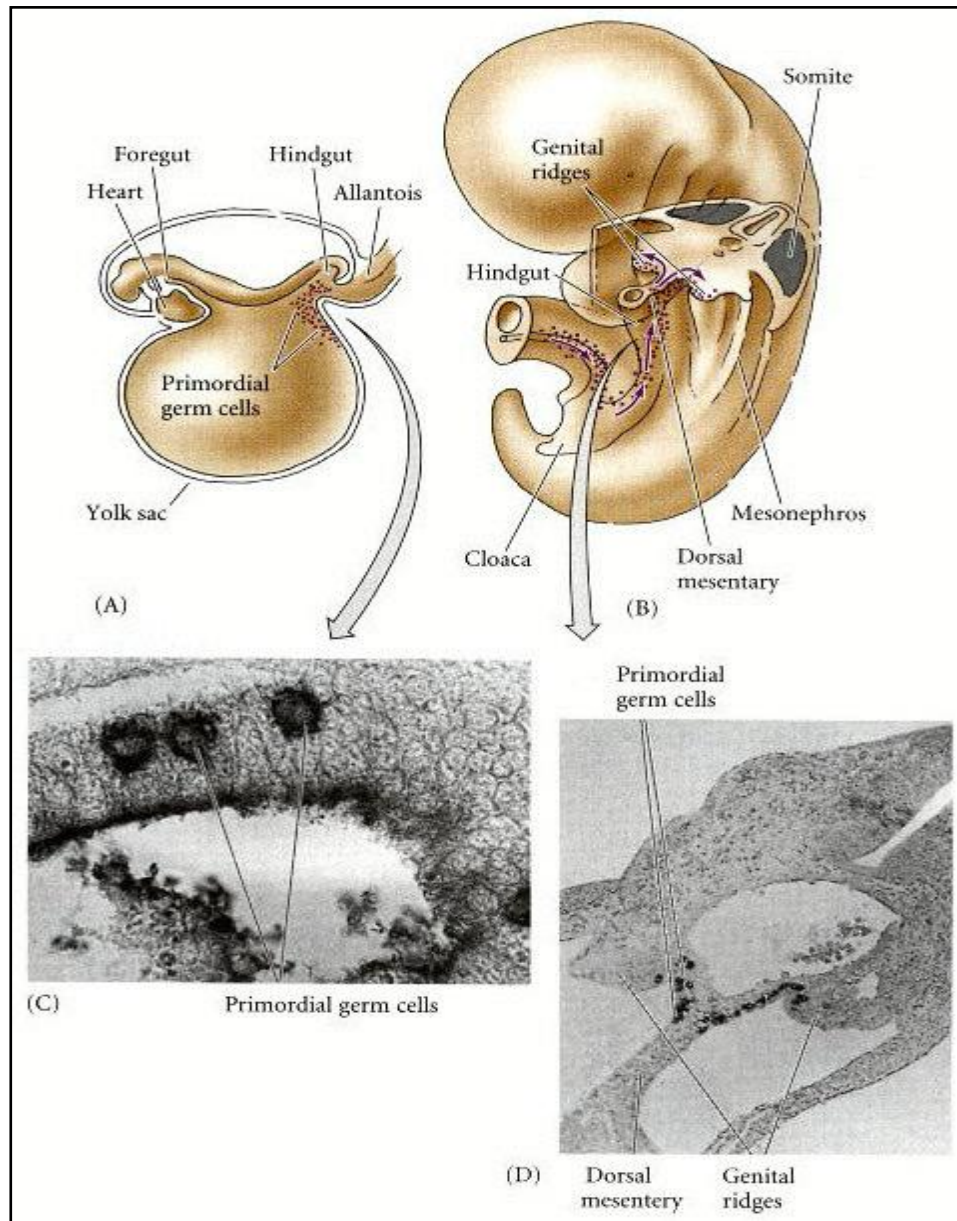
Figure 1.4: Migratory pathways of Primordial Germ Cells in mammalian embryos:

A: PGCs locate inside of yolk sac close to the region of hindgut and allantois. B: PGCs actively migrate along the dorsal mesentery to implant in the genital ridges. C: PGCs are at the level of hindgut close to yolk sac and allantois. D: PGCs use the dorsal mesentery as a migratory route. From (Gilbert, 2003)

The second process is sex determination of the embryos, this process occurs at around three or four days of gestation and is followed by the final process of gonadal differentiation. This process takes place at around 5-6 days of gestation and after all three processes are complete, the mature gonads finally function to produce efficient gametes.

1.1.6. Gonadogenesis in chicken embryos

How do the gonads form during embryonic development? The gonads develop from intermediate mesoderm forming bulged structures, the genital ridges, situated at the medioventral edge of the mesonephros and developing together with it (Browder et al., 1991). It has been reported that the group of cells differentiating into the gonads differentiate by thickening of the mesenchymal blastema of the genital ridges, with contributions from the coelomic epithelium and mesonephros (Martineau et al., 1997).
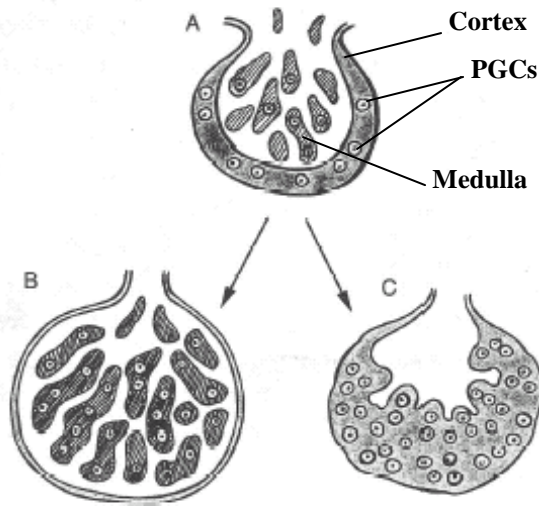
Figure 1.5: Diagram illustrating sex differentiation of the embryonic gonads:
A: the embryonic gonads at the indifferent stage consist of cortex and medulla
containing PGCs (grey) in both layers. B: Male embryonic gonads exhibit testicular
cords in medulla containing spermatogonia and spermatocytes inside the cords and
thin germinal epithelium in the cortex. C: Female embryonic gonads exhibit thick
cortex containing oocytes. From (McCarrey and Abbott, 1979).

In the beginning of gonadogenesis, the gonads of male and female embryos cannot yet be distinguished morphologically; this stage is therefore known as the "indifferent stage" (see Figure 1.7 and 1.8). When the gonads enter the sex differentiation process (see Figure 1.8), the male genital ridges develop into the testes while the female genital ridges developed into the ovaries. The somatic cells in the genital ridges at the indifferent stage become steroidogenic or hormone producing cells and supporting cells in both sexes. These somatic cells surround primordial germ cells (PGCs) inside and further develop into primary sex cords in both sexes (Romanoff, 1960).

Generally, chicken embryonic gonads consist of two layers: the cortex and medulla (see Figure 1.5A), as in mammals (Maraud et al., 1987); moreover, primary sex cords exist in both cortical and medullary regions in embryonic ovaries and testes. There are however some differences in gonad development between male and female embryos. In embryonic testes, cells in the medulla proliferate much more than in the cortex; this leads to thinning of the cortex in male gonads (Romanoff, 1960) (see Figure 1.5B). Furthermore, medullary cords develop further into secondary sex cords or testicular cords (see Figure 1.5B). In contrast, embryonic ovaries develop by regression of the medullary cords, a secondary sex cord arises, and this further develops leading to the thickening of the cortex (Romanoff, 1960) (see Figure 1.5C). These processes are controlled by genes in the sex chromosomes (Chue and Smith, 2011; Smith et al., 2007; Smith and Sinclair, 2001; Smith and Sinclair, 2004). In summary, gonadogenesis in the male is medullary, while gondanogenesis in the female is cortical.
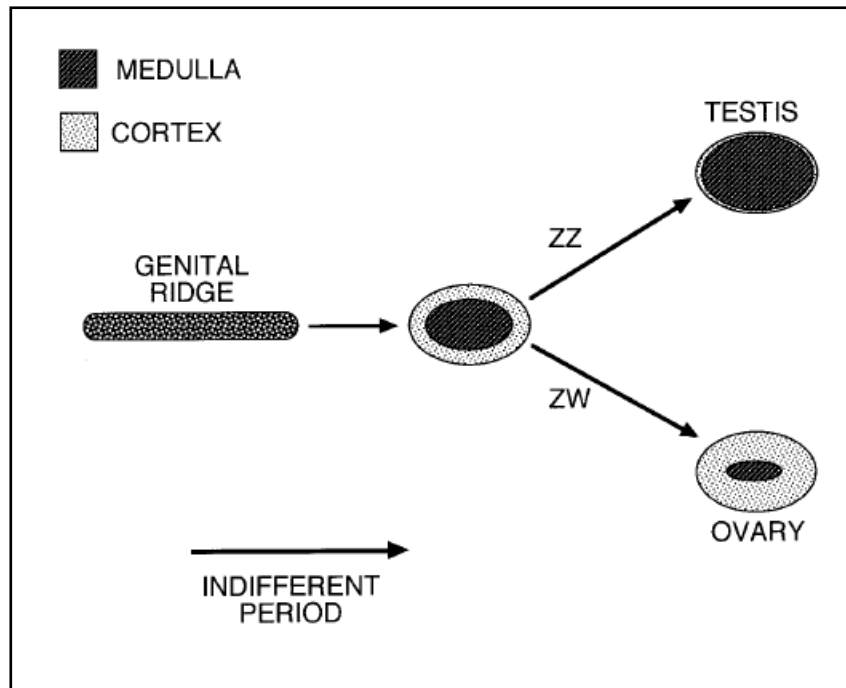
Figure 1.6: Diagram showing the development of cortex and medulla in the chick: Embryonic gonads (cortex (grey) and medulla (black)) at the indifferent stage while the genital ridges or bipotential gonads differentiate into embryonic testes in male (ZZ) and embryonic ovaries in female (ZW) under the influence of sex chromosomes. From (Clinton and Haines, 2001).
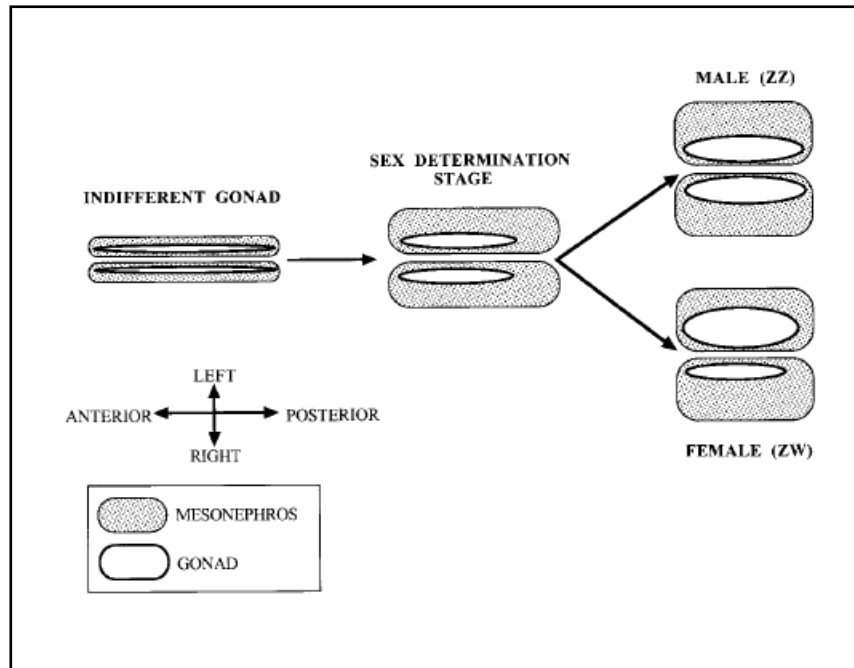
Figure 1.7: Diagram of gonadogenesis in the chick embryo: embryonic gonads (white) situated on the mesonephros (grey) at the indifferent stage when sexes cannot yet be distinguished morphologically. At sex determination or different stage, male gonadogenesis is bilateral (ZZ) while female gonadogenesis is unilateral (ZW). From (Clinton and Haines, 2001).

It has been described that gonadal differentiation in chicken embryos is controlled by two molecular mechanisms: the gene cascade mechanism (see Figure 1.10), controlling cellular differentiation of bipotential cells in the genital ridges to differentiate into testicular cells or ovarian cells, and the sex-determining mechanism (see Figure 1.9), controlling the differentiation of the genital ridges into embryonic testes (ZZ) or ovaries (ZW) (see Figure 1.10) (Smith et al., 2007; Smith and Sinclair, 2001; Smith and Sinclair, 2004).
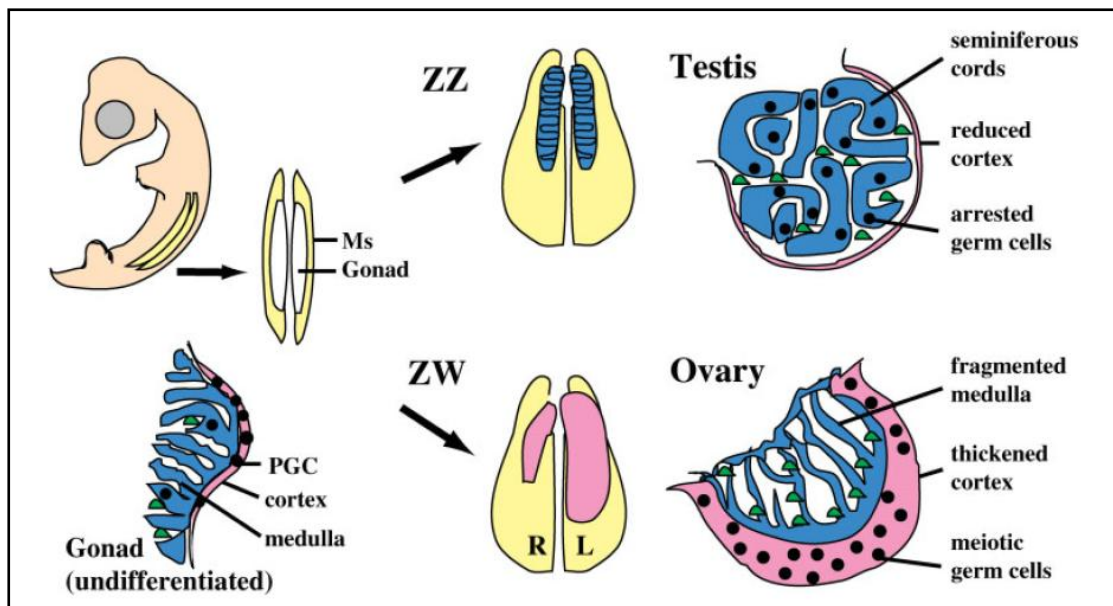
Figure 1.8: Gonadal differentiation in chick embryos: this process starts at 3.5 days of incubation (HH20). The genital ridges (white) situated at medioverntral of mesonephros (yellow). At the undifferentiated or indifferent stage, the genital ridges consist of the cortex (pink), medulla (blue) and PGCs located in both layers; moreover, the embryonic sexes cannot be distinguished morphologically by the gonadal appearance. At 6.5 days of incubation (HH30), the embryonic sexes begin to differentiate into male (ZZ) testes (blue) and female (ZW) ovaries (pink). The embryonic testes develop symmetrically containing a thick medulla, testicular cord (blue) and male germ cells inside the cord (black). In contrast, the embryonic ovaries develop asymmetrically, only the left side differentiates into a functional ovary containing a thick cortex (pink) containing the female germ cells (black), while the right side regresses and forms cavities of dead germ cells (lacunae). From (Smith and Sinclair, 2004).
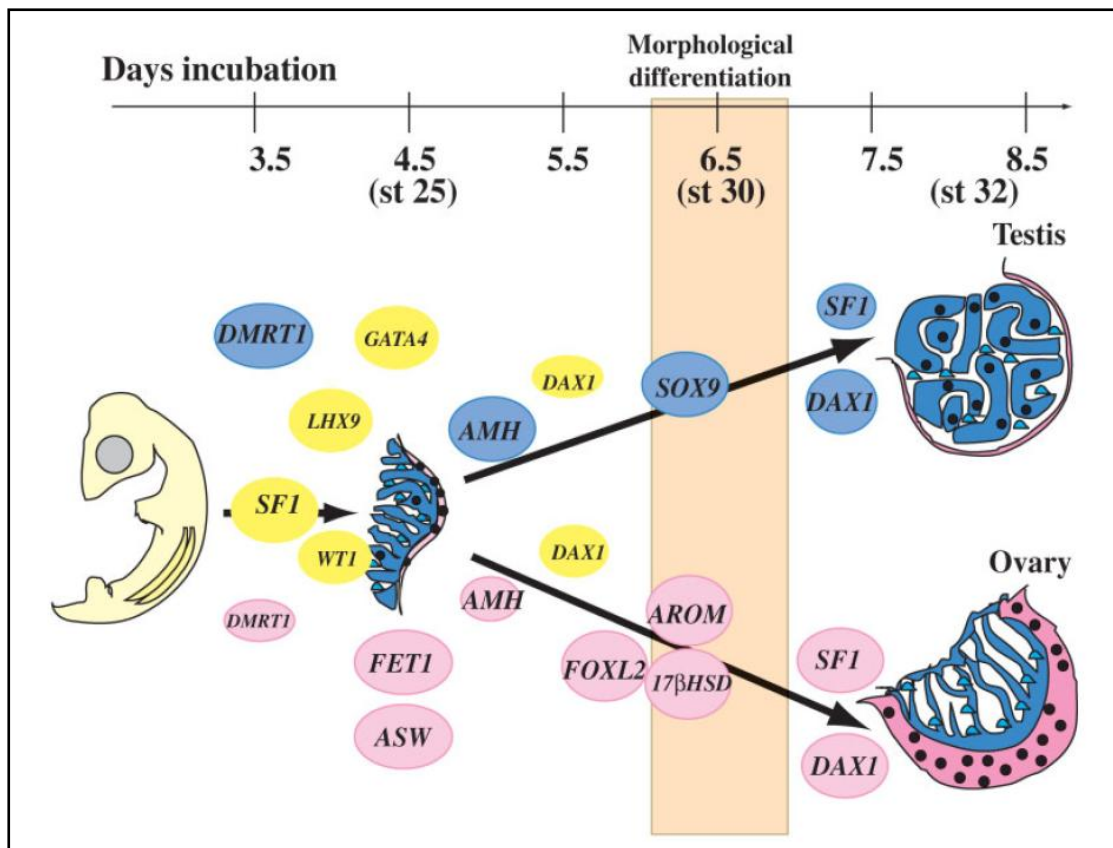
Figure 1.9: Diagram summarising differential sex-determining gene expression in chick embryos: genetic cascade of testis-determining genes regulating cellular differentiation in embryonic testis (blue circles) and ovarian-determining genes (pink circles) regulating cellular differentiation in embryonic ovaries (yellow circles) are shown during gonadal differentiation. The size of each circle indicates the level of gene expression in different period of times and sexes. The pale orange box represents an initial indication of gonadal differentiation between male and female embryos by gonadal morphology at day 6.5 (HH30) (abbreviation, st = stage). From (Smith and Sinclair, 2004).

1.1.7. Development of the chicken reproductive system

The chicken reproductive system consists of gonads functioning for gamete production and accessory ducts conducting gametes to the region of fertilization (see Figure 1.11A). In male embryos, the testes develop symmetrically (symmetrical or bilateral gonads), whereas in female embryos, only the left ovary functionally develops and able to produce female gamete but the right ovary regresses (asymmetrical gonads) (see above). The testes contain seminiferous tubules convoluted inside the testes, while the ovary contains somatic follicular cells and gametic ovarian cells such as oogonia and oocytes in different stages. The accessory embryonic duct in the male is called "Wolffian duct" (see Figure 1.11B), which differentiates from the mesonephric duct. Like embryonic testes, Wolffian ducts develop both on the left and right sides and contribute to the vas deferens which connected to the opening region of the cloaca. In addition, anterior accessory embryonic ducts develop into the epididymis (Lilie, 1919). The accessory embryonic ducts in female are called "Mullerian ducts" (see Figure 1.11C); in vertebrates, Mullerian ducts regress in male embryos under the influence of male sex hormone. On the other hand, Mullerian ducts in female embryos are retained and develop into the female oviduct in adults, whereas Wolffian ducts regress. In birds, like the right ovary, the right Mullerian duct regresses during development (see in Figure 1.10) (Romanoff, 1960).
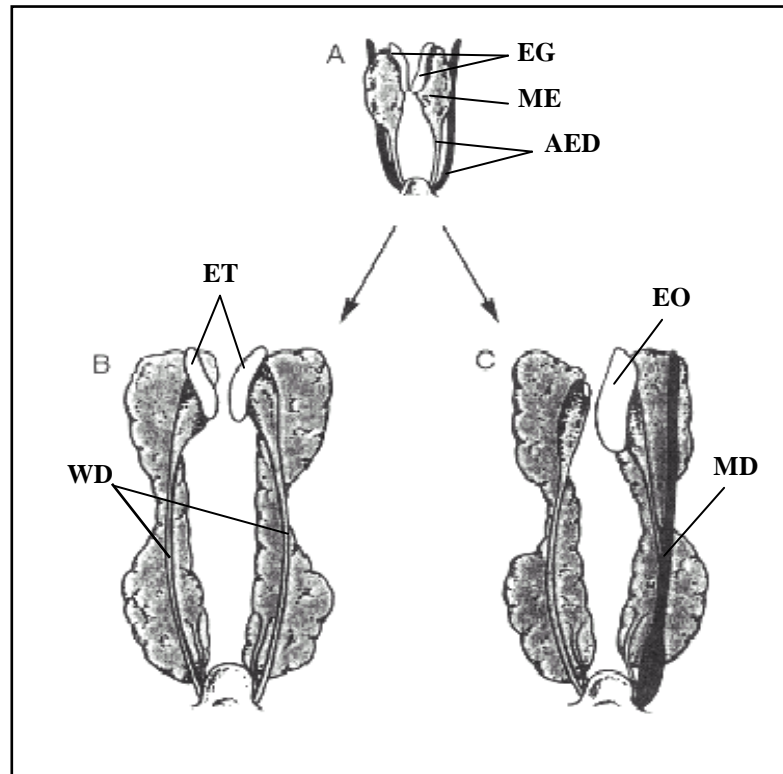
Figure 1.10: The reproductive system in chick embryos: A: Embryonic reproductive system at indifferent stage consists of embryonic gonads (white) and accessory embryonic ducts (grey and black). B: Chicn male at hatching stage has Wolffian ducts (grey) in both left and right sides of. C: Chick female at hatching stage has only the left Mullerian duct (black). From (Romanoff, 1960) (Abbreviations, EG: Embryonic Gonads; ET: Embryonic Testes; EO: Embryonic Ovary; AED: Accessory Embryonic Duct; WD: Wolffian Duct; MD: Mullerian duct; ME: Mesonephros).

1.1.8. Evolution of germ cells: invertebrate and vertebrates

What mechanisms make cells become the germ line, and how do germ line cells become different from somatic cells? It has been reported that a "germline gene set" exists, controlling germ line fate among organisms. The first germline gene described was "*vasa*", deadbox helicase which acts as a translational regulator (Hay et al., 1988; Lasko and Ashburner, 1988). It has been proposed that the occurrence of *vasa* (*vas*)-related genes is universal among metazoan (Gustafson and Wessel, 2010; Mochizuki et al., 2001). In invertebrates, the *vasa* (*vas*) related genes and other member of DEAD box proteins from sponge, *Hydra* and planaria family have been cloned; *vas*-related genes in *Hydra*, *Cnvas1* and *Cnvas2* are strongly expressed in germline cells (Mochizuki et al., 2001). This suggests that *vas*-related genes occur universally among metazoans (Mochizuki et al., 2001). Other previous studies focused on *vasa* in Cnidarians. It was discovered that germline-soma segregation existed in Hydrozoa, *Hydractinia echinata* using *in situ* hybridization and immunohistochemistry for Vasa (Rebscher et al., 2008). *Hevas* is expressed in the interstitial stem cells, while the *Hevas* transcript is not detectable in developing gametes. This suggested that maternal Vasa protein, but not the mRNA, is a maternal constituent of germ plasm in this species (Rebscher et al., 2008). Moreover, it has been reported that not only *vasa* but also *nanos* play a role for germ cell specification in the sea anemone, *Nematostella vectensis* (Extavour et al., 2005). In addition, *vasa* expression is detected in both presumptive PGCs late in embryonic development and multiple somatic cell types during early embryogenesis. This suggested that preformation in germ cells might have evolved from ancestral epigenesis (Extavour et al., 2005). In the roundworm (Nematode) *Caenorhabditis elegans*, a *vasa* homlog (*Glh*), has been shown to play a

crucial role for germline segregation (Gruidl et al., 1996). P granules, which are cytoplasmic structures associated with germ nuclei in the *C. elegans* gonad, are localized exclusively to germ cells, or germ cell precursors, throughout the life cycle (Schisa et al., 2001). It has also been reported that VBH-1, a *C. elegans* protein closely related to *Belle* and *Vasa*, is expressed specifically in the *C. elegans* germline, where it is associated with P granules (Salinas et al., 2007). Another *Vasa*-related gene family- *glh*, which encode protein components of P granules, did not appear essential for RNA to concentrate in P granules suggesting a function in transportating RNA to the nucleus. This suggested that P-granules related to *Vasa* associated proteins do not function only for the germline in *C. elegans* but have other functions (Schisa et al., 2001). The *vasa* homologue *macvasa* has been identified in the flatworm *Macrostomum lignano*; it is expressed in testes, ovaries, eggs and somatic stem cells (Pfister et al., 2008). In segmented worm (Annelid), *vasa* orthologues *CapI-vasa* from Polychaete and *Capitella sp.I* have been found to be expressed in developing gametes of sexually mature adults (Rebscher et al., 2007). It has also been reported that expression of *vasa*, *Pdu-vasa* from another Polychaete, *Platynereis dumerilii* is unveiled in germ cells and somatic stem cells at the posterior growth zone (Rebscher et al., 2007). These results suggested a common origin of germ cells and somatic stem cells, similar to Flatworm and Cnidarians, which may imply that this is the ancestral mode of germ cell specification in Metazoa.

In arthropods, especially insects, *Drosophila melanogaster*, the *vasa* gene is responsible for a maternal-effect mutation that causes a deficiency in pole cells, germline precursor cell formation (Hay et al., 1988). Moreover, the possible function of *vasa* in *Drosophila* has been reported that its protein, VASA, binds to target

mRNAs involved in germline determination (Hay et al., 1990). In other arthropods, amphipod crustacean, *Parhyale hawaiensis*, it has been reported that a single blastomere relates to germ cell formation since the localized Vasa protein has been detected at the eight-cell stage in one blastomere of *P. hawaiensis* using immunohistochemistry (Extavour, 2005). However, this finding has been challenged by another new finding that germ cells in *P. hawaiensis* depend on Vasa protein for their maintenance but not for their formation (Ozhan-Kizil et al., 2009). Since the function of *vasa* in *P. hawaiensis* has been knocked down by MO injection, MO-mediated inhibition of *vasa* translation caused germ cells death after gastrulation. This indicated that in *Parhyale*-Vasa protein is not required for germ cell establishment but is required for their subsequent proliferation and maintenance (Ozhan-Kizil et al., 2009). Im mollusk, bilvaves, the oyster *vasa*-like gene, *Oyvlg* has been reported as a specific marker of the germ line in *Crassostrea gigas* (Fabioux et al., 2004a; Fabioux et al., 2004b); moreover, its expression is restricted to germline cells in both males and females, including germinal stem cells and auxiliary cells (Fabioux et al., 2004a; Fabioux et al., 2004b). This finding suggested a role for *Oyvlg* in germline development. Strikingly, germ line determinants in echinoderms like the sea urchin *Strongylocentrotus purpuratus* are not localized early in sea urchin development, but do accumulate in the small micromere lineage (Juliano et al., 2006). It has also been reported that in embryos of this species, Vasa protein is post-transcriptionally enriched in the small micromere lineage, which results from two asymmetric cleavage divisions early in development (Juliano et al., 2006; Juliano and Wessel, 2009). They also reported that although there are similarities between the *vasa* mRNA expression patterns of several sea urchins and sea stars, the time frame of enriched protein expression differs significantly (Juliano and Wessel, 2009). In summary in

invertebrates, i.e. *C. elegans* and *Drosophila*, the precursors of the germ line, PGCs, are specified by maternal components present in the cytoplasm. Moroever, the function of the germline determinant, *vasa*, is not restricted only to the germline but also has functional roles in the somatic stem cells. The relationship between germ line and somatic cells in terms of their function and distribution needs to be elucidated.

In protochordates like the ascidian, *Ciona interstinalis* (another urochordate)*,* the germline cells, PGCs originate from the endodermal strand cells; a *vasa* homologue (*CiVH*) of this species has been cloned from ovarian tissue by PCR and it was shown that its expression is specific to germ cells in adult and juvenile gonads (Takamura et al., 2002). Futhermore, it has been demonstrated by immunoflorescence that Vasa protein is expressed in the gastrula embryo with both puctate and diffuse patterns (Shirae-Kurabayashi et al., 2006).

Vasa is highly conserved among vertebrates. *vasa* has been reported to be a member of a novel gene family of DEAD box proteins in different species. *XVLG1*, a *Xenopus laevis vasa*-like gene, is specifically expressed in the adult testis and ovary (Komiya et al., 1994). More recentwork from the same group led to the isolation and cloning of *vasa* homologues in mammals. The mouse *vasa* homologue, *Mvh* encoding a DEAD-family protein, exhibits higher degree of similarity with the product of the *Drosophila vasa* gene (*vas*) than other previously reported mouse genes (Fujiwara et al., 1994). Furthermore, its expression was exclusively detected in testicular germ cells such as spermatocytes and round spermatids in perinuclear granules (Fujiwara et al., 1994). In addition, immunohistochemical analyses of MVH protein has been carried out: the protein was exclusively expressed in primordial germ cells just after their colonization

of embryonic gonads and in germ cells undergoing gametogenesis in both males and females (Tanaka et al., 2000; Toyooka et al., 2000). The rat *vasa*-like gene, RVLG has been cloned and its expression detected specifically in the gonads of male and female adult rats (Komiya and Tanigawa, 1995). The zebrafish homologue of the *Drosophila vasa* gene has also been cloned; its transcript was present in embryos just after fertilization and zebrafish *vas* RNA from the 1-cell stage to 10 days of development has been detected (Yoon et al., 1997). In contrast to findings in zebrafish, it has been shown that teleost medaka, *Oryzias latipes vasa* gene, *olvas*, is expressed in a somatic structure, the embryonic shield, this finding, coupled with the fact that *vasa* mRNA, which is localized to the germ plasm of zebrafish but does not label a similar structure in medaka (Herpin et al., 2007). This suggested the possibility of fundamentally different mechanisms governing PGC specification in these two fish species. However it is also possible that the expression in the shield in medaka is due to non-specific labelling, because this is not seen in other teleost fishes. In Nile tilapia, *Vas* (a Drosophila *vasa* homologue) is expressed in germ cells during oogenesis and spermatogenesis in both females and males (Kobayashi et al., 2000) and in grass carp, *Ctenopharyngodon idella*, *Civasa* (*Ctenopharyngodon idella vasa*) transcripts have been detected in ovaries and testes but not in somatic tissues (Li et al., 2010).

In pig, the *vasa* homolog (*Pvh*) expression at mRNA and protein levels is expressed specifically in the ovary and testis (Lee et al., 2005). In human, an ortholog of the *Drosophila* gene *vasa* has been isolated and its expression found to be restricted to the ovary and testis (Castrillon et al., 2000). Furthermore, its protein is not only expressed in human normal germ cells but also malignant germ cells (Zeeman et al., 2002).

In chicken, Gallus gasllus, germline-specific expression of a chicken vasa homolog protein (CVH) has been demonstrated throughout all stages of development, from uterine-stage embryos to spermatids and oocytes in adult gonads (Tsunekawa et al., 2000). Moreover, in the same study, anti-CVH staining demonstrated specific expression in the gonads of other species including the adult testes of quails (*Coturnix coturnix japonica*), turtles (*Pelidiscuc sinensis*) and snakes (*Trimeresurus flavoviridis*) (Tsunekawa et al., 2000). The results of these studies reveal that CVH and its expression is highly conserved among vertebrates (indeed among all metazonas) and specifically expressed in the germline.
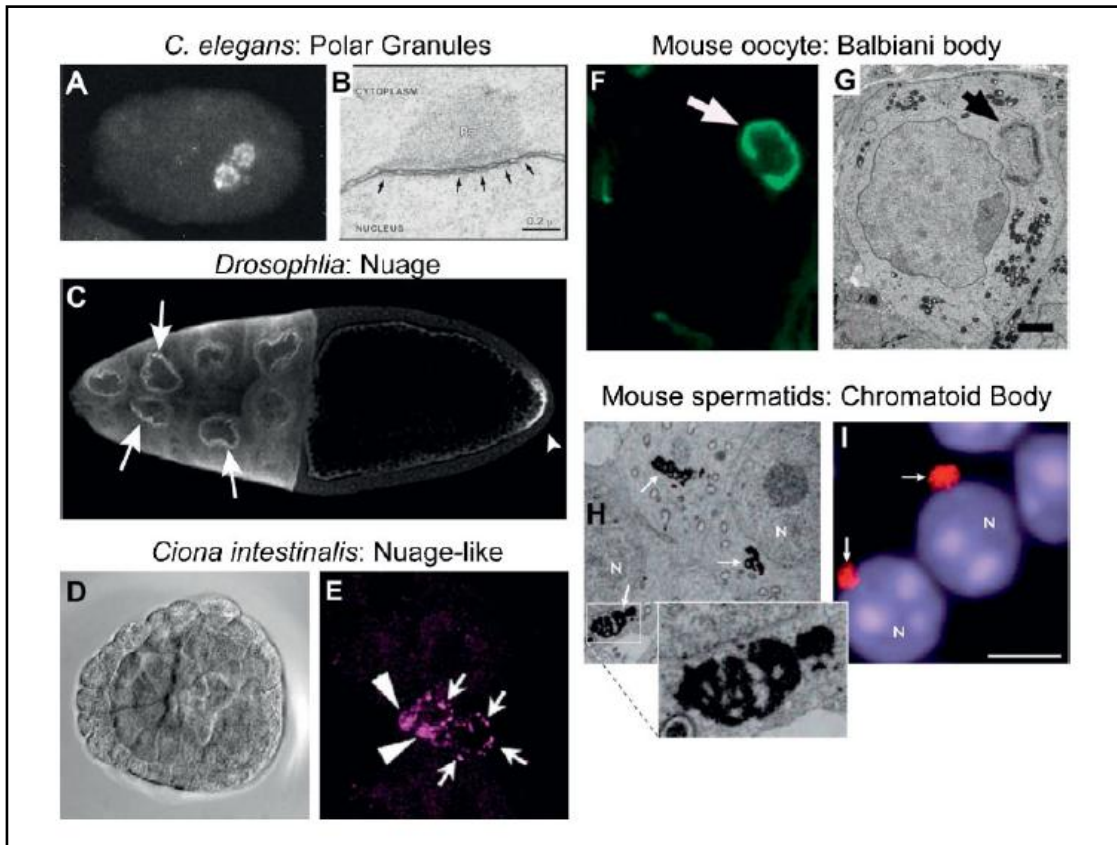
Figure 1.11: Expression of Vasa protein associated with nuage-like structures in different areas of invertebrates and vertebrates: A, B: in polar granules of *C. elegans* (black arrows indicate nuclear pores), C: in developing egg chamber of Drosophila (white arrowhead indicates the pole plasm in the oocyte) and the perinuclear nuage in nurse cells (white arrows) D, E: in gastrula embryo of ascidian, *Ciona intestinalis* shows punctate (arrows) and diffuse (arrowheads) expression. F,G,H: in Mouse oocytes, Vasa protein (white arrow) (F: immunofluorescene) localizes to the Balbiani body (G: TEM) and chromatoid bodies (black arrows) of spermatids (H: TEM) contain Vasa protein (white arrows) (I: immunoflourescence). From (Gustafson and Wessel, 2010).

1.1.9. Applications and technologies related germ cells

: Isolation and derivation of chicken germ cells

Is there a reliable source of chick germ cells, and can their germline-contributing properties be maintained *in vitro*? There have been several attempts to isolate and grow chick germ cells. It was first demonstrated that chick PGCs can be cultured from pre-streak stage embryos (Karagenc et al., 1996); factors secreted by STO cells (a mouse embryonic fibroblast cell line) were found to enhance PGCs maintenance *in vitro* (Karagenc and Petitte, 2000). It was also shown that PGCs can be obtained from the germinal crescent and that these can be successfully transfected by retroviruses. Moreover, chimaeras have been produced after injecting such transfected PGCs cells into recipient embryos, which grew to sexual maturity and produced offspring containing the foreign DNA (Vick et al., 1993).

Chick PGCs have some unique characteristics that distinguish them from their mammalian counterparts, such as the fact that they use the blood circulation as migratory routes during their migration. This allows chick PGCs to be isolated from embryonic blood. This was achieved relatively recently (van de Lavoir et al., 2006a), which established the protocol for isolating and deriving blood-derived PG cells from chick embryos. Blood derived PG cells remained undifferentiated after prolonged culture in the presence of LIF, SCF and bFGF (van de Lavoir et al., 2006a). The cells established by this protocol exhibited a good germline transmission after injection into stage 13-15 (HH) embryos, but do not contribute to somatic tissues. These results suggest that the factors such as LIF, SCF and FGF are required for survival of PGCs *in vitro* and for them to retain the property of germline transmission. Moreover,

genetically modified PGCs have been created by this protocol (van de Lavoir et al., 2006a), which opens the way for future production of transgenic lines of birds using this methodology.

There have also been several attempts to isolate gonadal-derived germ cells from later stages, using embryonic gonads (Chang et al., 1997; Chang et al., 1995b; Ha et al., 2002; Park and Han, 2000; Park et al., 2003a; Park et al., 2003b; Shiue et al., 2009; Suraeva et al., 2008; Wang et al., 2009; Wu et al., 2010). A comparison in isolation and derivation of blood-derived and gonadal-derived PGCs line is shown in Table 1.1.

However, very few germline chimaeras have been achieved by injecting cultured gonadal-derived germ cells into the recipients (Chang et al., 1997; Chang et al., 1995b; Ha et al., 2002; Park et al., 2003a; Park et al., 2003b). IGF and IL-11 were found to be essential for cultured gonadal PGCs to maintain germline competence and colony formation (Chang et al., 1995a; Park and Han, 2000). Furthermore, production of germline chimaeras has been achieved from gonadal cells cultured in the presence of these factors. These results suggested that there are differences between blood-derived and gonadal-derived germ cells in terms of long-term culture *in vitro* for germline chimaera production.

Table 1.1: A comparison in methods of isolation and derivation of blood-derived and gonadal-derived PGCs

Cells	Feeder cells[1]	Sera/Growth factors/Cytokines[2]
blood-derived PGCs		
References		
(van de Lavoir et al., 2006a)	irradiated BRL	FBS, CS/ bFGF, SCF/ secreted LIF by BRL
(Yamamoto et al., 2007)	-	FBS, CS/ - / -
(Choi et al., 2010)	-	FBS, CS/ bFGF, SCF/ hLIF
(Macdonald et al., 2010)	irradiated STO	FBS, CS/ FGF, SCF/ secreted LIF by BRL
gonadal-derived PGCs		
References		
(Park and Han, 2000)	CEF	FBS, CS/ bFGF, SCF, IGF-I/ mLIF, IL-11
(Suraeva et al., 2008)	GSC	FBS, CS/ bFGF, SCF/mLIF
(Shiue et al., 2009)	CEF	FBS, CS/ bFGF, SCF, IGF-I/ mLIF, IL-11
(Wang et al., 2009)	inactivated MEF	FBS, CS/ bFGF, SCF, IGF-I/ mLIF
(Wu et al., 2010)	CEF	FBS/ bFGF/ mLIF

Abbreviations:

[1] BRL = Buffalo rat liver cells, STO = Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant fibroblast, CEF = Chicken embryonic fibroblasts, GSC = Gonadal stromal cells, MEF = Mouse embryonic fibroblasts

[2] FBS = Fetal bovine serum, CS = Chicken serum, bFGF = Basic fibroblast growth factor, SCF + Stem cell factor, IGF-I = Insulin growth factor type I, mLIF = murine Leukaemia inhibitory factor, IL = Interleukin type 11

: The production of transgenic birds

Apart from being able to carry genetic inheritance from parents to offspring, chick germ cells can also be used as a vector for creating transgenic birds. Germ cell-based transgenesis was first described by using the germinal crescent-PGCs as a target along with a replication-deficient retroviral vector (Vick et al., 1993). Lentivirus vectors have also been used successfully for chick transgenesis; moreover, this vector can also be used to introduce transgenes into gonadal-PGCs (Shin et al., 2008). Blood-PGCs were also described as a target for making transgenic chickens, using electroporation for gene transfer (van de Lavoir et al., 2006a). Methods using PGCs for creating transgenic birds have been called "embryo-mediated system" (Han, 2009). A "testis-mediated system" has also been described (Lee et al., 2006); this method is said to be advantageous because it eliminates the need for PGC retrieval and reduces the time for the test cross (Han, 2009). However, a comparison between embryo-mediated system and testis mediated system for obtaining high yields and efficient production of transgenic chickens needs further evaluation.

1.2. Stem Cells

1.2.1. Definition and terminology

What are stem cells and what makes this type of cell different from other cell types? Stem cells are defined as cells that are able to self-renew and proliferate indefinitely without becoming malignant or having any abnormalities of their phenotype or

karyotype. Some (but not all) stem cells are multipotent, meaning that they can differentiate *in vitro* into other cell types. Further, some (pluripotent) can contribute to somatic or germline cells after reinjected into the recipients and may be able to form teratocarcinomas when injected into the nude mice or other adult organisms. (Atala, 2005; Gardner and Brook, 1997; Lavial and Pain, 2010; Loeffler and Roeder, 2002; Smith, 2001).

1.2.2. Biology of stem cells: types of stem cells

Stem cells can be isolated from embryos (embryonic stem cells; ESCs) or from adult tissues (adult stem cells; ASCs). In mammals, ESCs have been derived from the inner cell mass (ICM) of the blastocyst stage while ASCs can be isolated from many adult tissues and organs including the intestine (Potten and Morris, 1988), bone marrow containing haematopoietic stem cells (Graham and Wright, 1997) and the basal layer of the skin (Watt, 1998). Due to the advances of molecular technology, the reliable marker used to identify the source of ASCs in the body is crucial. For example, it has been reported that Lgr5 is a specific marker for intestinal stem cells since Lg5 positive cells are expressed in crypt base columnar cells, suggesting that it represents the stem cell of the small intestine and colon (Barker et al., 2007). In summary, these previous reports indicated that each organ system may contain specific types of stem cells for replenishing damaged tissues or supporting tissue turnover.

1.2.2. Biology of stem cells: characteristics and properties of stem cells

Mouse embryonic stem cells (mESCs), can self-renew, contribute to somatic and germ line lineages *in vivo* and *in vitro* and form teratomas. Self-renewal is the ability of stem cells to divide indifnitely either *in vivo* or *in vitro* giving rise to new identical cells without an alteration of genotype or phenotype. ESCs are pluripotent (or even totipotent): they are able to differentiate into many cell types, including derivatives of any of the three embryonic germ layers (ectoderm, mesoderm and endoderm). *In vivo,*ESCs can contribute to any cell type in embryos and also to germline cells after injection into blastocyst stage recipient embryos. Finally, ESCs have the ability to form tumours (teratocarcinomas) when injected into the adult (i.e. nude mice whose their immune system has been suppressed).When these tumors are analyzed histologically, they exhibit different types of tissues representative of primary germ layers including ectoderm, mesoderm and endoderm.

The morphological characteristics of mESCs include having a round shape and small size, large nucleus with one or two prominent nucleoli and a small amount of cytoplasm (Robertson, 1987). In addition, biochemical characteristics of mESCs by expressing different makers have been described since mESCs were first isolated from ICM of E3.5-4.5 days post coitum (dpc) blastocyst (Evans and Kaufman, 1981; Martin, 1981). In addition, mESCs exhibit high level of endogenous alkaline phosphatase (Strickland et al., 1980) and this has been reported to be stage specific in mouse embryos (Hahnel et al., 1990). These cells express several antigenic epitopes including SSEA1, ECMA7, EMA1, EMA6, (Hahnel and Eddy, 1987; Kemler et al.,

1981; Solter and Knowles, 1978), characteristic of undifferentiated ESCs. These characteristics are used to assess the ability of the cells to self-renew and/or be able to differentiate *in vitro* (Evans and Kaufman, 1981). Growing conditions for maintaining these properties have been developed including the use of feeder layer cells and other factors such as supplemented ESC culture medium (Smith and Hooper, 1987). These studies suggested that *in vitro*, ESCs have unique morphological and biochemical characteristics accompanying the undifferentiated state, which define ESCs as distinct from other cell types. However, the evidence supporting such characteristic of ESCs *in vitro* still need to be compared to the *in vivo* situation.

1.2.2. Biology of stem cells: stem cells in vertebrates

Apart from mouse ESCs have been successfully isolated from Syrian hamster (Doetschman et al., 1988) and rat (Iannaccone et al., 1994). Embryoid body (EB) formation has been shown to be a common ES-like characteristic in both species; however, the expression of AP activity, SSEA1 expression and production of coat colour chimaeras have been only reported in rat-ESCs. The establishment of mink ESCs from 7-day blastocyts has been reported (Sukoyan et al., 1993). They can form EBs or teratomas, although *in vivo* differentiation has not been tested. In pig, pluripotent stem cells have been derived from blastocysts (Wheeler, 1994) as well as from 25-27 day genital ridges (Piedrahita et al., 1998). These cells form EBs and also express AP; moreover, coat colour chimaeras and transgenic chimaeric piglets have also been produced from them. In sheep, ESCs have been isolated from 8-day blastocysts (Wells et al., 1997). Their characteristics have been identified by morphology and cloned lambs have been created after transfer to synchronized

recipient ewes. ESCs have also been established from cattle blastocysts by fibroblast cloning (Cibelli et al., 1998) but ES-like characteristics have been defined only by morphology. However, *in vivo* differentiation of cattle ESCs has been used to produce transgenic chimaeric calves. In primates, the first primate-ESCs were isolated from 6-day blastocysts of Rhesus monkey (Thomson et al., 1995). Their ESCs characteristics were defined based on AP activity and SSEA3, SSEA4 expression and being able to differentiate *in vitro*. However, chimaera production has not yet been reported. One year later, ESCs of the common marmoset, were established from 8-day blastocysts (Thomson et al., 1996). They exhibited differentiation *in vitro*, EB and teratoma formation.The first human pluripotent stem cells were isolated from IVF blastocysts (Thomson et al., 1998); similar cells derived from 5-9 week embryonic genital ridges have been reported (Shamblott et al., 1998). These two pluripotent ESCs shared commom ES-like characteristics by having AP activity, expressing SSEA3, SSEA4. In addtiiton, pluripotent ESCs isolated from IVF blasotocysts exhibited telomerase activity while pluripotent ESCs isolated from genital ridges are capable of EB formation. Chimaeras created by these two sources of pluripotent ESCs has not yet been attempted.

In non-mammalian sepecies, isolation of ESC-like cells from Medaka fish (Hong et al., 1998) and Zebrafish (Sun et al., 1995) has been reported. They have AP activity and are able to form EB-like structures, while zebrafish ESC-like cells have been shown to be able to differentiate *in vitro*. Furthermore, transgenic chimaeric fry and juveniles have been produced by ESCs from these two species. In conclusion, vertebrates-ESCs share common ES-like characteristics including exhibiting AP activity, being able to form EB or teratoma. However, the study on the difference of

ES-like characteristics among vertebrates in terms of differential ESCs gene expression need to be further investigated.

1.2.3. Chick embryonic stem cells (ESCs): isolation, culture and characterization

Unlike murine ESCs, chick ESCs have been shown to be able to contribute only to somatic lineages but not the germline (Lavial and Pain, 2010). Chick blastodermal cells retrived from the area pellucida of stage X (Eyal-Giladi and Kochav, 1976; EG&K) embryos have been shown to be able to contribute to all somatic tissues and the germline after injection into the subgerminal cavity of stage X (EG&K) recipient embryos (Carsience et al., 1993; Kagami et al., 1995; Kino et al., 1997; Petitte et al., 1990). Thus, cESCs are more similar to murine epiblast stem cells (EpiSCs) (Lavial and Pain, 2010) than to mESCs. This could be due to the fact that stage X is relatively more advanced than the mouse ICM, from which mESCs are derived.

Chicken ESCs were first isolated from stage X blastodermal cells by culturing them on inactivated STO feeder cells (Pain et al., 1996). Blastodermal cells derived cESCs have been cultured in embryonic stem cell medium (ESA) in the presence of growth factors and cytokines including bFGF, IGF-1, mSCF, IL-6, IL-11, CNTF, OSM and LIF (Pain et al., 1996). Like mESCs, cESCs can be maintained in an undifferentiated state in the presence of LIF. It has been shown that chicken LIF maintained cESCs in such state *in vitro* (Horiuchi et al., 2006; Horiuchi et al., 2004).

Several characteristics have been shown to be shared btween cESCs and their mESC counterparts. First, alkaline phosphatase (AP) activity is exhibited by cESCs (Pain et al., 1996; van de Lavoir and Mather-Love, 2006; van de Lavoir et al., 2006b). Immunological markers are also expressed, including stage-specific embryonic antigen (SSEA) SSEA1, SSEA3 and SSEA4 (Knowles et al., 1978; Pain et al., 1996; Shevinsky et al., 1982; Solter and Knowles, 1978). Lastly, expression of chick homologues of Oct3/4 (*cPouv*) and *cNanog* has also been reported in cESCs (Lavial et al., 2007).

Being able to differentiate is one of the characteristics of ESCs; it has been reported that cESCs can successfully differentiate into nerve cells, haematopoietic cells and muscle cells and that they can also form embryoid bodies if plated onto low adherence plates in medium without LIF (Pain et al., 1996). Moreover, the removal of LIF from culture medium causes loss of SSEA1 (Pain et al., 1996) and pluripotent stem cell markers *cPouV* and *cNanog* expression (Lavial et al., 2007).

The most important characteristics of ESCs is somatic and germline contribution. Although freshly isolated chicken blastodermal cells have been shown to be able to contribute extensively to the somatic as well as the germline after injected into the subgerminal cavity of stage X (EG&K) recipient embryos (Carsience et al., 1993; Kagami et al., 1995; Kino et al., 1997; Petitte et al., 1990), cESCs can only produce somatic chimaeras (Pain et al., 1996; van de Lavoir et al., 2006b). The possible explanation of the failure to colonize the germline may be an inherent attribute of the cells, a consequence of the very rapid pace at which the germline segregates from the somatic tissues and predetermination of the germline in birds (van de Lavoir et al.,

2006b). The reasons why cESCs cannot contribute to the germline need to be investigated.

1.2.4. Evolution of stem cells: stem cells in lower organisms and regeneration

What is the function of stem cells in lower organisms and when are the stem cells established in those organisms? Do stem cells in such organisms differ from those of higher organisms? The evolutionary origin of stem cells based on molecular and cellular bases still remains unclear. However, it has been proposed that adult stem cells of lower organisms support asexual reproduction (Agata et al., 2006).

In Planarians, it has been reported that stem cells called "neoblasts" can give rise to all cell types (Slack, 2011). Planarains contain neoblast into adulthood to regenerate missing body parts by fission which is essential for asexual reproduction in planarians (Agata et al., 2006). Moreover, their stem cells located in the internal mesenchymal pace from head to tail also produced epithelial cells (Hori, 1978). Interestingly, *Hox* genes are increasingly expressed along a spatial gradient in the posterior region of this animal. Moreover, its expression has been rearranged along the anterior-posterior axis during regerneration, suggesting that *Hox* genes may be involved in the regulation of differentiation of pluripotent stem cells in a position-dependent manner (Orii et al., 1999). This evidence comes from grafting experiments, suggesting that intercalation between dorsal and ventral positions induces blastema formation (Kobayashi et al., 1999) and that anterior–posterior intercalation may be essential for rearrangement of *Hox* gene expression (Agata et al., 2003).

Hydra reproduces asexually by budding and their stem cells are called "interstitial cells" since they have been found to locate between ectoderm and endoderm (Bode, 1996). These cells have been reported to differentiate into neurons, nematocytes and other cell types (Bode et al., 1987; David and Gierer, 1974; David and Murphy, 1977). The importance of these cells in *Hydra* has been demonstrated because interstitial cell-less mutants of *Hydra* were unable to catch food by themselves due to the lack of neurons and nematocytes (Marcum and Campbell, 1978; Sugiyama and Wanek, 1993).

Adult pluripotent stem cells are not restricted only to lower organisms but can be found in primitive chordates. In colonial ascidians, they are capable to proliferate asexually their colonies by budding (Nakauchi and Takeshita, 1983). It has been reported that "haemoblasts" participate in bud formation to generate a new ascidian (Kawamura and Sunanaga, 2010). The haemoblasts have been shown to be ascidian pluripotent cells by differentiating into inner epithelia and different cells types including neurons, pharynx, heart and other undifferentiated blood cells (Freeman, 1964; Kawamura et al., 1991; Kawamura and Sunanaga, 2010). However it is not known whether they can self-renew.

Interestingly, sponge, the lowest metazoan organism in animal kingdom has been found to have a stem cell system contained in "gemmules". Different types of cells have been found in primordial gemmules including "archeocytes" (which are thought to be sponge pluripotent cells), trophocytes (cells that have archeocyte features but also include numerous cytoplasmic lipid inclusions) and spongioblasts (gemmule coat forming cells). These stem cells play a role for asexual reproduction to survive

sponges from severe environment (Agata et al., 2006; Funayama et al., 2005a; Funayama et al., 2005b). These previous studies indicated that the stem cells in lower organisms are responsible for asexual reproduction to produce new offspring to ensure survival during unfavorable conditions.

1.3. Pluripotency

1.3.1. Definition and terminology

The ability of cells forming a whole conceptus is defined "totipotent", whereas "pluripotent" is defined as the ability to contribute to different tissues of the fetus and in some cases to the extraembryonic membrane when used for chimaera formation (Campbell and Wilmut, 1997) (see Table 1.2).

Table 1.2: A comparison of totipotency and pluripotency of the cells Adapted from (Campbell and Wilmut, 1997)

	Cells	
	Totipotency	**Pluripotency**
Definition	Ability to form a whole Organism	Ability to differentiate into many or all tissues including the germ line of chimaeric animals
Examples	Blastomeres of early cleavage stage embryos*	ICM, EC cells, ES cells and EG cells
Technology	Embryo splitting, blastomere separation	Aggregation with morulae, injection into blastocysts

Abbreviations:

ICM = Inner cells mass, EC = Embryonic carcinoma cells, ES = Embryonic stem cells, EG = Embryonic germ cells

* Germ line-competent ES and EG cells were also classified as totipotent by some authors.

1.3.2. Pluripotency

Pluripotency was first studied from murine germ cell tumors (or teratocarcinomas) since they have been shown to be pluripotent cells by exhibiting undifferentiated cells among many differentiated cell types derived from primary germ layers after transplanted into mouse recipients (Martin and Evans, 1975). The pluripotent cell lines isolated from these tumors have been called embryonic (embryonal) carcinoma cells (ECCs). However, disadvantages of these cells have been reported by often being aneuploid and rarely giving rise to the germline (Illmensee and Mintz, 1976).

The second type of pluripotent stem cell is called embryonic stem cells (ESCs) which are able to maintain the undifferentiated stage *in vitro*, retain the ability to form tumors upon transplantation, differentiate into other cell types and form embryoid bodies *in vitro*. The most important characteristic of these cells is their ability for somatic and germline contribution. Regarding an undifferentiated state, it has been shown that LIF (either synthetic or secreted by BRL) is responsible for maintaining ESCs in an undifferentiated state (Nichols et al., 1994; Smith and Hooper, 1987); however, LIF can be substituted by other cytokines including IL-6, oncostatin M and CNTF to maintain mouse ESCs in such state (Conover et al., 1993; Nichols et al., 1994; Rose et al., 1994; Wolf et al., 1994).

The third type of pluripotent stem cell is embryonic germ cells (EGCs). These cells can be isolated from post-migratory germ cells from 10.5-11.5 dpc embryos. Moreover, these cells can be derived *in vitro* by culturing gonadal cells with culture medium supplemented with LIF, FGF-2, SCF and plated them onto feeder cells

(Matsui et al., 1992; Resnick et al., 1992). It has been shown that these cells exhibit pluripotency since they differentiate *in vitro* and contribute somatic and germline chimaeras (Matsui et al., 1992).
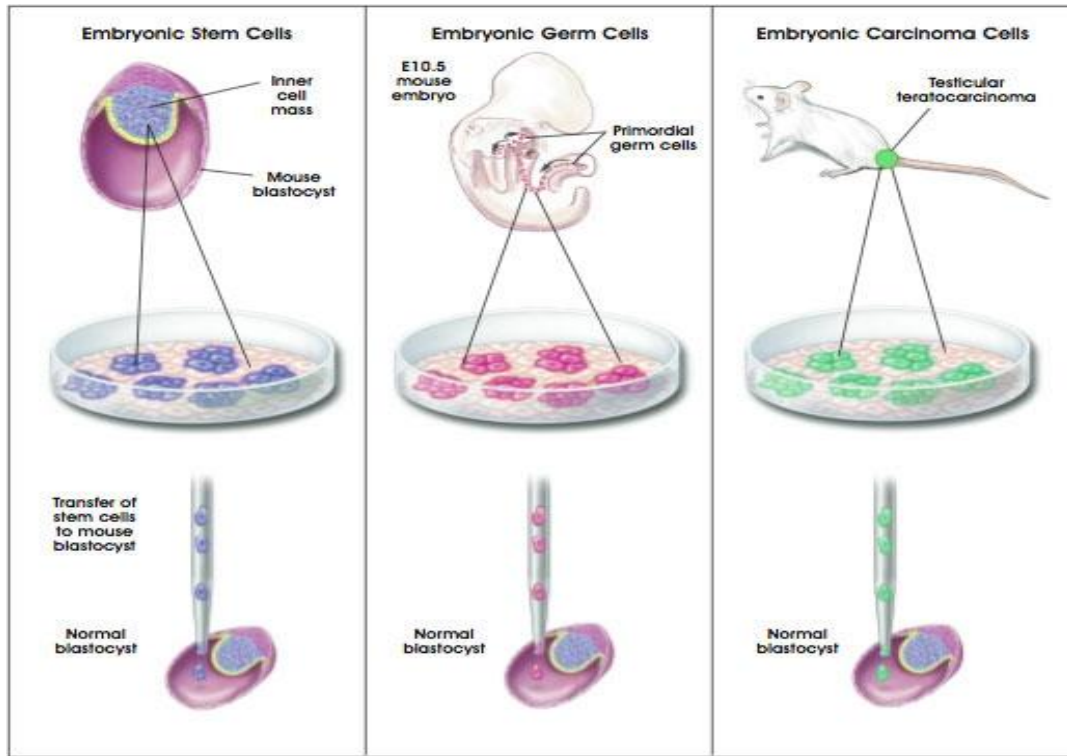
Figure 1.12: Three types of mouse pluripotent stem cells isolated from different sources such as embryonic stem cells from ICM of blastocysts, embryonic germ cells from post-migratory PGCs and embryonic carcinoma cells from germ cells tumors. (Source: http://stemcells. nih.gov/info/scireport/appendixb.asp)

In chick, pluripotent cells have been isolated from several sources (see Figure.113). The types of pluripotent cells, sources of pluripotent cells, and confirmation methods of pluripotentcy are summarized in Table 1.3.

Table 1.3: Pluripotent cells in chicken embryos

Cells types[1]	Sources[2]	Confirmation methods	References
ESCs	Stage X (EG&K)	EB formation, *in vitro* differentiation somatic chimaera	(Pain et al., 1996) (van de Lavoir et al., 2006b) (Petitte et al., 2004)
EpiSCs	Stage X-XIII (EG&K)	N.D.	(Boast and Stern, Unpublished work)
PGCs	Stage 14-17 (H&H)	germline chimaera	(van de Lavoir et al., 2006a)
EGCs	Stage 28 (H&H)	EB formation, *in vitro* differentiation somatic chimaera	(Park and Han, 2000)
GSCs/SSCs	Juvenile-6 wk old and adult (24-wk old) make roosters	EB formation, *in vitro* differentiation germline chimaera	(Lee et al., 2006) (Jung et al., 2007)
iPSCs	N.D.	N.D.	N.D.

Abbreviations:

[1] ESCs = Embryonic stem cells, PGCs = Primordial germ cells, EGCs = Embryonic germ cells, GSCs = Germline stem cells, SSCs = Spermatogonial stem cells, iPSCs = Induced pluripotent stem cells.

[2] EG&K = stage according to Eyal-Giladi and Kochav, H&H = stage according to Hamburger and Hamilton, EB = Embryoid body, N.D. = Not done.
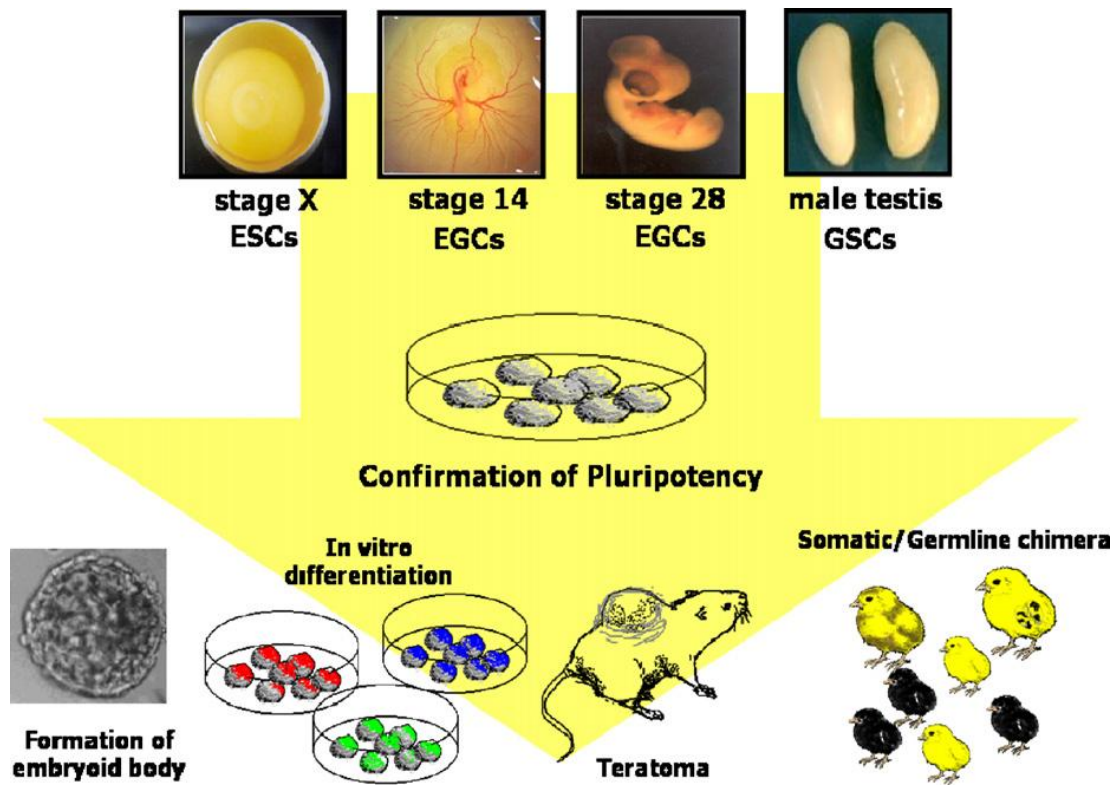
Figure 1.13: Avian pluripotent cells in different stages of development and confirmation of pluripotency. From (Han, 2009).

1.3.3. Gene and molecular mechanism regulating pluripotency

:*Oct3/4* and chicken *Oct4* homologue *cPouV*

Oct3/4 (octamer-binding transcription factor 3/4) also known as POU5F1 (POU domain, class 5, transcription factor 1) belongs to POU family members act as transcriptional repressors or activators depending on their co-factors. It has been reported that adenovirus E1A works as a co-factor of Oct3/4 by linking between Oct3/4 and its transcription machinery (Scholer et al., 1991) and Sox2. In addition, the control of Oct-3/4-Sox-2 complex identified a Sox-2 regulatory region (Tomioka et al., 2002); moreover, Oct-3/4 and Sox2 regulates the Oct-3/4 gene in embryonic stem cells (Okumura-Nakanishi et al., 2005). It has been reported that Octamania, the POU factors including Oct4 and 6, are expressed as early as in the preimplantation embryo and thus may regulate early events of murine development (Scholer, 1991). Mouse pluripotent ES cells are controlled by the POU transcription factor Oct3/4 and its expression is restricted to pluripotent cells (Pesce et al., 1998a) and downregulated during spermatogenesis and oogenesis (Pesce et al., 1998b). In addition, it has been reported that Oc3/4 is important for setting founder pluripotent cells during murine ESC establishment. (Nichols et al., 1998; Niwa et al., 2002). Moreover, Oct3/4 expression is able to prevent ESCs differentiation upon withdrawal of LIF, and if its expression maintained at a critical level, it can prevent ESC differentiation (Niwa et al., 2000). Furthermore, Oct-3/4 maintains the proliferative embryonic stem cell state via specific binding to a variant octamer sequence in the regulatory region of the UTF1 locus (Nishimoto et al., 2005). In chicken, recently, a gene named *cPouV* has been identified as the chick Oct3/4 homologue by sequence homology, synteny and

functional conservation (Lavial et al., 2007). It can rescue mouse ES cells deprived of Oct3/4 and the downregulation of *cPouV* caused cESCs differentiation (Lavial et al., 2007).

: *Nanog*

Nanog (or Tir Na Nog, after the mythological Celtic land of the 'ever young') is one of key factors expressed in pluripotent cells and downregulated during differentiation; moreover, it plays a role for the maintenance of self-renewal and pluripotency (Chambers et al., 2003; Darr and Benvenisty, 2006). This gene encoded a 26 homeodomain-containing transcription factor and has been shown to be able to maintain mESCs cell-renewal and pluripotency by inhibiting NFkappaB and cooperating with Stat3 (Torres and Watt, 2008). Interestingly, normal levels of Nanog did not prevent ESCs differentiation after withdrawal of feeders, although undifferentiated ESCs express this gene (Yasuda et al., 2006). It has been reported that Nanog expression is responsible for the maintenance of the primitive ectoderm in the mouse embryo (Mitsui et al., 2003). It has been experimentally confirmed by *in vitro* that Nanog deficient mouse ESCs slowly differentiate into extra-embryonic endoderm lineages, which correspond to the absence of a primitive ectoderm in Nanog–/– mutant (Mitsui et al., 2003). On the other hand, over-expression of Nanog renders mouse ES cells resistant to differentiation following the removal of LIF (Darr et al., 2006). Importantly, it has been reported that the level of Nanog is crucial for maintaining ESCs in an undifferentiated state since the reduction of Nanog+/- ESCs caused spontaneous differentiation in long term culture (Hatano et al., 2005). Similarly to Oct4, Nanog acts by repressing the transcription of differentiation-

promoting genes; moreover, Octamer and Sox elements are required for transcriptional cis-regulation of Nanog expression (Kuroda et al., 2005). Recently, it was reported that Nanog is required for primitive endoderm formation through a non-cell autonomous mechanism, since outgrowths of mutant ICMs give rise to PE (primitive endoderm), but not EPI (epiblast) derivatives. Surprisingly, Gata4 expression in mutant ICM cells is absent or strongly decreased, thus loss of Nanog did not result in precocious endoderm differentiation. This report proposed a non-cell autonomous requirement of Nanog for proper PE formation in addition to its essential role in EPI determination.

: *Sox2*

SRY (sex determining region Y)-box 2, also known as SOX2, is a transcription factor belonging to the SoxB1 subfamily of genes (Miyagi et al., 2009), essential to maintain self-renewal of undifferentiated embryonic stem cells. Sox2 is involved in maintaining pluripotency through Oct3/4 (Chickarmane et al., 2006). In early mouse development, Sox2 is first expressed in the Inner Cell Mass (ICM) where its role was proposed to maintain cells in undifferentiated state (Wegner, 1999; Wood and Episkopou, 1999) and in the early neural plate (Uwanogho et al., 1995; Wood and Episkopou, 1999). It has been shown that Sox2 is required for very early embryonic development since Sox2 null mutant mice faild to develop beyond implantation (Wegner, 1999). Sox2 is also expressed in three types of stem cells including neural, embryonic (ES cells) and trophoblast stem cells (Wiebe et al., 2000; Yuan et al., 1995; Zappone et al., 2000). Regarding the mechanism of action of Sox2, it has been reported that Sox2-Oct3/4 complex regulates Nanog transcription (Kuroda et al.,

2005; Rodda et al., 2005). The main role of Sox2 in mouse ES cells is maintenance of the level of Oct-3/4 expression, since Sox2-null ESCs failed to maintain pluripotency and exhibit alteration in the expression of factors acting Oct3/4 upstream (Gu et al., 2005; Schoorlemmer et al., 1994). In conclusion, it seems that the key stem cell regulators bind and regulate genes encoding other transcriptional regulators then allow the determination of developmental potency of pluripotent stem cells.

ERNI :

ERNI, an early response gene to signals from the organizer (Hensen's node), was first used as marker to show that neural induction begins before gastrulation in chick embryos (Streit et al., 2000). The same gene was also isolated from chick ES cells by a gene trap strategy, and suggested to define a novel gene family named *cENS* (chicken Embryonic Normal Stem cells gene) (Acloque et al., 2001). It has been reported that expression of cENS-1/cERNI genes are expressed very early during chicken embryonic development as well as in pluripotent chicken embryonic stem (CES) cells. The regulation of these genes has been studied molecularily by identifying a promoter region, which is specifically active in cESCs compared to differentiated cells (Acloque et al., 2004). The results have been used to demonstrate that mutation of the B region in the cENS-1 promoter strongly decreases promoter activity in CES cells, suggesting that this region is essential for activating transcription. In addition, a cESCs line exhibiting high-grade transgenic somatic

70

chimaera contribution also expresses *ERNI* (van de Lavoir et al., 2006b). These results suggest that *ERNI* expression is a marker of chick pluripotent embryonic cells.

1.3.4. Evolution of pluripotency

The concept of totipotency/pluripotency arises from the classical experimental embryology experiments done by (Driesch, 1891) on sea urchin embryos. This experiment demonstrated when a sea urchin embryo at the 2 cell stage is cut in half (separating the two blastomeres), both halves of the cut embryo develop into complete larvae. The same result is obtained in newt embryos of up to 16 blastomeres: the cut embryo regenerated two complete embryos (Spemann, 1902). These studies first established that cells can have a range of differentiated repertoires ("potency") greater than their normal fate.

The study of evolution of pluripotency need other information from different fields, Recently, the expression of genes associated with a pluripotency gene regulatory netwok has been studied in mammals and birds using *in situ* hybridisation, microarrays and bioinformatics (Fernandez-Tresguerres et al., 2010). The authors claim that multiple components of this network are either novel to mammals or have acquired novel expression domains in early stage of mouse embryos; moreover, the downstream action of pluripotency factors is mediated largely by genomic sequence elements that are not conserved in chick. Furthermore, elements driving expression of *Sox2* and *Fgf4* are proposed to have evolved by the change of a small number of nucleotides and the authors proposed that the network in charge of embryonic pluripotency was an evolutionary novelty of mammals (Fernandez-Tresguerres et al.,

2010). However it is important to note that these conclusions about pluripotency are based entirely on gene expression and prediction of regulatory elements rather than on any functional studies.

The evolution of regulatory elements driving expression of Oct4, Sox2 and Nanog, has also been studied by bioinformatics. It was proposed that these genes share an ancient evolutionary origin because they are highly conserved (Fuellen, 2011; Fuellen and Struckmann, 2010).

However, a combination of integrated results done by multidisciplinary works such as developmental biology, molecular biology and bioinformatics to answer the evolution of the embryonic pluripotency gene regulatory network needs further investigation.

1.4.   Aims of this thesis

Chick embryos are a powerful model to study developmental biology, including stem cell biology (Stern, 2005). Little is known about the biology of chick stem cells regarding the similarity and/or difference between chicken ES cells and germ cells. cES cells have been shown to be able to contribute only to the somatic but not the germ line (Pain et al., 1996), while chick germ cells do contribute to the germ line (van de Lavoir et al., 2006a). Since both ES and EG cells are generally considered to be pluripotent (Petitte et al., 2004), the similarity and difference of these two types of cells needed to be studied. Chicken germ cells have unique characteristics including that they migrate via the embryonic blood circulation before settling in the gonads

(Niewkoop and Sutasurya, 1979). In addition, the right ovary regresses in most female birds with only the left ovary remaining functional in the adult. It is therefore clear that the biology of germ cells in the chick system requires further study.

Taking advantage of the availability of established chicken ES cells, gonadal-derived PG cells and primary gonocytes, we asked the question of to what extent these lines differ from each other either *in vivo* (embryos) or *in vitro* (cells). To compare these scenarios, we used *in situ* hybridisation rather than the more commonly used RT-PCR methods. *In situ* hybridisation allows us to see the localization of genes in specific cells rather than in the population as a whole, as well as to discriminate expression within specific regions within organs like the embryonic gonads. Moreover, this method provides information about whether the cells are homogeneous or heterogeneous in terms of gene expression both *in vivo* and *in vitro*. Here we undertook an extensive comparison of gene expression *in vivo* and *in vitro* focusing on a number of genes usually thought to mark pluripotent cells (*cPouV*, *cNanog*, *cSox2* and *ERNI*), germ cell markers (*Cvh* and *cDazl*) and a number of genes expressed by embryonic cells in various states of differentiation.

This thesis has the following major aims:

1) To compare different methods for deriving cell lines from chicken embryonic germ cells from different embryonic sources.

2) To investigate whether placing germ cells in culture is accompanied by characteristic changes in gene expression of pluripotency-associated and other genes.

3) To compare the gene expression profiles of cultured embryonic germ cells with those of the parent gonadal tissue.

4) To investigate whether there are left-right and/or male-female differences in gene expression in embryonic gonads and whether any such differences reflect the ability of cells obtained from such gonads to grow *in vitro*.

Chapter 1 briefly reviews the literature by introducing chicken germ cells in general with a focus on cES cells. The concept of pluripotency is also introduced in Chapter 1. Chapter 2 summarises the common experimental methods used in this thesis, including the methods used for isolation and culture of chicken embryonic germ cells (gonocytes). More specific methods are described within individual chapters. Chapter 4 then examines the dynamics of changes of gene expression of stem cell- and pluripotency-associated genes during derivation of embryonic germ cells from chicken gonocytes. Chapter 5 provides a comparison *in vivo* and *in vitro* of pluripotent stem cell markers in embryonic gonads and primary cultures, which is extended in Chapter 5 to a fuller analysis of the dynamics of gene expression in gonads, ES, PG cells and primary gonocytes. Chapter 6 then compares both the expression of pluripotency-associated markers and differences in growth rate between left and right and male and female gonads and cultured cells derived from them.

Chapter 2. Methods

2.1. Isolation of chicken gonadal stromal cells (GSCs) and chicken embryonic fibroblasts (CEFs)

To isolate GSCs, the gonads were dissected from the embryos at stage 26-28 (Hamburger and Hamilton, 1951) (5-6 days of incubation). Cells were dissociated with 0.25% trypsin/0.025% EDTA solution (GIBCO™, UK) at room temperature for 5 min and then centrifuged at 200g for 5 min (Park and Han, 2000). The cell suspension containing both PGCs and somatic cells (including GSCs) was seeded onto gelatin-coated 24-well plates (Becton Dickinson) and cultured with GSCs culture medium consisting of DMEM (GIBCO Invitrogen) medium supplemented with 10% FBS (GIBCO), 1 mM sodium pyruvate (GIBCO), 2 mM L-glutamine (GIBCO), 0.16 mM β-mercaptoethanol (Chemicon), 1% MEM Non-Essential Amino Acids 10 mM (100X) (GIBCO). The 24-well plates were incubated at $39.5^{o}C$ in 5% CO_2 until somatic cells (GSCs) had formed a monolayer. For subculture, gPGC colonies were dislodged by washing 3 times with Ca/Mg-free PBS. The GSC monolayer was detached with 0.05% trypsin-EDTA (GIBCO) at room temperature for 10 min and dislodged by gentle pipetting. Cells were resuspended in GSC culture medium to make 1:2 dilution and then seeded onto 100 x 20 mm tissue culture dishes (Falcon) and incubated at $39.5^{o}C$ in 5% CO_2 until confluent.

The method used for isolating CEFs was based on a modified version of the protocol for isolation and handling of primary mouse embryonic fibroblasts (MEFs) by Matise

et al. (2000). The culture medium used for isolating primary CEFs (CEF medium) consisted of DMEM (GIBCO) medium supplemented with 10% FBS (GIBCO). To isolate CEFs, two 50 ml tubes of DMEM medium (GIBCO) were prepared. Another two tubes of 50 ml CEF culture medium containing 500 μl 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO) were also prepared and kept at room temperature before use. The eggs were opened and the embryos placed into a 100 x 20 mm tissue plastic dish. The embryos were cleaned by washing in Tyrode's solution (see preparation below). The body of the embryo was placed into another dish containing DMEM. The viscera were removed using sterile forceps. The remaining carcass was transferred to a new sterile 100 x 20 mm dish containing DMEM and cut into small pieces with sterile scissors. The tissues were dissociated mechanically using a 21G sterile needle attached to a 10 ml syringe. The tissue homogenate was divided into two groups (destined for separate treatments, with and without trypsin). 5 ml of this was collected and into a 50 ml tube containing 0.25% trypsin/0.025% EDTA solution (GIBCO™, UK) for the trypsin treatment group. The tube was incubated at 39.5 ^{o}C in 5% CO_2 for 30 mins. The remaining tissue (about 5 ml) was placed directly into a tissue culture dish containing 20-25 ml CEF culture medium, 500 μl 100 U/ml penicillin and 100 mg/ml streptomycin and 0.25% trypsin/0.025% EDTA solution (GIBCO). After 30 mins, the 50 ml tube containing trypsinized tissues was dissociated by pipetting up and down vigorously and then divided into two tubes (15 ml per tube), which were then centrifuged at 1,100 rpm for 10 mins. The supernatant was removed and the pellet resuspended in CEF culture medium. The cell suspension was seeded onto a tissue culture dish (Becton Dickinson, USA) containing 20-25 ml CEF culture medium, 500 μl 100 U/ml penicillin and 100 mg/ml streptomycin and incubated at 39.5^{o}C in 5% CO_2 overnight. For subculture, the CEF monolayer was

rinsed by washing with Ca/Mg free PBS twice. Cells were detached with 0.25% trypsin-EDTA at room temperature for 5 min., resuspended in 5 ml CEF culture medium and then dissociated by gentle pipetting. Cells were seeded onto 100 x 20 mm tissue culture dishes at 1:5 or 1:10 dilution and incubated at 39.5^{o}C in 5% CO_2 until confluent. For cryopreservation, after centrifugation, the pellet was resuspended in freezing medium consisting of 10% Dimethyl Sufoxide (Sigma-Aldrich, USA), 50% FBS (GIBCO™, UK) up to volume with DMEM medium (GIBCO™, UK). The pellet was agitated by gentle pipetting and cells ($1x10^6$ cells/100 mm tissue culture dish) were aliquotted into cryogenic vials (Nalgene, USA) (1 ml per vial). Cells were frozen at -80 ^{o}C and then kept in liquid nitrogen tank.

2.2. Preparation of culture dishes and feeder cells

Tissue culture dishes were coated with 0.1% gelatin (Millipore, USA). 0.5 ml of the gelatin solution was added to each well of 48 well dishes and 1 ml/well for 24 well dishes. The gelatin-coated dishes were incubated at room temperature for 30 minutes, and the gelatin then removed from each well by vacuum suction just before use.

STO feeder cells (American Type Culture Collection No. CRL 1503) used for culturing cESCs were mitotically inactivated with Mitomycin C (10 μg/ml; Sigma) for 1.5 h at 37 °C and then rinsed 3 times with 1X Ca/Mg free PBS before use. The cells were detached from the culture dishes using 0.25% trypsin/0.025% EDTA solution and incubated at 37°C for 5 mins until the feeder cells detached from the culture dishe. The trypsin was inactivated by adding 5 ml of STO culture medium (DMEM containing 10% FBS and 2 mM L-glutamine) to the culture dishes. The cells

were resuspended in STO culture medium and then transferred to a 15 ml tube and washed by centrifugation at 1,100 rpm for 10 minutes. The supernatant was aspirated, the pellet resuspended in STO culture medium and the cells counted with a haemocytometer . The cells were seeded on gelatin-coated dishes at a density of $1x10^5$ cells/ml as described above and incubated at 37^oC in 5% CO_2 for one to two days before use.

BRL-3A feeder cells (American Type Culture Collection No. 43 CRL 1442) used for culturing cPGCs were mitotically inactivated by irradiation according to Crystal Bioscience Standard Operating Protocol (M.C. van de Lavoir, personal communication) (BRL irradiation Number 215-01). On day 1, cryopreserved BRL cells were thawed and transferred to a 15 ml centrifuge tube. The cells were centrifuged for 10 mins at 1,000 rpm and the pellet was seeded onto six tissue culture dishes with 20 mm at a density of $1.5-1.6x10^7$ cells/cellstack. The cells were cultured for 5 days and incubated at 37.0^oC in 5% CO_2 until confluent. On day 5, confluent cells were washed twice with Ca/Mg-free PBS and then trypsinized. The cell suspension was transferred to 50 ml tubes and centrifuged for 6 mins at 1,000 rpm. The pellet was resuspended in 20 ml manipulation medium (CO_2 independent medium (GIBCO) containing 10% FBS, 2mM Glutamax and 1% Pen/Strep). The cells were adjusted to a density of $2-3x10^8$ cells/stack and transferred to 50 ml tubes for X-irradiation at 5,000-10,000 rads for 1 hr. After irradiation, cells were centrifuged for 6 mins at 1,000 rpm. The pellet was seeded onto 100 x 20 mm tissue culture dishes and incubated at 37.0^oC in 5% CO_2. After 24 hr, the cells had usually formed a monolayer. The medium was changed every few days and monitored for breakthrough growth.

GSC and CEF feeder cells used for culturing EGCs were prepared without Mitomycin C treatment. The feeder cells were maintained at 39.5°C in 5.0% CO_2 in GSC culture medium consisting of DMEM supplemented with 10% FBS and 2 mM L-glutamine. The cells were detached from the culture dishes using 0.25% trypsin/0.025% EDTA solution and incubated at 37°C for 5 mins. The trypsin was inactivated by adding 5 ml of GSC culture medium as described above, the cells resuspended in GSC culture medium, transferred to a 15 ml tube and washed by centrifugation at 1,100 rpm for 5 minutes. The supernatant was removed, the pellet resuspended in GSC culture medium and the cells counted with a haemocytometer. GSC feeder cells at a density of 2.5×10^4 cells/ml were seeded onto gelatin-coated 24 well dishes while CEF feeder cells at density of 2.5×10^3 cells/ml were seeded onto gelatin-coated 48 well dishes and incubated at 37.0^oC in 5% CO_2 for one day before use.

2.3. Preparation of media for culturing chicken embryonic stem cells (cESCs), chicken primordial germ cells (cPGCs) and chicken embryonic germ cells (cEGCs)

Culture medium for cESC (ESM) was prepared by conditioning Buffalo Rat Liver (BRL) cells (American Type Culture Collection No. 43 CRL 1442). First, BRL-3A cells were cultured in DMEM containing 10% FBS and 2 mM L-glutamine. BRL cells were grown for three to four days to confluence, the primary medium replaced with knockout DMEM containing 5% FBS and 2mM L-glutamine and cells cultured at 37°C in 5% CO_2 for 3 days. Thereafter, the conditioned medium was removed after three days then put into a 50 ml tube and stored at 4°C. The collection of BRL conditioned medium was repeated for another two new batches following the same

steps as above. To make complete medium, BRL conditioned medium was filtered through a 0.2 μM filter (Thermo Scientific) and diluted to 50% or 80% with knockout DMEM with 15% FBS and supplemented with 1 mM sodium pyruvate, 1% MEM Non-Essential Amino Acids Solution 10 mM (100X), 1% MEM Vitamin Solution (100X) (GIBCO), 1 mM of each nucleotide (adenosine, guanidine, cytosine, uridine, thymidine; Chemicon), 0.16 mM β-mercaptoethanol (Chemicon), 100 U/ml penicillin and 100 mg/ml streptomycin. The medium was stored at 4°C and used within 7 days.

To prepare culture medium for cPGCs (PGM), we followed a protocol provided by Crystal Bioscience (M.C. van de Lavoir, personal communication). PGM was prepared by conditioning Buffalo Rat Liver (BRL) cells to make the conditioned medium which is similar to ESM except for some minor changes described below. On day 1, BRL-3A cells were conditioned in Cell Growth Medium containing DMEM supplemented with 10% FBS and 2 mM L-glutamine and 1% penicillin/streptomycin and maintained for three days to confluence. On day 4, the primary medium was replaced with Cell Growth Medium containing DMEM supplemented with 5% FBS and 2 mM L-glutamine and 1% penicillin/streptomycin and maintained for 4 days to confluence. The confluent cells were dissociated with 0.25% trypsin-EDTA and then seeded onto three tissue culture dishes with 20 mm. Grid to make three batches of BRL conditioned medium and maintained for 4 days to confluence. On day 8, three batches of BRL cells were cultured in KO-DMEM conditioning medium containing 5% FBS and 2 mM L-glutamine medium for three days; conditioned medium was collected three times. BRL conditioned media were stored at 4°C before use. cPGC culture medium (PGM) consists of 35% KO-DMEM conditioned medium as described above, 52.5% KO-DMEM, 7.5% FBS, 2.5% Chicken serum (GIBCO), 2

mM L-glutamine , 1 mM sodium pyruvate, 1x MEM Non-Essential Amino Acids Solution 10 mM (100X), 0.1 mM β-mercaptoethanol, 4 ng/ml recombinant human fibroblast growth factor (rhFGF) (R&D Systems) and 6 ng/ml recombinant mouse stem cell factor (rmSCF) (R&D Systems). The medium was filtered through a 0.2 μM filter (Thermo Scientific) and aliquotted into 50 ml tubes. The medium was stored at 4°C for up to 7 days. Before use, 16 μl of SCF and 16 μl of FGF were added to a 50 ml tube.

To prepare cEGC culture medium (EGM), we followed a previously described protocol (Park and Han, 2000). cEGCs were cultured and maintained in EGM containing DMEM, 10% FBS, 2.5% Chicken serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X MEM Non-Essential Amino Acids Solution 10 mM (100X), 0.1 mM β-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin, 1 M HEPES, 5 ng/ml recombinant human stem cell factor rhSCF, 10 U/ml murine leukemia inhibiting factor (mLIF) (Chemicon), 10 ng/ml basic-fibroblast growth factor (bFGF) (R&D Systems), 0.04 ng/ml human interleukin-11 (hIL-11) (R&D Systems), and 10 ng/ml insulin-like growth factor-I (IGF-I) (R&D Systems).

2.4. Culture and maintenance of cESCs, cPGCs and cEGCs

To culture and maintain cESCs, cryo-preserved cESCs from an established line (9N2, generated and kindly provided by Dr. Bertrand Pain) (Pain et al., 1996) were thawed and maintained in culture according to published protocols (Pain et al., 1996; van de Lavoir and Mather-Love, 2006). The cells were grown on a mitotically inactivated STO feeder layer with a concentration of cESCs : STO of 10:1. When cESCs reached

80–100% confluence they were passaged in a 1:2 – 1:3 ratio. To maintain the cell line and prevent differentiation, the cells were passaged by transferring 20% of the ESM from the old well to the new well covered with the new STO feeder layer. The cells were washed in Ca/Mg free PBS for 1 min and then dissociated mechanically by pipetting up and down gently with a 1 ml Gilson pipette tip. 80% of the new ESM was added into the new well (1 ml per well in total). The old well was replenished by adding 1 ml the new ESM. The viability and morphology of cESCs was assessed daily under an Axiovert 100 inverted microscope (Zeiss) to confirm that the cells had not differentiated, using the criterion of a large nucleus with a prominent nucleolus and relatively little cytoplasm (Figure 2.1).

To culture and maintain cPGCs, cryo-preserved cells from several established lines (527 [gonadal-derived], 162 [blood-derived] and NuGFP-02 [a blood-derived line which did not contribute to the germ line], generated and kindly provided by Dr Marie-Cécile van de Lavoir, Crystal Bioscience, California, USA) (Figure 2.1) were thawed and maintained in culture according to published protocols (van de Lavoir et al., 2006). For subculture, the cells and medium were agitated gently and transferred to centrifuge tubes. The cells were spun at 300 g for 5 min, the pellet resuspended in PGM and seeded onto new wells containing a feeder layer of irradiated BRL cells at a concentration of 25,000 cells cm^2. For cryopreservation, the cells were resuspended in freezing medium containing 10% FCS, 1.0% penicillin/streptomycin and 10% DMSO. The vials were frozen at -80 ^{o}C and transferred to liquid Nitrogen after 24 hr.

To culture and maintain cEGCs, cells were cultured in EGM. For primary culture, gonocytes and somatic cells (gonadal stromal cells) were seeded onto 24 well plates

and incubated at 39.5 ^{o}C in 5% CO_2 until the somatic cells (GSCs) had formed a monolayer and gonocytes had colonized the top of the GSCs. For subculture, the colonies of gonocytes were agitated by gentle pipetting without trypsin-EDTA treatment and then seeded onto mitomycin C-treated (10 µg/ml; Sigma-Aldrich) inactivated chicken embryonic fibroblast (CEF) feeders. For long term culture, gonocytes derived cEGCs were grown on GSC feeder cells without mitomycin C-treatment (Figure 2.1). Cells were passaged when confluent, 1:2 dilution, as described above for cESCs.
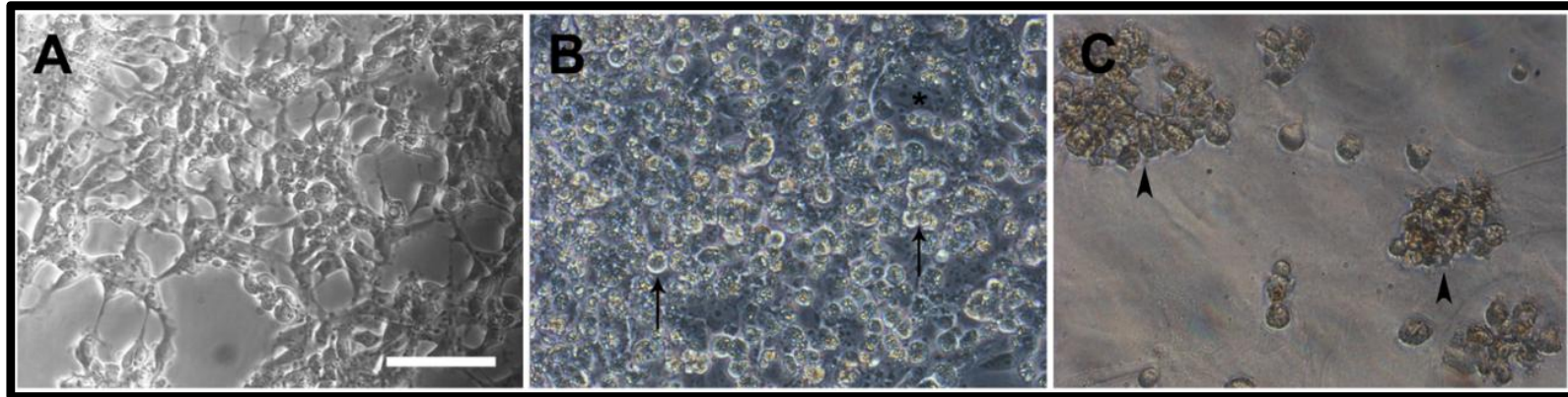
Figure 2.1: Morphology of cESCs cPGCs and cEGCs:

A: the established cESCs line 9N2 (Pain et al., 1996) has typical characteristics of undifferentiated embryonic stem cells with prominent large nucleus and relatively little cytoplasm. B: the established cPGCs line NuGFP-02, isolated from embryonic blood (van de Lavoir et al., 2006) can easily be distinguished from BRL feeder cells (asterisk) by having large cells with large nuclei and refractive granules in the cytoplasm (arrows). C: long term cultured gonocytes derived from EGCs formed colonies that were multi-layered and well delineated (arrowheads). (Scale bar = 50 μm).

2.5. *In situ* hybridization (ISH) of chick embryonic stem cells (cESCs)

2.5.1. Transcription of DIG-riboprobe

To make DIG-riboprobe, vectors, restriction enzymes, RNA-polymerases and transcription temperature used in this study were described in the table below. Briefly, vectors were cut with the appropriate restriction enzyme for 4-5 hours or overnight at $37^{o}C$ and plasmid DNA was checked by agarose gel electrophoresis. The DNA was then extracted with Phenol:Chloroform followed by Sodium-Acetate/Ethanol precipitation after which the DNA was dissolved at about 1mg/ml. The DIG-riboprobe was then transcribed with the appropriate enzyme (T3, T7 or SP6) at $37^{o}C$ for 2 hours (for SP6 transcription 2-3 times the amount of DNA template was used and transcription done at $40^{o}C$). The remaining DNA template was then digested with DNase I for 30 minutes and the DIG-riboprobes checked by agarose gel electrophoresis. The riboprobe was precipitated with Lithium Chloride (LiCl) and ethanol, washed in 70% ethanol and re-dissolved in water at about 1 mg/ml. Then, riboprobe was diluted about 5-10x (to 100-200 μg/ml) in hybridization buffer and kept at $-20^{o}C$ for use multiple times.

Table 2.1: Gene markers used for *in vivo* and *in vitro* comparison of gene expression in chicken embryonic gonads, primary gonocytes, primordial germ cells and embryonic stem cells.

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
BERT	Chick BERT	*EcoRI*	T3		(Papanayot ou et al., 2008)
cBMP4	Chick BMP4	*BamHI*	T3		(Streit and Stern, 1999)
pFLBMP8-1	Chick BMP8	*NcoI*	T7		(Lavial et al., 2007)
***cBra9* (mesoderm)**	Chick Brachyury	*XbaI*	T3	Gift from V. Cunliffe	(Smith et al., 1991)
***cCdx* (extra-Embryonic, caudal)**	Chick Cdx2	*ClaI*	T3		(Pernaute et al., 2010)
***cChCh* (early neural plate)**	Chick Churchill	*XhoI*	T3		(Sheng et al., 2003)
***Connexin43* (gap junctions**					
pFLCripto2	Chick Cripto	*SacII*	SP6		(Lawson et al., 2001)
pFLEomes	Chick Eomeso dermin	*SalI*	T7		(Pernaute et al., 2010)
ERNI Wpst	Subclone for ERNI for ISH	*KpnI*	T3		(Streit et al., 2000)
***cGata2* (epidermis)**	Chick Gata2	*NdeI*	T7		(Sheng and Stern, 1999)

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
cGata6	Chick Gata6	*NcoI*	SP6		(Chapman et al., 2007)
cGeminin	Chick Geminin	*XhoI*	T7		(Papanayot ou et al., 2008)
HP1-α	Chick HP1-alpha				(Papanayot ou et al., 2008)
cKlf2	Chick Klf4	*NotI*	T3	Gift from P. Antin	(Antin et al., 2010)
cKlf4 ("Yamanaka factor")	Chick Klf4	*NotI*	T3	Gift from P. Antin	(Antin et al., 2010)
pFL Nanog ("Yamanaka factor")	Chick Nanog	*ApaI*	SP6		(Lavial et al., 2007)
pFLIp06 ("Yamanaka factor")	Chick Oct3/4 homologue	*ApaI*	SP6		(Lavial et al., 2007)
cOtx2 (early embryo organier, prosencephalon)	Chick Otx2	*XhoI*	T3	Gift from L. Bally-Cuif	(Bally-Cuif et al., 1995)
cPdx1 (endoderm)	Chick Pdx	*HindIII*	T3	Gift from Grapin Lab	
cRunx2	Chick Runx2	*XhoI*	SP6	Gift from A.H. Monsoro-Burq	(Holleville et al., 2007)
cSox1 (mature neural plate)	Chick Sox1	*XhoI*	T7	Gift from H. Kondoh	(Kamachi et al., 1998)

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
cSox2 ("Yamanaka factor", neural plate)	Chick Sox2	*PstI*	T7	Gift from P. Scotting	(Uwanogho et al., 1995)
cSox3 (pre-neural)	Chick Sox3	*PstI*	T7	Gift from P. Scotting	(Uwanogho et al., 1995)
pBSXsox17α (endoderm)	Chick Sox17	*SmaI*	T7	Gift from Woodland	Hudson et al., 1997
cSlug (neural crest; ingressing cells)	Chick Snail-2	*NotI*	T3		(Sefton et al., 1998)
cSna (neural crest; ingressing cells)	Chick Snail-1	*NotI*	T3		(Sefton et al., 1998)
cTbx3	Chick Tbx3	*XhoI*	T3	Gift from C. Tickle	(Tumpel et al., 2002)
Cvh* (germ cell marker)	Chick Vasa Homologue	*NcoI*	SP6		(Tsunekawa et al., 2000)
cDAZL* (germ cell marker)	Chick Deleted in Azoospermia Like	*NdeI*	T7	Gift from J. Petitte	(Rengaraj et al., 2010)

(* = not tested in early embryos but specific patterns shown in embryonic gonads).

2.5.2. Preparation of cESCs, cPGCs and cEGCs for ISH

Cells were fixed in freshly made 4% paraformaldehyde (PFA) in PBS and EGTA overnight at 4°C. PFA was then replaced with absolute methanol and cells kept for up to 1 week at -20°C. For longer storage before hybridisation, cells were taken through day 1 of the *in situ* protocol and stored in pre-hybridisation mix at -20°C until required. The ISH procedure is a modification of the standard method used for whole mounts of embryos (Stern, 1998) and is described below.

DAY 1:

- The cells were rehydrated through graded series of methanol (75%, 50% and 25%, respectively) in Ca/Mg-free PBS containing 0.1% Tween-20 (PTW) at room temperature and washed twice with PTW at room temperature for 5 min.

- Cells were then post-fixed for 30 minutes at room temperature in 4% paraformaldehyde in PTW containing 0.1% glutaraldehyde and rinsed three times with PTW at room temperature.

- Cells were then washed twice with hybridization solution (see Table 2 for composition) before incubation in a hybridisation oven at 68°C for 2 hours. The hybridization mix was then replaced with the appropriate pre-warmed probe in hybridization mix and left to hybridise in the oven at 68°C overnight.

Table 2.2: Composition of the hybridization solution

Component (stock conc.)	Final concentration	Volume to add
Formamide	50%	25 ml
SSC (20x, pH 5.3 adjusted with citric acid)	1.3x SSC	3.25 ml
EDTA (0.5M, pH 8.0)	5 mM	0.5 ml
Yeast RNA (20mg/ml)	50 µg/ml	125 µl
Tween-20	0.002	100 µl
CHAPS (10%)	0.005	2.5 ml
Heparin (50 mg/ml)	100 µg/ml	100 µl
H_2O		~18.4 ml
Total:		50 ml

DAY 2:

- The cells were rinsed once and washed twice (30 min each) in pre-warmed hybridization solution at 68°C, then a further 20 min in 1:1 hybridization solution: TBST (See Table 3 for composition) at 68°C, followed by 3 1-hour washes in TBST at room temperature.

- Cells were then incubated in blocking buffer (TBST containing 5% heat inactivated sheep serum and 1 mg/ml BSA) for 1 hour before incubation overnight at 4°C in a 1:5,000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche) in blocking buffer.

Table 2.3: Composition of 10x TBST

NaCl	8 g
KCl	0.2 g
1M Tris-HCl pH 7.5	25 ml
Tween-20	11 g
H_2O	~64 ml
Total:	**100 ml**

DAY 3:

- Cells were rinsed three times and then washed three times (one hour each) in TBST.

- After two 10 min washes in NTMT (See Table 4 for composition), alkaline phosphatase activity was revealed by incubation at room temperature 60 in NTMT containing 4.5 μl nitro-blue Tetrazolium (NBT; 75mg/ml in 70% DMF) and 3.5 μl bromo-chloro-indole phosphate (BCIP; 50mg/ml in 100% DMF) per 1.5 ml. Staining required between 15 min and occasionally up to 48 hours at room temperature.

- The staining reaction was then stopped by washing twice for 10 min in TBST. The resulting stained cultures were photographed using bright field microscopy.

Table 2.4: Composition of NTMT

5M NaCl	1 ml
2M Tris HCl (pH 9.5)	2.5 ml
2M $MgCl_2$	1.25 ml
10% Tween-20	5 ml
H_2O	44.75 ml
Total:	**50 ml**

2.5.3. Whole-mount in situ hybridization of embryonic gonads

Chicken embryonic gonads at Hamburger and Hamilton (1951) (HH) stages 26-28 (5.5-6 days' incubation) and 35 (9 days' incubation) were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4 ^{o}C. The fixed embryos were then transferred to absolute methanol and embryos stored in this for up to 1 week (or in hybridisation mix for longer; see above) at -20°C. On day 1, the gonads were rehydrated through graded series of methanol, 75%, 50% and 25% , respectively in Ca/Mg free PBS containing 0.1% Tween-20 (PTW) at room temperature and washed three times with PTW at room temperature for 5 min. The gonads were then post-fixed for 30 minutes at room temperature in 4% paraformaldehyde in PTW containing 0.1% glutaraldehyde and rinsed twice with PTW at room temperature. The gonads were then washed three times with hybridization solution (See Table 4 for composition) for 1 hour each at room temperature before incubation in a water bath at 70°C for at least 3 hours. The hybridization mix was then replaced with the

appropriate pre-warmed probe in hybridization mix and left to incubate in the oven at 70°C overnight. In subsequent days the protocol was similar to that described above for cell cultures and as described in (Stern, 1998) for whole mounts of embryos.

2.6. Immunoperoxidase and immunofluorescence staining of cESCs, cPGCs and cEGCs

2.6.1. Immunoperoxidase staining of cESCs, cPGCs and cEGCs

Chicken embryonic stem cells (cESCs), primordial germ cells (cPGCs) and embryonic germ cells (cEGCs) were fixed with 4% PFA in 48 or 24 well plates at 4 $^{\circ}$C for 15 min (Stern and Holland, 1993). Fixed cells were washed three times for 5 mins each with Ca/Mg-free PBS, incubated in 3% hydrogen peroxide (H_2O_2) in methanol at room temperature for 20 min and washed three times for 5 mins each with Ca/Mg-free PBS. They were then placed in blocking buffer (PBST; Ca/Mg-free PBS containing 0.1% Triton X-100 and 1% BSA) at room temperature for 30 min. Stage specific embryonic antigen type-1, SSEA-1 (MC-480) antibodies (dilutions, 1:50 and 1:10 in blocking buffer, respectively) at 4 $^{\circ}$C overnight. The cells were washed three times for 5 mins each with Ca/Mg-free PBS and then incubated goat anti-mouse IgM-HRP (Jackson Immunoresearch) (dilution 1:500 in blocking buffer) at room temperature for 2 hr. The cells were washed 3 times for 5 mins each with Ca/Mg-free PBS and incubated in DAB (3,3'-diaminobenzidine tetrahydrochloride (Roche, Germany) (diluted in peroxidase buffer (Roche, Germany) at room temperature for 5-15 mins until brown colour develops. The reaction was then stopped by washing the cells with Ca/Mg-free PBS three times. The cells were observed under an Axiovert 100 inverted microscope under bright field optics.

2.6.2. Immunofluorescent staining of cESCs, cPGCs and cEGCs and staining with DAPI

Chicken embryonic stem cells (cESCs), primordial germ cells (cPGCs) and embryonic germ cells (cEGCs) were fixed with 4% PFA in 48 and 24 well plates at 4 $^{\circ}$C for 15 min (Stern and Holland, 1993). Fixed cells were washed three times for 5 mins each with Ca/Mg free PBS. After washing, the cells were then placed in blocking buffer (see above) at room temperature for 30 mins and then incubated in primary antibodies as described below at 4 $^{\circ}$C overnight. The cells were washed three times for 5 mins each with Ca/Mg-free PBS and then incubated in the appropriate secondary antibodies (see below) at room temperature protected from light. The cells were washed 3 times for 5 mins each with Ca/Mg free PBS and counterstained with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) (Roche) (Russell et al., 1975). They were then washed in Ca/Mg free PBS three times and mounted in Citifluor before observation by fluorescence microscopy.

2.6.3. Whole-mount immunocytochemistry of embryonic gonads

2.6.3.1 Immunofluorescent double staining with SSEA-1 and VASA antibodies in chicken embryonic gonads.

Chicken embryonic gonads at HH stage 28 (5.5-6 days' incubation) were dissected and then fixed with 4% PFA in 48 well plates for 30 min (Stern and Holland, 1993). Fixed gonads were rinsed with Tyrode's solution and washed 3 times for 1 hour with PBST, with gentle rocking, at room temperature and then blocked in blocking buffer

(PBST containing 1% BSA) at 4 $^{\circ}$C overnight. The blocked gonads were incubated in rat anti-Cvh (see above) and Stage Specific Embryonic Antigen type-1, SSEA-1 (MC-480; see above) antibodies (dilutions, 1:50 and 1:10 in blocking buffer, respectively) at 4 $^{\circ}$C for 48 hours. The gonads were then washed 3 times for 1 hour with PBST, with gentle rocking, at room temperature and then incubated in Cy3 -conjugated goat-anti-mouse-IgM (Jackson) and goat-anti-rat-IgG-FITC (Sigma) (dilutions, 1:100 and 1:50 in blocking buffer, respectively) at 4 $^{\circ}$C overnight. The next day, the gonads were washed 3 times for 1 hour with PBST, with gentle rocking, at room temperature and observed by fluorescence microscopy.

2.6.4. Antibodies

The primary antibody for Chicken vasa homologue (Cvh) (germ cell specific marker) (Nakamura et al., 2007; Tsunekawa et al., 2000; van de Lavoir et al., 2006) was a kind gift of Professor Takahiro Tagami from NILGS (Japan) and antibodies against other putative embryonic germ cell markers including SSEA-1, SSEA-3, SSEA-4, IGA6, INTEGRIN-B1 (Jung et al., 2005; Park and Han, 2000) were obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD). The details of antibodies used in this study are described in Table 2.6 below.

Table 2.5: Cell lines description

Name of cell lines	Source	Type of cells	Details
9N2	Provided by Dr. Bertrand Pain	Chicken Embryonic stem cells (cESCs)	Not commercial cell line
162-2	Crystal Bioscience Company	Chicken Primordial Germ cells (cPGCs)	Blood derived line, Good germline transmission, Not commercial cell line
NuGFP-02	Crystal Bioscience Company	Chicken Primordial Germ cells (cPGCs)	Blood derived line, Does not go germline transmission, Not commercial cell line
GFP-527	Crystal Bioscience Company	Chicken Primordial Germ cells (cPGCs)	Gonadal germ cell derived line, Good germline transmission, Not commercial cell line

Table 2.6: Antibodies description

Antibody	Antigen	Source	Cells/ Flourophore detection colour	Species	Host	Type
Primary	Chicken vasa homologue (Cvh)	NILGS§ Japan	Germ cells	Chick	Rat	IgG
Primary	MC-480 (SSEA-1)	DSHB* USA	Germ cells	Mouse	Mouse	IgM
Primary	MC-631 (SSEA-3)	DSHB* USA	Germ cells	Mouse	Rat	IgM
Primary	MC-813-70 (SSEA-4)	DSHB* USA	Germ cells	Human	Mouse	IgG3
Primary	P2C62C4 (INTEGRIN-A6)	DSHB* USA	Germ cells	Chick	Mouse	IgG1
Primary	V_2E_9 (INTEGRIN-B1)	DSHB* USA	Germ cells	Chick	Mouse	IgG1
Primary	Rabbit Polyclonal Serum	Millipore	Anti-phospho Histone H3 (Ser10)		Rabbit	IgG
Secondary	Rat IgG (H+L)	Sigma-Adrich	FITC (green)	Rat	Goat	IgG
Secondary	Rat IgM (H+L)	Invitrogen UK	Alexa Fluor 594 (Red)	Rat	Goat	IgM
Secondary	Mouse IgG (H+L)	Invitrogen UK	Alexa Fluor 594 (Red)	Mouse	Donkey	αIgG
Secondary	Mouse IgG (H+L)	Invitrogen UK	Alexa Fluor 488 (Green)	Mouse	Donkey	αIgG
Secondary	Rabbit IgG	Invitrogen	Alexa Fluor 546 (Red)	Rabbit	Donkey	IgG

§NILGS: National Institute of Livestock and Grassland Science (Japan), *DSHB: Developmental Studies Hybridoma Bank (Iowa University, USA)

Chapter 3. Isolation and culture of chicken embryonic

germ cells

3.1. Introduction

Since chicken embryonic stem cells (cESCs) can contribute only to somatic but not germline lineages (Pain et al., 1999; Pain et al., 1996; van de Lavoir et al., 2006b), chicken primordial germ cells (cPGCs) have been explored as a genetic tool for germline transmission (van de Lavoir et al., 2006a). Because of the complex migration routes of cPGCs through the embryo during development, there are several potential sites from which cPGCs could be isolated from the embryo, such as the central area pellucida of the pre-primitive streak blastoderm (Eyal-Giladi et al., 1976; Ginsburg and Eyal-Giladi, 1987; Nakamura et al., 2007), the germinal crescent of the late primitive streak stage embryo (Fujimoto et al., 1976b; Nakamura et al., 2007), the blood vessels at somite stages (Fujimoto et al., 1976a; Nakamura et al., 2007; van de Lavoir et al., 2006a; Yamamoto et al., 2007) and the embryonic gonads during organogenesis (Fujimoto et al., 1976b; Meyer, 1964; Nakamura et al., 2007; Park et al., 2003; Ukeshima et al., 1987; Zaccanti et al., 1990). To date, two methods have been used for isolating cPGCs which have successfully been transmitted through the germline. van de Lavoir et al. (2006a) isolated cPGCs from the embryonic blood and injected them into stage X (Eyal-Giladi and Kochav, 1976) embryos. Park et al. (2003) isolated cPGCs from the embryonic gonads and transferred them into the dorsal aorta of White Leghorn (WL) recipient embryos. These reports indicate that

both chicken embryonic blood and the gonads are potential sources for isolating cPGCs capable of germline transmission.

In addition to being a tool for avian germline transmission, cPGCs have also been shown to be able to generate pluripotent cells. Pluripotent cells (referred to as embryonic germ cells, EGCs), can be obtained from PGCs *in vitro* (Northrup et al., 2011). Park and Han (2000) were the first to describe a method for deriving pluripotent EGCs from chicken gonadal primordial germ cells (gPGCs): EGCs produced by this method can form embryoid bodies which can differentiate into a variety of cell types and produce chimaeric chickens with EGC contribution to many somatic tissues after injected into stage X host embryos. This indicates that gPGC-derived EGCs isolated from the embryonic gonads are pluripotent.

This part of the study was designed to find a simple method for deriving chicken EGCs from embryonic gonads. A second aim is to characterize chicken gonocyte-derived-EGCs to study their gene expression dynamics *in vitro* (described in Chapter 4) and to compare the expression of various markers in chicken gonocyte-derived EGCs with cESCs and cPGCs *in vitro* (described in Chapter 5).

3.2. Methods

To characterize chicken gonocytes in the embryonic gonads (*in vivo*), chicken embryonic gonads with their attached mesonephroi at HH stages 25-28 embryos were collected using sharp tweezers with needle under a stereo microscope. The gonads

were rinsed with Tyrode's solution and then fixed with 4% PFA in a glass vial at 4°C for 30 min (for whole-mount staining, Stern and Holland, 1993) or at 4°C overnight (for whole-mount *in situ* hybridization (Stern, 1998). Immunofluorescence staining was performed with for Stage Specific Embryonic Antigen type 1 (SSEA-1) (MC-480) and Chicken vasa homologue (Cvh) (rat anti-Cvh antibody). *In situ* hybridisation was used to detect *Cvh* transcripts. *Cvh* (1,994 bp) had been subcloned into pGEMT-easy I (3,015 bp) (kind gift of Dr. Bertrand Pain, Clermont-Ferrand, France). Selected hybridized and post fixed embryos were embedded in Fibrowax (BDH) for histological sections and then cut on a Zeiss MICROM microtome at 10 μm thick.

To isolate and culture chicken gonocytes from the embryonic gonads, the gonads were dissected from the embryos at HH stage 26-28 (5-6 days of incubation). Cells were dissociated with 0.25% typsin-EDTA (Gibco Invitrogen) at room temperature for 5 min and then centrifuged at 200g for 5 min (Park and Han, 2000). The cell suspension containing both PGCs and somatic cells was seeded onto gelatin-coated 24 well plates (see Chapter 2) and cultured with EG cell culture medium (Park and Han, 2000) consisting of DMEM medium supplemented with 10% FBS (PAN), 2% chicken serum, sodium pyruvate, glutamax, β-mercaptoethanol, penicillin-streptomycin, 5 ng/ml hSCF (R&D systems), 10 units/ml mLIF (Chemicon), 10 ng/ml bFGF (R&D systems), 0.04 ng/ml h-IL-11 (R&D systems), and 10 ng/ml IGF-1 (R&D systems) (see Chapter 2). The 24 well plates were incubated at 39.5 ^{o}C in 5% CO_2 until the somatic cells had formed a monolayer and gPGCs had colonized as a primary culture. For subculture, the colonies of chicken EG cells were dislodged by gentle pipetting (without trypsin treatment) and then seeded onto mitomycin C-treated (10 μg/ml; Sigma-Aldrich) inactivated chicken embryonic fibroblast (CEF) feeders.

To characterize cultured chicken gonocytes (*in vitro*), the cultures were fixed in 4% PFA in 24 well plates at 4°C overnight. The following day, the fixed cells were processed for Periordic Acid Shiff (PAS) staining (Park and Han, 2000), Alkaline Phosphatase (AP) assay (Stern and Holland, 1993), SSEA-1 immunostaining (Park and Han, 2000) or cell *in situ* hybridization for *Cvh* (adapted from (Stern, 1998); see Chapter 2).

To characterize chicken gonocyte-derived-EGCs (*in vitro*), long term cultured EGCs were fixed in 4% PFA in 48 well plates at 4°C for 30 min. The fixed cells were then processed for immunofluorescence staining for SSEA1, SSEA3, SSEA4, Integrin α6 (IGA6) or Integrin β1 (IGB1), all previously reported markers for chicken embryonic germ cells (Jung et al., 2005). Images were collected using a Retiga 2000R camera (Q imaging) attached to an inverted microscope (Zeiss Axiovert) or an upright microscope (Olympus Vanox).

3.3. Results

3.3.1. Morphology of cultured chicken gonocytes

In this study, cultured chicken gonocytes isolated from 5.5-6 day old embryos were plated together with their surrounding somatic cells as primary cultures. The somatic cells (gonadal stromal cells) attached to the surface of the culture dish and were used directly as the initial feeder cells. On day 1 of culture, the cultures contained gonocytes, somatic cells including embryonic stromal cells (thin and long shape) and nucleated red blood cells (RBCs; an ovoid or rugby shape) which can be recognised by morphological characteristics (Chang et al., 1997; Chang et al., 1995; Park and

Han, 2000) (Fig. 3.1A). The embryonic stromal cells slowly spread as a monolayer within 5 days, to which the gonocytes adhered and grew (Fig 3.1B). Gonocytes form clear colonies around day 7 (Fig. 3.1C); the colonies were uniformly round and did not tightly adhere to embryonic stromal cells. For subculture, gonocytes were passaged and grown on chicken embryonic fibroblasts (CEFs) (Fig. 3.1D). Gonocyte colonies were multi-layered and well delineated and could therefore be distinguished from embryonic stromal cells, CEFs and nucleated RBCs by the large size, large nucleus and relatively small amount of cytoplasm of the gonocytes, as previously described (Chang et al., 1997; Chang et al., 1995; Park and Han, 2000).
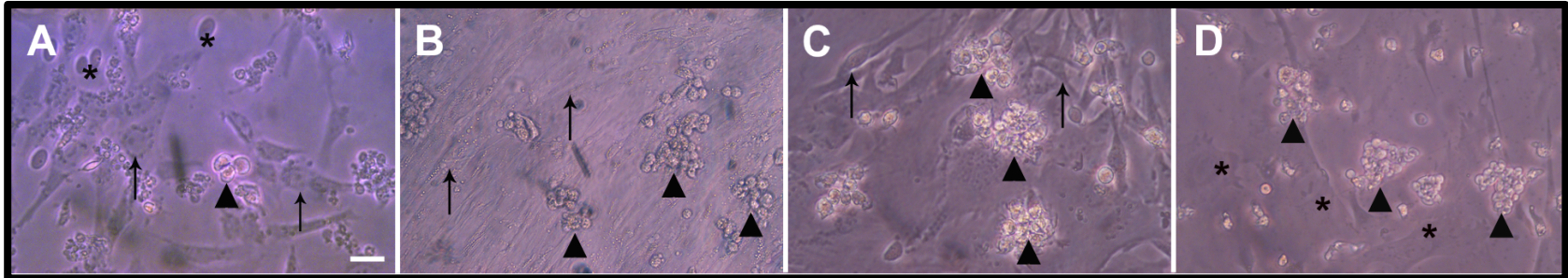
Figure 3.1: Morphology of cultured chicken gonocytes:

A) On day 1, the cultures contained gonocytes (arrowhead), embryonic stromal cells (arrows) and nucleated RBCs (asterisk). B)

On day 5, the colony of gonocytes start to be observed (arrowheads) growing on the stromal cells (arrows). C) On day 7, gonocyte

colonies (arrowheads) are well formed; colonies do not tightly adhere to the stromal cells (arrows). D) Gonocytes were

successfully formed multi-colonies (arrowheads) on CEF feeders (asterisk) after the first passage of subculture.

3.3.2 Characterization of gonocytes in chicken embryonic gonads (*in vivo*)

Chicken embryonic gonads at HH stage 25-28 were stained by immunofluorescence or *in situ* hybridisation for two reported germ cell markers: SSEA-1 and Chicken vasa homologue (Cvh protein or mRNA). SSEA-1 (Fig. 3.2A) and Cvh (Fig. 3.2B) positive cells were detected in the embryonic gonads. *Cvh* positive cells (Fig. 3.2C) were also detected the embryonic gonads in both left and right gonadal ridges and in the dorsal mesentery (Fig. 3.2C').
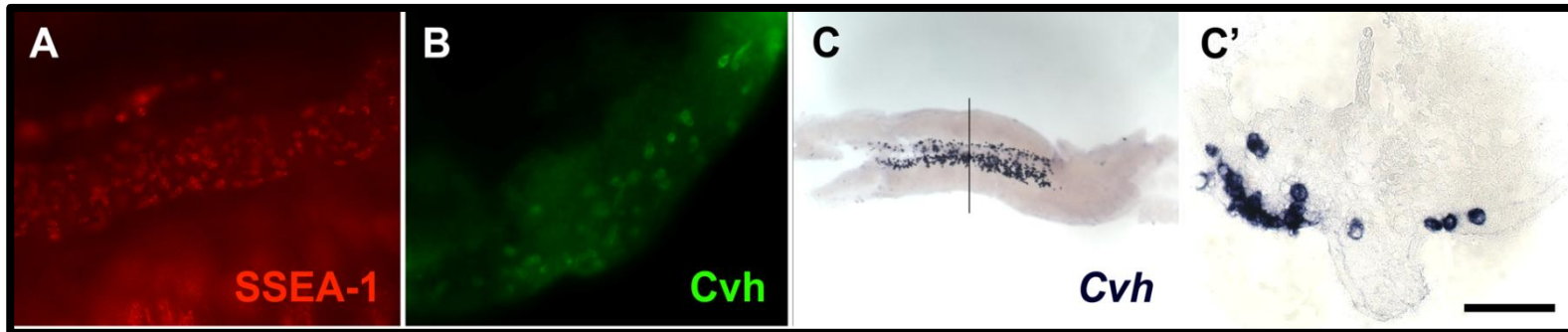
Figure 3.2: Characterization of gonocytes in chicken embryonic gonads (*in vivo*):

Chicken embryonic gonads containing gonocytes express the chicken germ cell markers SSEA-1 (A), Cvh (B) and *Chicken vasa homologue* (*Cvh*) (C). A and B are immunofluorescence images, C is a whole-mount *in situ* hybridisation for *Cvh* and C' is a transverse section through C showing *Cvh* positive cells in both left and right gonadal ridges and in the dorsal mesentery (C'). (Scale bar = 50 μm).

3.3.3. Characterization of cultured chicken gonocytes (*in vitro*)

To distinguish chicken gonocytes from somatic cells as described above, cultured chicken gonocytes were stained with several methods including Periodic Acid Schiff (PAS) (PAS positive cells were 197 cells/197 (100%)), Alkaline Phosphatase (AP) (AP positive cells were 113 cells/134 (84%)), SSEA-1 (SSEA-1 positive cells were 53 cells/59 (90%)) and *in situ* hybridization for *Cvh* (*Cvh* positive cells were 84 cells/93 (90%)). Gonocytes cultured for 7 days are positive for PAS (Fig. 3.3A), AP (Fig. 3.3B), while somatic cells are negative and SSEA-1 (Fig. 3.3C)*. Cvh*-expressing cells were detected on 7 days-cultured gonocytes by cell *in situ* hybridization (Fig. 3.3C). The percentage of cells stained by each of the 4 methods was 100%, 84%, 90% and 90%, respectively. These differences suggest that cells are heterogeneous; since all cells are Alkaline Phosphatase positive, the proportions above show that only subsets of them express PAS, SSEA-1 and/or *Cvh*. To establish how many different cell types (or states) exist, a more detailed study with double and/or triple staining is required.
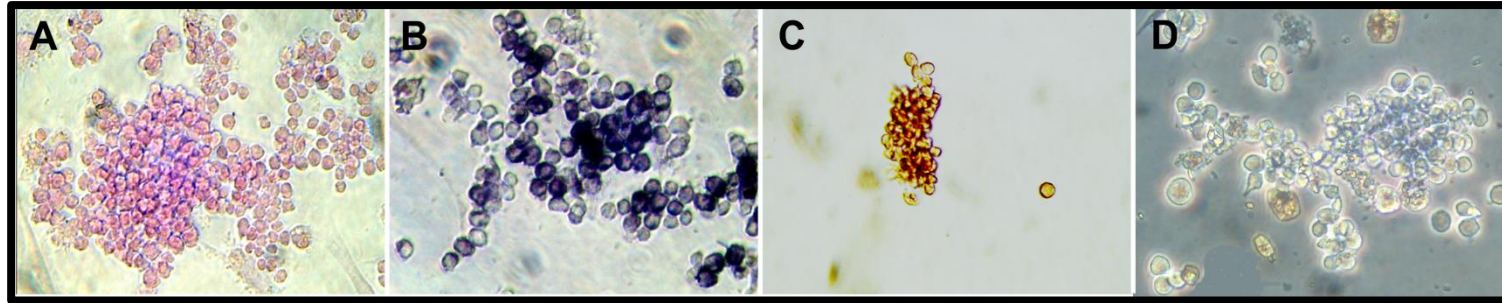
Figure 3.3: Characterization of cultured chicken gonocytes (*in vitro*):

Cultured chicken gonocytes were stained by Periodic Acid Schiff (PAS) staining (A), Alkaline Phosphatase (AP) assay (B), Stage specific embryonic antigen type 1 (SSEA-1) (C) and cell *in situ* hybridization for *Chicken vasa homologue* (*Cvh*) (D).

3.3.4. Characterization of cultured chicken gonocytes derived-EGCs (*in vitro*)

To determine whether the culture medium (EGM) used in this study allows derivation of chicken gonocytes into putative EGCs, cultured chicken gonocytes were stained for markers expressed in chicken EGCs (Jung et al., 2005). The expression of these markers was also compared between primary gonocytes, cESCs and chicken EGCs (cultured gonocytes). We find that SSEA-1 is expressed in cEGCs (123 cells/129 (95%)) (Fig. 3.4.1C), primary gonocytes (8 cells/13 (62%)) (Fig. 3.4.1F) and cESCs (5 cells/38 (13%)) (Fig. 3.4.1I). SSEA-3 is expressed in both cEGCs (47 cells/68 (69%)) (Fig. 3.4.2C) and primary gonocytes (14 cells/28 (50%)) (Fig. 3.4.2F) but not in cESCs (0 cells/81 (0%)) (Fig. 3.4.2I). SSEA-4 is expressed only in primary gonocytes (12 cells/16 (75%)) (Fig. 3.4.3F) but not in either cEGCs (0 cells/74 (0%)) (Fig. 3.4.3C) or cESCs (0 cells/18 (0%)) (Fig. 3.4.3I). Integrin-α6 is expressed in both cEGCs (87 cells/102 (85%)) (Fig. 3.4.4C) and primary gonocytes (2 cells/18 (11%)) (Fig. 3.4.4F) but not in cESCs (0 cells/75 (0%)) (Fig. 3.4.4I) and Integrin-β1 is expressed in both cEGCs (37 cells/46 (80%)) (Fig. 3.4.5C) and primary gonocytes (9 cells/24 (38%)) (Fig. 3.4.5F) but not in cESCs (0 cells/102 (0%)) (Fig. 3.4.5I), similar to Integrin-α6.
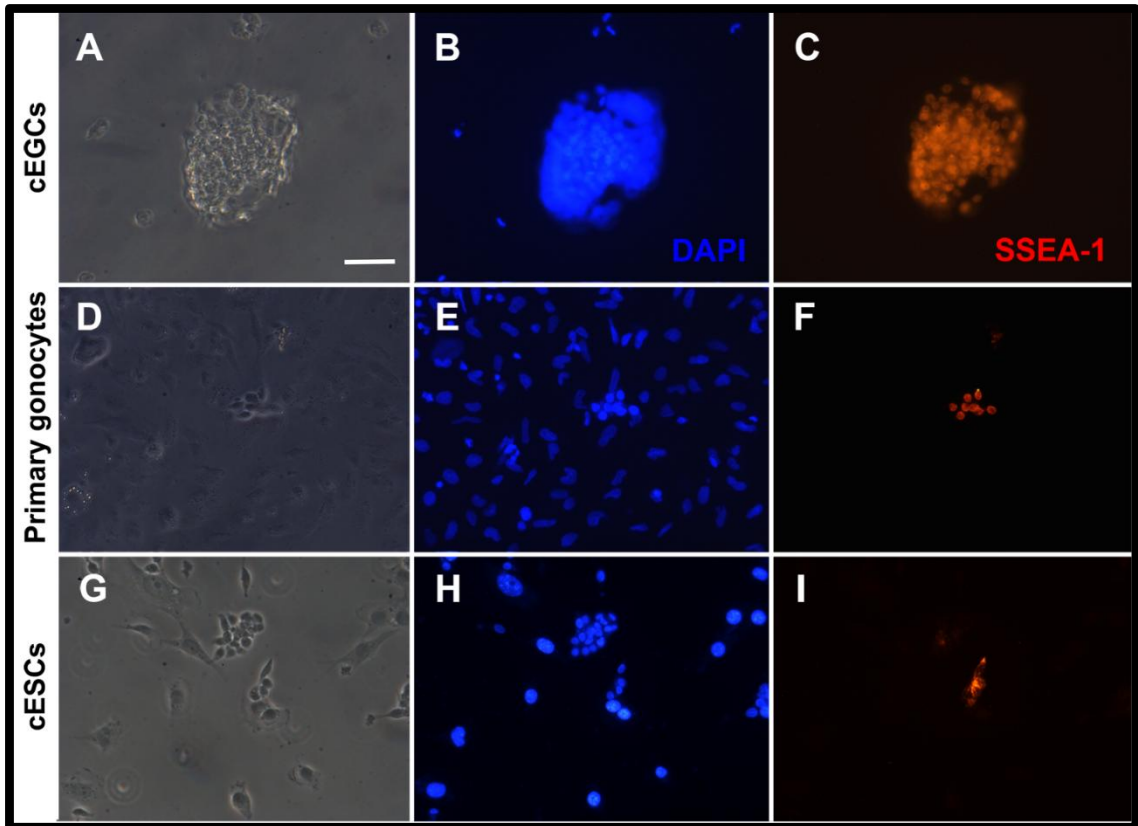
Figure 3.4: SSEA-1 expression in three cultured cell types: A-C) cEGCs, D-F) primary gonocytes and G-I) ESCs. A, D and G are Phase Contrast views, B, E and H are the cultures stained with DAPI to label the nuclei, and C, F and I show SSEA-1 staining. (Scale bar = 50 μm).
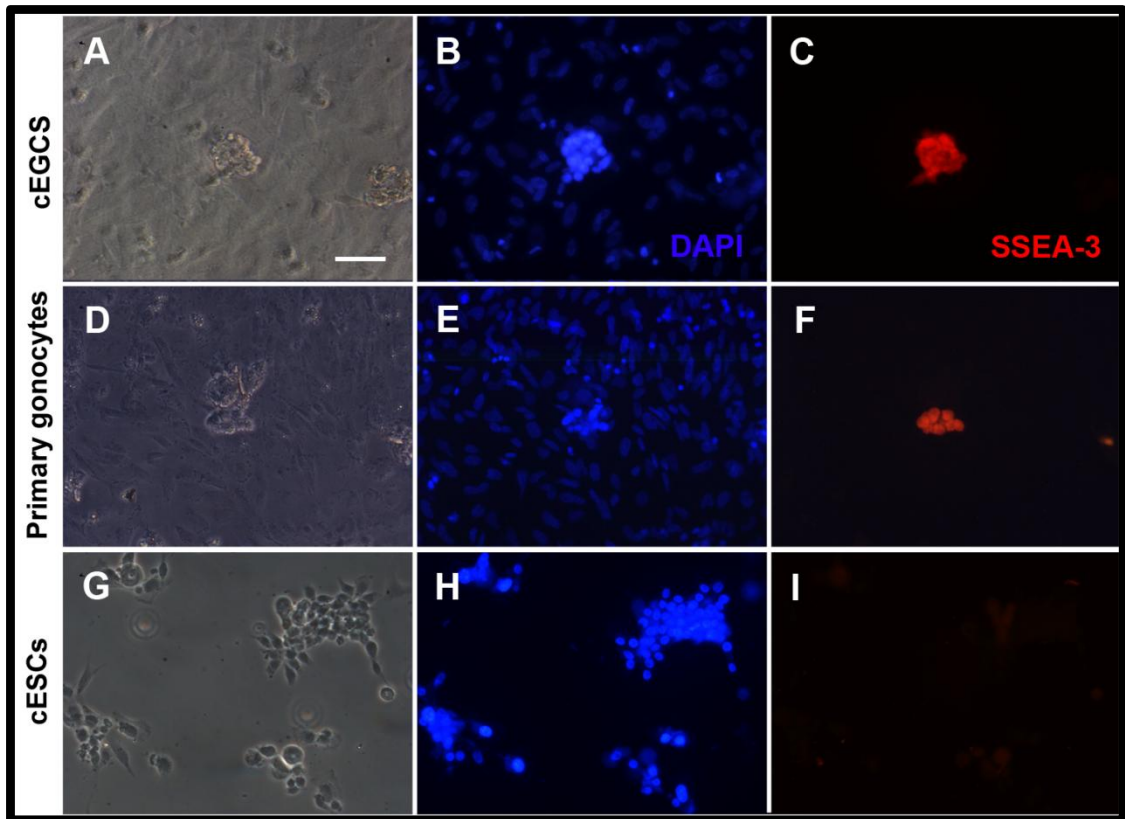
Figure 3.5: SSEA-3 expression in three cultured cell types: A-C) cEGCs, D-F) primary gonocytes and G-I) ESCs. A, D and G are Phase Contrast views, B, E and H are the cultures stained with DAPI to label the nuclei, and C, F and I show SSEA-3 staining. (Scale bar = 50 μm).
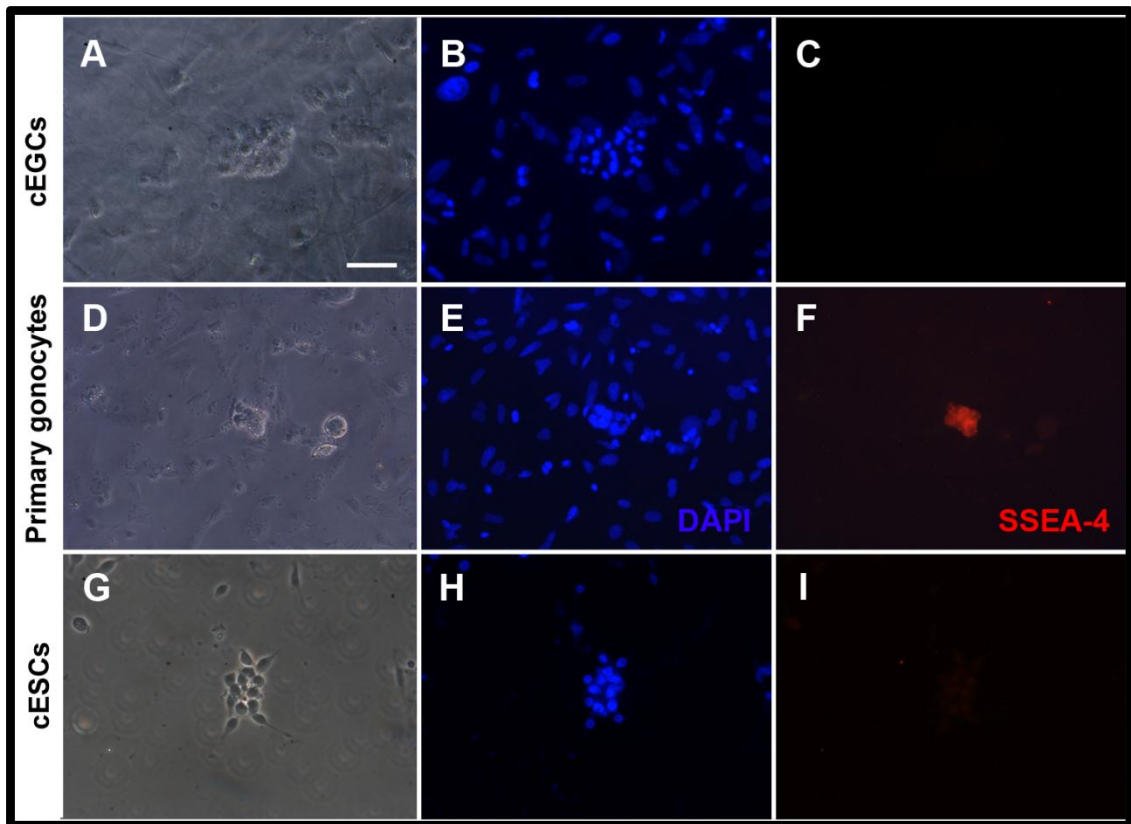
Figure 3.6: SSEA-4 expression in three cultured cell types: A-C) cEGCs, D-F) primary gonocytes and G-I) ESCs. A, D and G are Phase Contrast views, B, E and H are the cultures stained with DAPI to label the nuclei, and C, F and I show SSEA-4 staining. (Scale bar = 50 μm).
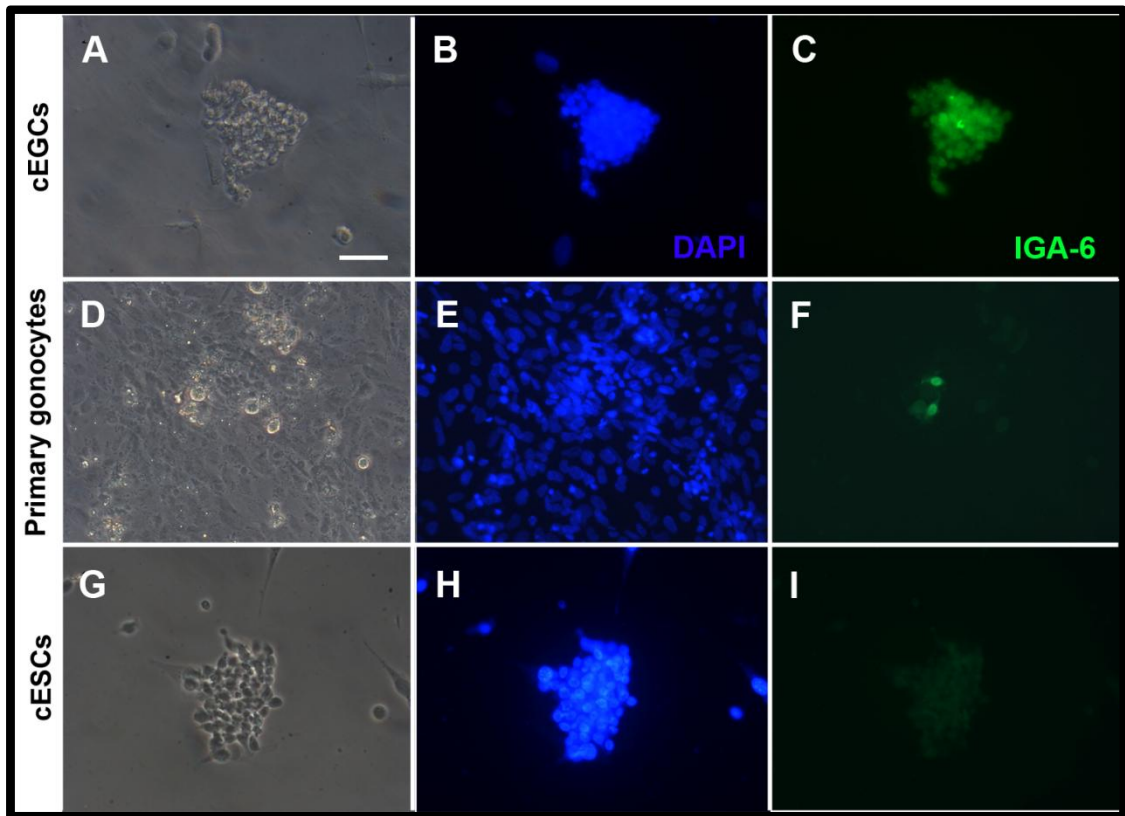
Figure 3.7: Integrin-α6 expression in three cultured cell types: A-C) cEGCs, D-F) primary gonocytes and G-I) ESCs. A, D and G are Phase Contrast views, B, E and H are the cultures stained with DAPI to label the nuclei, and C, F and I show Integrin-α6 staining. (Scale bar = 50 μm).
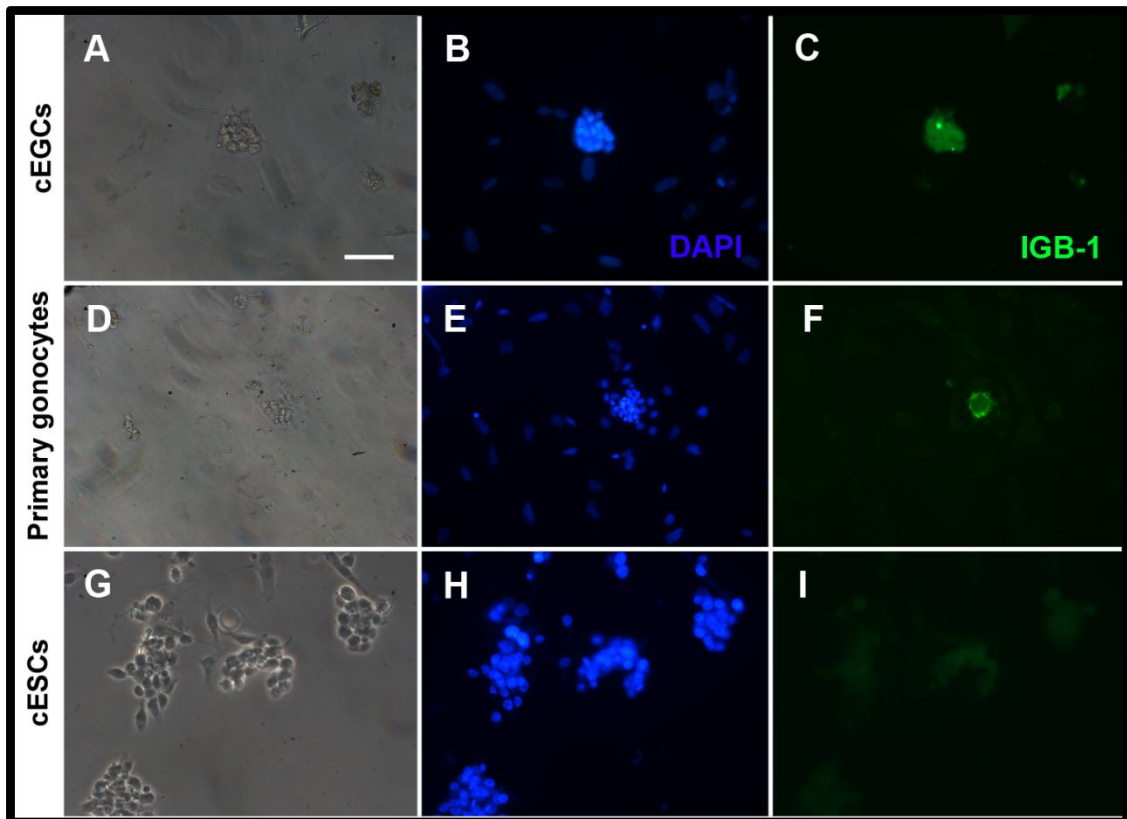
Figure 3.8: Integrin-β1 expression in three cultured cell types: A-C) cEGCs, D-F) primary gonocytes and G-I) ESCs. A, D and G are Phase Contrast views, B, E and H are the cultures stained with DAPI to label the nuclei, and C, F and I show Integrin-β1 staining. (Scale bar = 50 μm).

3.4. Discussion

3.4.1. Morphology of cultured chicken gonocytes

The morphology of cultured chicken gonocytes was monitored after 7 days of plating the cells. In the present study, the colonies of chicken gonocytes were first observed at day 5 and appeared to have matured by day 7 of culture (based on the presence of larger cells which often formed clumps, suggesting clonal expansion). The colonies grew on top of the embryonic stromal cells which were used as an endogenous feeder layer during the primary culture. The colonies were uniformly round and did not tightly adhere to embryonic stromal cells. These characteristics differ from the morphology of blood-derived PGCs which form colonies that grow without adhering to the feeder cells (Choi et al., 2010; Macdonald et al., 2010; van de Lavoir et al., 2006a). The significance of this difference is unclear but it seems likely that adhesion of germ cells to the feeders is an early sign of differentiation. Therefore, this characteristic of both types of germ cells might be related to the process of sustaining the undifferentiated state of the cells.

3.4.2 Characterization of gonocytes in chicken embryonic gonads (*in vivo*)

This experiment aimed to use embryonic gonads as a source of post-migratory gonadal PGCs or gonocytes in order to derive embryonic germ cells from chicken gonocytes. The expression of SSEA-1 and Cvh (by antibody staining and *in situ* hybridisation) was used to identify the population of gonocytes located in the

embryonic gonads. Both markers are expressed in the embryonic gonads. SSEA-1 has been used as a marker of chicken ES cells (Pain et al., 1996; van de Lavoir et al., 2006b) as well as germ cells (Karagenc and Petitte, 2000; Mozdziak et al., 2005; Mozdziak et al., 2006). In this study, SSEA-1 positive cells were found to be expressed in the embryonic gonads differently from Cvh positive cells. Double immunostaining for SSEA-1 and rat anti-Cvh was performed; some co-localization cells was observed in the embryonic gonads but there are also some Cvh-expressing cells that do not express SSEA-1 and vice-versa . This experiment shows that the studies of identification of chicken gonocytes using SSEA-1 and Cvh as markers for germ cells. *In situ* hybridisation with a *Cvh*-riboprobe was the best marker to reveal germ cells in the embryonic gonads probably because of better penetration of the riboprobe into the gonadal matrix than antibodies. In sections through whole-mount *in situ* hybridised gonads, *Cvh* positive cells were detected in both left and right gonadal ridges and also in the dorsal mesentery where germ cells migrate towards the gonad *in vivo* (Ukeshima et al., 1987). These findings indicates that the *Cvh*-riboprobe is a good marker to identify germ cells both within the gonad and along the migratory route of these cells to their final gonadal location (Nakamura et al., 2007).

3.4.3. Characterization of cultured chicken gonocytes (*in vitro*)

The present results reveal that chicken gonocytes can be successfully isolated from chicken embryonic gonads and cultured *in vitro*. At the beginning of this study, it was also attempted to isolate cPGCs from embryonic blood (data shown). However this

was not successful. There are several explanations for why cPGCs could not be successfully isolated from embryonic blood.

First, the number of PGCs in the blood is much lower than that in the gonads. Blood vessels contain about 194-285 cells per embryo (Nakamura et al., 2007) while the embryonic gonads contain about 385 PGCs per embryo in males and 947 in females during sexual differentiation (about 6-6.5 days of incubation) (Zaccanti et al., 1990). These observations suggest that PGCs divide 1-3 times between the stage of their migration within the circulation and the stage at which they have colonised the gonad.

The different types of feeder cells used in the two experiments could also partly account for the difference. Here STO cells were used as feeders for growing PGCs from the blood, whereas chicken embryonic fibroblasts were used as feeders for growing PGCs from the gonads. Mouse fibroblast (STO) cells are necessary for the proliferation and survival of mouse PGCs *in vitro* (Resnick et al., 1992). In another study, fibroblasts isolated from the gonadal ridges of chicken embryo were used as a feeder layer for supporting the survival and proliferation of PGC isolated from the blood cultures *in vitro* (Chang et al., 1995). The species specificity of and other parameters of growth factors secreted by the feeder cells for survival and proliferation of PGCs still requires further study.

This study therefore confirmed the method of Park and Han (2000) for isolation and culture of chicken gonocytes from embryonic gonads. In addition, cultured-gonocytes were cryopreserved for future experiments. Since chicken PGCs have unique characteristics such as refractive granules in the cytoplasm, it has been reported that

chicken PGCs contain glycogen granules in their cytoplasm (Fujimoto et al., 1976a; Fujimoto et al., 1976b; Meyer, 1964; Singh and Meyer, 1967). PGCs use glycogen granules as an energy source during their migration; Alkaline phosphatase (AP) has been reported to play an important role for enzymatic activity during the passive and active phases of migration (Swartz, 1982). Therefore, PAS, a histochemical technique for detecting glycogen granules, and AP are generally considered to be an indication of PGCs in culture. Human PGCs are also PAS and AP positive (Fuyuta et al., 1974; Shamblott et al., 1998). Thus, PAS and AP may represent evolutionarily conserved markers for PGCs among the vertebrates.

A previous study (Park and Han, 2000) also reported that chicken embryonic germ cells derived from gPGCs are PAS- and AP-positive. However the present study differs from Park and Han's in that in the former, mitomycin-C-inactivated chicken fibroblasts were used as feeders, rather than the mitotically active fibroblasts used by Park and Han. In the present study, the percentage of cells stained by each of the 3 methods (PAS, AP and *Cvh*) was also different (100%, 84% and 90%, respectively). Since AP activity is only present in a subset of *Cvh*-positive cells, the present findings suggest that *Cvh* and PAS are more reliable markers than AP to identify the gonocyte population.

3.4.4. Characterization of cultured chicken gonocyte-derived-EGCs (*in vitro*)

The present study succeeded in deriving chicken EGCs (stable for at least 5 passages) from gonocytes grown in embryonic germ cell culture medium (EGM). Chicken

gonocyte-derived-EGCs in this study form uniformly round, multilayered and well delineated colonies which have also been reported in other previous studies (Park and Han, 2000; Wang et al., 2009; Wu et al., 2010). The colonies of cEGCs are easily dislodged from the feeder layers since they are not tightly attached to the feeder cells. This suggests that cEGCs share common characteristics with cPGCs and gonocytes in that they can sustain themselves at an undifferentiated state, a characteristic of true stem cells.

Although there is no definitive marker for chicken embryonic germ cells, it has been reported that SSEA-3, SSEA-4, Integrin-α6 and β1 can be used for this purpose (Jung et al., 2005). These integrins have been reported to be expressed in the gonadal ridges of mouse embryos (Anderson et al., 1999) and to play a key role in the migration of PGCs to the embryonic gonads (De Felici and Dolci, 1989). Here, cultured chicken gonocyte-derived EGCs were found to express all the above markers except SSEA-4. Previous studies reported that PGCs express SSEA-1 and Integrins α6 and β1. However, both SSEA-3 and SSEA-4 were described as markers for PGCs (Choi et al., 2010; Jung et al., 2005). The different result obtained by Choi et al. (2010) could be explained by the fact that they used blood-derived PGCs whereas gonocyte-derived cells were used here, and that Choi et al. (2010) examined cells cultured for a short time, whereas the present study used gonocytes cultured for 1 month. However, Jung et al. (2005) used gonadal-derived cells cultured for many passages, therefore neither factor can account for the difference. Since there is no reliable marker to identify EGCs as distinct from their gonocyte precursors, we cannot determine whether EGCs are present from the beginning of the culture or whether they arise *in vitro*.

The results from this study suggest that SSEA-3, SSEA-4, Integrin-α6 and –β1 are not good markers for chicken ES cells. However, SSEA-1 might be. Expression of the SSEA-1 (Le^X) oligosaccharide epitope (Streit et al., 1996) is common to chicken ES, EG cells and primary gonocytes. The key surface and matrix molecules playing an important role in the conversion of primary gonocytes into EGCs and definitive germ cells for chicken EGCs need to be further investigated.

Chapter 4. Gene expression dynamics of pluripotent stem cell markers during derivation of embryonic germ cells from chicken gonocytes

4.1. Introduction

Pluripotent stem cells were first established from a murine teratocarcinoma cell line isolated from a transplantable teratoma whose cells had the ability to grow in clonal culture (Evans, 1972). Three years later, the differentiation *in vitro* of clonal pluripotent teratocarcinoma cells was reported and the cells named "embryonal carcinoma cells (ECCs)" (Martin and Evans, 1975). Culturing cells from tumour L8402C-168 showed it to be composed only of ECCs. These cells revealed characteristics of pluripotency, with the formation of simple and complex cystic embryoid bodies and subsequent differentiation into multiple cell types *in vitro* (Martin and Evans, 1975).

Another type of pluripotent stem cells are embryonic stem cells (ESCs), which were first established from blastocyst stage mouse embryos (Evans and Kaufman, 1981). Other than mammals, embryonic stem cell lines have only been established successfully from domestic fowl. The first report of their derivation involved their isolation from the stage X (Eyal-Giladi and Kochav, 1976) chick blastoderm (Pain et al., 1996). Such cells exhibited several characteristics in common with mouse ES cells including embryoid body formation (Pain et al., 1996), differentiation into derivatives of all three primary germ layers *in vitro* (Pain et al., 1996) and *in vivo* (van de Lavoir

et al., 2006) and production of somatic chimaeras (Pain et al., 1996; van de Lavoir et al., 2006) indicating that cESCs are pluripotent stem cells.

Embryonic germ cells (EGCs) constitute a third type of pluripotent stem cells. They were first derived from post-migratory primordial germ cells (PGCs) isolated from the 10.5–11.5 dpc mouse embryo using a combination of feeder layers, LIF, fibroblast growth factor-2, and stem cell factor (SCF) (Matsui et al., 1992; Resnick et al., 1992). These cells also exhibit the property of pluripotency including being able to differentiate *in vitro* and contribution multiple lineages (generating both somatic and germ line chimaeras) when injected into host blastocysts (Matsui et al., 1992)**.**

In the chick, the derivation of pluripotent embryonic germ cells has been reported (Park and Han, 2000). For the present study, we consider that EGCs have been established from the parent PGCs after 5 passages in culture. Such cells can be maintained as undifferentiated stem cells, can form embryoid bodies when cultured in suspension, can differentiate into a variety of cell types and can also produce chimaeric birds after injection into stage X embryos (Park and Han, 2000). These results show that chicken EGCs are pluripotent. However, the cellular and molecular characteristics of cultured chicken EGCs or gonocytes has hardly been investigated, including the expression of makers for pluripotency. Moreover, there have been few if any studies of the changes in expression of pluripotency-associated genes that accompany the establishment of EGCs from the parent gonocytes isolated form the embryo. Such a study of the dynamics of gene expression would be particularly valuable for understanding the changes that cells undergo as they become established as a pluripotent stem cell line.

The chick embryonic gonads contain large numbers of post-migratory germ cells settled in both sides, as revealed by WISH for the germ cell definitive marker, *Cvh* (see Chapter 3). This provides an abundant source of germ cells to start *in vitro* cultures of chick gonocyte-derived EGCs. Here, taking advantage of the method described in Chapter 3, the gene expression dynamics of markers associated with pluripotency and differentiation is studied during the establishment of EGCs from cultured chicken gonocytes.

These experiments are designed to test the following hypothesis:

- Derivation of EGCs from cultured chicken gonocytes is accompanied by changes in the expression profile of gene/markers of pluripotency and lineage commitment as well as proliferative characteristics.

The experiments in this chapter are aimed at studying the changes in expression of genes/makers described in Table 4.1, related to pluripotency, pre-neural and neural state and germ cells markers before the establishment of EGCs. Most comparable studies use PCR-based strategies for analysing gene expression. Although this method is quantitative for the whole culture, it cannot reveal the extent of cell diversity (mosaicism) or distinguish between eg. a few cells expressing a gene at high level and many cells expressing at lower level. For this reason we decided to use cell *in situ* hybridisation and antibody-staining methods to visualise the cell diversity of gene expression and how this changes with time during the establishment of self-renewing cell lines from chick embryonic gonocytes.

4.2. Methods

4.2.1. Gene expression in cultured chick gonocytes

The set of genes used in this study was chosen to include some considered to be markers of pluripotency and/or ESC markers such as the chicken Oct3/4 homologue (*cPouV*) and *cNanog* (Lavial et al., 2007), *cKlf4* (Macdonald et al., 2010) as well as markers of different stages of neural plate development such as *cSox2*, *cSox3* (Uwanogho et al., 1995) and *ERNI* (Acloque et al., 2001; Streit et al., 2000). The expression profiles of the definitive germ cell marker chicken *Vasa* homologue (CVH) (Tsunekawa et al., 2000) was also studied. Changes in proliferation activity in the cultures were assessed using a mitotic marker, anti-phospho Histone H3 (Ser10) (Adams et al., 2001; Canela et al., 2003; Oike et al., 2003; Siegel et al., 2003). Expression profiles of mRNA were studied by *in situ* hybridization as described in Chapter 2. The probes, sources and references are listed in Table 4.1. Subcultured cells from passage 0 (P0) to passage 5 (P5) were subjected to cell-ISH for different markers and immunofluorescent staining for anti-phospho Histone H3 (Ser10), as described below.

Regions of the cultures containing cell masses and single cells were scored using a 20X objective. The scoring system was modified from a previous study (Ghanem, 2010) as described below.

−: no expression detected.

+: the marker is expressed in small cohorts of cells (<50% of the cells scored).

++: the marker is expressed more than half of the cells.

+++: expression in almost all of the cells

++++: as above, but also indicating particularly strong levels of expression.

4.2.2. Immunofluorescence staining for H3 in cultured chicken gonocytes

Cultured chicken gonocytes used for this part were cultured and maintained *in vitro* as described in Chapter 2. Subcultured cells from P0 to P5 were fixed in 4 well plates at 4 $^{\circ}$C for 15 min (Stern and Holland, 1993). Fixed cells were washed three times for 5 mins each with Ca/Mg free PBS. After washing, the cells were blocked in blocking buffer (PBST, Ca/Mg free PBS containing 0.1% triton X-100 and 1% BSA) at room temperature for 30 mins. The cells were washed three times for 5 mins each with Ca/Mg free PBS. The blocked cells were incubated in Rabbit anti-phospho histone H3 (see Table 4.1) diluted 1:500 at 4 $^{\circ}$C overnight. The cells were washed three times for 5 mins each with Ca/Mg free PBS and then incubated in donkey anti-rabbit IgG (see Table 4.1) at room temperature for 1 hr covered with foil to protect it from light. The cells were washed 3 times for 5 mins each with Ca/Mg free PBS and stained with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) (Roche Diagnostics , Germany) (Russel et al., 1975), washed with Ca/Mg free PBS three times and observed under fluorescence using a Zeiss Axiovert 100 inverted microscope.

4.3. Results

4.3.1. Patterns of gene expression during derivation of cultured chicken gonocyte-derived-EGCs

4.3.1.1. Gene expression profile of pluripotency markers

In this experiment, cultured chicken gonocytes were cultured in embryonic germ cell culture medium (EGM) as described in Chapter 2 to examine changes in expression of pluripotency-related and other markers *cPouV*, *cNanog*, *cSox2*, *cKlf4*, *ERNI*, *cSox3* and *Cvh*.

Cultured chicken gonocyte-derived-EGCs express pluripotency-related markers *cPouV* (Fig. 4.1A,A'-F,F'), *cNanog* (Fig. 4.1G,G'-L,L'), c*Sox2* (Fig. 4.1A,A'-F,F'), *ERNI* (Fig. 4.2A,A'-F,F') and *cKlf4* (Fig. 4.2G,G'-L,L'). Gene expression was followed over 5 passages in time course. Culture of gonocytes in embryonic germ cell culture medium (EGM), which successfully supported the derivation of chicken EGCs from gonocytes (Chapter 3) was accompanied by changes in expression of pluripotency markers over time. Expression of *cPouV* (P0 = 20 cells/80 (23%)) (Fig. 4.1A,A') and *cNanog* (P0 = 16 cells/66 (24%)) (Fig. 4.1G,G') are observed at Passage 0 [scored as ++]. From Passage 1 to Passage 4, *cPouV* is progressively downregulated and disappeared (P1 = 4 cells/115 (3%)) (Fig. 4.1B,B'-E,E'), [scored as -], while *cNanog* starts to downregulate at Passage 1 (P1 = 81 cells/226 (36%)) (Fig. 4.1H,H'), [scored as +]. Expression of *cNanog* is progressively downregulated at Passage 2 (P2 = 0 cells/60 (0%); P3 = 0 cells/46 (0%)) to Passage 4 (P4 = 0 cells/65 (0%)) ((Fig. 4.1I,I'-K,K'), [scored as -]. At Passage 5 both *cPouV* (P5 = 35 cells/120 (29%)) and

cNanog (P5 = 30 cells/69 (43%)) are upregulated again (Fig. 4.1F,F' and Fig. 4.1L,L'), [scored as ++].

At Passage 0, both *cSox2* (P0 = 225 cells/247 (91%)) (Fig. 4.2A,A'), [scored as ++++] and *cKlf4* are expressed strongly (P0 = 94 cells/138 (68%)) (Fig. 4.2G,G'), [scored as +++]. Expression of both markers is maintained more or less constant throughout the culture period (*cSox2*, P1 = 52 cells/90 (58%); P2 = 45 cells/74 (61%); P3 = 29 cells/45 (64%); P4 = 125 cells/145 (86%); P5 = 202 cells/210 (96%)); *cKlf4*, P1 = 425 cells/500 (85%); P2 = 18 cells/188 (10%); P3 = 85 cells/111 (77%); P4 = 130 cells/178 (73%); P5 = 322 cells/352 (91%)) (Fig. 4.2).

4.3.1.2. Gene expression profile of pre-neural markers

The expression of pre-neural and neural, *ERNI* (P0 = 95 cells/148 (64%)) and *cSox3* (P0 = 90 cells/145 (62%)) was observed at Passage 0 (Fig. 4.3A,A' and G,G'). *ERNI* expression seems to be maintained throughout the culture period. *cSox3* expression is strongest at Passage 0 (P0 = 90 cells/145 (62%)) and Passage 5 P0 = 65 cells/101 (64%)), with some downregulation and some variation at intermediate passages (P1 = 50 cells/141 (35%); P2 = 138 cells/249 (55%); P3 = 0 cells/52 (0%); P4 = 72 cells/96 (75%)) (Fig. 4.3).

4.3.1.3. Gene expression profile of the germ cell marker *Cvh*

Cvh is strongly expressed throughout the period of culture (P0 = 436 cells/564 (77%); P1 = 216 cells/311 (69%); P3 = 87 cells/120 (73%); P4 = 144 cells/162 (89%); P5 = 202 cells/213 (95%)), except perhaps for a slight downregulation in Passage 2 (P2 = 36 cells/90 (40%)) (Fig. 4.4).

Figure 4.1: The expression of pluripotency markers, *cPouV* and *cNanog*:

The first column shows phase contrast views of the cells (A, G) and expression of *cPouV* and *cNanog* in bright field (A', G'), respectively at Passage 0. *cPouV* is downregulated at Passage 1 (B, B'), Passage 2 (C, C'), Passage 3 (D, D') and Passage 4 (E, E') and upregulated again at Passage 5 (F, F'). *cNanog* expression appears low at Passage 1 (H, H') and downregulated at Passage 2 (I, I'), 3 (J, J'), 4 (K, K'). *cNanog* expressing cells are seen again at Passage 5 (L, L'). (Scale bar = 50 μm).

Figure 4.1: The expression of pluripotency markers, *cPouV* and *cNanog*
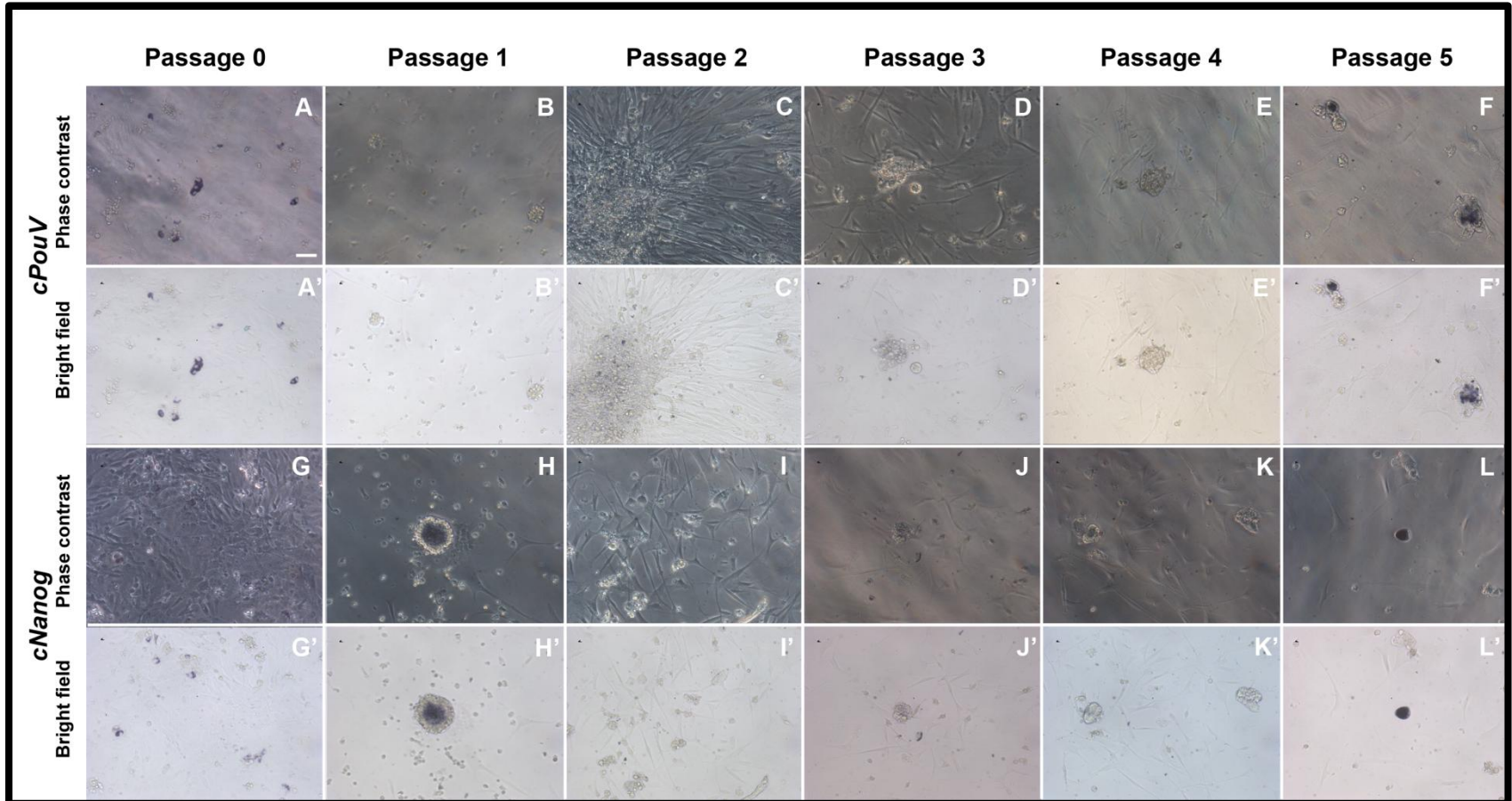
Figure 4.2: The expression of pluripotency markers, *cSox2* and *cKlf4*:

The first column shows phase contrast views of the cells (A, G) and expression of *cSox2* and *cKlf4* in bright field (A', G'), respectively at Passage 0. *cSox2* Expression of both markers appears to be maintained throughout culture. (Scale bar = 50 μm).

Figure 4.2: The expression of pluripotency markers, *cSox2* and *cKlf4*
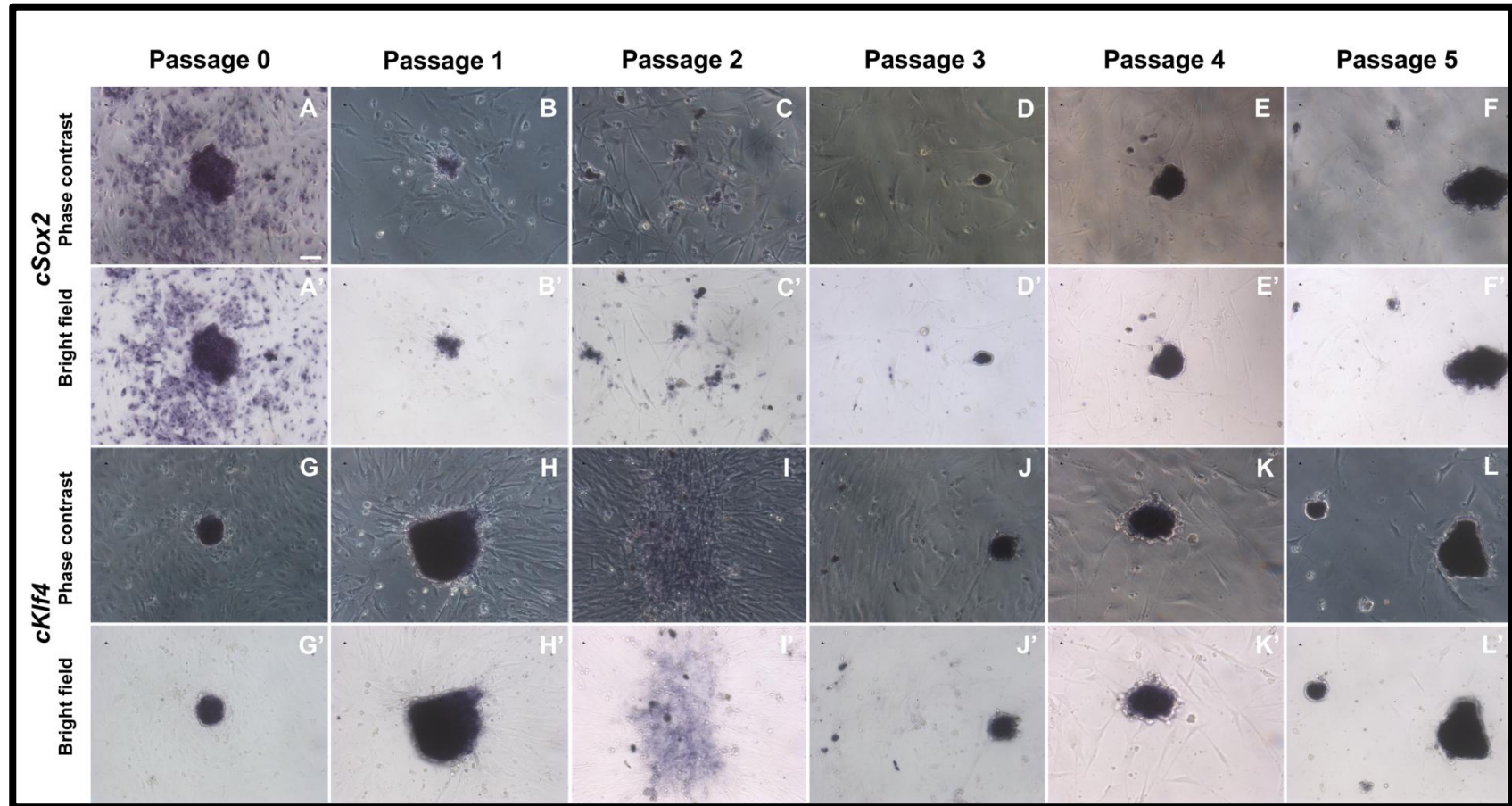
Figure 4.3: The expression of pre-neural markers, *ERNI* and *cSox3*:

The first column shows a phase contrast view of the cells (A, G) and expression of *ERNI* and *cSox3* in bright filed (A', G'), respectively at Passage 0. Expression of *ERNI* seems to be maintained throughout the culture period. *cSox3* is strongest at Passage 0 (G, G') and Passages 4 (K, K') and Passage 5 (L, L'). (Scale bar = 50 μm).

Figure 4.3: The expression of pre-neural markers, *ERNI* and *cSox3*
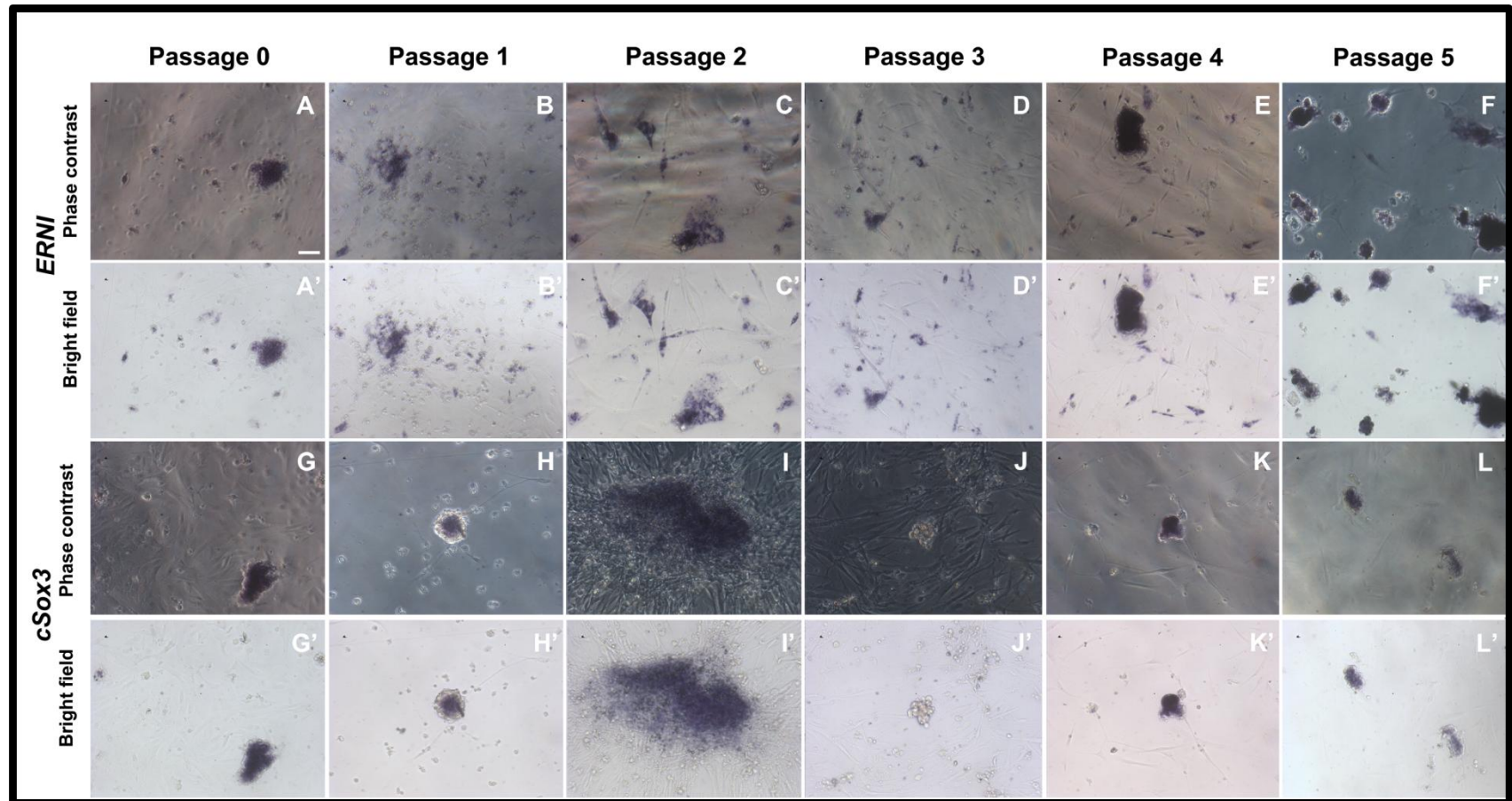
Figure 4.4: The expression of germ cell marker, *Cvh*:

The first column shows the cells in phase contrast view (A) and expression of *Cvh* in bright field (A') as observed at Passage 0. This expression remains throughout the culture, except for possible slight downregulation at Passage 2 (C, C'). (Scale bar = 50 μm).

Figure 4.4: The expression of germ cell marker, *Cvh*
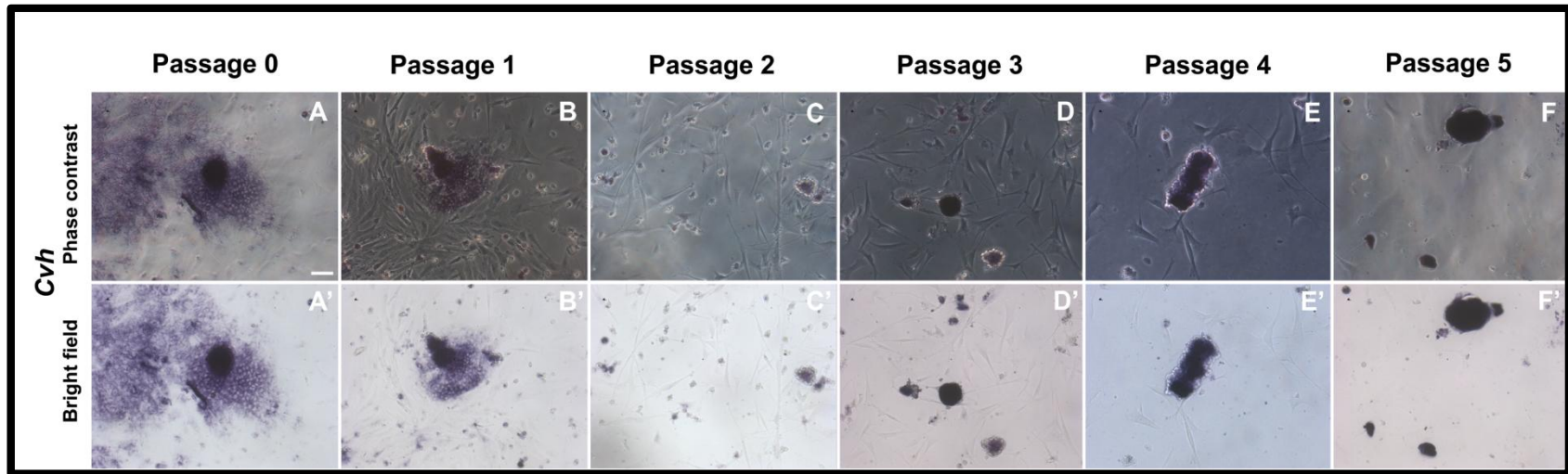
4.3.2. Proliferation assessed by phospho Histone H3 during derivation of cultured chicken gonocyte-derived-EGCs

Staining with anti-phospho Histone H3 was used as a marker of mitosis to identify whether cell proliferation changes accompany their establishment as EGCs from chicken gonocytes over 5 passages. Positive cells (Mitotic cells) are abundant at Passage 0 (Fig. 4.5C). Although observation of the fields suggests that the number of dividing cells progressively decreases (Fig. 4.5 F, I, L, O, R; 90 cells/1,667 (5%) for P0, 7 cells/70 (10%) for P1, 4 cells/42 (10%) for P2, 4 cells/21 (19%) for P3, 2 cells/16 (13%) for P4 and 0 cells/16 (0%) for P5, respectively), there is also a reduction in overall cell number in the cultures (Fig. 4.5 E, H, K, N, Q). The proportion of dividing cells over the total is difficult to estimate but there appears to be no significant change in the division rate.

Figure 4.5: Mitosis revealed by staining with anti-phospho Histone H3:

The first column shows phase contrast views of cultured gonocytes at Passage 0 (A), Passge 1 (D), Passage 2 (G), Passage 3 (J), Passage 4 (M) and Passage 5 (P). The second column, shows the same field stained by DAPI to visualise all nuclei at the same stages. The third column reveals anti-phospho-Histone H3 staininig in the same fields . Cell density, cell number and the overall number of mitotic cells progressively decreased between Passages 1-4 (O). No dividing cells were seen at Passage 5 in these cultures (R). (Scale bar = 50 μm).

Figure 4.5: Mitosis revealed by staining with anti-phospho Histone H3.
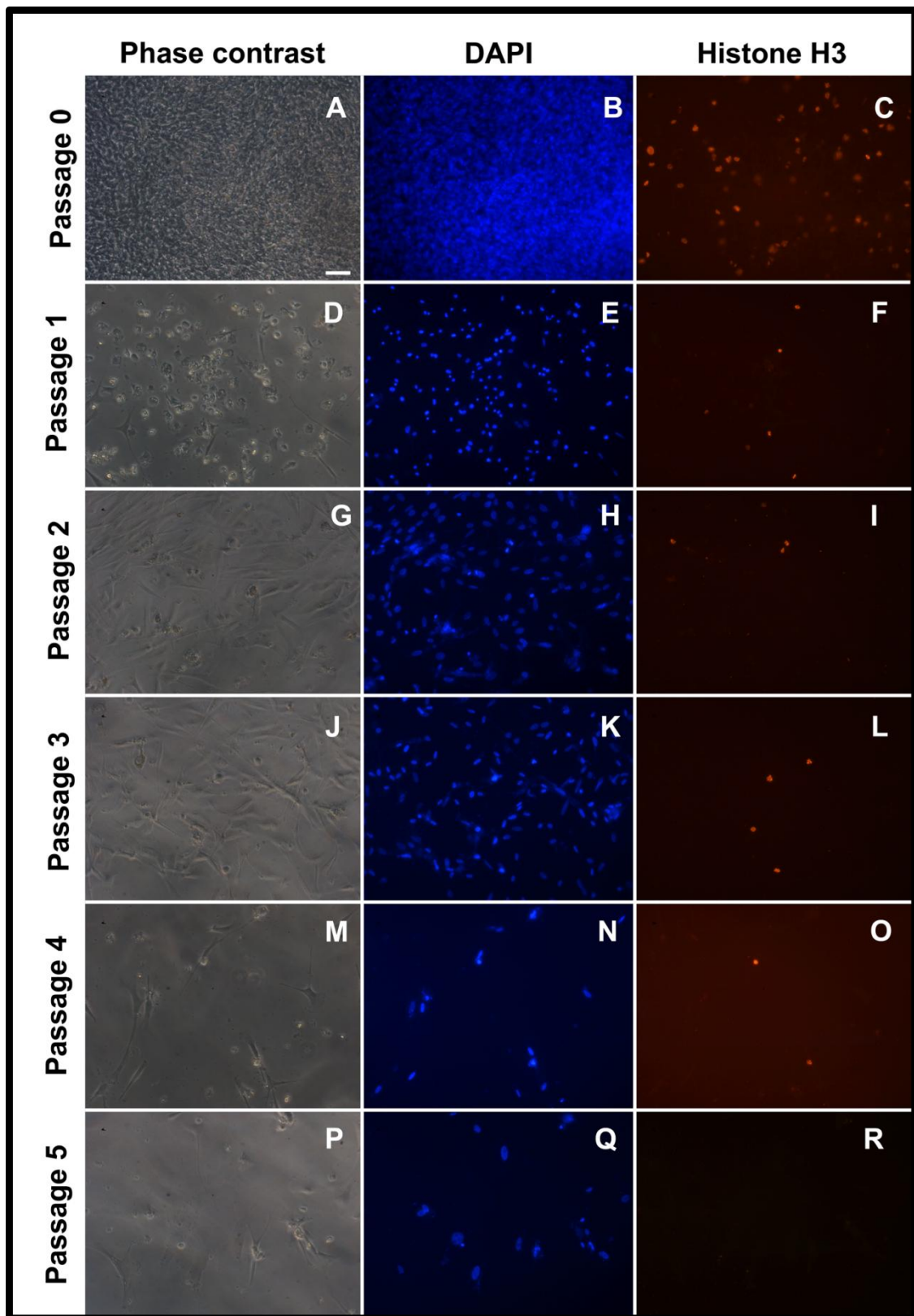
4.4. Discussion

4.4.1. Patterns of gene expression during derivation of cultured chicken gonocytes derived-EGCs

4.4.1.1. Gene expression profile of pluripotency markers

Here, three genes generally considered to be markers for pluripotency, the so-called "Yamanaka factors" (Takahashi and Yamanaka, 2006) (*cPouV*, *cSox2* and *cKlf4*) (Lavial et al., 2007; Macdonald et al., 2010; Takahashi and Yamanaka, 2006) were used for studying the changes which chicken gonocytes undergo during their spontaneous conversion into EGCs in culture. Upon first plating the gonadal cells (Passage 0), *cPouV*, *cNanog*, *cSox2* and *cKlf4* are expressed differently: while *PouV*, *Sox2* and *Klf4* are all expressed fairly strongly (along with another marker of chick ES cell pluripotency, *ERNI*; Acloque et al., 2001), *cNanog* and *cPouV* are almost absent. Several studies have reported that *cPouV*, *cNanog*, *cSox2*, *cKlf4* and *ERNI* are all expressed in chicken PGCs (Canon et al., 2006; Lavial et al., 2007; Macdonald et al., 2010) (see also Chapter 3). Since these genes are generally considered as markers of pluripotency (Lavial et al., 2007; Macdonald et al., 2010; Takahashi et al., 2007), it is tempting to speculate that gonadal cells, gonadal-derived-PGCs or gonocytes that express those makers might be pluripotent. The different expression of *Nanog* to all the other markers is striking, but also consistent with findings in mouse and human ES cells where *Nanog* expression does not always accompany pluripotency (Nichols and Smith, 2009; Wray et al., 2010). *Nanog* may also be dispensable for inducing pluripotency in somatic cells (Takahashi and Yamanaka, 2006).

Culture in the embryonic germ cell culture medium (EGM) used in this study induces changes in the expression of pluripotency markers. The results suggest that there is some variation in the dynamics of how expression of these markers changes. Several of them appear to be downregulated or even disappear in the middle passages (2-4) during the time course but these reappear by Passage 5. This is a somewhat surprising result and it should be pointed out that this is derived from a single experiment. To confirm this, it will be necessary to repeat the study.

Other markers appear to be maintained throughout the culture, e.g. *Sox2*, *Klf4* and *ERNI* and perhaps *Sox3*. The proportion of cells expressing different markers also seems to vary during culture. In general, observation of the cultures suggests that cell clusters tend to show stronger expression of these markers as well as higher numbers of expressing cells than isolated cells. These features could suggest that pluripotency of cultured chicken gonocytes *in vitro* is dynamic, but it also seems likely that not all of these genes accurately reflect pluripotency by themselves. To resolve this, it will be necessary to test pluripotency directly by determining the contribution of cells from different passages to different somatic tissues in chimaeras, which is beyond the scope of the present study.

4.4.1.2. Gene expression profile of pre-neural and neural markers

ERNI was originally isolated and named as an early response gene to neural induction from the organizer (Hensen's node) (Streit et al., 2000). It was also later identified as being downregulated in chicken ES cells that had been induced to differentiate by Retinoic Acid and is therefore also a marker for the undifferentiated, proliferating

state (and possibly pluripotency) of chick ES cells, where it is expressed highly (Acloque et al., 2001). It would be interesting to perform a similar experiment using EGCs.

A similar relationship between early pre-neural expression with pluripotency and expression in chick ES cells is seen for *cSox3*, an early neural marker expressed in prospective neural plate from very early stages in chick (Albazerchi and Stern, 2007; Uwanogho et al., 1995) (Streit et al., 1998). *Sox3* is initially expressed in a very broad territory of the early (pre-streak) chick embryo, very similar to *ERNI*. Like *ERNI*, it is induced by FGF8 produced by the hypoblast (Streit et al., 1998; Streit et al., 2000; Albazerchi and Stern 2007). In mouse embryos, it is *Sox2* rather than *Sox3* that displays this pattern of expression which led to the suggestion that the functions of *Sox2* and *Sox3* have been swapped during vertebrate evolution (see Uwanogho et al., 1995; Rex et al., 1997; Stern, 2006). In both classes, *Sox2* is later expressed throughout the neural plate and forming neural tube and is generally considered to be the earliest definitive neural marker (see also Papanayotou et al., 2008). Here, their expression also appears similar. However in both cases *in situ* hybridisation produced quite a lot of background signal, both in embryos and in culture, and it is still impossible to be certain that all of the expression observed for these markers is specific.

During primary screening of genes in chicken embryonic gonads (see Chapter 5), *cSox3* was found to be expressed in chicken embryonic gonad; in sections, *cSox3* positive cells were detected in left and right gonadal ridges and in the dorsal mesentery where chicken PGCs reside (data not shown). As discussed above, *ERNI*

and *Sox3* have similar expression patterns in very early (pre-streak) embryos, and their expression in the gonads and throughout culture of gonocytes and EGCs is also comparable. This suggests that they may play similar functions in these various cells, or at least that their expression may be regulated by common mechanisms. Further study is required to determine their functional connections in gonocytes and the EGCs derived from them.

4.4.1.3. Expression profile of the germ cell marker *Cvh*

Germ cells are relatively unusual in having at least one unambiguous marker gene that is completely specific– it is never expressed in any other cell type, and it appears that it is expressed by all germ cells and throughout metazoan evolution, including invertebrates (Lasko and Ashburner, 1988). Chicken vasa homologue (Cvh) has been isolated and characterized in chick PGCs (Tsunekawa et al., 2000) – it is among a very small number of genes that are true markers for a particular cell type or state, as it is never expressed in any cell type except germ cells. Therefore Cvh is the best marker to identify germ cells objectively. PGCs have an unusual status, in that they are set aside very early during development (therefore in some sense a very early embryonic cell) and totipotent (they can give rise to the entire body including more germ cells), yet at the same time they are highly specialised – their function is to produce the germ line and they are therefore in some sense "committed". It is therefore particularly interesting to look at possible changes in expression of this unambiguous germ cell marker during the derivation of pluripotent EGCs from chicken gonocytes in culture. It was found that *Cvh* is expressed in cultured gonocyted in all passages (except perhaps for a very slight and transient downregulation at Passages 2-3). By Passages 4 and 5 the expression appears similar

142

to Passage 0. This finding indicates that these cells maintain their germ cell identity, as assessed by their expression of *Cvh*, throughout the derivation. This raises the interesting paradox: it may be possible for cells to retain a unique identity ("differentiation", or "commitment") while apparently gaining pluripotency.

Interestingly, expression of all markers used in this study are most obviously seen in cell colonies rather than in single cells. Although this was not quantified systematically, it was a marked trend observed in almost all cases of stained cultures, with virtually all markers. This could suggest that the most actively proliferating cells (since the colonies are most likely clonal) are those that tend to express pluripotency-associated markers. On the other hand, it was observed that the number of dividing cells decreases with time in culture and passage number.

4.4.2. Proliferation assessed by phospho Histone H3 during derivation of cultured chicken gonocyte-derived-EGCs

The pattern of cell division of germ cells in chick embryos has been studied previously (Swartz and Domm, 1972). Here we find that the number of cells staining for anti-phospho Histone H3 (Ser10) decreases during successive passages, concomitant with a reduction in both cell number and cell density. This accounts for the extremely slow growth of these cells *in vitro* (see Chapter 6). There may also be some left-right and sex differences in the proliferation rate which were not assessed in this initial study. Further insights into these questions are provided in Chapter 6.

A further factor to consider is that it is generally believed that establishment of immortal cell lines *in vitro* is accompanied by the cells going through a "crisis" where the rate of proliferation decreases dramatically before increasing once again (Hayflick, 1965; Hayflick and Moorhead, 1961; Shay and Wright, 2000; Wright et al., 1989). It seems likely the apparently virtual cessation of cell proliferation at Passages 4-5 may represent such a crisis, presaging the more robust establishment of self-renewal capacity of the cells. Consistent with this, it is at this point that most pluripotency-associated markers also become re-expressed since expression of pluripotent markers, particularly *cPouV* and *cNanog* has been observed again at passage 5 (see above).

Chapter 5. Molecular characterisation of embryonic stem cells and primordial germ cells *in vivo* and *in vitro*

5.1. Introduction

As discussed in Chapter 1, it is possible to establish self-renewing, pluripotent stem cell lines from the early mammalian inner cell mass (to generate Embryonic Stem cells, or ES cells) as well as from gonocytes (for embryonic germ cells, or EG cells). Cells isolated from the early chick embryo prior to gastrulation can also be established in culture and are pluripotent (ESCs; however these cannot contribute to the germ line) (Pain et al., 1996). As presented in previous chapters, EG-like cells can be similarly established in culture; however it is not yet known whether these cells are truly pluripotent, and they have not yet been characterised molecularly.

A more fundamental question is whether any of these established cell lines represent a natural cell population from the tissue source of origin, or whether the cells acquire a new state upon culture which defines them as self-renewing and pluripotent. To begin to answer these questions, we undertook a detailed molecular analysis of cESCs compared with the blastoderms from which they are derived, and of EG cells compared to the gonads from which they are obtained. We chose to examine the expression of 30 molecular markers for various fates and cell states, in time course following the isolation of primary cells *in vitro*.

That ESCs change in culture from their natural state is already obvious from the observation that although freshly isolated cESCs can contribute to all somatic cell types as well as to the germline after injection into stage X recipient chick embryos, (Petitte et al., 1990), chicken ESCs (after culture) appear to be unable to colonize the germline (Etches, 2006). In contrast, PGCs have been shown to contribute to the germline (Sang, 2004), but suggested not to contribute to somatic tissues (Etches, 2006). However, chicken PGCs can differentiate into EGCs after removal of SCF and FGF from the culture medium, which causes them to resemble ESCs morphologically as well by their ability to contribute to somatic cell types (van de Lavoir et al., 2006). Conversely, it has been reported that chicken ESCs could be reprogrammed to a germ cell fate by electroporation of Cvh, after which descendants of the transfected ESCs can be found in the gonad (Lavial et al., 2009). It remains to be determined whether these transfected cells can indeed contribute to the germ line. Despite these pioneering studies, the relationships between a somatic fate of ESCs and a germ cell fate of PGCs have not yet been unveiled, and we know remarkably little about the properties of these cell types at the molecular level, either *in vivo* or *in vitro*.

For the various reasons explained above, it is interesting to examine whether there are differences in the expression of pluripotentcy-associated and other differentiation genes between somatic and germline cells and their tissues of origin. Here we undertake such an analysis, using chicken embryonic gonads and cultured gonocytes and compare them with cES cells. We also compare these cultured cells ("*in vitro*") with their tissue of origin ("*in vivo*"). Does the profile of gene expression change upon culture, and if so how quickly? Using *in situ* hybridisation, we are also able to

determine the degree to which the cell populations are uniform or heterogeneous, *in vivo* and *in vitro*.

5.2. Methods

5.2.1. Gene expression profiles of in chicken embryonic gonads, primary gonocytes, established primordial germ cells and embryonic stem cells

The methods used for studying gene expression *in vivo* and *in vitro* using *in situ* hybridization were described in detail in Chapter 2. We studied an established gonadal-PGC line, GFP-527, shown to be capable of efficient germline trasmission (M.C. van de Lavoir, Crystal Bioscience, personal communication). The line was kindly provided by Dr. Marie-Cecile van de Lavoir. An established cESCs line, 9N2 (Pain et al., 1996) was also used, obtained from Dr. Bertrand Pain (Université Lyon 1, France). The riboprobes for characterizing 30 genes studied in this chapter are listed alphabetically in Table 5.1 below. Expression of these was also tested on early chick embryos at different stages, both to use as controls and for comparison with the cell lines; embryos were staged according to (Hamburger and Hamilton, 1992).

5.2.2. Cell counting and statistical analysis of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells.

To count cells expressing the markers listed in Table 5.1 in the embryonic gonads, the positive cells expressing those markers were counted in every section from the

gonadal ridges (where the germ cells localize) in both left and right sides at stage 25-28HH. The average number of cells expressing the markers in the embryonic gonads was plotted. To count expressing cells in primary gonocytes (passage 0) and established PGCs (GFP-527), tissue culture wells of each experiment were selected. Expression of each marker was assessed only in cells showing gonocyte-like morphology located in the centre of each well. Within each series of experiments, three replicates were done for each assay. For each well, representative fields showing a number of cells expressing in each marker were recorded, transferred to a spreadsheet before performing statistical analysis to assess the percentage of positive cells expressing the markers (mean and standard deviation), which was then plotted.

Table 5.1: Gene markers used for *in vivo* and *in vitro* comparison of gene expression in chicken embryonic gonads, primary gonocytes, primordial germ cells and embryonic stem cells.

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
BERT	Chick BERT	*EcoRI*	T3		(Papanayot ou et al., 2008)
cBMP4	Chick BMP4	*BamHI*	T3		(Streit and Stern, 1999)
pFLBMP8-1	Chick BMP8	*NcoI*	T7		(Lavial et al., 2007)
cBra9 (mesoderm)	Chick Brachyury	*XbaI*	T3	Gift from V. Cunliffe	(Smith et al., 1991)
cCdx (extra-Embryonic, caudal)	Chick Cdx2	*ClaI*	T3		(Pernaute et al., 2010)
cChCh (early neural plate)	Chick Churchill	*XhoI*	T3		(Sheng et al., 2003)
Connexin43 (gap junctions					
pFLCripto2	Chick Cripto	*SacII*	SP6		(Lawson et al., 2001)
pFLEomes	Chick Eomeso dermin	*SalI*	T7		(Pernaute et al., 2010)
ERNI Wpst	Subclone for ERNI for ISH	*KpnI*	T3		(Streit et al., 2000)
cGata2 (epidermis)	Chick Gata2	*NdeI*	T7		(Sheng and Stern, 1999)

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
cGata6	Chick Gata6	*NcoI*	SP6		(Chapman et al., 2007)
cGeminin	Chick Geminin	*XhoI*	T7		(Papanayot ou et al., 2008)
HP1-α	Chick HP1-alpha				(Papanayot ou et al., 2008)
cKlf2	Chick Klf4	*NotI*	T3	Gift from P. Antin	(Antin et al., 2010)
cKlf4 ("Yamanaka factor")	Chick Klf4	*NotI*	T3	Gift from P. Antin	(Antin et al., 2010)
pFL Nanog ("Yamanaka factor")	Chick Nanog	*ApaI*	SP6		(Lavial et al., 2007)
pFLIp06 ("Yamanaka factor")	Chick Oct3/4 homologue	*ApaI*	SP6		(Lavial et al., 2007)
cOtx2 (early embryo organier, prosencepha lon)	Chick Otx2	*XhoI*	T3	Gift from L. Bally-Cuif	(Bally-Cuif et al., 1995)
cPdx1 (endoderm)	Chick Pdx	*HindIII*	T3	Gift from Grapin Lab	
cRunx2	Chick Runx2	*XhoI*	SP6	Gift from A.H. Monsoro-Burq	(Holleville et al., 2007)
cSox1 (mature neural plate)	Chick Sox1	*XhoI*	T7	Gift from H. Kondoh	(Kamachi et al., 1998)

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
cSox2 ("Yamanaka factor", neural plate)	Chick Sox2	*PstI*	T7	Gift from P. Scotting	(Uwanogho et al., 1995)
cSox3 (pre-neural)	Chick Sox3	*PstI*	T7	Gift from P. Scotting	(Uwanogho et al., 1995)
pBSXsox17α (endoderm)	Chick Sox17	*SmaI*	T7	Gift from Woodland	Hudson et al., 1997
cSlug (neural crest; ingressing cells)	Chick Snail-2	*NotI*	T3		(Sefton et al., 1998)
cSna (neural crest; ingressing cells)	Chick Snail-1	*NotI*	T3		(Sefton et al., 1998)
cTbx3	Chick Tbx3	*XhoI*	T3	Gift from C. Tickle	(Tumpel et al., 2002)
*Cvh** (germ cell marker)	Chick Vasa Homologue	*NcoI*	SP6		(Tsunekawa et al., 2000)
*cDAZL** (germ cell marker)	Chick Deleted in Azoospermia Like	*NdeI*	T7	Gift from J. Petitte	(Rengaraj et al., 2010)

(* = not tested in early embryos but specific patterns shown in embryonic gonads).

Gene expression patterns of these markers (Table 5.1 above) in control embryos are shown in Figure 5.1 below.

Figure 5.1: Expression patterns of markers listed in Table 5.1 in control

embryos at stages 4-11HH.
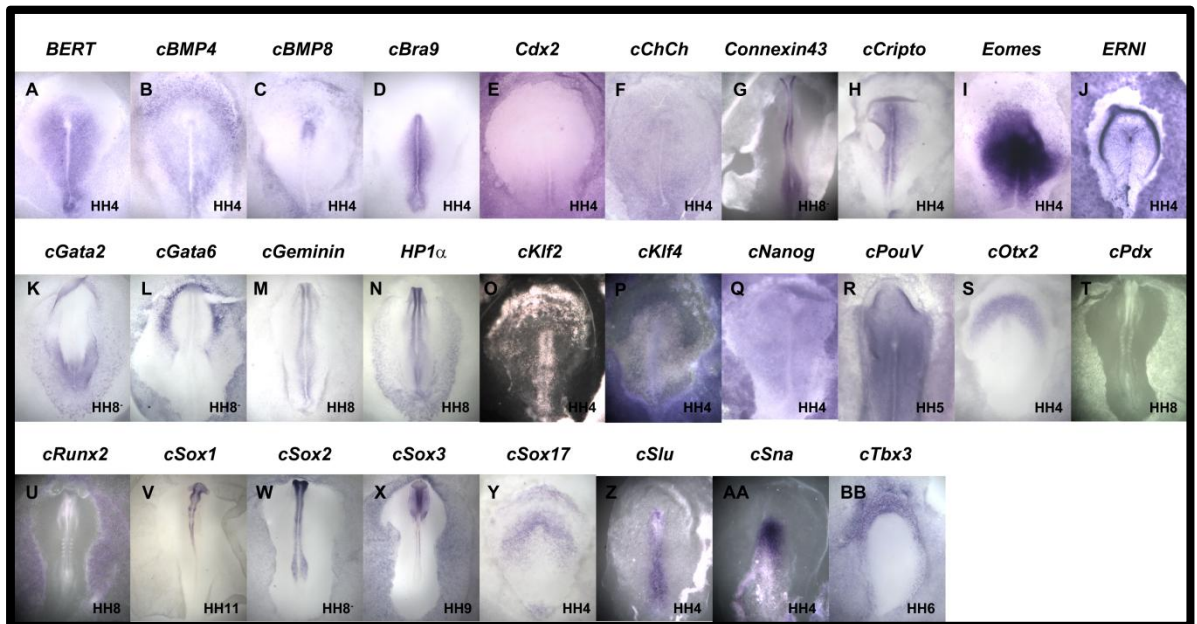
5.3. Results

5.3.1. *In vivo* and *in vitro* comparisons of gene expression in chicken embryonic gonads, primary gonocyctes and established primordial germ cells and embryonic stem cells.

To compare gene expression profiles *in vivo* and *in vitro*, the embryonic gonads at stage 25-28 (4.5-5 days' incubation), primary gonocytes (passage 0), established PG and ES cells were used. *In vitro,* all cell types express *cPouV*, *cNanog*, *cSox2* and *ERNI* (Figure 5.2F-S). *cPouV*, *cNanog* and *ERNI* are all expressed in the embryonic gonads (Figure 5.2A, B and D), whereas *cSox2* expression is not (Figure 5.2C). In

sections of embryonic gonads, expression of *cPouV*, *cNanog* and *ERNI* (Figure 5.2A',

B' and D') can be detected in both left and right gonadal ridges and dorsal mesentery,

the migratory route of germ cells. On the other hand, expression of another

"Yamanaka factor", *cSox2*, cannot be detected in gonadal sections at all (see Figure

5.2.C').

Quantification of cells expressing these genes bears out the above qualitative

assessment: *cNanog* is expressed in the largest number of cells (Mean $\pm$ SD) = 2,040

$\pm$ 1,358 per gonad (n=3), Table 5.2 and Figure 5.3), whereas the numbers of cells

expressing *cPouV* and *ERNI* were 1,089 $\pm$ 142 (n=3) and 953 $\pm$ 1,030 (n=3) per

gonad, respectively (Table 5.2 and Figure 5.3) and there were no cells expressing

cSox2 (counted from 5 embryonic gonads, n=5). The average number of cells

expressing *Cvh* was 885 $\pm$ 639 (n=5) per gonad, Table 5.2 and Figure 5.3). Since Cvh

is a reliable germ cell marker (see above), These results imply that many of the cells

expressing each of the "pluripotency"-related genes *cNanog*, *cPouV* and *ERNI* are not

germ cells, and that the populations of cells expressing these markers only partially

overlap. However, establishing how many different subpopulations of cells are

present in these cultures will require double *in situ* hybridization, which turned out to

be very difficult *in vitro*.

In vitro quantification of cells expressing these genes show that the percentage (only

cells with gonocyte-like morphology) of primary gonocytes expressing *ERNI* was 60

$\pm$ 39%, Table 5.3 and Figure 5.4) and established-PGCs expressing *ERNI* = 60/83

cells (72%). The proportion of primary gonocytes expressing *cPouV* and *cNanog* were

54 $\pm$ 22% and 41 $\pm$ 34%, respectively (Table 5.3 and Figure 5.4) and for established-

PGCs it was 63 cells/103 (61%) for c*PouV* and 47 cells/121 (39%) for *cNanog* . While 35 $\pm$ 22% primary gonocytes expressed *cSox2* (Table 5.3 and Figure 5.4), and 16 established-PGCs out of 101 (16%) did so. *Cvh* was expressed in 54 $\pm$ 34% of cells with gonocyte-like morphology in primary gonocyte cultures, Table 5.3 and Figure 5.4) and 42/59 cells (71%) of established PGC cultures. Thus for these *in vitro* cells, the number of cells expressing each marker are approximately the same, including for *Sox2* which is now expressed in a proportion of both primary and established PGC-derived cells.

Figure 5.2: *In vivo* **and** *in vitro* **comparisons of gene expression in chicken embryonic gonads, primary gonocytes, established primordial germ cells and embryonic stem cells:** *cPouV* (A, A'), *cNanog* (B,B'), *cSox2* (C, C'), *ERNI* (D, D') and *Cvh* (E, E') are shown in embryonic gonads and gonadal sections, respectively. In culture, these genes are expressed in primary gonocytes (F, G, H, I, J), established-PGCs (K, L, M, N, O) and ESCs (P, Q, R, S, T). (Scale bar in A' = 50 μm, F, K, P = 100 μm, arrow = positive cells).

Figure 5.2: *In vivo* and *in vitro* comparisons of gene expression in chicken embryonic gonads, primary gonocytes, established primordial germ cells and embryonic stem cells.
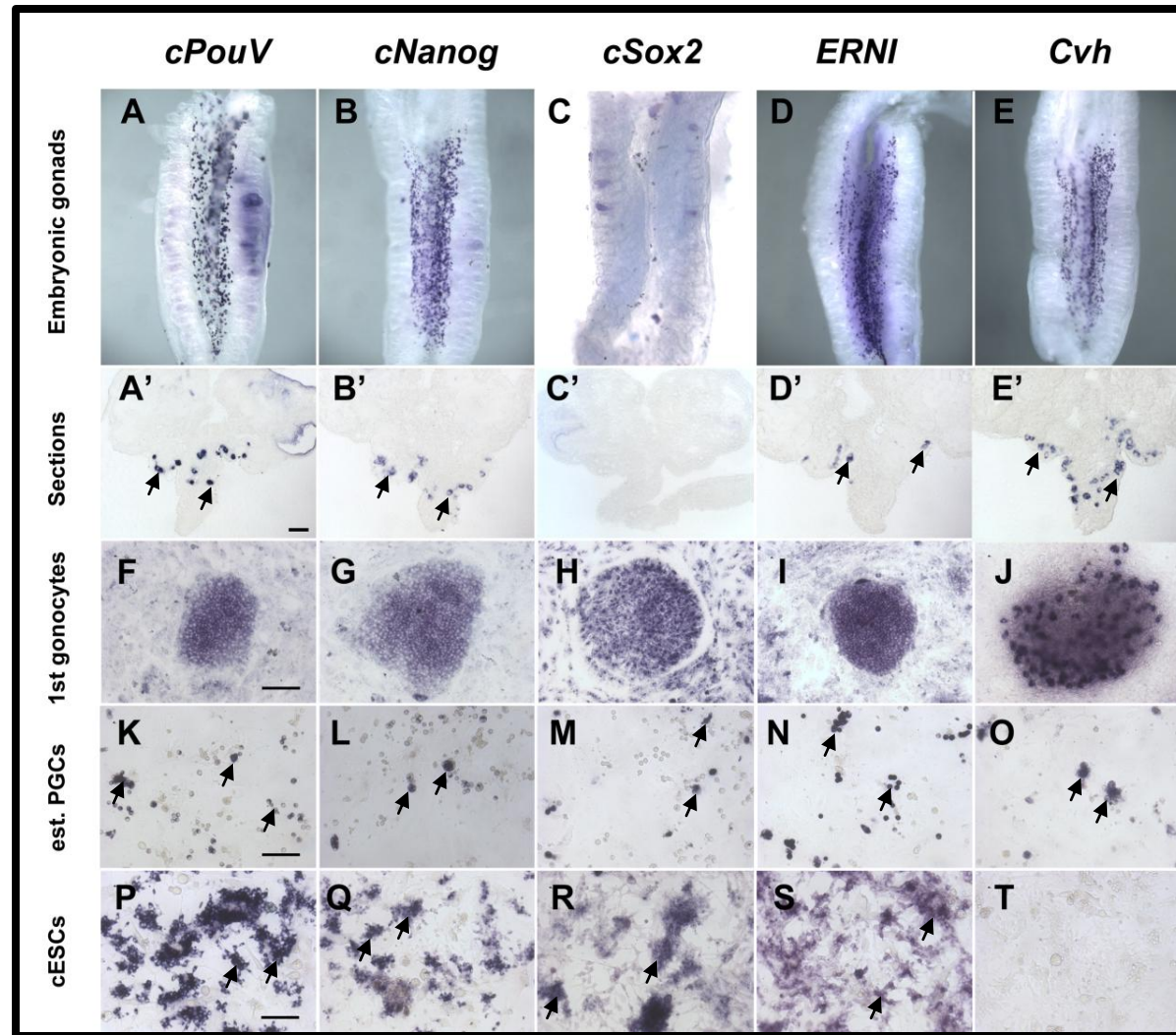
Table 5.2: Quantification of cells expressing *cPouV*, *cNanog*, *cSox2*, *ERNI* and *Cvh* in embryonic gonads.

Marker	Embryonic gonads	Expressing cells per gonad
cPouV	1	995
	2	1,253
	3	1,020
	Mean	**1,089**
	SD	**142**
cNanog	1	566
	2	2,314
	3	3,239
	Mean	**2,040**
	SD	**1,358**
cSox2	1	0
	2	0
	3	0
	4	0
	5	0
	Mean	**0**
	SD	**0**

Marker	Embryonic gonads	Expressing cells per gonad
ERNI	1	139
	2	609
	3	2,110
	Mean	**953**
	SD	**1,030**
Cvh	1	810
	2	340
	3	787
	4	515
	5	1,972
	Mean	**885**
	SD	**639**

Figure 5.3: Quantification of cells expressing various genes in embryonic gonads.
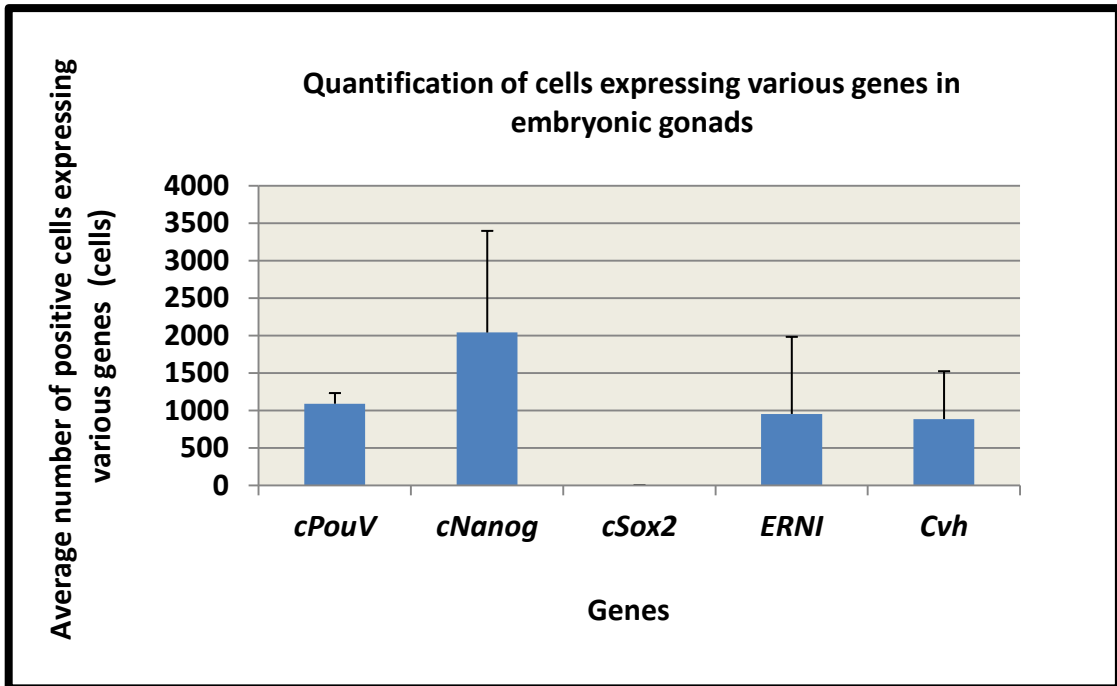
Table 5.3: Expression of *cPouV*, *cNanog*, *cSox2*, *ERNI* and *Cvh* in primary gonocytes.

Marker	Exp	Positive cells	Total cells counted	%
cPouV	1	456	577	79%
	2	256	600	43%
	3	488	1233	40%
			Mean	**54%**
			SD	**22%**
cNanog	1	693	866	80%
	2	174	754	23%
	3	103	559	18%
			Mean	**41%**
			SD	**34%**
cSox2	1	210	373	56%
	2	74	635	12%
	3	136	370	37%
			Mean	**35%**
			SD	**22%**

Marker	Exp	Positive cells	Total cells counted	%
ERNI	1	636	657	97%
	2	194	995	20%
	3	114	176	65%
			Mean	**60%**
			SD	**39%**
Cvh	1	268	620	43%
	2	172	650	27%
	3	1288	1392	93%
			Mean	**54%**
			SD	**34%**

Figure 5.4: **Quantification of cells expressing various genes in primary gonocytes.**
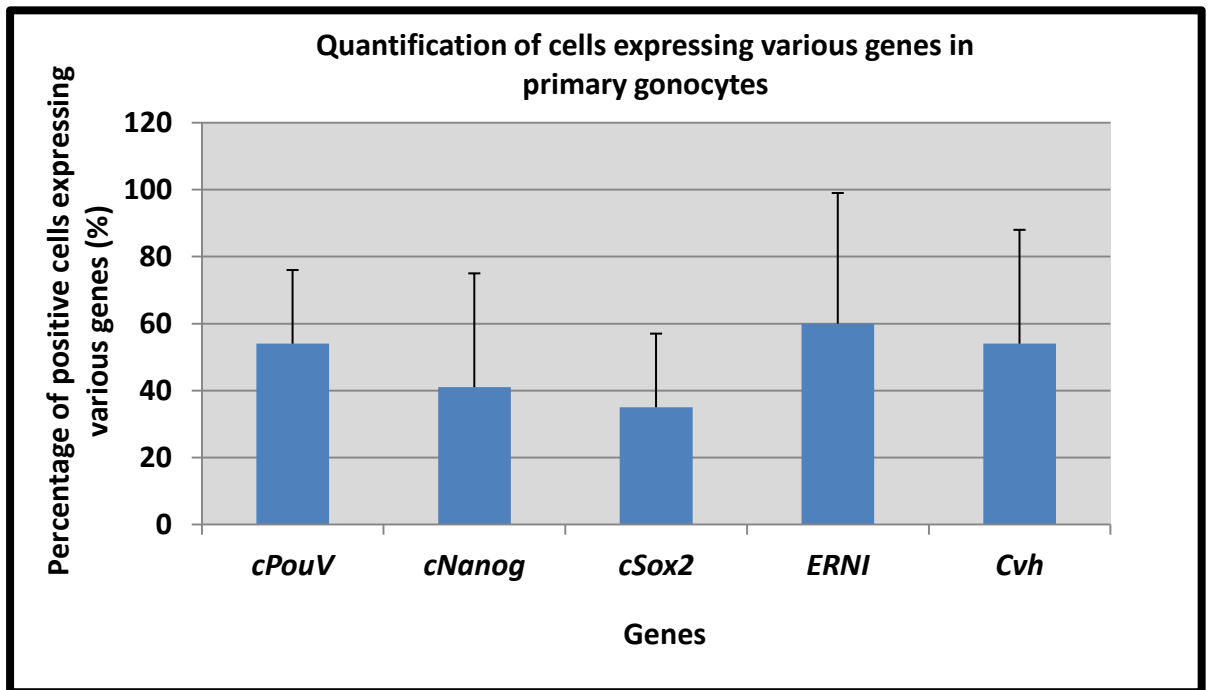
Next, the expression of a number of genes that had previously been associated with various properties of stem cells was studied in the same cell populations. *cKlf4* (n=3), *cKlf2* (n=3) are expressed in embryonic gonads (see Figure 5.5C, C' and D, D') but *cTbx3* (n=4) and *cRunx2* (n=2) are not (see Figure 5.5A, B) (see Figure 5.5E, E'). *cTbx3* is expressed in primary gonocytes (73/324 cells (23%)) (see Figure 5.5F) but not in established-PGCs (see Figure 5.5J).*cRunx2* and *cKlf4* are expressed in primary gonocytes (53/141 cells (38%) and 28/66 cells (42%), respectively) (see Figure 5.5G, H). The proportion of primary gonocytes expressing the germ cell marker, *cDAZL* was (252/428 cells (59%)) (see Figure 5.5I). Expressions of *cRunx2* (22/47 cells (47%)), *cKlf4* (12/44 cells (27%)), *cKlf2* (49/80 cells (61%)) and *cDAZL* (42/111 cells (38%)) are expressed in established-PGCs except *cTbx3*. Furthermore, *cKlf4* is expressed in cESCs (see Figure 5.5P) but *cTbx3* is not (see Figure 5.5O).

Figure 5.5: *In vivo* **and** *in vitro* **comparisons of gene expression for stem-cell-related genes in chicken embryonic gonads, primary gonocytes, established primordial germ cells and embryonic stem cells:** Expressions of *cKlf4* (C, C'), *cKlf2* (D, D') and *cDAZL* (E, E'). No expressions of *cTbx3* (A, A') and c*Runx2* (B, B') in embryonic gonads and gonadal sections, respectively. *cTbx3* (F), *cRunx2* (G), *cKlf4* (H), *cDAZL* (I) are expressed in primary gonocytes . *cRunx2* (K), *cKlf4* (L), *cKlf2* (M) and *cDAZL* (N) are expressed in established-PGCs but not *cTbx3* (J). *cRunx2* is expressed in ESCs (P) but not *cTbx3* (O). (Scale bar in A' = 50 μm, F, J, O = 100 μm, arrow = positive cells).

Figure 5.5: *In vivo* and *in vitro* comparisons of gene expression for stem cell related genes in chicken embryonic gonads, primary gonocytes, established primordial germ cells and embryonic stem cells.
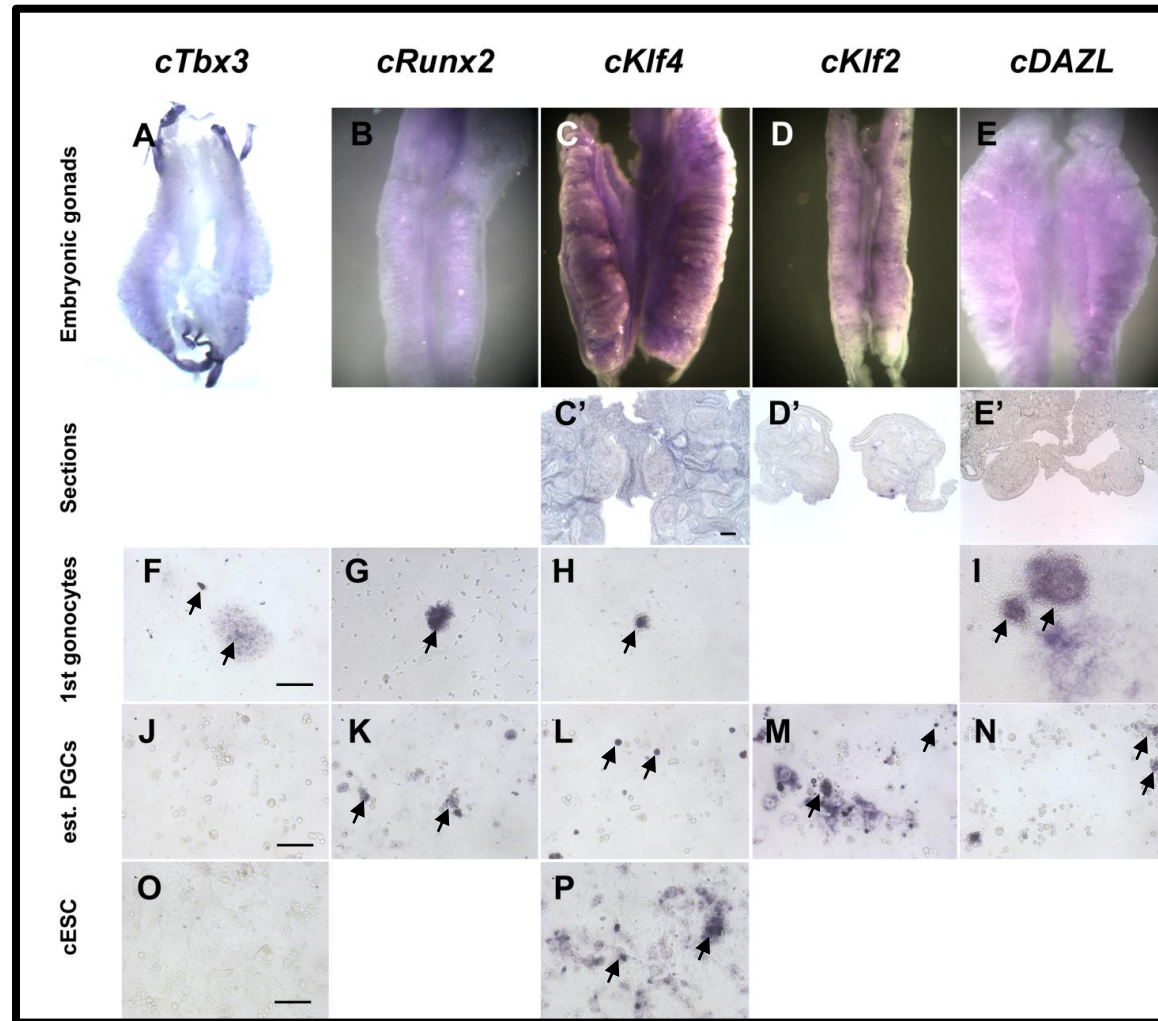
Next we examined several genes connected with neural induction and/or very early stages of neural plate development in the same populations. *Eomes* (n=3), *cSox3* (n=4) and *cChCh* (n=3) are expressed in embryonic gonads and their sections (see Figure 5.6B, B', C, C' and E, E') but *Cdx2* (n= 2) and *HP1α* (n=4) are not (see Figure 5.6A, A' and D, D'). On the other hand, primary gonocytes expressed *Cdx2* and *cSox3* (88/147 cells, 60% and 35/88 cells, 40%, respectively; see Figure 5.6F and H) while there were no primary gonocytes expressing *Eomes*, *HP1α* and *cChCh* (see Figure 5.6G, I and J) suggesting that the cell population only contains undifferentiated cells. Interestingly, expressions of *Cdx2* (37/48 cells (77%)), *Eomes* (31/64 cells (48%)), *HP1α* (53/75 cells (71%)) and *cChCh* (36/58 cells (62%)) are expressed in established-PGCs (see Figure 5.6K, L, M, N and O). However, this study did not check differentiation potential and this should be done in the future.

**Figure 5.6:** *In vivo* **and** *in vitro* **comparisons of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells:** Expressions of *Eomes* (B, B'), *cSox3* (C, C') and *cChCh* (E, E') in embryonic gonads and gonadal sections. No expressions of *Cdx2* (A, A') and *HP1α* (D, D') in embryonic gonads. *Cdx2* (F) and *cSox3* (H) are expressed in primary gonocytes but not *Eomes* (G)*, HP1α* (I) *and cChCh* (J). While, *Cdx2 (K), Eomes* (L), *cSox3* (M), *HP1α* (N) and *cChCh* (O) are expressed in established-PGCs. (Scale bar in A' = 50 μm, F, K = 100 μm, arrow = positive cells).

Figure 5.6: *In vivo* and *in vitro* comparisons of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells.
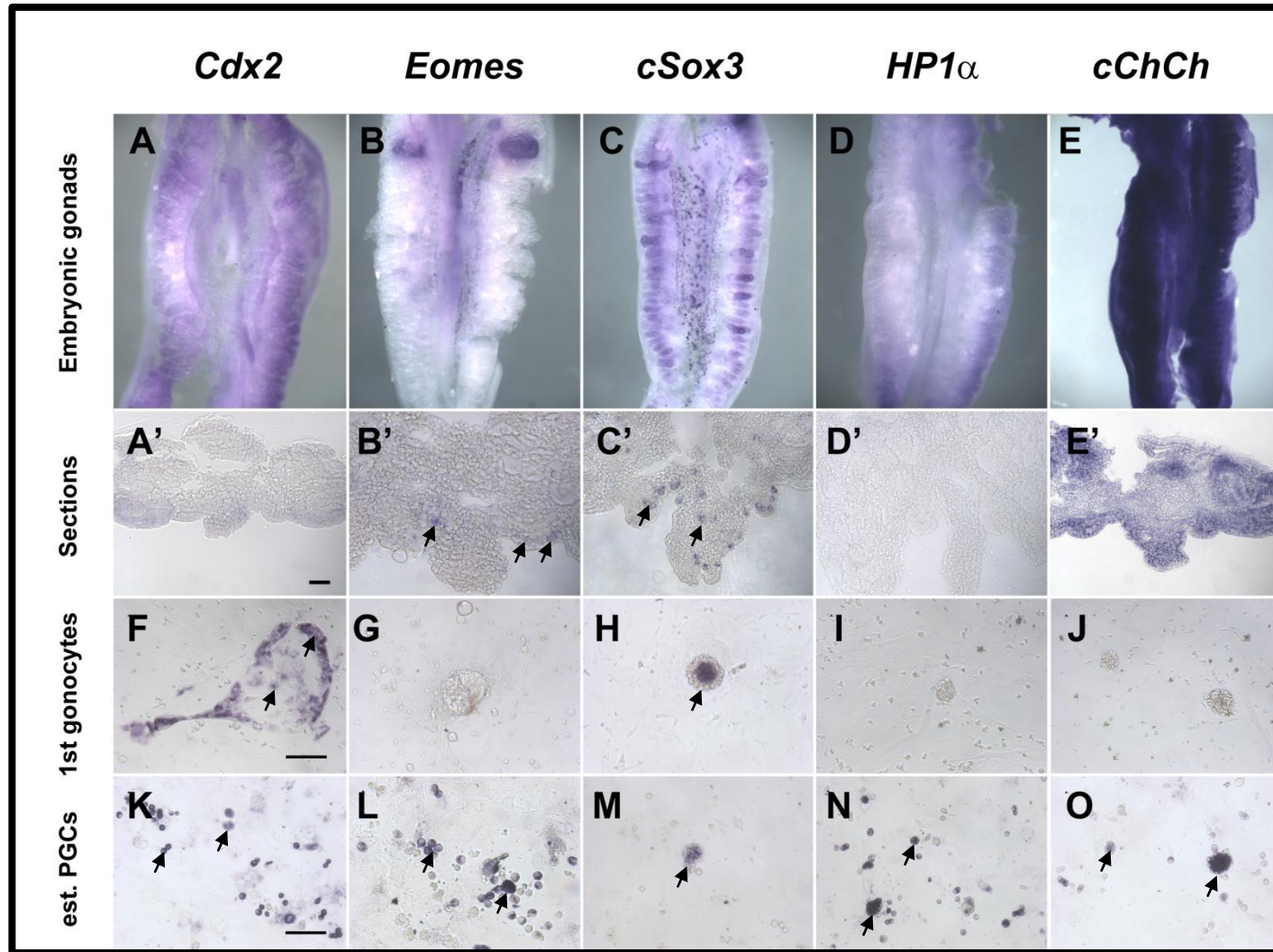
Finally several other genes connected with early neural and/or mesodermal development were examined. These genes were *BERT* (n=3)*, cBMP4* (n=3), *cBMP* (n=3), *cBra9* (n=3), *Connexin43* (n=3), *cCripto* (n=2), *cGata2* (n=3), *cGata6* (n=3), *cGeminin* (n=3), *cOtx2* (n=5), *cPdx* (n=2), *cSox1* (n=4), *cSox17* (n=5), *cSlu* (n=3) *and cSna* (n=2). There was no clear expression of any of these markers in embryonic gonads (Figure 5.7 A, A' to H, H' and Figure 5.8 A, A' to G, G'), in primary gonocytes (Figure 5.7 I to O and Figure 5.8 H to K) or in established PGCs (Figure 5.7 P to W and Figure 5.8L, M, O, P, Q, R). *cPdx* was expressed in 60/150 established PGCs (40%) (Figure 5.8N). In future it would be interesting to test the ability of these various cells to differentiate into various cell types for example after exposure to retinoic acid.

Figure 5.7: *In vivo* **and** *in vitro* **comparisons of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells:** There was no expression of *BERT* (A, A'), *cBMP4* (B, B'), *cBMP8* (C, C') *cBra9* (D, D'), *Connexin43* (E, E'), *cCripto* (F, F'), *cGata2* (G, G'), *cGata6* (H, H'), in embryonic gonads . No expression of *BERT* (I), *cBMP4* (J), *cBMP8* (K) *cBra9* (L), *cCripto* (M), *cGata2* (N), *cGata6* (O) in primary gonocytes and no expression of *BERT* (P), *cBMP4* (Q), *cBMP8* (R) *cBra9* (S), *Connexin43* (T), *cCripto* (U), *cGata2* (V), *cGata6* (W) in established-PGCs. (Scale bar in A' = 50 µm, I, P = 100 µm).

Figure 5.7: *In vivo* and *in vitro* comparisons of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells.
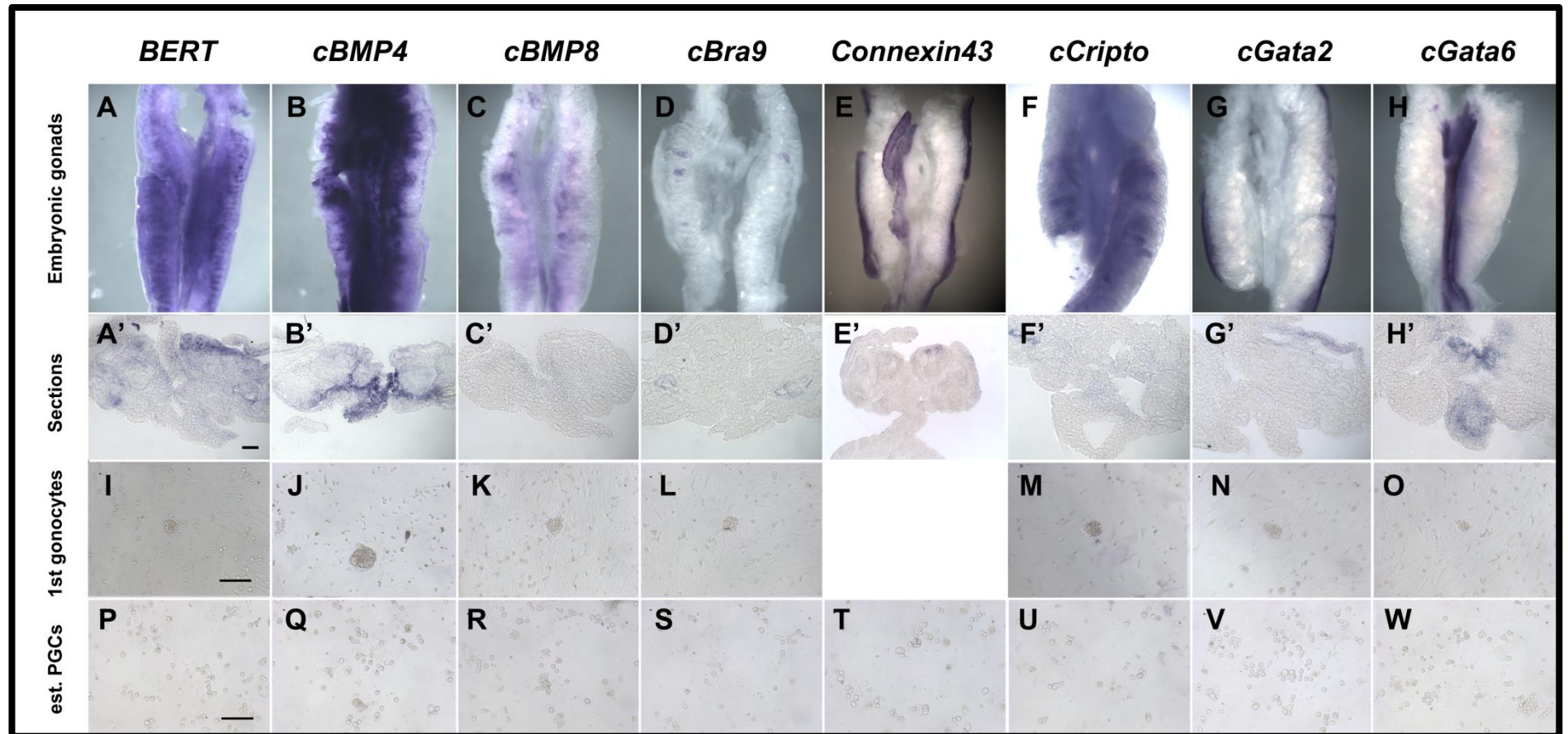
Figure 5.8: *In vivo* **and** *in vitro* **comparisons of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells:** There were no expressions of *cGeminin* (A, A'), *cOtx2* (B), *cPdx* (C, C'), *cSox1* (D), *cSox17* (E), *cSlu* (F, F') *and cSna* (G, G') in embryonic gonads and their sections. No expressions of *cGeminin* (H), *cOtx2* (I), *cSox1* (J), *cSox17* (K) in primary gonocytes and no expressions of *cGeminin* (L), *cOtx2* (M), *cSox1* (O), *cSox17* (P) *cSlu* (Q) *and cSna* (R) in established-PGCs, except *cPdx* (N). (Scale bar in A' = 50 μm, H, L = 100 μm, arrow = positive cells).

Figure 5.8: *In vivo* and *in vitro* comparisons of gene expression in chicken embryonic gonads, primary gonocytes and established primordial germ cells.
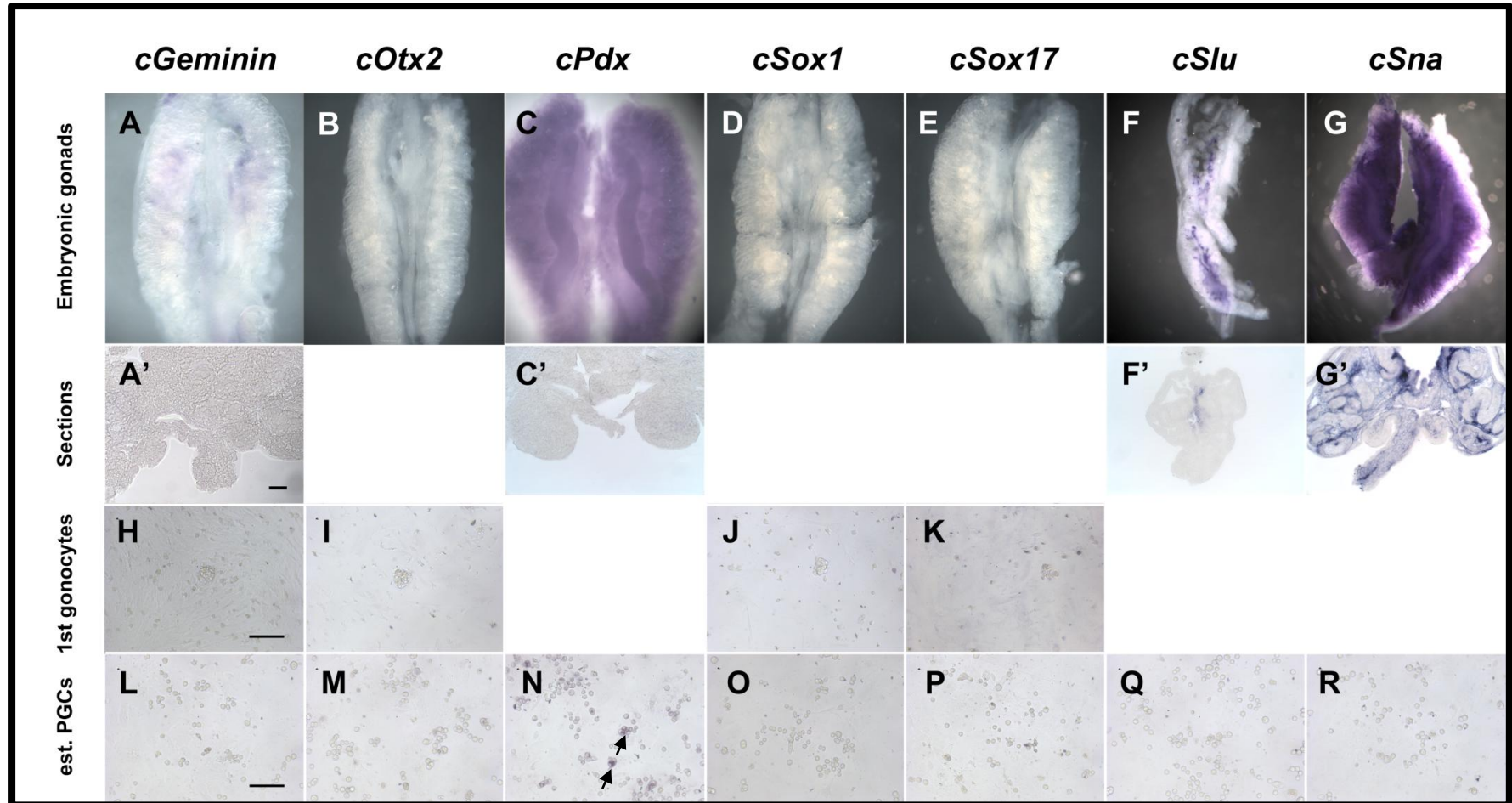
5.4. Discussion

This study reports several findings: first, there is no *cSox2* expression in the embryonic gonads (25-28HH). Conversely, *cSox3* is expressed in the gonads; moreover, in their sections, *cSox3* positive cells were detected in both left-right gonadal ridges and dorsal mesentery. Such regions of the gonads where *cSox3* positive cells were detected are the same regions where *Cvh and cDazl* (germ cells markers) positive cells were also detected, suggesting that *cSox3* positive cells localized in both left-right gonadal ridges and dorsal mesentery may be germ cells. Moreover, early chick embryos do not express *cSox2* before about stage 5, but both primary gonocytes and established PGCs and ESCs do. Conversely for *cSox3*, early embryos express (gonads a little less so, as shown as in Figure 5.6) but EGCs, ESCs and PGCs do not. This suggests that *cSox2* may be substituted by *cSox3* in embryonic chick gonads. This is almost the opposite situation as found in mouse, where *Sox2* is expressed in the very early embryo. It seems possible that different SoxB1 class genes may have exchanged some of their functions during evolution, or at least in chick and mouse. In addition, it has been reported that SOX2 is not expressed in human germ cells *in vivo* (Perrett et al., 2008) as well as porcine embryonic germ cells (Petkov et al., 2011), suggesting that this gene may have different role and other function in development of non-murine germ cells.

Secondly, *cSox2* is expressed immediately after taken from gonocytes *in vivo* and put them to the culture. Presumably, culture conditions i.e. growth factors or other cytokines in the culture medium may turn on *cSox2* expression. This is one of the

most dramatic indicators that cells change their molecular properties upon being placed in culture.

In this study, the result of *in vivo* quantification shows that the majority of cells in the gonads express *cNanog* and *cPouV* (average number is 2,040 and 1,089 cells/ gonad, respectively). This is consistent with previous reports that these genes are highly expressed in embryonic chick gonads (Canon et al., 2006; Lavial et al., 2007). However the number of cells expressing these markers, especially *cNanog*, is generally greater than that those expressing *Cvh* (885 cells per gonad), suggesting that at least some *cNanog* expressing cells in the gonad are not germ cells, consistent with previous findings in embryos (Canon et al., 2006; Lavial et al., 2007). Interestingly, quantification of the proportions of cells expressing the various markers in the present study suggest a marked change in the relationship between the proportion of cells expressing Cvh and other markers *in vivo* and *in vitro*. In the gonad, there are many more cells expressing the other markers than Cvh-positive cells (gonocytes). *In vitro*, however, Cvh cells are more numerous than those expressing other markers. The most likely explanation for this difference is that *in vitro* conditions favour the proliferation of gonocyte-derived cells, whilst cells expressing other markers are more likely to correspond to stromal cells and cease to expand *in vitro*.

A third important finding is that all three cultured cell populations (ES cells, primary gonocytes and established-PGC lines) are highly heterogeneous with respect to the markers they express. This is particularly evident for putative porcine EGCs show that *c-Myc* and *Klf4* were expressed in primary culture while *Oct4*, *Nanog*, *Sox2* were not (Petkov et al., 2011). In addition, rat EGC, ESC lines expressed high levels of *Oct4*, *Sox2*, *Klf4* and *Mvh* but low levels of *Nanog*, *Rex-1* and *c-Kit* (Northrup et al., 2011),

175

suggesting that these differences could be species-specific or different culture conditions.

Finally, it is interesting to observe that only about half of the cells with gonocyte-like morphology express *Cvh* or *cDazl*. This could suggest either that some gonocytes lose expression of these markers in culture or, perhaps more likely, that some cells considered to have gonocyte-like morphology (rounded, relatively non-adherent, appearing singly or in small clumps) may be gonadal stromal or other cells other than gonocytes. Consistent with this, sections reveal that some cells expressing these markers are seen in areas of the gonad other than those containing *Cvh-* or *cDazl-* positive cells, For example *ERNI* which is expressed in lager percent in culture than *Cvh* and *cDazl* and also appeared in the regions other than the gonadal ridges in section (Figure 5.2D').

cPouV and *cNanog* have already been reported to be expressed in early chick embryos (Canon et al., 2006; Lavial et al., 2007); Both genes are expressed in both area pellucida and area opaca of the epiblast in pre-streak embryos (Canon et al., 2006; Lavial et al., 2007). The same studies also demonstrated that *cPouV* and *cNanog* are expressed in the germinal crescent where germ cells are located at stages 4-9HH (Canon et al., 2006; Lavial et al., 2007). Moreover, *cNanog* is expressed in the genital ridges at stage 20HH at later stages of chick development (Canon et al., 2006) while *cNanog* and *cPouV* were detected in developing gonads at stage 33HH (Lavial et al., 2007). In this study, *cPouV*, *cNanog* and *ERNI* positive cells were found not only in left and right gonadal ridges of chicken embryonic gonads at stage 25-28HH consistent with previous studies (Canon et al., 2006; Lavial et al., 2007) but also in

the dorsal mesentery which is part of the migratory route of germ cells . These findings are consistent with at least some of the *cPouV*, *cNanog* and *ERNI* positive cells being germ cells. However, double *in situ* hybridization for *Cvh* and other pluripotent markers is necessary to test this possibility directly.

Chapter 6. Left-right asymmetric and sex-specific properties in chicken embryonic gonads and germ cells

6.1. Introduction

The vertebrate body plan is usually viewed as being divided into 3 axes: anterior-posterior (A-P), dorsal-ventral (D-V) and left-right (L-R). Left-right patterning plays important roles for internal organ formation, positioning and embryonic turning (Levin, 2005; Raya and Izpisua Belmonte, 2006; Shiratori and Hamada, 2006). The process is regulated by genes encoding transcription factors and secreted growth factors, but surprisingly there are important differences among different vertebrates in terms of which specific genes are involved (Levin, 2005; Raya and Izpisua Belmonte, 2006). To date only two main players have been found to be conserved in all vertebrates: Pitx2 and Nodal (Levin, 2005; Levin et al., 1995; Zhu et al., 1999). PITX2, a member of the conserved bicoid-type homeobox gene family plays a role for establishing L-R asymmetry through its expression in the left lateral plate mesoderm and in a number of organs such as the heart and head (Gage et al., 1999a; Gage et al., 1999b; Zhu et al., 1999). *Pitx2*-knockout mice have abnormalities of internal organ asymmetry (Lin et al., 1999; Lu et al., 1999), showing that this gene plays an essential role in controlling laterality in mice.

Unlike mammals, which have apparently symmetric gonads, most female bird species develop asymmetrically, generating a functional ovary only on the left side, whereas males develop bilateral testes (Romanoff, 1967). Before sexual differentiation (the

"indifferent stage"), there are no asymmetric morphological differences between left and right embryonic gonads in either sex. The gonads contain two layers, the cortex and medulla (Smith and Sinclair, 2001; Smith and Sinclair, 2004). Morphological differences in embryonic gonads appear after sexual differentiation, male embryos (which are the homogametic sex, ZZ) develop bilateral testes, while female embryos (heterogametic, ZW) develop a functional left ovary and the right ovary regresses (Smith and Sinclair, 2004).

The embryonic gonads of male and female embryos become different during gonadal differentiation. Embryonic testes exhibit greater medullary development by the appearance of testicular cords containing male germ cells, supporting Sertoli cells inside and hormone producing Leydig cells outside the cords. On the other hand, the ovary exhibits greater cortical development by proliferation and expansion of the cortex, and female germ cells locate in this layer (Smith et al., 2007). Early differences between male and female embryos also include a greater number and size of female germ cells at an earlier stage than in males. This suggests that female germ cells proliferate and grow faster, or perhaps start differentiating later, than their male counterparts (Zaccanti et al., 1990).

There are also molecular differences between male and female embryonic gonads, some of which include sex- and laterality-specific differences in the endocrine signalling system. For example, estrogen receptor alpha (ERα) is expressed in the left but not the right cortex of both sexes (Andrews et al., 1997; Nakabayashi et al., 1998) but aromatase, a key enzyme for converting testosterone into estrogen is expressed and detected only in female gonads (Andrews et al., 1997; Smith et al., 1997). *PITX2*

is preferentially expressed in the left female gonad, where it induces gonadal cell proliferation and morphogenesis (Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008).

Several genes underlie sexual differentiation and lie near the top of a genetic hierarchy governing sex specific differences. During sexually dimorphic gene expression, *DMRT1* (Smith et al., 2003; Smith et al., 2009a) and *Sox9* genes (Kent et al., 1996; Morais da Silva et al., 1996) are preferentially expressed in sexually dimorphic (ZZ) male embryos. In contrast, *HINTW* (Smith, 2007; Smith et al., 2009b), *FET1* (Reed and Sinclair, 2002) and *FOXL2* (Hudson et al., 2005) genes are expressed in female (ZW) embryos. Even a relatively common secreted molecule like BMP7 can have both sex- and laterality differences in expression; *Bmp7* has been shown to be expressed asymmetrically at the beginning of genital ridge formation and also after sexual differentiation. Moreover, a sex-specific expression pattern of *Bmp7* was observed in the ovarian mesenchyme (Hoshino et al., 2005).

Although several differences of sex-specific gene expression in male and female embryonic gonads have been reported, the expression of genes associated with pluripotency has not been examined in detail in embryonic testes and ovaries in chick. This is of particular interest first because of the above-mentioned observations that female PGCs are more numerous than male ones at an equivalent stage, because it is widely believed that only male gonocytes can establish immortal cell lines in culture, and also because if there are left-right differences in the ability of gonocytes to become established in culture, knowledge of such differences might turn out to be useful for improving the establishment of such cultures in the laboratory for

transgenesis or other purposes. This chapter therefore aims to study asymmetric gene expression in left and right gonads from male and female embryos both before and after gonadal sex differentiates morphologically into male and female (which occurs at day 9 of development), as well as to assess the growth potential of gonocytes *in vitro* according to sex and laterality. For the gene expression study we concentrate on genes related to pluripotency in other systems: *Nanog*, *PouV* (*Oct4*), *Sox2* and *ERNI*.

6.2. Methods

6.2.1. Gene expression in chicken embryonic testes and ovaries

Chicken embryonic gonads at stage 35 (H&H) (about 9 days' incubation), a stage at which the sex of male and female embryos can be distinguished by morphological appearance of the embryonic gonads, were dissected and then fixed with 4% PFA/EGTA at 4°C overnight. The fixed embryonic testes and ovaries were subjected to whole-mount *in situ* hybridization (WISH) as described in Chapter 2. After WISH and photography, selected hybridized and post fixed embryonic testes and ovaries were embedded in FibrowaxTM (BDH^{GUN}, UK) for histological sections and then cut on a MICROM (Type HM315) microtome at 10 μm thickness.

6.2.2. Cell counting and statistical analysis of gene expression in embryonic testes and ovaries

To assess the proportion of cells expressing the genes listed in Table 6.1 in the embryonic testes and ovaries, expressing cells were counted starting from the first

181

section of the first slide containing gonadal tissue. To avoid counting the same cells more than once, one in three sections were counted until the last section of the gonad was reached.

To obtain the average number of expressing cells from the outer layer (cortex) and inner layer (medulla) in embryonic testes and ovaries, three sections representing the anterior, middle and posterior regions from each left-right side were randomly selected from male and female embryos.

Expressing cells located in the cortex and the medulla were counted separately. In males, expressing cells were located in one or two outer thin layers of the germinal epithelium of the cortex; this is the area containing cortical male germ cells while expressing cells located next to the cortex are considered as medullary male germ cells. In female, cells expressing the genes assessed are located in thick germinal epithelium (about 5-6 layers) of the cortex are likely to be cortical female germ cells while expressing cells counted next to the thick cortex are considered as medullary female germ cells. The average number of positive cells expressing those markers in the cortex and medulla of embryonic testes and ovaries from both sexes were analyzed and plotted. The unpaired Student's t-Test with two-tailed distribution and two-sample unequal variance was used to compare (pairwise) the number of cells expressing germ cell marker, *Cvh* with various genes such as *cPouV*, *cNanog*, *cSox2* and *ERNI* between left-right sides, cortex-medulla layers in male and female embryonic gonads.

Table 6.1: Probes of gene markers used to study expressions of pluripotency markers in chicken embryonic testes and ovaries compared to germ cell marker (*Cvh*).

Insert name (marker)	Description	Cut enzyme	Transcribe enzyme	Source	References
ERNI Wpst	Subclone for ERNI for ISH	*KpnI*	T3		(Streit et al., 2000)
pFL Nanog	Chick Nanog	*ApaI*	SP6		(Lavial et al., 2007)
pFLIp06	Chick Oct3/4 homologue	*ApaI*	SP6		(Lavial et al., 2007)
cSox2	Chick Sox2	*PstI*	T7	Gift from P. Scotting	(Uwanogho et al., 1995)
***Cvh* (germ cells)**	Chick Vasa Homologue	*NcoI*	SP6		(Tsunekawa et al., 2000)

6.2.3.   Sex genotyping of chicken embryos by PCR

The method used for sexing chicken embryos described in (Clinton et al., 2001) was modified as described below.

6.2.3.1. Tissue collection and genomic DNA preparation

After dissecting the embryonic gonads at stage 26-27 (H&H) (5 days' incubation) to process derivation of male and female germ cells *in vitro*, the posterior part of the

embryo containing embryonic tail tip was collected and placed in 50 μl of digestion

buffer (10 mM Tris, 1mM EDTA, 1% SDS, pH 8.0 containing 10 μg/ml Proteinase-

K) and incubated overnight at 45 ^{o}C. The following day, 170 μl of 5 M NaCl was

added into the Eppendorf tube. The tubes were mixed on the rocker for 5 min and

spun 5-10 min at full speed in an Eppendorf centrifuge at room temperature. The

supernatant was removed (without salt) to a new tube and 500 μl of 2-Isopropanol

added. The tubes were mixed vigorously by inversion and genomic DNA floating in

the tubes was spooled out using the blunt end of a glass Pasteur pipette (flamed using

a Bunsen burner). The excess liquid in the Pasteur pipette removed and the genomic

DNA resuspended in 150 μl of TE buffer. The tubes containing genomic DNA were

warmed in a 37 ^{o}C heat block for 2 hr and kept at room temperature until use.

6.2.3.2. PCR primers

The primers used in this study were synthesized by Invitrogen Custom Primers.

W chromosome sequence

Primers were designed to amplify 415 bp product of the *XhoI* repeat sequence (Tone

et al., 1982):

5' primer: 5' CCCAAATATAACACGCTTCACT 3'

3' primer: 3' GAAATGAATTATTTTCTGGCGAC 5'

Ribosomal gene sequence

Primers were designed to amplify a 256 bp product of the 18S ribosomal gene from position 1267 to 1522 (Hedges et al., 1990).

5' primer: 5' AGCTCTTTCTCGATTCCGTG 3'

3' primer: 3' GGGTAGACACAAGCTGAGCC 3'

6.2.3.3 PCR conditions

PCR reactions were performed on 1 μl of diluted DNA solution. The reactions were performed in 25 μl 1.5 mM $MgCl_2$, 10x PCR buffer containing 200 μM dNTPs, 1 μM *XhoI*-repeat primers, 0.5 μM 18-primers and 1 U Taq polymerase (Boehringer Mannheim). The PCR reactions were standardized at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min. A final extension step of 72 °C for 10 min was carried out for all reactions. PCR reactions were performed in a PTC-150 Thermal Cycler (Minicycler, MJ research). PCR products were analyzed by agarose gel electrophoresis (1 μl of PCR product containing 5 μl of loading buffer) and visualized under UV light after ethidium bromide staining (Sambrook et al., 1989). 1 kb ladder was used as a molecular size marker (MS in all figures). The genomic DNA prepared from male and female embryos at stage 35 (H&H) (9 days' incubation) was used a positive control.

6.2.4. Growth rate of male and female germ cells *in vitro*

To study the growth rate of male and female germ cells *in vitro*, the method for isolating and culturing chicken germ cells described in Chapter 2 was used with slight

modifications. The left and right embryonic gonads (stage 35, 9 days' incubation) from male and female whose sexes had been determined by PCR were labelled and followed in culture to assess their growth rate from passage 0 (P0) to passage 5 (P5). Before starting to subculture male and female germ cells, differences of germ cell morphology, health and degree of confluence (cell density on the plates) between male and female were recorded every day. Cells isolated from left-right gonads of male and female embryos that were unable to grow in culture were not examined while cells grown up to P5 were selected and recorded. The day since the last passage from P0 to P5 was recorded to analyze any differences in growth rate between left-right gonads and male-female embryos. An average of the day since the last passage from P0 to P5 from left-right gonads and male-female embryos was analyzed and plotted.

6.3. Results

6.3.1. Left-right asymmetric gene expression and quantification of cells expressing various genes in male and female embryonic gonads

To compare the left-right asymmetric gene expression in male and female embryos, the gonads at stage 35 (9 days' incubation) were used. WISH shows that male embryonic testes and female embryonic ovaries express the germ cell marker, *Cvh* (Figure 6.1E, 6.2E) and various genes such as *cPouV* (Figure 6.1A, 6.2A), *cNanog* (Figure 6.1B, 6.2B)*, cSox2* (Figure 6.1C, 6.2C) and *ERNI* (Figure 6.1D, 6.2D). In testicular sections, average number of germ cells expressing *Cvh* in left and right male gonads (Mean $\pm$ SD) was 21 $\pm$ 16 and 11 $\pm$ 11 per gonadal section ($p = 0.01$, n=3)

186

respectively, Figure 6.3). Cells expressing *Cvh* are located both in cortex and few cells in the medulla (Figure 6.1E'): an average of 5 ± 2 and 5 ± 4 cells (per section) were found in the cortex of left and right gonadal sections (n=3), Figure 6.4), and 16 ± 9 and 16 ± 17 were found in the left and right medulla respectively (n=3), Figure 6.4).

In the female the differences in average number of germ cells between left and right gonads is greater than in the male: 62 ± 29 were found per gonad on the left and 2 ± 4 on the right; $p = 0.01$, n=3; Figure 6.3). Here, almost all the germ cells are located in the ovarian cortex: 54 ± 34 on the left and 3 ± 3 on the right cortex; $p = 0.01$, n=3; Figure 6.5), and 9 ± 14 on the left and 12 ± 8 on the right medulla (n=3), Figure 6.5).

Having established the distribution of *Cvh*-positive cells (germ cells) in male and female gonads, we next assessed the number and distribution of cells expressing four genes that have been associated with pluripotency in other systems: *PouV*, *Nanog*, *Sox2* and *ERNI*. Cells expressing *cPouV* were located in both cortex and medulla in the left testicle but there were very few cells in medulla in the right testicle (Figure 6.1A'). Average number of cells expressing *cPouV* in left and right male gonads was 65 ± 27 and 22 ± 12 per gonad ($p = 0.01$, n=3); Figure 6.3). Significantly more *cPouV* expressing cells were found in the left cortex than on the right: 8 ± 5 and 4 ± 3 per section respectively, $p = 0.05$ (n=3); Figure 6.4)). The medulla also showed left-right differences: 49 ± 24 for the left and 12 ± 12, for the right, $p = 0.01$, (n=3); Figure 6.4). In female gonads, average number of cells expressing *cPouV* in the left gonad was significantly higher than the right (104 ± 47 and 32 ± 30; $p = 0.01$, n=3; Figure 6.3). In the cortex there were 70 ± 33 and 32 ± 29 for left and right respectively ($p=0.01$, n=3; Figure 6.5) whereas in the medulla 38 ± 29 and 8 ± 7, $p = 0.01$ were counted on

the left and right respectively, (n=3; Figure 6.5). Given that females have very few germ cells in the medulla at this stage (see above), the majority of these c*PouV* expressing ovarian medullary cells (Fig. 6.2 A') are likely to be stromal cells.

cNanog (Figure 6.1B') has a pattern of localization similar to that of *cPouV*. Average number of cells expressing *cNanog* in left and right male gonads was 69 ± 26 and 21 ± 11 per gonad ($p = 0.01$, n=3; Figure 6.3). In the cortex 16 ± 8 were observed on the left and 5 ± 2 on the right ($p = 0.01$, n=3, Figure 6.4), whereas the medulla contained 50 ± 23 on the left and 17 ± 9 on the right ($p = 0.01$, n=3, Figure 6.4). As with *cNanog* positive cells were detected in both cortex and medulla in the left and the right female gonads (Figure 6.2B') with significant left-right differences: 89 ± 33 per gonad on the left and 32 ± 16 per gonad on the right ($p = 0.01$, n=3; Figure 6.3). In ovarian cortex, there were 45 ± 21 *cNanog*-expressing cells on the left and 13 ± 9 on the right ($p = 0.01$, n=3; Figure 6.5). In the medulla, 43 ± 15 were counted on the left and 21 ± 13 on the right ($p = 0.01$, (n=3); Figure 6.5). As with *cPouV*, therefore the majority of the medullary ovarian cells expressing *cNanog* are unlikely to correspond to germ cells. Moreover, there appear to be more *cNanog* expressing cells in both cortex and medulla of both male and female gonads than *Cvh*-expressing cells, suggesting that the left-right differences in expression of these genes are not confined to the germ cells but also to surrounding stromal cells.

cSox2 positive cells were hardly observed in the cortex in testicular sections (Figure 6.1C') while *cSox2* positive cells were detected in both cortex and medulla in ovarian sections (Figure 6.2C'). In male, average number of cells expressing *cSox2* in the left gonad was significantly higher than that on the right (34 ± 13 and 23 ± 12

respectively; $p = 0.01$, n=4; Figure 6.3). The cortex contained 7 ± 6 and 6 ± 4 per section on the left and right respectively (n=4; Figure 6.4), whereas the testicular medulla contained 35 ± 13 on the left and 28 ± 12 on the right (n=4; Figure 6.4). In female, average number of cells expressing *cSox2* in the left and the right gonads were 37 ± 16 and 19 ± 8 respectively ($p = 0.01$, n=3; Figure 6.3). The cortex contained 11 ± 22 on the left and 1 ± 2 on the right per section (n=3; Figure 6.5) whereas the left and right ovarian medulla contained 22 ± 6 and 13 ± 4 respectively ($p = 0.01$, n=3; Figure 6.5). These numbers reveal that there is little or no correlation between *cSox2* expression and the distribution of *Cvh*-positive germ cells. This is consistent with idea that that germ cells do not express *cSox2 in vivo* (see also Chapter 5). Moreover the morphology of *cSox2* expressing cells is more akin to tubular cells than germ cells especially in the ovarian medulla (eg. see Fig. 6.2 C'). However, significant left-right differences are observed for *cSox2* expression in both sexes, although this is less marked than for the other genes studied here. In fact, some whole mount embryos even show greater numbers of *cSox2* expressing cells in the right ovary than on the left (eg. Fig. 6.2C).

ERNI expressing cells were localized in both cortex and medulla in testicular sections (Figure 6.1D'), while in ovarian sections, *ERNI* positive cells were detected in cortex and few cells in medulla (Figure 6.2D'). In male, average number of cells expressing *ERNI* in the left was significantly higher than that in the right gonads (36 ± 21 and 14 ± 8; $p = 0.01$, n=3; Figure 6.3); in the left and right cortex 8 ± 4 and 3 ± 3 cells were counted respectively ($p = 0.01$, n=3; Figure 6.4) whereas the medulla contained 34 ± 18 on the left and 15 ± 9 on the right ($p = 0.01$, n=3; Figure 6.4). In female, average number of cells expressing *ERNI* in the left and the right gonads was 25 ± 14 and $10 \pm$

8 respectively ($p = 0.01$, n=3; Figure 6.3). In cortex, 28 ± 9 were counted on the left and 9 ± 5 on the right ($p = 0.01$, n=3; Figure 6.5) whereas the medulla contained 9 ± 4 on the left and 8 ± 6 on the right (n=3; Figure 6.5). Thus, although *ERNI* also seems to be expressed in stromal cells in addition to germ cells, its expression most closely reflects the distribution of *Cvh*-positive cells in the left and right gonads of both sexes.

Figure 6.1: Left-right asymmetric gene expression in male embryonic gonads:

cPouV (A), *cNanog* (B), *cSox2* (C), *ERNI* (D) and *Cvh* (E) positive cells are expressed in both left and right testes**.** Testicular sections exhibit *cPouV* (A'), *cNanog* (B'), *cSox2* (C') and *ERNI* (D') positive cells and germ cells, *Cvh* (E'). Abbreviations: RT = Right testes, LT = Left testes. (Scale bar = 50 µm, arrow = positive cells).

Figure 6.1: Left-right asymmetric gene expression in male embryonic gonads.
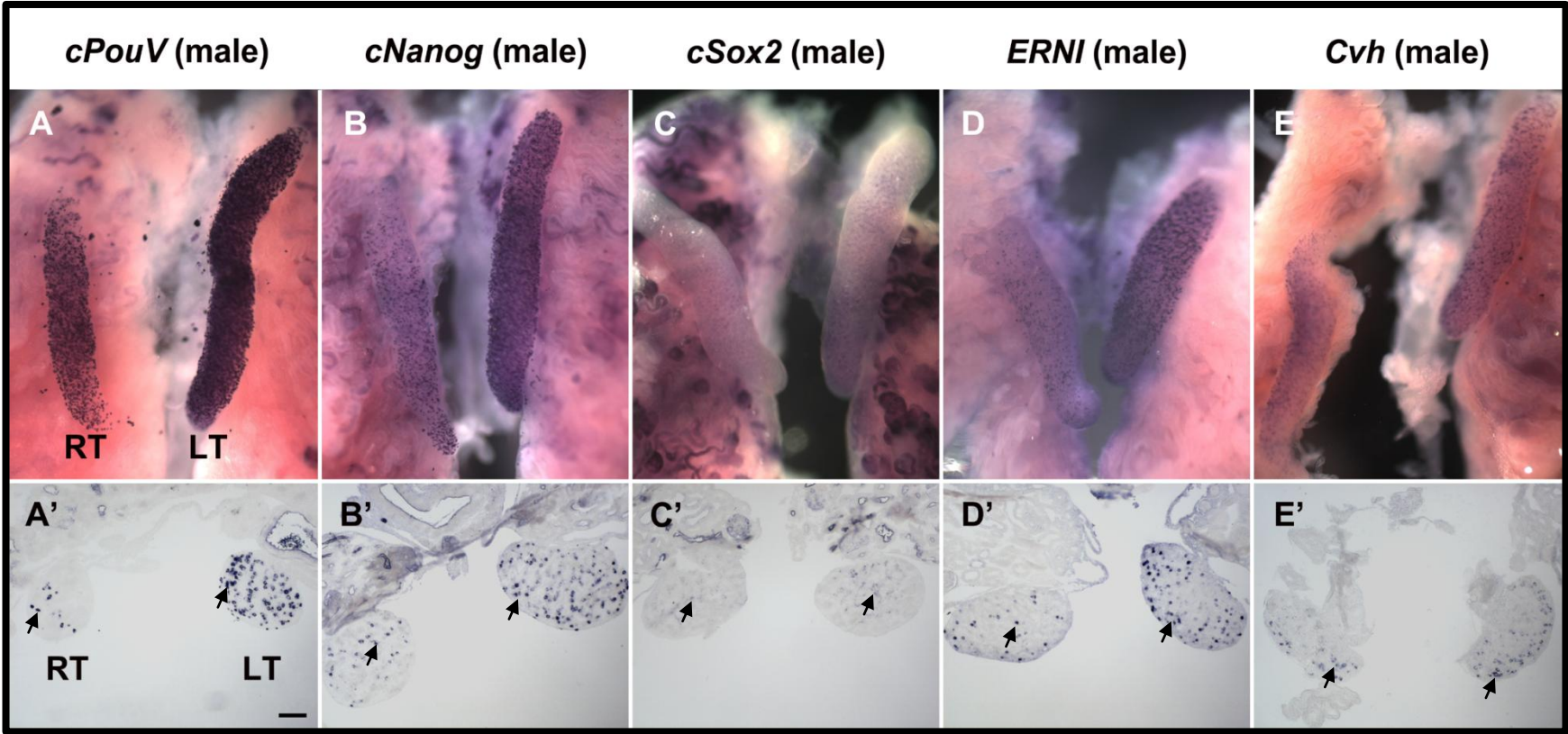
Figure 6.2: Left-right asymmetric gene expression in female embryonic gonads:

cPouV (A), *cNanog* (B), *cSox2* (C), *ERNI* (D) and *Cvh* (E) positive cells are expressed in both left and right ovaries**.** Ovarian sections exhibit *cPouV* (A'), *cNanog* (B'), *cSox2* (C') and *ERNI* (D') positive cells and germ cells, *Cvh* (E'). Abbreviations: RO = Right ovary, LO = Left ovary. (Scale bar = 50 µm, arrow = positive cells).

Figure 6.2: Left-right asymmetric gene expression in female embryonic gonads.
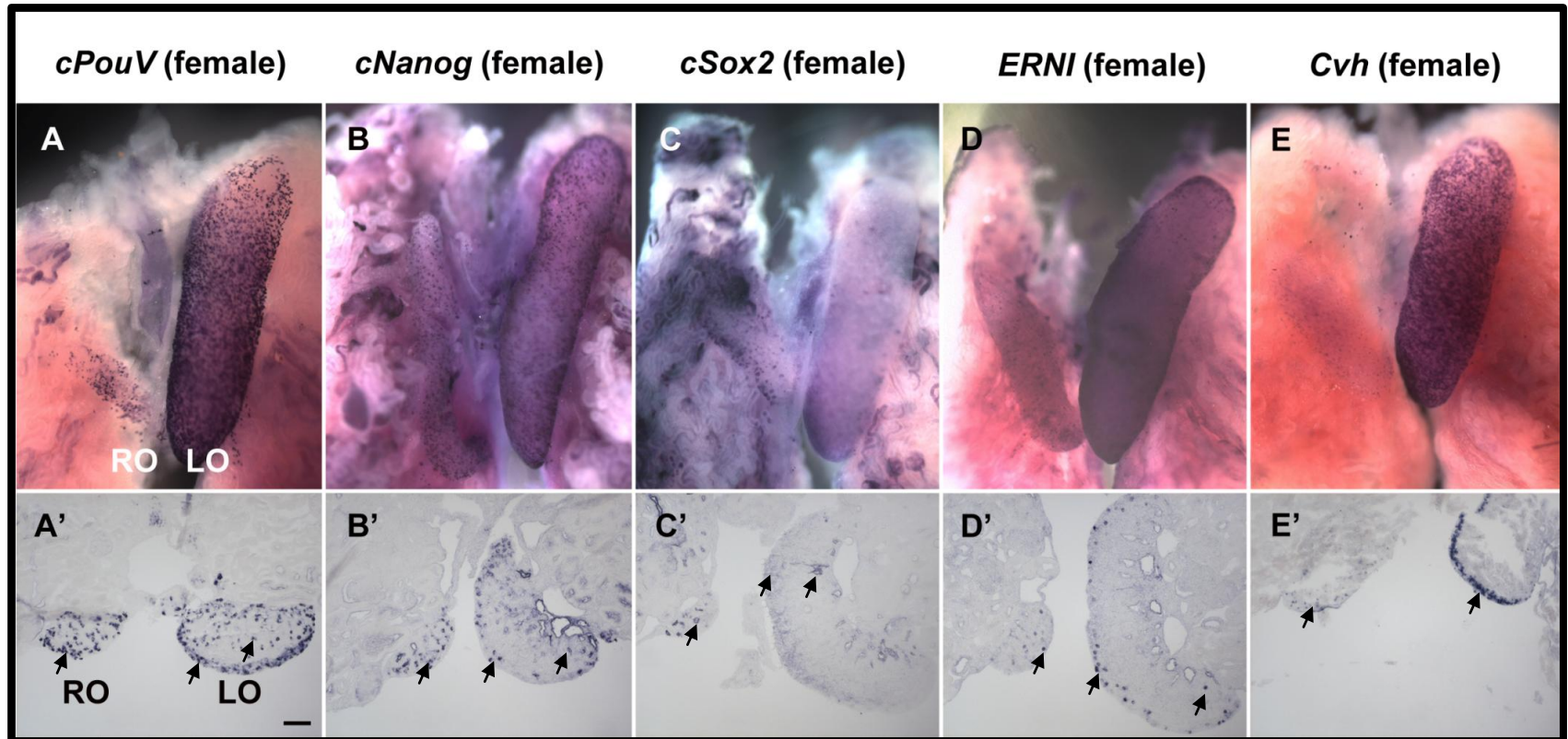
Figure 6.3: Quantification of cells expressing various genes in male and female embryonic gonads
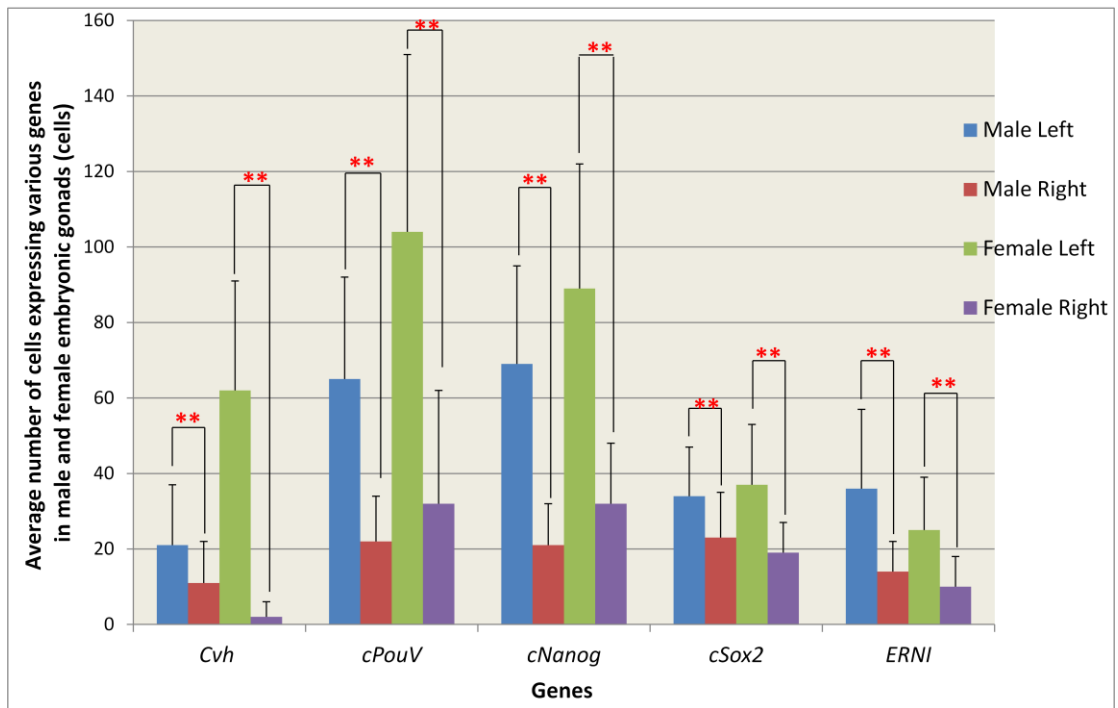
(**significant different at $p < 0.01$)

Figure 6.4: Quantification of cells expressing various genes in male cortex and medulla.
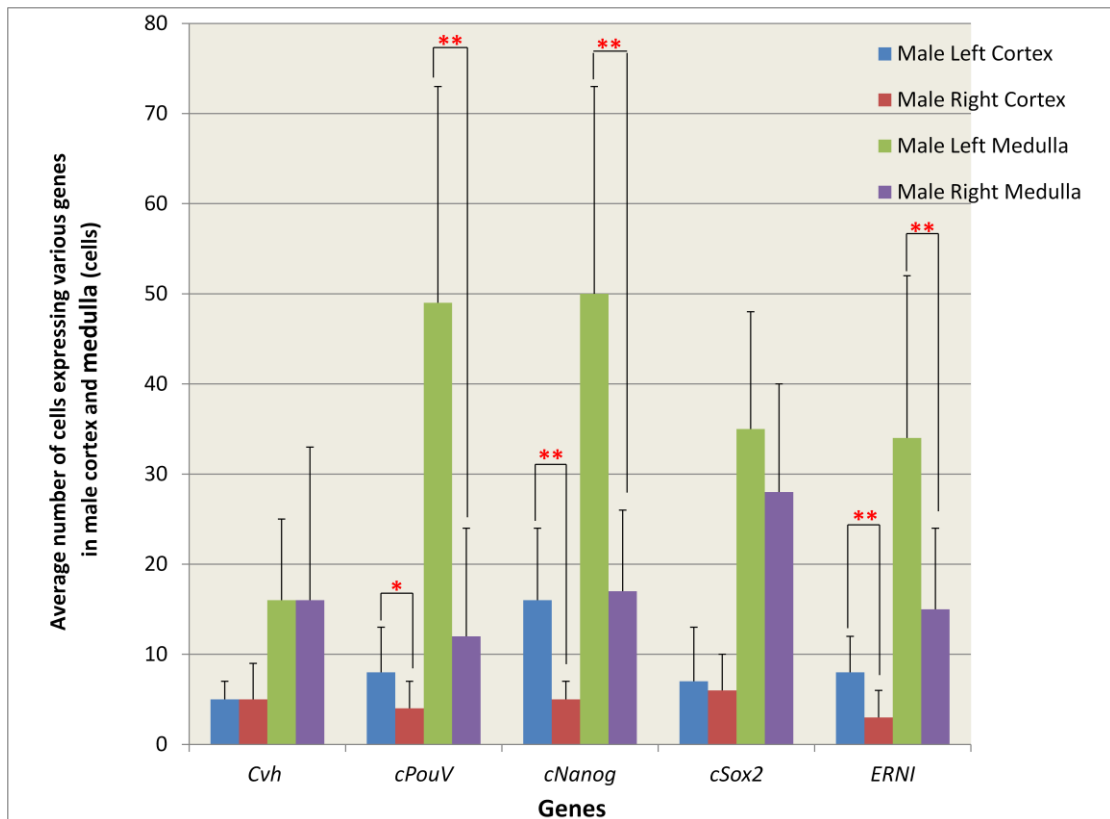
(*significant different at $p < 0.05$, **significant different at $p < 0.01$)

Figure 6.5: Quantification of cells expressing various genes in female cortex and medulla.
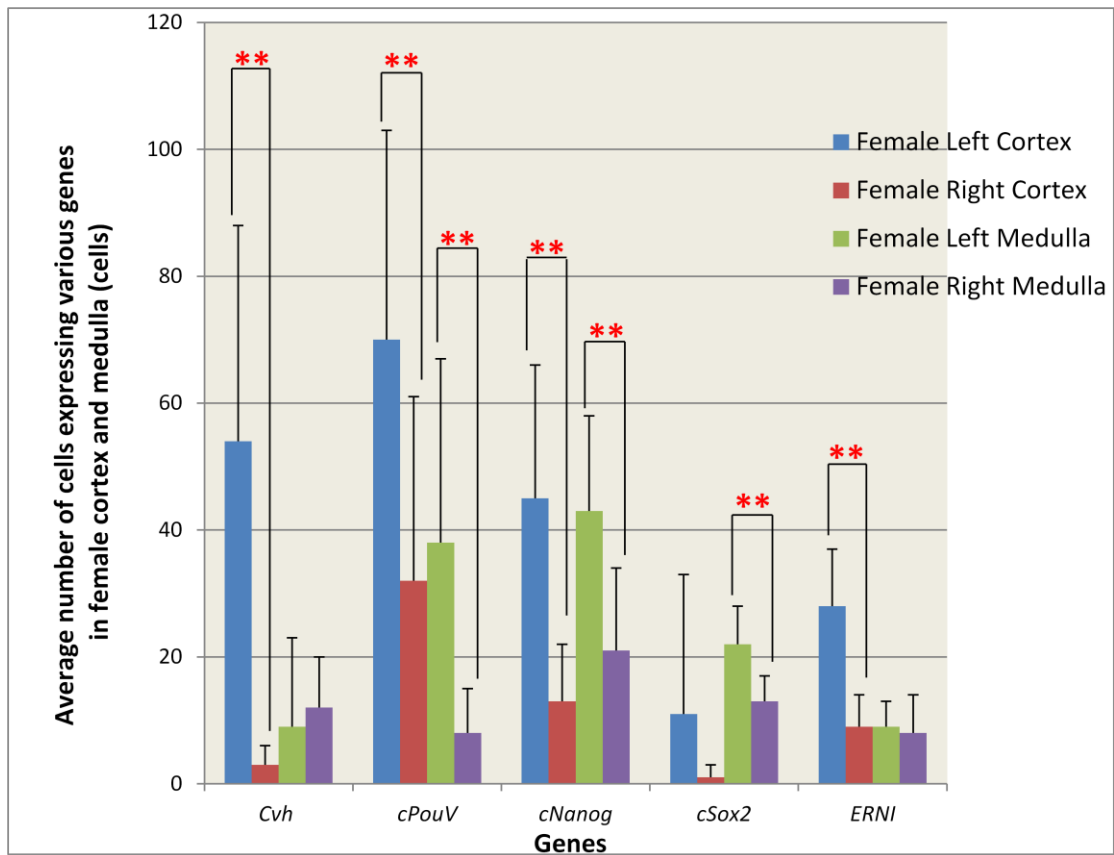
(**significant different at $p < 0.01$)

6.3.2. Left-right and sex-related differences in growth rate of germ cells *in vitro*

To study whether there are differences in growth rate of gonadal cells between male and female and/or left and right embryonic gonads after placing them in culture, embryonic gonads at the indifferent stage (26-27HH, 5 days' incubation) were collected from 24 embryos. Before taking the embryonic gonads to culture, left and right embryonic gonads were separated and some posterior tissue containing the tail tip of the embryos was collected for sexing the embryos by PCR. There were 15 male embryos and 9 female embryos (see Table 6.2).

During *in vitro* culture of gonadal cells isolated from indifferent gonads, useful results could be obtained from 9 left gonads and 9 right gonads obtained from the 24 samples (see Table 6.3). Likewise, 9 male gonads and 9 female gonads were also obtained from 24 samples (see Table 6.4). The total of 18 samples described above (comprising 5 left male gonads, 4 left female gonads, 4 right male gonads and 5 right female gonads) survived in culture up to passage 5; the remaining samples died. Growth curves showing average day since the last passage and passage number between left-right gonads and male-female embryos were plotted. There were no differences in growth rate between left and right gonadal cell cultures (irrespective of sex of embryo). The time required for passaging (number of days required to attain confluence) increased greatly from P1 to P2. Right gonadal cells were in a stationary phase while left gonadal cells were in a log phase at P3 to P4. The growth curve of both left and right gonadal cells increased at P5 and they were not different (see Figure 6.7).

On the other hand, there were differences in growth rate between male and female gonadal cells. The curve shows that at P1, the time required for passaging of both male and female gonadal cells increased greatly from P1 to P2, and both cell types decreased at P3. Between P3 and P4, female gonadal cells were in a stationary phase while male gonadal cells were in a log phase. Interestingly, at P4, the day since the last passage exponentially increased in female gonadal cells while it decreased in male gonadal cells (see Figure 6.8). In conclusion therefore, male gonad-derived cells appear to grow faster *in vitro* than their female counterparts, the difference becoming evident around the fifth passage. In contrast there seem to be no significant left-right differences in the rate of growth of cells derived from the left and right gonads of either sex. The latter finding is surprising because of the difference in *Cvh*-positive cells found on the two sides of both sexes, as presented earlier in this chapter. However it should be noted that these are preliminary observations based on very few experiments and therefore need to be repeated with larger numbers of gonads, followed over a longer period of time *in vitro*. It would also be useful to compare these results with estimates of the proportion of dividing cells as assessed by staining with PCNA or BrdU-positive cells.

Table 6.2: Sex identification of DNA samples isolated from 24 chick embryos by PCR.

Embryo	Stage (HH)	Sex (M/F)	Embryo	Stage (HH)	Sex (M/F)
1	27	M	13	26	M
2	26	M	14	26	M
3	26	M	15	26	M
4	27	F	16	27	M
5	27	M	17	27	F
6	27	M	18	27	F
7	27	M	19	27	M
8	27	M	20	27	F
9	27	F	21	26	M
10	27	F	22	27	F
11	27	F	23	27	M
12	27	M	24	27	F

Figure 6.6: Specificity of PCR primer. PCR reactions were performed using W-repeat (W) primer on female and male DNA: PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 3 min. The 1st lane includes molecular size (MS) markers. M = male DNA control, F= female DNA control. A: DNA samples 1 to 12, B: DNA samples 13 to 24. The PCR result demonstrates that after using W-primer which is specific to female, bands of 415 bp (W) can be seen on lanes 4, 9, 10 and 11 of the first group of DNA samples compared to male and female control DNA samples (A). In the second group of samples, bands of 415 bp (W) appear on lanes 17, 18, 20, 22 and 24 compared to male and female control DNA samples (B).

Figure 6.6: Specificity of PCR primer. PCR reactions were performed using W-repeat (W) primer on female and male DNA samples.
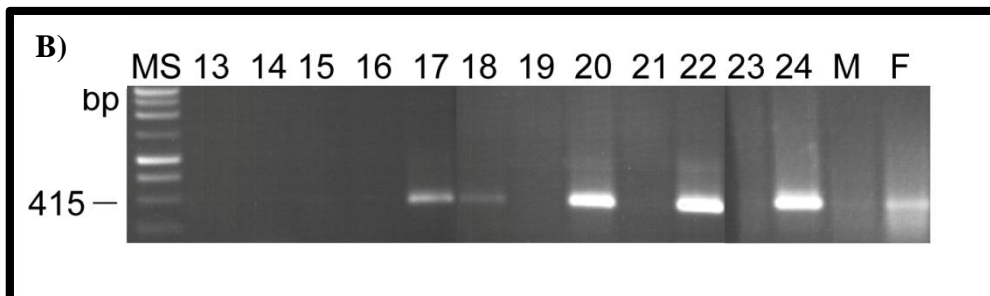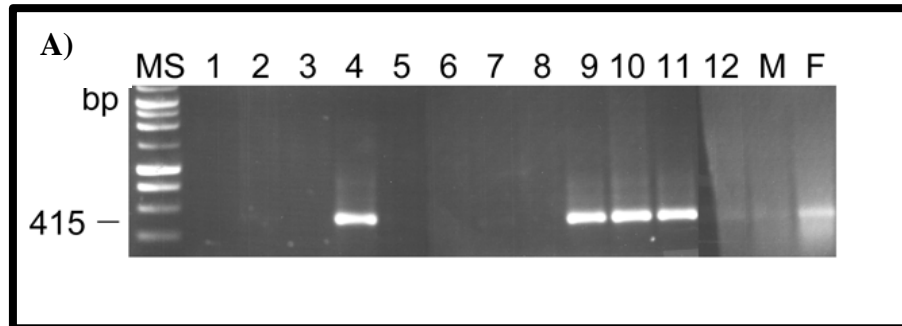
Table 6.3: Data represents day since the last passage and passage number of left and right gonadal cells.

Gonad number and Type	Days since the last passage (time to confluence)				
	P1	P2	P3	P4	P5
L8-M	3	10	7	9	32
L15-M	3	9	6	38	8
L16-M	3	8	5	4	4
L19-M	3	12	5	3	3
L23-M	3	7	5	4	7
L4-F	3	12	5	5	7
L9-F	3	12	3	7	6
L17-F	3	7	7	5	34
L24-F	3	8	4	4	3
Mean	**3**	**9.4**	**5.2**	**8.8**	**11.6**
SD	**0**	**2.1**	**1.3**	**11.1**	**12.3**
R16-M	3	7	5	4	4
R19-M	3	8	4	4	3
R21-M	3	7	5	5	6
R23-M	3	7	5	4	3
R9-F	3	12	5	6	8
R11-F	3	9	7	7	30
R17-F	3	7	5	5	34
R22-F	3	7	5	5	4
R24-F	3	8	4	4	3
Mean	**3**	**8**	**5**	**4.9**	**10.6**
SD	**0**	**1.7**	**0.9**	**1.1**	**12.3**

Figure 6.7: Growth curve plotted by average day since the last passage and passage number of left and right gonadal cells.
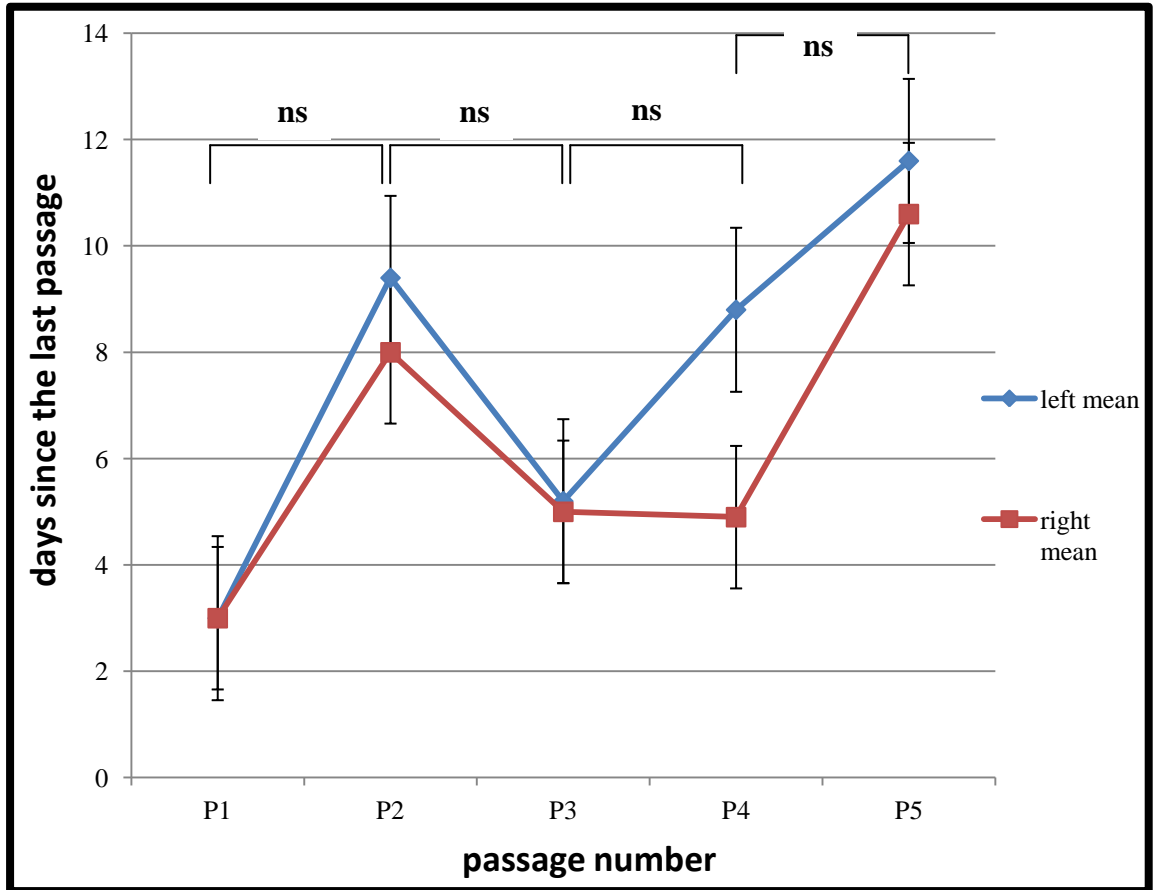
(ns : not significant different)

Table 6.4: Data represents day since the last passage and passage number of male and female gonadal cells.

Gonad number and Type	Days since the last passage (time to confluence)				
	P1	P2	P3	P4	P5
L8-M	3	10	7	9	32
L15-M	3	9	6	38	8
L16-M	3	8	5	4	4
L19-M	3	12	5	3	3
L23-M	3	7	5	4	7
R16-M	3	7	5	4	4
R19-M	3	8	4	4	3
R21-M	3	7	5	5	6
R23-M	3	7	5	4	3
Mean	**3**	**8.3**	**5.2**	**8.3**	**7.8**
SD	**0**	**1.7**	**0.8**	**11.3**	**9.3**
L4-F	3	12	5	5	7
L9-F	3	12	3	7	6
L17-F	3	7	7	5	34
L24-F	3	8	4	4	3
R9-F	3	12	5	6	8
R11-F	3	9	7	7	30
R17-F	3	7	5	5	34
R22-F	3	7	5	5	4
R24-F	3	8	4	4	3
Mean	**3**	**9.1**	**5**	**5.3**	**14.3**
SD	**0**	**2.3**	**1.3**	**1.1**	**13.9**

Figure 6.8: Growth curve plotted by average day since the last passage and passage number of male and female gonadal cells.
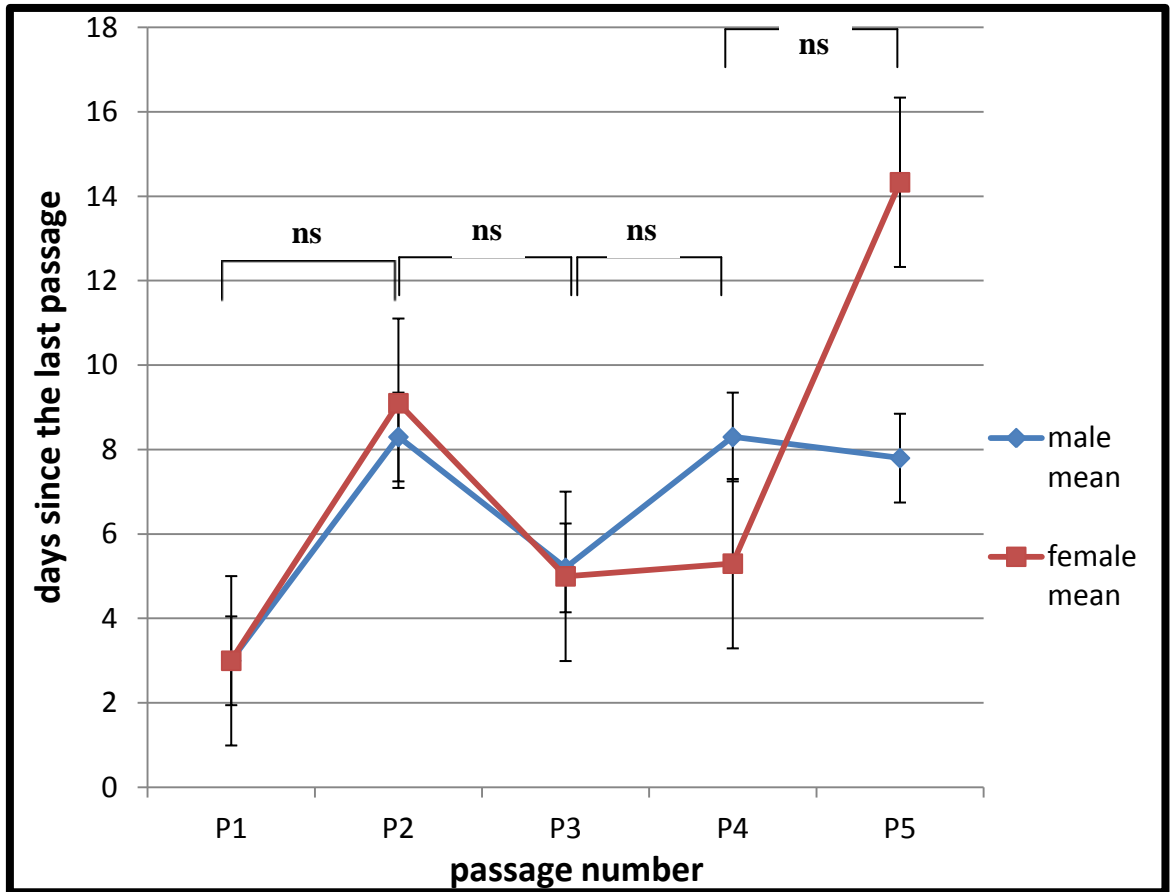
(ns : not significant different)

6.4. Discussion

A distinctive characteristic of gonadal development in chick embryos is that female embryos develop gonads asymmetrically: only the left side forms a functional ovary while the right side regresses (Smith and Sinclair, 2004). The molecular mechanisms underlying asymmetric development of female embryonic chick gonads is still unclear. However, it has been reported that *PITX2* plays a role in ovarian asymmetric development in female embryos; moreover, this gene is preferentially expressed in the left gonads, where it may regulate gonadal cell proliferation and morphogenesis (Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008). Other previous studies also reported asymmetric gonad development in chick embryos, suggesting that 70% of PGCs are found on the left side (Witschi, 1935). Furthermore, it was proposed that chicken left presumptive gonads secrete chemotactic factors at a higher level than the right; this was proposed to be involved in regulating the mitotic activity of PGCs (Swartz and Domm, 1972). These previous studies suggest the idea that early differences exist between male and female embryos. It has also been found that female germ cells are larger and that they increase in number earlier than those in males, suggesting higher proliferation or later differentiation of female germ cells than their male counterparts (Zaccanti et al., 1990).

The present findings on early differences between male and female chick embryos regarding germ cell morphology and number strongly supports the result of this study since the WISH of male and female embryonic gonads for germ cell marker, *Cvh*, demonstrates that *Cvh* positive cells exist in higher numbers on the left than the right

ovary. However this phenomenon is also seen in male embryos, since the differences of *Cvh* positive cells between the left and the right testis are slight and not statistically significant, indicating that asymmetric germ cell distribution is not entirely related to the sex of the embryo. It would be interesting to investigate this issue in mature adults to determine whether the left-right differences in germ cell numbers persist and eventually translate into differences in the rate of sperm production in roosters.

The present study also provides novel information about the expression of genes associated with pluripotency in embryonic gonads of both sexes and between left and right gonads. For all 4 genes studied (*cPouV*, *cNanog*, *cSox2* and *ERNI*), the number of cells expressing *cSox2* and *ERNI* genes are significantly higher on the left than the right gonads in male embryos. However this does not correlate directly with the number of germ cells present in each gonad or region. This suggests that stromal cells express these genes and that this expression is also left-right asymmetric. The functional significance of this complex expression pattern for left-right or sex differences in gonadal development is unclear.

These left-right differences are seen both in the cortex of female embryonic gonads. It has been reported that there were abandonment of germ cells in the embryonic chick ovary (Ukeshima, 1994). Since a lacunar structure has been found in medulla of both left and right ovaries, related to the reduction of germ cell number and germ cell apoptosis in the medulla (Ukeshima, 1996), this may play a role for reducing female germ cells in the medulla. Future experiments should address the question of this phenomenon only takes place on the left embryonic gonads in both sexes.

This study was also designed to ask the question of whether pluripotency markers are still expressed at later stages in embryonic gonads (35HH), after their differentiation into testes and ovaries. This extends the results of Chapter 5 for *cPouV*, *cNanog*, *cSox2* and *ERNI*. Studies in human gonads have been reported that both human fetal testicular and ovarian germ cells express pluripotent stem cell markers including OCT4 and NANOG, suggesting that both male and female fetal germ cells maintain expression of pluripotent stem cells markers during and after sexual differentiation of the gonads (Kerr et al., 2008a; Kerr et al., 2008b). The present study raises the question of what is the functional significance of this expression, which will require further investigation.

We were unable to find significant differences in growth rate between male and female or left and right gonadal cells *in vitro*. However a single observation at passage 5 raises the possibility that male gonadal cells may increase their growth rate while female gonadal cells slow down. This tantalizing preliminary observation needs to be pursued by repeating the experiment with larger numbers of gonads as well as by extending the analysis beyond passage 5.

Due to the larger size of female germ cells, it has been reported that there was a Balbiani body in female meiotic germ cells which was composed of a concentration of cell organelles shifted to one pole of the cells and that this is always seen in left ovarian cortex but not in medulla or male germ cells (Ukeshima and Fujimoto, 1991). This structure is also related to germ cell degeneration, which was frequently observed in the right ovary, but rarely in the left (Ukeshima and Fujimoto, 1991). Whether, and if so how, these observations could relate to any differences in growth

rate between male and female germ cells is unclear. Future studies are essential to determine whether there are indeed such differences in growth rate, and especially attempt to establish permanent cell lines from single gonads, to determine whether there are left-right and/or sex differences in these properties that arise later in the culture period and which may relate to the feasibility of establishing permanent cell lines from particular gonads.

Chapter 7. General Discussion

Avian germ cell-related pluripotency is an interesting issue for stem cell biology in chick; this has been reviewed a few years ago (Petitte et al., 2004). Avian pluripotent cells can be obtained from both early and adult stages of chick embryonic development (Han, 2009). One source of putative pluripotent cells is chicken embryonic germ cells (cEGCs) (Han, 2009; Petitte et al., 2004). Even though there have been several attempts to establish a method for deriving chicken embryonic germ cells from embryonic gonads (Park and Han, 2000; Shiue et al., 2009; Suraeva et al., 2008; van de Lavoir et al., 2006, Wang et al., 2009; Wu et al., 2010), very few have been successful. Here we undertook to characterise cells obtained from the embryonic gonads of chicken embryos both *in vitro* (following the method described in the above report) and in comparison with the tissue of origin, at a molecular level. In Chapter 3, we described the successful isolation and culture of chicken gonocytes and derived embryonic germ cells from chicken gonocytes isolated from indifferent gonads (25-28HH) of chick embryos by using Park and Han's protocol. Chicken gonocyte-derived embryonic germ cells were cultured successfully for up to 5 passages. The cultured cells were shown to express markers usually used to identify germ cells such as SSEA-1, SSEA-3, SSEA-4, Integrin-β6 and Integrin-α6 (Han, 2009; Jung et al., 2005). Moreover, chicken embryonic gonads at the indifferent stage (25-28HH) were shown to contain cells expressing the specific germ cell marker, *Cvh*, assessed both by *in situ* hybridisation with *Cvh* riboprobe and using an anti-Cvh antibody. These results reveal the existence of substantial numbers of *Cvh* positive cells in both left and right gonadal ridges and in the dorsal mesentery. This indicates that chicken embryonic gonads at stage 25-28HH are suitable source of chicken germ cells (gonocytes) for deriving chicken embryonic germ cells.

Taking advantage of using chicken embryonic gonads as a source of cPGCs, the methods described in Chapter 2 and 3 provide a path to study gene expression dynamics of pluripotent stem cells markers during derivation of embryonic germ cells from chicken gonocytes. This is described in a time course experiment in Chapter 4. The results presented in Chapter 4 reveal that there are changes in the gene expression profiles of a variety of markers including genes associated with pluripotency such as *cPouV*, *cNanog*, *cSox2*, *ERNI* and *cKlf4* during the derivation phase of EGCs. Changes in their expression were studied in time course, following primary cultured gonocytes over 5 passages.

Most previous studies have used "cell population" methods to study gene expression, such as RT-PCR. Although these methods do provide quantitative information about the whole culture, they cannot establish how homogenous or otherwise the cells within the culture are in terms of their expression of particular genes. Therefore they cannot distinguish moderate expresssion in a culture due to many cells expressing moderate levels of a marker from that due to a few cells expressing very high levels and others none. To study the degree of heterogeneity in cultures of chick gonocytes, we turned to *in situ* hybridisation for a large number of markers. These markers were chosen based on their reported value as indicators of pluripotency (eg. the "Yamanaka factors" *cPouV*, *cNanog*, *cSox2*, *cKlf4*, other pluripotency-associated genes like *ERNI*), as well as other genes whose expression is associated with various cell states in early embryos and during cell differentiation into a variety of lineages. *ERNI* and *cKlf4* have previously been reported to be expressed in chicken embryonic stem cells (Acloque et al., 2004; Acloque et al., 2001; van de Lavoir et al., 2006) and primordial germ cells (Macdonald et al., 2010).

Analysis of the expression profiles of these pluripotency-associated genes in Chapter 4, allowed us to ask the question of whether the establishment in culture of chicken pluripotent cells such as primary gonocytes, established-PGCs (*in vitro*) is accompanied by changes in the expression of these genes as compared to the parent tissue, chicken embryonic gonads (*in vivo*). This question was addressed in Chapter 5 by comparing gene expression patterns of 30 genes including pluripotency-associated genes such as *cPouV* (Lavial et al., 2007), *cNanog* (Lavial et al., 2007), *cSox2*, *ERNI* (Acloque et al., 2004; Acloque et al., 2001; van de Lavoir et al., 2006) and *cKlf4* (Macdonald et al., 2010) *in vivo* and *in vitro*. The results show that some of these genes, but not all, are expressed in both chicken embryonic gonads (*in vivo*) and pluripotent cells (*in vitro*). However, the study revealed some unexpected features. Among them we describe great heterogeneity in the numbers of cells that express different markers in culture, implying either that the cultures contain different cells, and/or that the expression of these markers is dynamic and changes constantly with time. This raises interesting questions concerning pluripotency of these cultures: is pluripotency a property of individual cells or only of whole cultures? Do protocols that generate different cell types *in vitro* rely on selection, rather than channeling, of different cell fates?

Another interesting finding afforded by the use of *in situ* hybridisation is that not all genes generally considered as markers of pluripotency are co-expressed. In particular *cKlf4* is barely expressed *in vivo* or *in vitro*. A particulary interesting change is observed for the *SoxB1* genes *Sox2* and *Sox3*. The former is not expressed in the parent gonads *in vivo* whereas the latter is expressed in a few cells. However upon being placed in culture, *Sox2* expression is initiated and *Sox3* is downregulated. Thus,

germ cells placed in culture adopt a new state more similar to that which characterises mammalian embryonic stem cells than their original state in the embryo.

Interestingly, of all the "pluripotency" markers studied, *ERNI* appears to be the one that most closely correlates with both *Cvh*-expressing cells *in vivo* and with cells with gonocyte-like morphology *in vitro* (Chapters 5 and 6). Many of the remaining "pluripotency" genes are expressed in many cells that are clearly not germ cells, as they exist in greater numbers than *Cvh* positive cells both *in vivo* and *in vitro* (Chapters 5 and 6).

Along with previous studies indicating that *ERNI* is strongly expressed in chicken ES cells (Acloque et al., 2004; Acloque et al., 2001; van de Lavoir et al., 2006), the expression of *ERNI* observed in chicken primary gonocytes, established-PGCs and embryonic stem cells in the present study suggests that *ERNI* is a useful marker for chicken pluripotent cells germ cells, perhaps better than other available markers commonly used for mammalian cells.

Finally, Chapter 6 addresses the question of whether pluripotency-associated markers studied in chicken embryonic gonads at the indifferent stage (25-28HH) in Chapter 5, are expressed in chicken embryonic testes and ovaries at later stages, following sexual differentiation, and how they correlate with the distribution of germ cells identified by *Cvh* expression. The results present the novel finding of left-right asymmetric expression both of the germ cell marker, *Cvh* as well as of *cPouV*, *cNanog* and *ERNI*. All of these are expressed in more cells on the left than the right gonads in both sexes. However, it was found that more cells express these genes than the number of *Cvh*-

positive cells, and that the genes are also expressed in regions (eg. the medulla of male embryonic testes) that contain few or no germ cells. Even these expression patterns are left-right asymmetric, suggesting that this is a property of the whole gonad (including stromal and tubule cells) rather than just germ cells. Whether this implies that some non-germ cell components contribute to establishing the long-term cultures, or whether "pluripotency" genes are not such good markers for this property, remains to be determined.

Finally, Chapter 6 reports differences in the rate of growth of cells derived from female and male embryonic gonads. Both left and right male gonads show a faster rate of proliferation (as determined by the time to attain confluence) than their female counterparts, which appear to slow down their growth at about passage 5. Future studies should be directed at extending this study to later passages including the establishment of permanent cell lines. If significant differences in growth rate between male and female gonocytes are found, this could partly explain the general belief in the field (M. McGrew and H Sang, R Etches, M.C. van de Lavoir, B. Pain, personal communications) that only male germ cells are capable of establishing long-term self-renewing cell lines *in vitro*.

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225

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GLOSSARY

Embryonic stem cells (ESCs): cell lines derived from very early embryos (in chick, stages before primitive streak formation; blastoderm, stage X-XIV, EG&K) which can be maintained *in vitro* indefinitely and can contribute to all somatic lineages. In mouse, ESCs can also contribute to the germ line.

Embryonic gonads (EGs): gamete-producing organs which develop as a part of the urogenital system from intermediate mesoderm. They arise from the gonadal ridge, a thickening of the germinal epithelium associated with the mesonephros and its duct (Wolffian duct)**.**

Primordial germ cells (PGCs): the precursor cells of gametes that will produce sperm in male and egg in female via gametogenesis. They can be found in embryos as early as the primitive streak stage by their expression of markers including Vasa (Cvh) and PAS-positivity. At this stage they reside in the hypoblast of the germinal crescent, from where they migrate to the blood circulation and later colonise the gonads.

Gonocytes: post-migratory or late primordial germ cells (PGCs) after they have settled inside the gonads.

Embryonic germ cells (EGCs): a stable, self-renewing cell line derived from gonocytes (or PGCs from another stage of development) that can be maintained in culture indefinitely. Some or all of these may be pluripotent or even totipotent (including the ability to contribute to the germ line). They may resemble ESCs by

morphology and have the ability to differentiate into derivatives of all three primary germ layers via embryoid body formation or monolayer differentiation and to generate chimaeras after injection into a blastoderm *in vivo*.