Investigating the genetic program of germ cell specification in pig

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Dissertation submitted to the University of Nottingham for the Degree of Research Master of Science in Advanced Genomic and Proteomic Science

August, 2010

ABSTRACT

At present, as the specification of germ cell in mouse well established, mechanisms of genetic and epigenetic regulation during germ cell development become important. Because whether they are identical in other mammals was unclear, detecting the expression of genes which are identified in mouse embryo in pig seems necessary. This project aims to investigate the expression of porcine PGC markers, trace PGC movement during porcine embryo development and gain understanding of the ontogeny of germ cells in pig. With the gene profiles of porcine, novel potential PGC markers can be identified, e.g., if we get a new antibody with no understanding of expression in pig germ cells, CT-1 (membrane protein) for instance, we can utilize oct-4 (nucleus protein) to identify CT-1. Specially, the approaches of porcine embryonic study can be spreaded to other mammalian species including human, which will facilitate us to understand human germ cell development.

Immunohistochemistry and in situ hybridization were used as main methods to detect gene expression during pig germ cell development in my experiment. The former focuses on cellular gene expression detection while the latter is mainly used to detection the expression in tissues. The immunohistochemistry results show that stella has no expression before E51 including E51, but was detected in foetal ovaries (E75-80). Oct-4 may be expressed in pig foetal testis and adult testis but very few positive cells were detected with the method immunofluorence. Expression of vasa was prior to dazl which is really different from in mouse. Besides, new primary antibody CT-1 was detected in various embryonic days and gonad, especially, it is expressed in E15, which is a really early stage, which suggests that it may be a novel PGC marker for early embryonic stage in pig.

ACKNOWLEDGEMENTS

The project started from February 2010 to August 2010 at the Department of Animal Science in Bioscience School, University of Nottingham. I gratefully acknowledge the advice offered by my project supervisor Dr. Ramiro Alberio (Department of Animal Science, School of Bioscience), and many suggestions and assistance by Contreras Caro Del Castillo David and Valdez-Magana Griselda during my project.

Xiaoni Sun, August 23th 2010

ABBREVIATIONS

AEC 3-amino-9 ethyl carbazoie

Blimp B-lymphocyte-induced maturation protein

BMP Bone morphogenetic protein

DAB Diaminobenzidine

DAZ Deleted in Azoospermia

DAZL Deleted in Azoospermia Like

DNA Deoxyribonucleic acid

Dpc Days post coitum

ES Embryonic stem

EG Embryonic germ

Hox Homeobox

ICM Inner cell mass

Ifitm Interferon- indeced transmembrane protein

PBS Phosphate-Buffered Saline

PFA Paraform aldehyde

PGC Primordial germ cell

POU Pit-Oct-Unc

RNA Ribonucleic acid

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Chapter 1: Introduction

1.1 General background of the project

Mature germ cells are also termed gametes, respectively male gametes, the spermatozoa (sperm cells), and female gametes, the oocytes (egg cells). When they are fused during fertilization, the first cell of a new individual, the zygote is created.

How does this single cell develop into a whole organism? This problem was first recognized in 1651 by William Harvey. The remarkable ability of germ cells to generate a complete organism has fascinated biologists for more than a century and remains a central question in research today, with the molecular mechanisms that underlie totipotency still unclear (Seydoux & Braun, 2006). Nowadays, scientists shift their focus onto germ cell precursors, which are also referred as primordial germ cells (PGCs), for their pluripotence and totipotence (Surani, 2007a).

The primordial germ cells are the common origins of spermatozoa and oocytes and thus represent the ancestors of the germ line. Germ line is considered as the development and movement of the (primordial) germ cells proceeding separately from the rest of the somatic cells.

The establishment of the germ cell lineage in mammalian is important for a great variety of reasons. Firstly, it may provide some information about the pluripotent state which is beneficial to us to know pluripotent stem cells well. Besides, the more knowledge of basic mechanism we master, the more methods may be found to restore pluripotency to somatic cells without the utilization of oocytes. Hence, generating human germ cells from the existing human ES cells may become not impossible any longer (Surani, 2007a). If oocytes can be generated from human ES cells, oocyte donors will be not essential. What's more, the oocytes generated through this way

maintain the whole genetic information of human pluripotent ES cells, so they can contribute to some cell therapy of diseases and medicine research (Surani, 2007a).

As to the mechanism of germ cell lineage establishment, it is not conserved amongst different model organisms. Usually, mouse model system is widely studied as mammal representative, for mouse belongs to eutherian (placental- not marsupial), which is the same as human , and its genetics is comparatively well developed (lots of mutants) and techniques exist for manipulating gene function (knocking genes out and turning them on), which are not available in any other model vertebrate.

(http://www.homepages.ucl.ac.uk/~ucbzwdr/teaching/b250-99/mouse.htm)

However, recent studies showed that mouse model cannot represent all of the vertebrates for various reasons. During early development, the morphology of the embryo itself which in mice becomes a cylinder (see figure1.1) whilst in the rest of species forms an embryonic disc (see figure 1.4C), including human. Hence, it can be suggested that PGCs development in mice might have developed a different mechanism to specify these cells from other mammals.

So, apart from mouse model, another mammal animal embryo model is really needed to establish as reference. Pig embryo is really a good model, because of the similarity of development and morphology within non rodent mammals including humans, and the number of available embryos that can be collected during early pregnancy.

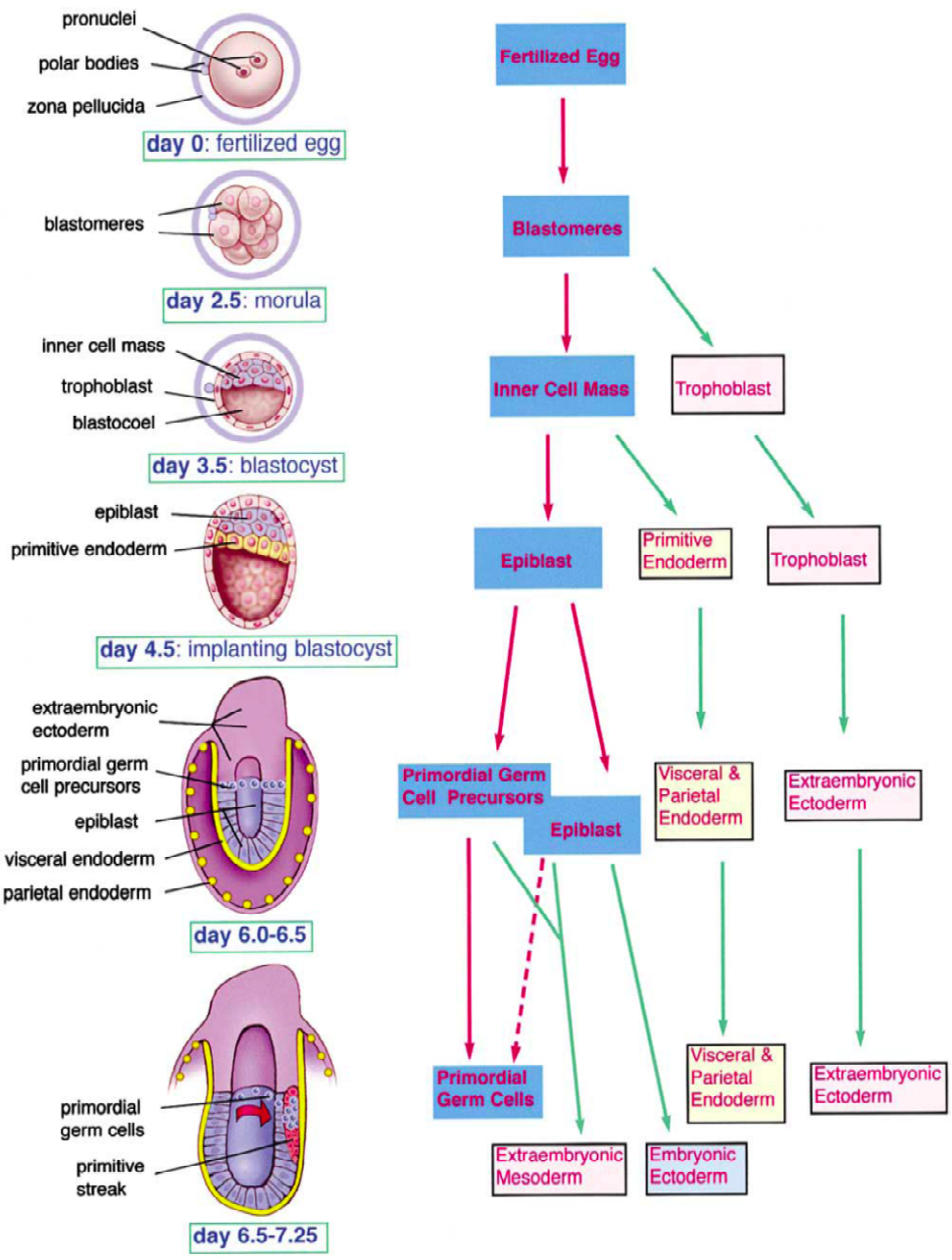


Figure1.1 Schematics from fertilized egg to primordial germ cell (PGC) formation in mouse. Mouse embryo development from fertilization (embryonic day 0) to PGC formation at day 7.25 is shown on the left part of the figure. Different cell types during early development are shown on the right as boxes. Arrows show differentiation: red arrows indicate the path from totipotency and pluripotency to PGCs, the dashed red arrow shows some epiblast cells have the potential to form PGCs in vitro and green arrows indicate differentiation of cells with no pluripotent character. Totipotent or pluripotent cells are boxed in blue including the fertilized egg, blastomeres, cells in the inner cell mass (ICM), epiblast, PGC precursors, and PGCs (Zhao and Garbers, 2002).

1.2 Germ cells

1.2.1 Origin of germ cells in vertebrate

In all sexually reproducing animals and plants, germ cells play a uniquely important role, namely the transmission of genetic information from one generation to the next generation, which are considered immortal. The final production of sperm and eggs has been widely studied by scientists in various species, both in vertebrates and invertebrates for many years (McLaren, 2003). The germ line is established in animal embryos with the formation of primordial germ cells (PGCs), which give rise to gametes and play an important role during germ cell development.

1.2.2 Primordial germ cells (PGCs)

Recently, to understand how the germ cell lineage is established in mammals, scientists focussed on the germ cell precursors, the founder cells of germ cell lineage, namely primordial germ cells (PGCs). PGCs are the embryonic precursors of the gametes which refer to male gametes, the spermatozoa (sperm cells), and female gametes, the oocytes (egg cells).

Different model organisms were utilized to investigate the mechanism of the development of PGCs. In all the model organisms, the place where PGCs form is far away from the developing gonads and they migrate to the developing ovaries or testes. In mouse, PGCs can first be detected at embryonic day 7.5 (E7.5) (see Figure 1.1) with the PGC marker gene alkaline phosphatase at the base of the allantois, subsequently were found to be present along the wall of the invaginating hind gut and migrate laterally to centralize in the genital ridges (Ginsburg et al., 1990).

In *Drosophila*, PGCs formed at the posterior pole of the developing embryo, but with the pole cells incorporated into the hindgut, they migrate out of the ventral side of the gut and then along the basal surface and finally reach the lateral mesoderm (reviewed in Starz-Gaiano and Lehmann, 2001).

In *Zebrafish*, four clusters of PGCs are formed during the cleavage stages. At the dome stage (4.3 hours post fertilization (hpf)), PGC clusters start to move dorsally and at 8.5 hpf, form a line at the border between the head and trunk mesoderm or line up within the lateral mesoderm, and then migrate towards an intermediate target, thereafter, at the 8-somite stage, migrate posteriorly to the developing gonad (Molyneaux KA & Wylie C, 2004).

1.2.3 Germ cell specification : mechanism of germ cell lineage establishment

Germ cells are different from somatic cells since they are unique for their ability to give rise to a new generation. It is this immortality that makes biologists engaged in the questions that how and when germ cells are segregated from somatic cells and how they are maintained during development until differentiation into sperm and egg. During this section, I intend to review some recent findings in some species.

Germ cells in the model systems such as *Drosophila*, *zebrafish* and *Caenorhabditis elegans* are specified by cytoplasmic determinants which are located in the posterior pole of the embryo, termed as pole plasm (germ plasm). The pole plasm is characterized by polar granules, structures which are present in great quantity of ribosome. In contrast, inductive signals play an essential role for germ cell specification in most mammals (Reviewed in Starz-Gaiano and Lehmann, 2001). So, segregation of primordial germ cells in animals can be classified into two major modes: one by maternally inherited determinates (‘preformation’) and another by inductive signals (‘epigenesis’) (Cassandra G. Extavour and Michael Akam, 2003).

1.2.3.1 Specification of germ cells in *Drosophila*

In *Drosophila*, germ cells, also called ‘pole cells’, are formed in a specialized cytoplasm, the germ plasm, at the posterior pole of the embryo. Germ plasm (pole plasm) plays an important role in germ cell specification and its transplantation to the anterior of the embryo can give rise to functional germ cells, which indicates that in

the germ plasm there are some factors sufficient to specify the germ cells. In another word, the pole plasm is a true germ cell determinant and not simply a germ cell marker.

A number of genes are required for germ plasm assembly, such as *Oskar, Vasa, Tudor* and *Aubergine*. The *oskar* gene plays a special role in germ plasm formation cause the site of *oskar* RNA localization and translation determines where germ plasm will assemble and the amount of *oskar* RNA and protein localized to the posterior determines the number of germ cells formed. (Reviewed in Starz-Gaiano and Lehmann, 2001) *Vasa* localizes with *oskar* to the polar granules, is related with *oskar* translation and identify germ cells in many organisms, including humans. *Tudor* localizes to the polar granules and the mitochondria and is involved in exporting mitochondrial ribosomal RNAs to the cytoplasm, which is necessary in the cytoplasm for germ cell formation. (Reviewed in Starz-Gaiano and Lehmann, 2001) *Aubergine* acts in miRNA pathway and is involved in both germ line determination and posterior patterning (Heather B. Megosh et al, 2006).

1.2.3.2 Specification of germ cells in *Zebrafish*

In *zebrafish*, similar as in *Drosophila*, PGCs are specified by maternal components including vasa. (Molyneaux KA & Wylie C, 2004) However, unlike in other organisms, where germ plasm is localized to a particular region of the egg (the posterior pole in *Drosophila* or *C. elegans*, or the vegetal pole in *Xenopus*), *vasa* RNA in *zebrafish* localizes to the edges of the cleavage planes in two- and four-cell embryos and segregates asymmetrically during subsequent divisions. (Reviewed in Starz-Gaiano and Lehmann, 2001) *Nanos* homolog, *nanos-1*, is expressed in the germ plasm and in the PGCs of the *zebrafish* and distributed throughout the oocyte cytoplasm. (Koprunner, 2001)

1.2.3.3 Specification of germ cells in mouse

Compared with the studies of PGC specification in the model systems mentioned above, the mouse utilizes different mode, induction by signalling molecules. In the

mouse embryo, the germ cells form at the border between epiblast and extraembryonic tissue. (Reviewed in Starz-Gaiano and Lehmann, 2001) The expression of Bmp4 (Lawson et al., 1999) and Bmp8b (Ying et al., 2000) in the extraembryonic ectoderm, and Bmp2 in the endoderm (Ying and Zhao, 2001), is required for the induction of germ cell fate among proximal epiblast cells in mouse. As to the regulatory genes involved in the specification process will be depicted in the lateral sections. Anyway, in mice, specification of primordial germ cells (PGCs) requires at least three key events: repression of the somatic program, reacquisition of potential pluripotency, and ensuing genome-wide epigenetic reprogramming.

1.3 Mammalian development

1.3.1 Mammalian embryogenesis — Gastrulation

Mammalian embryogenesis is often devided into two stages, respectively embryonic stage and a post-embryonic stage. Embryonic stage always attracts developmental biologists' interests because it is where the most exciting and dramatic events occur. Gastrulation is an important stage during mammalian embryonic development. The term gastrula refers to the appearance of the future craniocaudal axis of the foetus and the establishment of the three germ layers, ectoderm, mesoderm and endoderm, all of which are derived from the epiblast (Viebahn, 1999).

In mammals, the main body axes and the three germ layers are established during the gastrulation phase of early embryonic development. In higher primates and rodents, this phase is before implantation stage, while in other mammals they take the reversing time order (Viebahn, 1999).

The process of gastrulation is the most important step in the formation of the vertebrate body plan. The gastrulation is the correct placement of the precursor cells for subsequent morphogenesis and the body plan is established through inductive signals interaction between germ layer tissues and by the global patterning activity emanating from embryonic organizers (Tam & Behringer, 1997).

During gastrulation, series of morphogenetic processes take place, transforming the unstructured early embryo into a gastrula with several specific characteristics: (1) three germ layers, ectoderm, endoderm, and mesoderm are formed; (2) the basic body plan, inclusive of the construction of the main body axes, is established; and (3) the cells assume new positions, allowing them to interact with cells that were initially not close to them. This is the foundation of inductive signals interactions, which are the symbols of neurulation and organogenesis (Wang & Steinbeisser, 2009).

During this phase, the movement of cells reorganize the embryo from the blastula, a simple ball of cells, into a complex multilayered organism. Ectoderm, the outer germ layer, can generate the epidermis and nervous system; mesoderm, the middle germ layer, has the potential to become skeletal, muscle, blood, bone, and connective tissues; and endoderm, the inner germ layer, can give rise to the mucous membrane lining digestive and respiratory tract and digestive glands (Wang & Steinbeisser, 2009).

Vertebrate gastrulation includes four conserved morphogenetic movements. They are internalization, epiboly, convergence and extension. Internalization leads to cells which will generate the future mesoderm and endoderm beneath the prospective ectoderm by the blastopore, known as primitive streak in amniotes. Epiboly movements spread and make germ layers thin during gastrulation, whereas convergence and extension movements narrow the germ layers mediolaterally, and elongate the embryo from head to tail (Solnica-Krezel, 2005).

The placenta and foetal membranes, extraembryonic conceptus structures, are essential for the embryonic development of mammals in uterine. Generally speaking, during embryogenesis, the cell types which form these extraembryonic conceptus tissues are initially specified, since they must assume their functions early during the embryonic development. The placenta and foetal membranes play a protection function by forming a barrier which can protect the foetus from infection and maternal immune attack, and transport essential nutrients and waste betweem the foetus and its environment (Cross, 1998).

1.3.2 Vertebrate morphology

Along the anteroposterior axis, the vertebrate body can be divided into three regions, head, trunk, and tail, due to the patterning events during gastrulation. In amphibians, Spemann's organizer which refers to the area in the dorsal equator of the gastrula takes the responsibility to pattern the three primary germ layers. Besides, it is involved in the upper dorsal blastopore lip and the transplantation of the lip to the ventral side of the embryo can give rise to a complete secondary body axis (de Souza & Niehrs, 2000). In the frog *Xenopus laevis*, the future anteroposterior axis divides the organizer region into three parts: the anterior endoderm, the prechordal endomesoderm (prechordal mesoderm and pharyngeal endoderm) and the chordamesoderm. The anterior endoderm can form liver, while chordamesoderm can generate notochord. During gastrulation, these axial tissues migrate toward the anterior side with the neural-inducing signals which can pattern the ectoderm along the anteroposterior axis. (de Souza & Niehrs, 2000)

In 1931, Spemann pointed out that different parts of the organizer have different abilities to induce the formation of head or trunk or tail structures. The transplantation of organizers from early gastrulae can induce the formation of head, whereas the transplantation of organizers of older gastrulae can generate only secondary trunks or tails or both. The prechordal endomesoderm is considered having the ability to give rise to the head, while the posterior chordamesoderm has been considered to be trunk organizer (Niehrs, 1999). However, as to the anterior endoderm of the *Xenopus laevis* organizer, according to Bouwmeester et al. (1996), it seems related to head induction due to the finding that it expresses *cerberus*, a gene coding for a secreted factor with head-inducing properties.

Similar to amphibians, in mouse gastrula, anterior endodermal tissue is also play a role in the induction of the anterior central nervous system (CNS). The early mouse gastrula has a cylinder and contains two epithelial layers, an inner layer and an outer layer. The inner layer (epiblast) can give rise to all embryonic structures, whereas the outer layer, the primitive endoderm, cannot form the embryo proper. Gastrulation takes place at the posterior side of the embryo, with the formation of the primitive

streak and the node, a structure equivalent to the frog organizer. Axial endomesoderm is derived from the node and migrates toward the anterior side of embryo, replacing the visceral endoderm. Recent findings shows that the anterior visceral endoderm (AVE), which is related to the future anterior CNS during early gastrulation, has the ability to induce forebrain and midbrain. It suggests that head and trunk organizers are separated in the mouse embryo, the trunk organizer existing in the node but the head organizer in the AVE (de Souza & Niehrs, 2000).

1.3.3 Embryo development and implantation in animal models

Implantation is a necessary event during early embryo development of the animals which are viviparous birth. Although lots of differences exist in the mechanism of implantation amongst species, the processes from fertilization to the beginning of implantation seem similar (Lee & DeMayo, 2004). After fertilization, one single cell embryo is divided into two cells, four cells, eight cells, and then becomes a morula, subsequently forms the blastocyst, which consists of two cell types, the trophectoderm and the inner cell mass (ICM). The trophectoderm (outer layer) will generate the placenta and the inner cell mass (inner layer) that will become the embryo. Thereafter, the embryo gets rid of the zona pellucid and the blastocyst becomes ready for implantation. Different species have different duration of preimplantation stage. Based on the different ways of cell interactions between blastocyt and uterine, implantation can be classified into three types, respectively centric, eccentric and interstitial. When the blastocyst grows large and forms enough surface relation to fuse with the luminal epithelium, centric implantation occurs. For example, rabbits, dogs, domestic animals such as cows, pigs, and sheep, and many marsupials take this type. However, Mice, rats and hamsters take another type, eccentric implantation, which is characterized by that the luminal epithelium forms an invagination to surround the trophoblast. The last type of implantation is interstitial. During this type of implantation, the trophoblast goes through the luminal epithelium to invade the endometrial stroma and becomes imbedded into the uterus wall. Humans and guinea pigs belong to this type. (Lee & DeMayo, 2004)

1.3.4 Mouse development

1.3.4.1 Mouse embryogenesis

In mouse embryos, embryonic and extra-embryonic lineages are separated at the blastocyst stage before implantation and give rise to the foetus and the foetal part of the placenta respectively. Evidence can be given that at 3.5 days post-coitum (dpc) at the blastocyst stage, this become the inner cell mass (ICM) and the trophectoderm. Two trophectoderm cell types can then be distinguished: the cells which cover the ICM (polar trophoblast), and those which surround the blastocoelic cavity (mural trophoblast). (Cross et al. 2005)

Polar trophoblast cells proliferate actively under the stimulus of signals from ICM and give rise to the extra-embryonic ectoderm (ExE) and differentiate into the ectoplacental cone (EPC) at 5.5 dpc. However, mural trophoblast cells surround the blastocoelic cavity, cannot benefit from the trophoblast growth factors emitted by ICM. They get away from the mitotic cell cycle, experience rounds of DNA replication without mitosis and become polyploidy, forming primary giant cells at 5.5 dpc. (Cross, 2005)

However, in most mammals, the polar trophoblast, also called ‘‘Rauber’s layer’’, disappears quickly after blastocyst expansion and hatching (Viebahn, 1999), with no anatomical equivalent to the mouse ExE or EPC. Subsequently, the mural trophoblast grows and differentiates but not polar trophoblast.

After implantation in the uterus, the mouse embryo initially develops as a cylindrical structure which is rather different from most mammalian embryos. During the immediate post-implantation period (5-6 dpc), the mouse embryo changes in size and shape. The embryonic tissue volume increases by about 40-fold, largely due to the cell proliferation of the tissue that gives rise to the extraembryonic ectoderm (Tam & Behringer, 1997).

By E6.0 (the egg-cylinder stage) in the mouse, the cells of the epiblast begin to differentiate. Three differentiated cell types are formed: the trophoblast, the epiblast (also called the embryonic ectoderm or primitive ectoderm at this stage), and the

primitive endoderm (see figure 2). During the next major phase of development, termed gastrulation, the embryonic ectoderm will differentiate into the three primary germ layers—endoderm, mesoderm, and ectoderm. Thus, the embryonic ectoderm has succeeded the epiblast as the tissue that will generate the body of the embryo. The primitive endoderm differentiates into parietal and visceral endoderm, the anterior region of which will help regulate the development of the body plan during gastrulation.

The process of gastrulation begins at about E6.5 in the mouse. At that time, a primitive streak forms in a specific region of the epiblast along the posterior axis of the embryo. At the anterior end of the primitive streak is the node, a two-layered structure and important signaling center in the embryo. The ventral layer of cells in the node comes from the epiblast and generates the notochordal plate, which then forms the notochord. Endoderm, which will give rise to the gut, also develops near the node, along the sides of the notochord. By E8.0 in the mouse, the primitive ectoderm of the postimplantation blastocyst has generated the ectoderm, mesoderm, and endoderm of the gastrula.

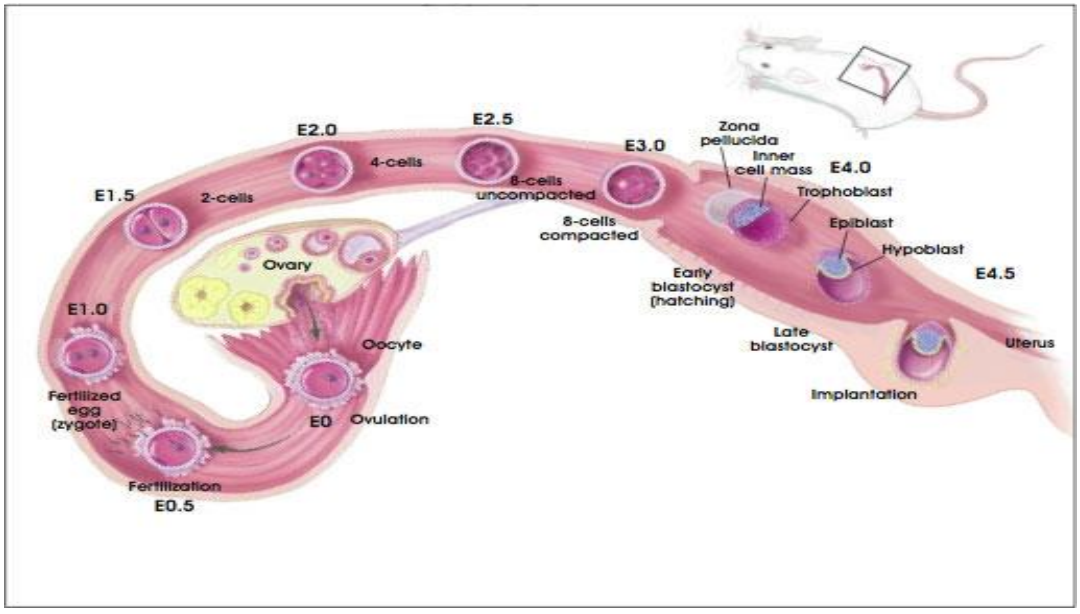


Figure 1.2: Development of the Preimplantation Blastocyst in Mice from Embryonic Day 0 (E0) Through Day 5 (E5.0) (copy 2001 Terese Winslow).

1.3.4.2 PGC development in mouse

In the mouse, primordial germ cells (PGCs) develop from the pluripotent epiblast and are first detected as alkaline phos-phatase-positive cells in the embryo at 7.25 days post coitum (dpc). PGCs rapidly proliferate although migrating along the hindgut and dorsal mesentery (8.5–9.5 dpc), reaching the genital ridges by 10.5–11.5 dpc. After colonizing the genital ridges, they continue proliferating until 13.5 dpc. Female germ cells then enter meiotic prophase and undergo meiotic arrest, whereas male germ cells remain mitotic until 15.5 dpc, when they enter a state of mitotic arrest (Yukiko, 2005).

Recent study shows that in mouse, specification of primordial germ cells (PGCs), occurs through the integration of three key events: the repression of a somatic mesodermal program (Saitou et al. 2002), re-acquisition of a potential pluripotency (Yabuta et al. 2006), and ensuing genome-wide epigenetic reprogramming during their migration period (Seki et al., 2007). Blimp1, a key regulator of the germ cell, in a few epiblast cells of early post-implantation embryos, represses the incipient somatic program in these cells and promotes progression toward the germ cell fate. (Ohinata et al, 2005)

Expression of Fragilis increases during migration and induces expression of other germ cell-specific genes such as Stella and the VASA homolog (Mvh). DAZL is another gene expressed exclusively in germ cells, and one of the proteins products of this gene is expressed throughout the life of germ cells and is required for the development, differentiation and maturation of germ cells (Lacham-Kaplan 2004).

1.3.5 Human development

1.3.5.1 Human embryogenesis

In human, after a successful fertilization in the ampullary part of the fallopian tube, the embryo migrates through the tube into the uterine cavity. This migration will cost 6 days. Along the way, the zygote divides several times, initially without increasing its volume because it is still compassed by the pellucid zone. This period is called the

blastomere stage. After that stage, the morula stage (16 cells) follows, around 2-3 days post-fertilization and by day 4 a fluid-filled space, the blastocyst cavity formed. People named now as a blastocyst. At this time, two layers of cells can be distinct in the embryoblast: the epiblast and the hypoblast. Thereafter the free blastocyst embeds itself with the pole and this is termed adplantation. With the blastocyst penetrating into the endometrium, the implantation has begun.

Implantation extends from the end of the first week of embryonic development (the moment of the hatching of the blastocyst) to the middle of the second development week (the formation of the primitive placental circulation system). From day 7 to day 12, it is a bilaminar embryo with primary yolk sac.

During the 3rd week of its development, the three embryonic germinal layers formed, with the appearance of the primitive streak. This streak is the location where laterally immigrating cells sink down to form the deep layers of the mesoblast and endoblast. Then the trilaminar germ disk formed. The mesoblast is divided into three parts: paraxial (surrounding the neural tube and later forms the somites), intermediate mesoderm (give rise to the urogenital system) and lateral plate mesoderm (divided into the somatopleural and splanchnopleural mesoderm). During this time the median part of the epiblast thickens and forms a groove and afterwards a tube (neural tube), out of which the central nervous system will arise.
(http://www.homepages.ucl.ac.uk/~ucbzwdr/teaching/b250-99/mouse.htm)

Gastrulation and the formation of notochordal process occur at day 16, and by day 18 the Hensen's node is evident. Appearance of the first somites occurs at day 20 (Carlson, 2009). The embryonic period is the first 8 weeks of pregnancy. The fetal period refers to roughly the last six months of the pregnancy.

1.3.5.2 PGC development in human

Gametes and the primordial germ cells are differentiated from other somatic cells very early, and migrate from the ectoderm (3^{rd} week) through the extraembryonic endoderm (5^{th} week) into the primordium of the future gonads, the genital ridge. At

the genital ridge, after an interaction with the coelomic epithelial cells, the primordium for the testis evolves in the 7th week, if a Y chromosome is present, or the primordium for the ovary in the 8th week, if it is not present.

In the 2nd week, PGCs can be found in the primary ectoderm (epiblast). In the 3rd week, they wander from the primary ectoderm into the yolk sac wall and collect near the exit of the allantois. During the 4th week, PGCs are situated among the endoderm cells in the secondary yolk sac wall. And in the 5th week, PGCs wander out of the yolk sac wall, along the vitelline and the dorsal mesentery, into the gonadal ridge. In the 6th week, the primordial germ cells become surrounded by the coelomic epithelial cells and the male / female gonadal primordia cannot be distinguished.

1.3.6 Pig embryo stage

Pig fertilization takes place in the oviduct, at the ampullary-isthmic junction. The two cell stage lasts for 6-8 hours, the 4 cell stage for 20-24 hours and this is when they entry into the uterus. The 8-16-cell-morula stage is reached around day 4, and the blastocyst staged at day 5-6; Hatching from the zona pellucida occurs at day 6-7 and this they remain free in the uterine lumen up to day 13 (Figure 1.3). Post-hatching embryos at the expanded hatched blastocyst stage can be collected around Days 7–8 in the pig, inside the blastocyst, differentiation of the hypoblast from the ICM is ongoing forming a confluent cell layer surrounding the blastocoel cavity (Stroband & Van der Lende 1990).

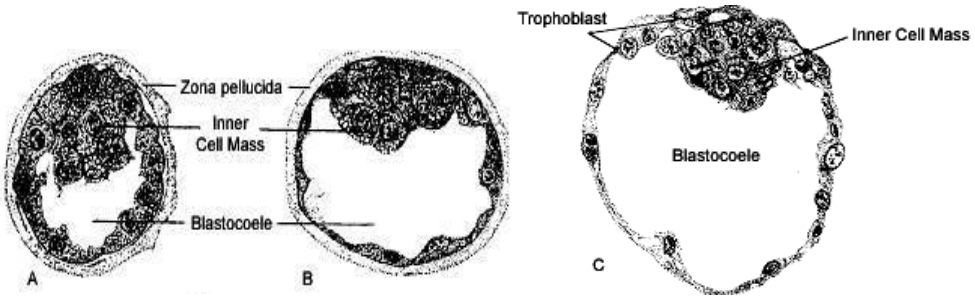


Figure 1.3. Schematics of a pig embryo from blastocyst to hatched stage (A&B: blastocyst, C: hatched embryo) these development stages are reached between days 5 and 8 after fertilization (Carlson, 1996).

Embryogenesis in pig can be usefully divided into two intervals, from 12 to 36 days and from 36 to birth at about 114 days. The first interval is often called prefoetal or embryonic and the second interval is described as the foetal period. Embryonic period is from the time of establishment of the body axis to when male and female can first be distinguished by the shape and position of their external genitalia (A.W.Marrable, 1971).

Pre-Streak Stage 1

At pre-streak stage 1 (Days 9–10) embryonic disk is formed, at one pole of the blastocyst (see Figure 1.4).

Pre-Streak Stage 2

This stage is characterized that a crescent-shaped thickening of the caudal portion of the embryonic disk appears. The posterior crescentic thickening appears to represent the first morphological sign of polarity in the pig as well as in cattle (Maddox-Hyttel et al. 2003) and sheep (Guillomot et al. 2004). Its formation may be accomplished through convergent differential growth of epiblast cells toward the caudal margin, as shown in the rabbit (Viebahn et al. 2002) and as indicated by findings in the pig (Flechon et al. 2004).

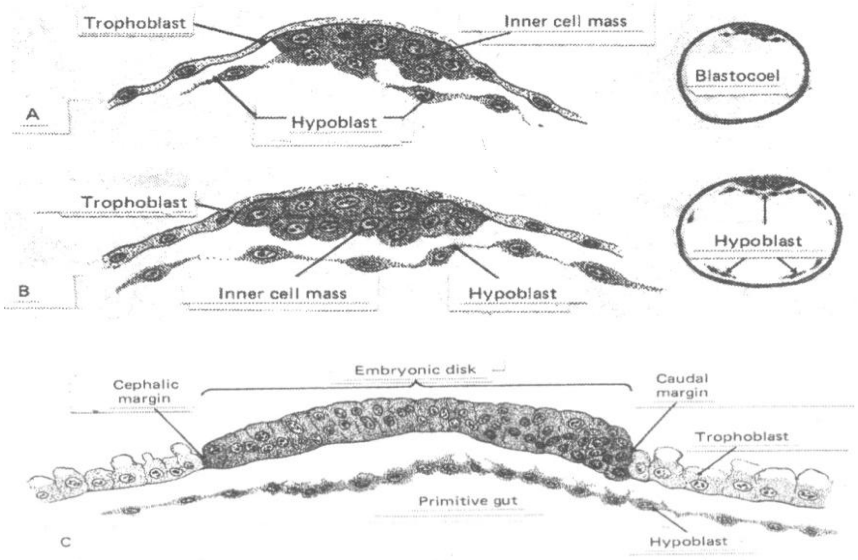


Figure 1.4. Schematics of pig embryo of E9 and E10. (A&B: showing whole embryos from 9 and 10 days respectively, C: section with the view of the embryonic disk and the primitive gut).

Primitive Streak Stage

As the name shows, this stage is characterized by occurrence of a primitive streak, but still no signs of a neural groove in the anterior part of the embryo proper. In this stage the primitive streak is extending from the posterior and towards the anterior pole of the embryonic disk (Figure 1.5), the crescent-shaped thickening, by stereomicroscopy, appeared to have gathered in the midline forming the primitive streak. At this stage, the primitive streak has developed as an axis of cell ingression of cells for meso- and endoderm formation. In pigs, the node appeared to be present from early during development of the primitive streak and formation of the notochord cannot be identified under the stereomicroscope. Later during the primitive streak stage, the streak appeared to have ‘‘regressed’’ while still no anterior signs of a neural groove are observed.

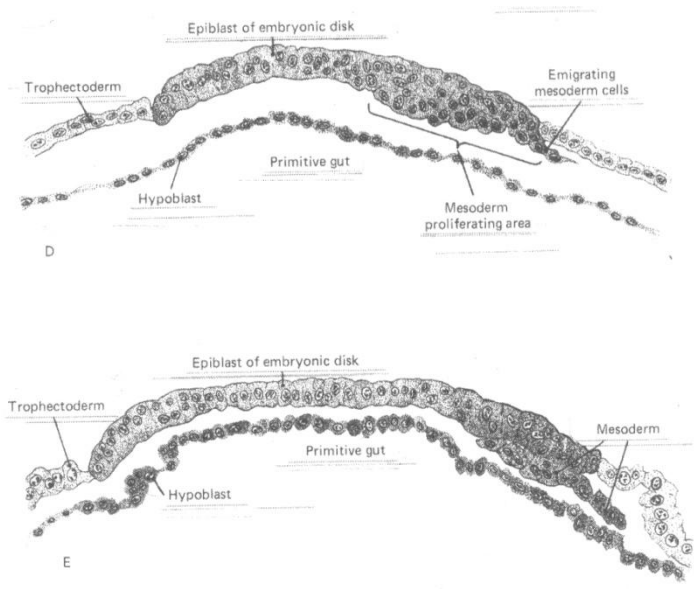


Figure 1.5. Schematics of section of developing pig embryos on the primitive streak. Orientation of the embryos are cranial to caudal from left to right with some migration of mesodermal cells.

Neural Groove Stage

Neurulation includes the formation of the neural plate (day 18-19), neural folds (day 20-21), and the neural tube (day 22-26). Neural plate is a thickening of the ectoderm caused when cuboidal epithelial cells become columnar. Shortly after its formation, the neural plate becomes folded through differential growth so that its medial portion is depressed, forming the neural groove, and its lateral elevated portions form neural folds. This stage is defined as the neural groove stage (Figure 1.6 A.). In porcine

embryos, at the stage where the neural groove extends almost to the posterior pole of the embryo proper, the mesoderm on either side of the notochord, i.e. the paraxial mesoderm, thickens to form the first pair of somites and thus this stage is termed the somite stage (Vejlsted et al. 2006).

Somite stage

It's marked by the appearance of a neural groove and adjoining neural folds. When the extension of the groove has almost reached the posterior pole of the embryo proper, the first pair of somites is observed. At this level of development and onwards, staging is proposed to be based on numbers of somites (Fig. 1.6 B).

The extraembryonic membranes develop from all three germ cell layers (ectoderm, mesoderm, and endoderm). These membranes include the yolk sac, amnion, chorion and allantois. The yolk sac is derived from the foregut and it is considered to be composed of splanchnopleure (endoderm and splanich mesoderm). The allantois is also derived from splanchnopleure, but it originates as an out-branching of the hindgut. Both the yolk sac and allantois are considered to be vascular because of the blood vessels within the splanich mesoderm. The other two extraembryonic membranes, chorion and amnion, are derived from somatopleure, which includes ectoderm and somatic mesoderm, and like this is devoid of blood vessels these two membranes are avascular (Figs. 1.6A&B) (Schaten & Constantinescu 2007).

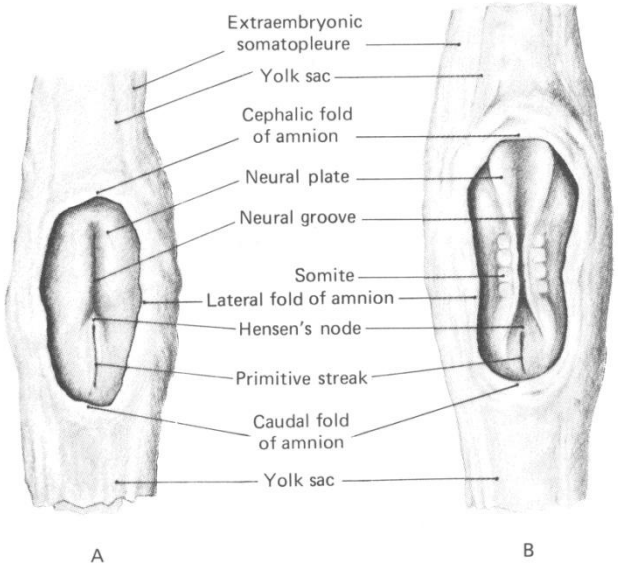


Figure 1.6. Schematics of pig embryos of neural groove and somite stage. Left at the first appearance of the neural groove and right at the somite stage (Carlson, 1996).

From day 11-12 the blastocyst start elongation and reduction of the diameter and implantation takes place at day 18 (Stroband & Van der Lende 1990).

The pig implantation is noninvasive and the blastocyst has a protracted period of expansion and undergoes a rapid elongation of its trophoblast before adhering to the lunal surface of the endometrium the morphology of the embryonic disc appears, at least superficially, similar to the chicken embryo and there is no "inversion of germ layers" as in the mouse, where conceptual flattening is necessary to obtain a comparable fate map of the embryonic disc (Lawson et al. 1991).

1.4 Germ line development

1.4.1 PGC signal pathway

Members of the bone morphogenetic protein (BMP) family play various roles in plenty of developmental processes. Binding of BMPs to their receptors induces phosphorylation of the BMP-specific smads (Smad1, Smad5, and Smad8). p-Smads1/5/8 then associate with Smad4, translocate into the nucleus, and activate the transcription of BMP-target genes (Dudley et al. 2007).

In mouse embryo, germ cell fate is determined in proximal epiblast by induction of BMP signals. BMP2, BMP4 and BMP8b control the formation and early proliferation of primordial germ cells (PGCs) and secreted by the murine extraembryonic ectoderm (ExE) at about E5.5. (Ohinata et al., 2009)

1.4.2 Epigenetics

Epigenetics refers to a collection of mechanisms and phenomena that define the phenotype of a cell without affecting the genotype. (Goldberg et al., 2007) At molecular level, it represents a range of DNA and chromatin modifications including DNA methylation, histone modifications, remodelling of nucleosomes and higher

order chromatin reorganization. The epigenetic profile of germ cells changes dynamically during their development (Sasaki & Matsui 2008). Recent studies have showed a series of epigenetic modifiers such as DNA methyltransferases, histone-modification enzymes and their regulatory factors (Reviewed by Hiroyuki Sasaki & Yasuhisa Matsui, 2008).

As mentioned above that the PGC precursors need to suppress the somatic gene expression programme, epigenetic modifications might be important for this suppression. PGC-like cells in Blimp1-null embryos have aberrant expression of the Hox genes, which are normally repressed in PGCs. This suggests that Blimp1 is essential for PGC specification. However, how Blimp1 regulates germ-cell specification and represses the somatic fate is still unclear now. It binds to a histone-arginine methyltransferase, PRmT5 (protein arginine N-methyltransferase 5), to repress premature expression of some germ-cell-specific genes in more advanced PGCs, and it is possible that epigenetic modification might also contribute to the somatic repression role of Blimp1(Reviewed by Hiroyuki Sasaki & Yasuhisa Matsui, 2008).

In mouse, with the number of germ cells increasing, germ cells start to migrate into the developing gonads (see Figure 1.7), and reach around E10.5. Soon afterwards, PGCs suffer a wave of genome-wide DNA demethylation, which is accompanied also by the profound changes in chromatin configuration and erasure of some histone modification marks (Surani et al. 2007). Evidence suggests that the entry of PGCs into the genital ridge may be important for the onset of the reprogramming process (Tam et al. 1994).

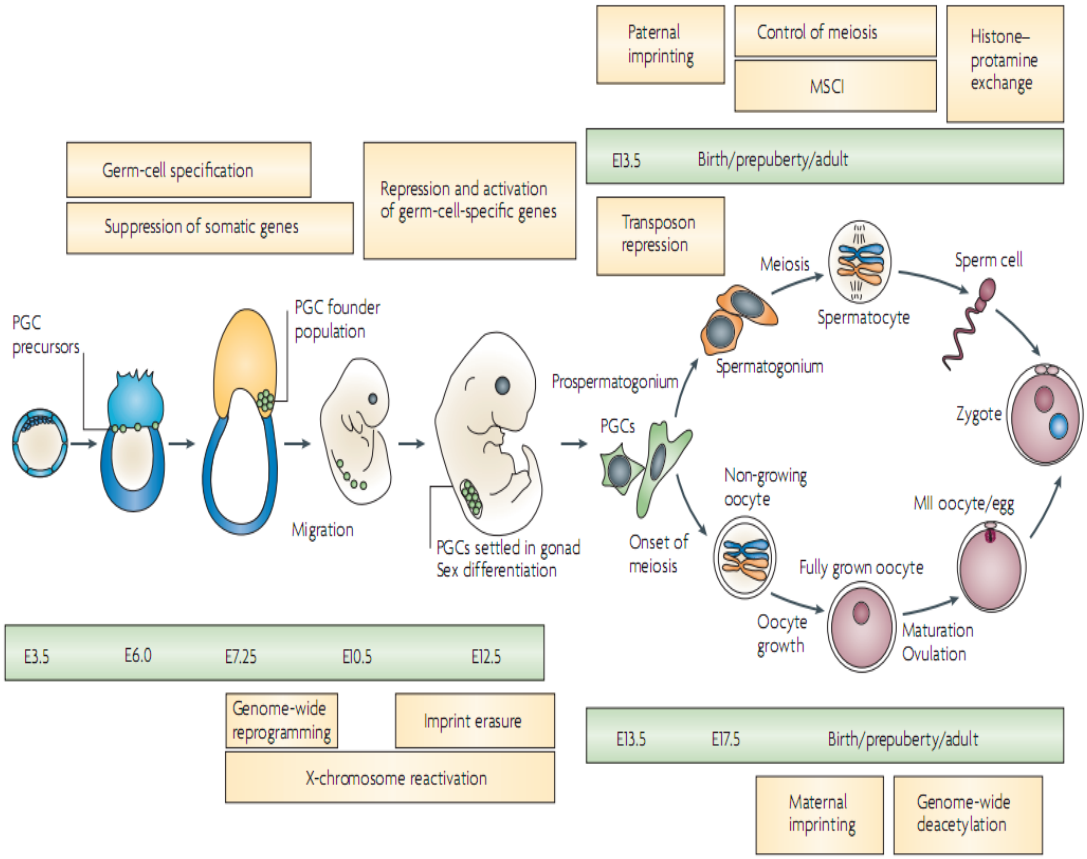


Figure 1.7. Germ cell development and associated epigenetic events in mice. Chronology of mouse germ cell development and the main epigenetic events that occur. PGCs (primordial germ cells) first emerge at embryonic day 7.25 (E7.25) as a cluster of about 20 cells. Subsequently, they rapidly proliferate with an average doubling time of approximately 16 hours. Before they stop dividing at E13.5, their number reaches up to about 26,000. MSCI, meiotic sex-chromosome inactivation. (Sasaki & Matsui 2008)

1.4.3 Imprinting

The term ‘genomic imprinting’ means whether a gene can be expressed in the embryo is depended on the parental genome, used to describe the functional differences between parental genomes during mammalian development. It is showed that there are a number of genes whose expression is strictly dependent on their parental origin. Some are expressed only when inherited from the father and others are expressed only when inherited from the mother (Surani, 2007).

Methylation of DNA is considered to be one of the major means of imprinting. DNA methylation results in the differential expression of paternal and maternal alleles of the imprinted genes. The imprinted genes operate during development and possibly into adulthood, but a given imprint is not passed on to that individual's progeny. Instead, the parental imprints on the genes are erased, and new imprints, corresponding to the sex of that individual, are established in the oocytes and sperm during gametogenesis. Not all genes are parentally imprinted. At the moment estimates suggest that up to 2% of all mammalian genes are imprinted (Carlson, 2009). Currently, approximately 50–100 imprinted genes have been identified in the mouse and human genomes (Surani, 2007).

1.4.4 Germ cell specific gene markers

1.4.4.1 *Stella*

Stella is the first gene known to be expressed in PGCs, and thereafter in the germ cell lineage. Motif analysis of *stella* (PGC7) suggested that it functions in DNA binding and RNA splicing, but the binding of *stella* to DNA and RNA seemed to be non-specific.

In mouse model system, immunohistochemistry analysis revealed that *stella* (PGC7) was specifically expressed in early pre-implantation embryos, PGCs, oocytes and pluripotent cells. The expression of *stella* (PGC7) is activated at E7.25 in mice (see Figure 1.1), specifically in the founder population of lineage-restricted primordial germ cells (PGCs). At the late bud stage (E7.5), *stella* is expressed highly with the intensity of the *stella* signal increased and some cells considered to be migrating from the initial cluster were also stained. At E8.5, groups of migrating PGCs were stained exclusively in the developing hindgut. Then, it is expressed in the germ line until about E15.5 in male and E13.5 in female gonads. While not detectable in adult testes, stella protein expression resumes in the immature oocytes in newborn ovaries, and it is subsequently detected in maturing oocytes and in preimplantation embryos. Soon after the formation of the zygote, stella accumulates in the pronuclei, and also detected in the cytoplasm, indicating stella antibody is both cytoplasmic and nucleus

protein. Both staining last during cleavage stages until the blastocyst stage, afterthen, *stella* is downregulated until its reappearance in the nascent PGCs.

1.4.4.2 *Fragilis*

The family of interferon-induced transmembrane protein (Ifitm/mil/fragilis) genes encodes cell surface proteins that may modulate cell adhesion and influence cell differentiation (Tanaka, 2005). Expression of *fragilis* is induced by extra-embryonic ectoderm through signalling molecules in mouse. *Fragilis* is the first gene to mark the onset of germ cell competence, and it follows a pattern consistent with the eventual segregation of germ cells from allantoic precursors.

At an earlier stage, only weak *fragilis* expression was seen throughout the epiblast in E6.0 mouse embryos. *Fragilis* expression persisted until the late bud stage (E7.5) , but gradually faded around the early head fold stage (E7.75), and was substantially reduced in migrating PGCs at E8.5 (Saitou, 2002).

1.4.4.3 *Blimp-1 (Prdm1)*

Blimp-1 (B-lymphocyte-induced maturation protein-1), also known as Prdm1, with Krüppel-type zinc fingers, is a known transcriptional repressor, which has a critical role in the foundation of the mouse germ cell lineage, since its disruption can result in a block early in the formation process of PGCs (Yasuhide Ohinata et.al, 2005). Specifically, its expression can drive B-cell differentiation into plasma cells by repressing the mature B-cell gene expression programme (Yasuhide Ohinata et.al, 2005), whereas inactivation of Blimp1 induces B-cell terminal differentiation to a halt. Blimp1 is widely expressed during development, including in migrating germ cells. The role of Blimp1 in the germ-cell lineage appears to be to repress the somatic-cell-specific Hox genes (Mclaren & Lawson, 2005). Blimp1 protein has a PR/SET domain, a proline-rich region, five C2H2 zinc fingers, and a C-terminal acidic domain (Hayashi et al., 2007). What's more, it is a key transcriptional regulator that is partly responsible for repressing the somatic program in PGCs while allowing establishment of germ cell character in these cells.

In mouse, the earliest Blimp-1 expression in the embryo proper was detected at E6.25 in the most proximal layer of the epiblast. These Blimp1-positive cells are restricted

to only the future posterior side of the shorter embryonic axis, which has recently been shown to form the primitive streak after dynamic morphological rearrangement (Yasuhide Ohinata et.al, 2005). At the early streak stage (E6.5), Blimp1 positive cells increase including a few within the nascent mesoderm. Then they relatively sharply increase due to a lag period, forming a tight cluster at the mid- to late-streak stage (E7.25) (Yasuhide Ohinata et.al, 2005).

It is noteworthy that Blimp1 expression is detected in many tissues and in many organisms. A variety of functions are attributed to Blimp1 protein, the diverse roles of this highly conserved gene suggest that it must have acquired new enhancers or regulatory elements for its role in germ cell specification in mammals (Surani et al. 2007).

1.4.4.4 *Vasa*

The *vasa* gene, first identified in Drosophila as a maternal-effect gene and subsequently in several invertebrate and vertebrate species such as frogs, fish, chickens, mouse, bovine and human, was demonstrated to be essential for germ cell development (Bartholomew, R.A, 2006). However, it has not been investigated in porcine embryo before.

Analysis of *vasa* homologs from various organisms demonstrates highly conserved regions among mammalian sequences and some important differences in its regulation (Reviewed by Raz, 2000).

Different from some genes like *Oct4* which is expressed in both germ cells and the inner cell mass (ICM) of developing embryos, *vasa* gene is exclusively expressed to the germ line. It is not expressed in either embryonic stem (ES) or embryonic germ (EG) cell lines derived from the ICM and migratory primordial germ cells (PGCs), and this suggested that *vasa* gene expression is a phenotypic characteristic of germ cells. Expression of *vasa* gene is mainly detected in post-migratory mammalian PGCs, whereas expression in Drosophila is maintained throughout migration to the embryonic gonads and beyond.

Vasa gene is expressed in mature oocytes but is down regulated during spermatogenesis. Besides, the expression in the developing embryo and adult is limited to the germ line of vertebrates. In humans, gene expression is minimal in spermatogonia but abundant in spermatocytes. Fetal oogonia and primordial and primary oocytes are positive for vasa protein. As oocytes continue to grow and develop in the postnatal mouse and human ovary, protein levels decrease. *Vasa* gene expression is absent in murine but not human and bovine antral follicles (Bartholomew & Parks 2007).

1.4.4.5 *Dazl*

The Deleted in Azoospermia Like (*DAZL*) gene is a member of the Deleted in Azoospermia (*DAZ*) family, which includes three genes: *BOULE*, *DAZ* and *DAZL*. The *DAZ* family genes encode proteins with a highly conserved RNA-binding motif and a unique DAZ repeat, and take a germ cell specific expression pattern. The DAZ proteins function in the post-transcriptional regulation of mRNA expression. The DAZL gene and its homologues are essential for germ cell development in several species. The DAZ genes are strong candidates for the AZFc azoospermia factor, one of the most common genetic causes of male infertility (Cauffman et al. 2005). Loss of function of the other autosomal DAZ family members results in failure to produce mature gametes in organisms as diverse as mouse, frog, fruit fly and nematode (Reynolds et al. 2005).

In mice, a knockout of the *dazl* gene results in meiotic arrest of male and female germ cells. In humans, *DAZL* is expressed early in PGCs in fetal gonads and then throughout gametogenesis. During spermatogenesis, expression has been shown in the nucleus and the cytoplasm of gonocytes, spermatogonia and primary spermatocytes. At the onset of meiosis, all *DAZL* is translocated from the nucleus to the cytoplasm.

DAZL is a cytoplasm protein. During oogenesis in mouse, DAZL is expressed in the cytoplasm of oogonia in fetal ovaries and developing follicular oocytes in adult ovaries. Moreover, transcripts of *DAZL* are also detected in Sertoli cells.

1.4.5 Pluripotency gene markers

1.4.5.1 *Oct4*

Oct4 (also known as *Oct-3* and *POU5f1*), a member of the Pit-Oct-Unc (POU) (Scholer et al. 1990a) family of transcription factors, is a marker for germ-line (oocytes), undifferentiated (pluripotent) and tumor cells. The gene structure, sequence, localization of the gene, and also regulatory regions, are highly conserved in mouse, human, and bovine, suggesting conservation of expression patterns and function (Nordhoff et al. 2001).

Its principle function is to block differentiation, meanwhile allowing proliferation. *Oct4* has been considered as a major regulator of cell pluripotency and lineage commitment, which means whether a cell maintains pluripotency or differentiates, is dependent upon the levels of *Oct4* expression. Levels above normal induce differentiation to primitive endoderm and mesoderm, while levels under normal result in loss of pluripotency and dedifferentiation towards trophectoderm (Niwa H, et al., 2000).

In mouse, the first expression of *Oct4* gene is in all blastomeres of the developing embryo, and then it becomes restricted to the inner cells of the blastocyst forming the inner cell mass (ICM), and is downregulated in the trophectoderm and the primitive endoderm. In the later development, *Oct4* expression is retained in the embryonic ectoderm at the egg-cylinder stage and downregulated at gastrulation in an anterior-posterior pattern. Primordial germ cells (PGCs) are the only cells which maintain Oct4 expression after this stage and arise at E7.2 of mouse embryo development within the extraembryonic mesoderm (Pesce & Scholer, 2001). Germ cells retain the expression of Oct-4 until the sexual differentiation of the gametes and meiosis which is at day 13-14 post-coitum (dpc) in the female and at the beginning of spermatogenesis in the newborn males (Pesce & Scholer, 2001).

1.4.5.2 *Nanog*

In mouse, nanog, a homeobox transcription factor, not only can blocks primitive endoderm differentiation, but also actively maintains pluripotency (Mitsui et.al, 2003).

Nanog plays a crucial role in the second embryonic cell fate specification event (Chambers et al. (2003) and Mitsui et al. (2003)). During preimplantation development, at morula stage, cells remain pluripotent as ICM or differentiate into trophectoderm. At this stage, nanog expression is low and Oct4 is the key determinant of cell fate. At blastocyst stage, when cells of ICM (epiblast) remain pluripotent or differentiate into primitive endoderm, *nanog* is expressed and becomes the crucial determinant of cell fate. Cells that express nanog remain pluripotent, while cells without *nanog* differentiate into primitive endoderm (Kaoru Mitsui, 2003).

1.4.5.3 *Sox2*

Sox2 is a member of the Sox (SRY-related High Mobility Group box) gene family that encodes transcription factors with a single HMG DNA-binding domain (Kamachi et al. 2000). Sox genes are expressed in various phases of embryonic development. Sox2 loss-of-function in the embryo and ES cells results in defective epiblasts and differentiation into trophectoderm lineages (Loh et al. 2008).

1.4.6 SRY gene

SRY gene code for a protein which contains a centrally located "high mobility group" domain (HMG box) (Whitfield et al., 1993). Further studies show there are no highly conserved sequence homologies between mammalian species for sequences outside the HMG box lacking of sequence conservation, despite conservation of HMG box sequences (Whitfield et al., 1993).

In male pig embryo, SRY gene is expressed between embryonic days 21 and 26 (E21-E26), obvious expression at E21 (when gentital ridge first appears) and E23 but faint expression on E26, and expression disappears on E31 when male testis determination is histologically evident (Daneau et al., 1996). The initial day of sppearence of genital ridge is E21, and at the period of E21 to E24, gonad still remains sexually undifferentiated. By E24 to E27, the tunica albuginea, a symbol of testis formation, can be identified.

Until now, the mouse is the only other species in which the Sry genital ridge transcript and its gene expression pattern have been described. In mouse, Sry expression can be detected from Days El0.5 to E11.5 in the undifferentiated genital ridge and expression almost disappeared at E12.5 (Adam Hacker et al., 1995).

1.4.7 Novel antibody CT-1

CT-1, also called β1, 4-N-Acetylgalactosaminyltransferase (β1, 4GalNAc-T, GM2/GD2 synthase; EC 2.4.1.94) is a key enzyme in the biosynthesis of complex gangliosides (Kogo Takamiya et al., 1998). The reason why it was considered as a key enzyme is that its direct products, GM2 and GD2, are essential for all the complex gangliosides synthesization.

Ganglioside is a molecule which is composed of a glycosphingolipid with one or more sialic acids, and the sialic acids are linked on the sugar chain. It can be found on the surface of oligosaccharide and provide cells with different surface markers which can play roles on cellular recognition and cell-to-cell communication. As to the location, they are abundantly concentrated in the brain of vertebrates and the composition is conserved among various species.

Nowadays, plenty of studies on gangliosides are conducted with kinds of methods such as addition to cells culture medium and injection into experiment animal. However, the biological functions of gangliosides are still not fully understood yet.

The experiment results from mutant mice which are CT-1 gene knockout, show no complex gangliosides synthesis, no obvious major abnormalities in the histological architecture of the brain or in gross behavior, but surprisingly, the male mutant mice became sterile and aspermatogenic and got serious defects in the testes. (Kogo Takamiya et al., 1998). It suggests CT-1 may have an important role on germ cell specification in mouse.

According to Kogo Takamiya et al., (1998), in the seminiferous tubules of the mutant mice, some multinuclear giant cells which are composed of immature round spermatids, vacuolated Sertoli cells which are abnormal too were obsearve and low testosterone level in the serum of mutant mice was tested. All the results suggest that complex gangliosides are essential in the transport of testosterone from Leydig cells to the seminiferous tubules. To convince this point, the ganglioside and testosterone mixture were injected into intratesticular and found they could cause partial restoration of spermatogenesis.

1.5 Aim of the project

The aim of the project is to investigate the expression of porcine PGC markers, trace PGC movement during porcine embryo development and gain understanding of the ontogeny of germ cells in pig. As to the investigation, it is at the foundation of information from mouse species model, by the means of comparison and assumption. Finally, it will improve our knowledge of human germ cell development. During the process of the project, two methods are mainly used, respectively immunohistochemistry and in situ hybridization.

Chapter 2: Materials and methods

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. It is performed by examining a thin slice (section) of tissue under a light microscope. Through the use of histological stains, plants or animals tissue section structure can be well visualized. Fixatives are used to maintain and prevent tissues from degradation, and to protect the structure of the cells with sub-cellular components such as cell organelles (e.g., nucleus, endoplasmic reticulum, mitochondria). Paraffin wax is most frequently used for light microscopy because it is immiscible with water, which can benefit for removing water in the process of dehydration with no damage to tissue. Samples are transferred through solutions of progressively more concentrated ethanol to get rid of the water, followed by a clearing agent, usually xylene, to remove the alcohol, and finally molten paraffin wax which replaces the xylene. For light microscopy, a glass knife mounted in a microtome is used to cut 5-10-micrometer-thick tissue sections which are mounted on a glass microscope slide. Hematoxylin and eosin (H&E) is the most commonly used light microscopical stain method in histology and histopathology. Hematoxylin stains nuclei blue while eosin stains the cytoplasm pink.

2.1 Embryo & Tissues Collection

The experiment embryos and foetus were provided by local abattoir from pregnant sows which gestational age was known. In this experiment, embryos at 28, 32 days after artificial insemination, foetus at age of 42, 51, 75 days and adult testis are needed. Due to these days are really late stages, the day 28 and 32 porcine embryos are harvested from uterus directly by eyes for the large enough size to differentiate and avoiding damages to the embryos, whereas the day 51 and 75 foetuses are unnecessary to harvest from uterus for its foetus structure but provided directly. After taking the embryos we need out of uterus, we fixed them immediately in 4% PFA (Paraformaldehyde) at 4 ℃ overnight and the next day washed them with PBS twice, dehydrated in 100% Methanol, finally stored at – 80^{o}C for use.

As for the pig genital ridge, foetal ovaries and foetal testis, they are obtained from the foetus directly. Besides, we get the adult testis and ovaries from the local abattoir

directly. The procedure after collecting is identical with the embryo and also stored at -80^{o}C for further use.

2.2 Embedding, paraffin blocks and collecting serial sections

After embryos and tissues collection, the next steps are embedding, tissue blocks and cutting serial paraffin section which are specimen preparation of immunohistochemistry. Firstly, the collected embryos were embedded in 3% (w/v) agarose gel, then the agarose blocks were processed in a histokinette (LEICA TP1020) at a programme set as:

70% EtOH	2hrs X 2
90% EtOH	2hrs X 2
100% EtOH	2hrs X 2
Toluene	2hrs X 2
Wax Paraffin	2hrs X 2

Thereafter, we need to take embryos out of the agarose blocks and put them in the blocks with paraffin because it can be heated to liquid state, and dissolved by xylene, and then quickly turned to a solid state again for maximum structural support when sectioning. Subsequently, cut the paraffin embryo into serials of sections at a nominal thickness of 5 or 6 μm using a rotary Microtome (LEICA RM2125), float them on cold distilled water and warm water about 36^{o}C and then smear them over the glass surface. Finally, put the slides on the hotplate in a sequence and dry the slides at 60^{o}C. As to tissues such as ovaries and testis, it is unnecessary to collect sections in a sequence.

2.3 Antibodies Details

As we know, immunological methods are quite valuable for the detection of specific proteins. If a protein or antigen is injected into an animal, a mouse for example, the mouse will generate an antibody in its serum. This antibody is a protein which reacts in a highly specific way with the antigen. The antigen-antibody complex is less soluble than the antigen and antibody. At this time, if something (second antibody) binds to the antibody, by whatever means, the cell which contain the antigen can be possible to detect. In this experiment, seven primary antibodies were used for

detecting protein expression of porcine PGCs. These antibodies' details were displayed in table 2.1.

Table 2.1 Details of primary antibodies used in this experiment.

Antigen	Brand / catalog / concentration	Dilution on sections (immunohistochemistry) V/V	Dilution on sections (immunofluoscence) V/V
Oct3/4	*OCT 3/4 (N-19) goat polyclonal anti human SC8628* (Santa cruz biotechnology) 200μg/ml	1:1000	
Dazl	*DAZL mouse monoclonal anti human mca2336* (AbDSerotec) 500μg/ml	1:1000	
Vasa	*VASA (DDX4/MVH) rabbit polyclonal anti human ab13840* (abcam) 500μg/ml	1:1000	
Stella	*STELLA rabbit polyclonal anti mouse ab19878* (abcam) 300μg/ml	1:1000	
Blimp1	*PRDM1/BLIMP1 goat polyclonal anti human ab13700* (abcam) 500μg/ml	1:1000	
Nanog	NANOG rabbit polyclonal anti mouse ab14959 (abcam) 500μg/ml	1:1000	
CT-1	β1,4GalNAc-T goat polyclonal anti mouse pure		1:50

2.4 Immunohistochemistry

Immunohistochemistry is a really useful technique for detection of specific proteins, with the principle combining both immunological and biochemistry. In this technique, an antibody is used to link a cellular antigen specifically to a stain which can be seen with a microscope. The principle of this technique is that the primary antibody is derectly labelled with fluorophore such as FITC, or unlabeled with detection by a labelled secondary antibody. If a second antibody is used, it must be generated against the immunogloblins of the first antibody source. For instance, if the first antibody is raised in rabbit, then the second antibody could be goat anti rabbit.

The applied biotinylated secondary antibody is for immunohistochemical staining to allow the colour reaction after developing with either DAB kit (DAB peroxidise substrate kit, Vector) or AEC kit (AEC red substrate kit, Invitrogen), whereas, the streptavidin (ABC Elite Kit, Vectastain) is for immnofluoscence colour reaction signal. For the sake of embryo structure distinguishment, H&E staining was used. The protocols of H&E staining, immunohistochamical staining and immunofluoscence will be shown as follows.

2.4.1 H&E stainng Protocol

Reagent preparation

70% IMS	90% IMS	1% acidified IMS (fumehood)
210 ml IMS	270% ml IMS	280 ml IMS
90 ml dH_20	30 ml dH_20	4 ml Hcl
		116 ml dH_20

Ammoniated water (fume hood)	1.5% Eosin stain
300 ml dH_20	3 g Eosin powder
3 ml ammonia solution	60 µl acetic acid
	300 ml dH_20

1. Xylene 3 x 5 min
2. 100% IMS x 5 min
3. 90% IMS x 5 min
4. 70% IMS x 5min
5. H_20 x 3 min
6. Harris Haematoxylin 2 min
7. Tap water until runs clear
8. 1 % acid IMS (2 dips)
9. Rinse in tap water

10. Ammoniated water (2 dips)
11. Rinse in tap water
12. Eosin 3 min
13. Tap water until runs clear
14. Mount in Hydromount, add coverslip and allow to airdry.

2.4.2 Immunohistochemical staining for paraffin sections Protocol

Day 1

1. Dewax & dehydrate

    - Xylene 3 x 5min
    - 100% EtOH 2 x 2min
    - 90% EtOH 2 x 2min
    - 70% EtOH 1 x 2 min
    - PBS 1x 10 min

2. Citrate antigen retrival step

    - Before adding slides, boil citrate buffer in plastic tray (5 min at 900W)
    - Immerse slides (fill all empty spaces in trough with blanks if necessary.)
    - Simmer on low for 10 min (check the sections after 6-7 mins, 1 min at 900W- 9 min at 450W)
    - Leave to cool for around 30 min
    - Wash in PBS 1x for 10 min

3. Peroxidase blocking step

    - Immerse in 3% H_2O_2 for 10 min
    - Wash in PBS 1x for 10min

4. Antigen blocking step

    - Add 3 drops of serum to 10 ml PBS (make up enough for 2° Ab)
    - Mark out section with liquid blocker pen
    - Block in serum for 2 hours
    - If possible blot excess serum from test slides but leave on control slide

5. Primary antibody

    - Dilute primary antibody 1:1000 with blocking serum on PBS
    - Add the primary antibody on the sections

- Incubate in humidified chamber overnight at +4°C

Day 2

6. Washing step

    - Wash in PBST : 1x for 10 min
    - Wash in PBS 1x for 5 min

    - Prepare AB complex
        - — Add 1 drop A to 5 ml PBS and vortex
        - — Add 1 drop B and vortex

7. Secondary antibody

    - Dilute secondary antibody (Biotinylated secondary antibody, Vectastain) 1:500 (V/V) in blocking serum (as prepared above)
    - Add the dilution to slides and incubate for 30 min
    - Wash in PBST for10min
    - Wash in PBS for 5 min

8. AB complex (This must be prepared 30 mins before use to allow avidin and biotin to bind together)

    - Add to slides and incubate for 30 min
    - Wash in PBS for 10 min

9. DAB substrate step

    - To 5 ml of distilled water add:
        - — 2 drops of buffer solution - vortex
        - — 4 drops of DAB substrate - vortex
        - — 2 drops of H2O2 – vortex
    - Add DAB and develop 2–3 min
    - Rinse in running tap water for 10 min

10. Counter stain & Dehydrate

    - MAYERS haematoxylin for 30 seconds
    - Rinse in running tap water for 5 min
    - Ammoniated IMS for 1 min
    - Rinse in running tap water for 5 min

11. Mount in Hydromount, add coverslip and allow to airdry.

12. Observe slides when dry by microscope and take photos for further use.

Preparation of reagents
PBS (10 litres 1x)
85g NaCl + 2.5 KH_2PO_4 + 35.8g $Na_2HPO_4.2H_2O$ pH 7.4

10mM Citrate buffer
Stock A citric acid buffer
4.2g citric acid + 200ml dH_2O
Stock B citrate buffer
14.7g sodium citrate + 500ml dH_2O
10mM citrate buffer
500ml containing 9ml stock A, 41ml stock B and 450ml dH_2O, pH 6

Peroxidase block
40ml H_2O_2 + 360ml d H_2O
To give 3% solution (Make up before use)

Blocking serum (Vectastain kit)
Add 3 drops normal serum to 10ml PBS

Primary antibody (goat polyclonal OCT-4)
1:1000 on 2% blocking serum

2.4.3 CT-1 Immunofluoscence Protocol

Day 1

1. De-wax and rehydrate sections

    - Xylene 2 x 10min
    - 100% EtOH 2 x 5min
    - 90% EtOH 2 x 5min
    - 70% EtOH 5 min

2. Wash the sections with PBS for 5 mins.
3. Boiled sections in a 0.01 molar sodium-citrate retrieval solution (PH=6) diluted in PBS using the microwave oven at 450w, 3 x 5mins. (add deionized water in between to replace the evaporated liquid).
4. Cooled down slides to room temperature for 30 mins and wash with PBS several times. For the last washing step use deionized water for 5 mins.
5. Tap blocking serum (goat serum) from the slide and dry around the section (do not touch the section).

6. Incubate the slides for 30 mins in blocking serum (goat serum). Add ~30ul per section.
7. Incubate the slides in a humidity chamber at 4℃ overnight, with the specific primary antibody CT-1 (1:50).

Day 2

8. Warmed up the sections for half an hour at room temperature and wash PBS for 5mins.
9. Incubate sections with the specific 2nd antibody for 30 mins. Use a black humidity chamber or cover it from night

Anti-mouse antiserum conjugated to Fitc (red), 1:200

Lectin, also called DBA 1:200

Add ~30ul per section

10. Wash the sections with PBS for 5 min, protect from light.

11. Add STREPTAUDINE (red)1:100 in PBS, incubate 15min
12. Wash in PBS 5 min, several times.
13. Mounting

Add 1-2μl Dappi to Vectashield and no press when mounting. Use nail oil to cover the border and put slides in envelope with cover away from light in fume hood around 30 min. Then observe and take photos. If the slides can't be observed immediately, they must be kept in freezer 20 below zero.

Sodium Citrate Buffer (10mM sodium citrate, 0.05% Tween 20, PH 6.0):

Tri-sodium citrate (dihydrate) 2.94g + Distilled water 1000ml

Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4℃ for longer storage.

Note: this buffer is commonly used and works perfectly with many antibodies. It gives very nice intense staining with very low background.

2.5 *In situ* hybridization

In situ hybridization, also referred to a hybridization histochemistry, takes advantage of the fact that DNA and RNA undergo hydrogen bonding to complimentary sequences of DNA or RNA. Suitable probes can be made to detect particular sequences of DNA or RNA by labelling sufficient length DNA or RNA sequence. The application of these probes on tissues allows DNA or RNA to be localized in tissue regions and cell types. This technique is a quite useful and powerful method for the ability to localize gene expression to specific cell types in specific regions and observe how changes in this distribution occur throughout development and correlate with behavioral manipulations (Sean M. Montgomery, 2002). During this project, this method was not used, but will be used for further study.

Chapter 3: Results

3.1 Antibody validation

In order to investigate whether the protein identified from mouse germ cells are also expressed in porcine germ cells, pig foetal ovary sections are utilized as positive control tissues. Pictures from pig foetal ovary sections with immunostaining from the different antibodies are shown below.

According to the experiment results, proteins of interest are all expressed in porcine primordial follicles. Through the comparison of images, it is obvious that Blimp1 and Oct-4 genes are nucleus expression, whereas, dazl and vasa are cytoplasmic expression. As to stella, it is expressed in both nucleus and cytoplasmic. All of these are consistent with gene profiles in mouse. In mouse, blimp1 and oct-4 are both identified in nucleus as transcriptional fators, dazl and vasa are cytoplasmic protein, and stella is speculated to shuttle between nucleus and cytoplasm (Saitou, 2002).

3.1.1 *OCT 3/4* goat polyclonal anti human

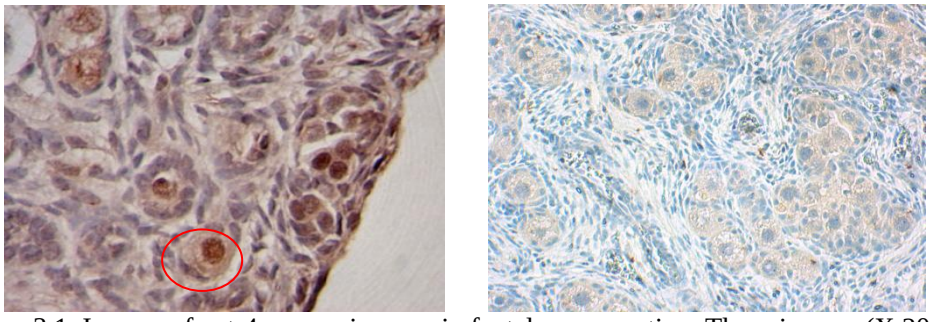


Figure3.1. Images of oct-4 expression on pig foetal ovary section. These images (X 20) were taken from optical Microscope (Olympus BH2). Red mark at left image shows Oct-4 expression in primordial follicle, right image is panel negative control.

3.1.2 *DAZL* goat polyclonal anti human

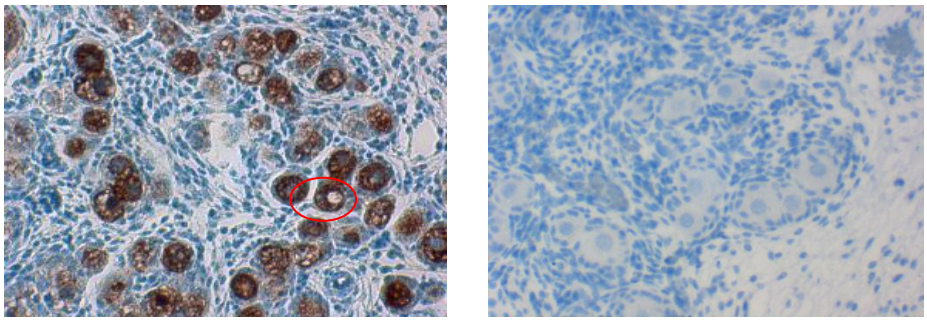


Figure3.2. Images of dazl expression on pig foetal ovary section. These images (X 20) were taken from optical Microscope (Olympus BH2). Red mark at left image shows dazl expression in primordial follicle, right image is panel negative control.

3.1.3 *VASA* rabbit polyclonal anti human

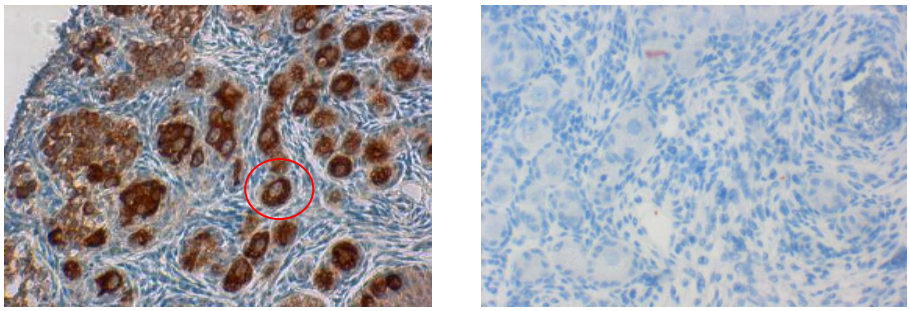


Figure3.3. Images of vasa expression on pig foetal ovary section. These images (X 20) were taken from optical Microscope (Olympus BH2). Red mark at left image shows vasa expression in primordial follicle, right image is panel negative control.

3.1.4 *STELLA* rabbit polyclonal anti mouse

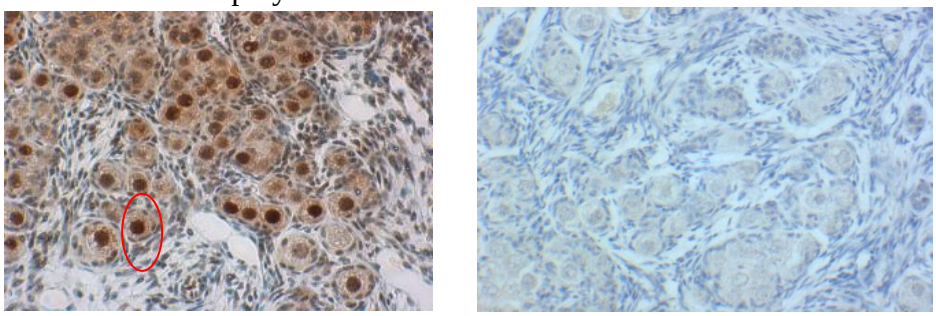


Figure3.4. Images of stella expression on pig foetal ovary section. These images (X 20) were taken from optical Microscope (Olympus BH2). Red mark at left image shows stella expression in primordial follicle, right image is panel negative control.

3.1.5 *BLIMP1* goat polyclonal anti human

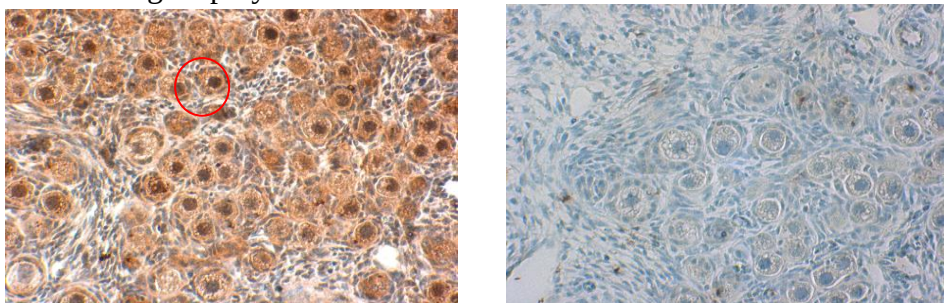


Figure3.5. Images of blimp1 expression on pig foetal ovary section. These images (X 20) were taken from optical Microscope (Olympus BH2). Red mark at left image shows blimp1 expression in primordial follicle, right image is panel negative control.

3.1.6 *Nanog* rabbit polyclonal anti mouse

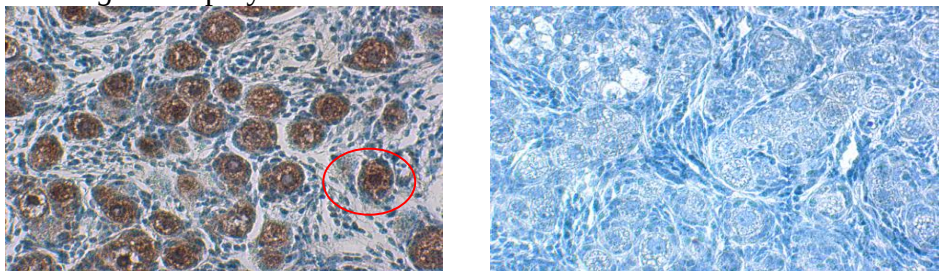


Figure3.6. Images of nanog expression on pig foetal ovary section. These images (X 20) were taken from optical Microscope (Olympus BH2). Red mark at left image shows nanog expression in primordial follicle, right image is panel negative control.

3.2 Stella was not expressed in pig embryo before E51 (including E51), but was detected in the fetal ovaries (E75-80).

According to the immunohistochemistry results that we have obtained, stella was not expressed in porcine gonad before E51 (including E51), but was detected in the feotal ovaries (E75-80) (see Figure 3.4.), that is, the first date of stella expression in pig embryo should be between E51 and E75. During this period, genital ridge is becoming mature gonad, ovary or testis, which suggests that stella gene may contribute to this process.

However, compared with the stella expression pattern in mouse mentioned above, it is expressed really later in pig organism. In mouse, the expression of *Stella* (PGC7) is activated at E7.25, and at E8.5, groups of migrating PGCs were stained exclusively in the developing hindgut, thereafter, it maintains expression in the germ line until about E15.5 in male and E13.5 in female. As to the relationship of embryonic date in mouse and pig, the table below display some important embryonic days amongst mouse, human and pig.

Table 2.2 Embryonic days in mouse, human and pig.

mouse	1	7	8	9	10	11	12	13	14	15	16
human	1	16	20	25	29	32	36	41	45	54	70
porcine	1	14	14-15	16	16-17		31	34		42	54

3.3 *Vasa* is expressed prior to *Dazl* gene in porcine which is opposite in mouse.

Through lots of trial, we discover that the first date of *dazl* expression in pig gonad is E31, however, *vasa* is detected at E28, although only several positive cells (PGCs) detected (see figure 3.7 &3.8). This phenomenon is really different from that in mouse. In mouse, *dazl* is expressed prior to *vasa* as a regulation factor (Reynolds et. al, 2005).

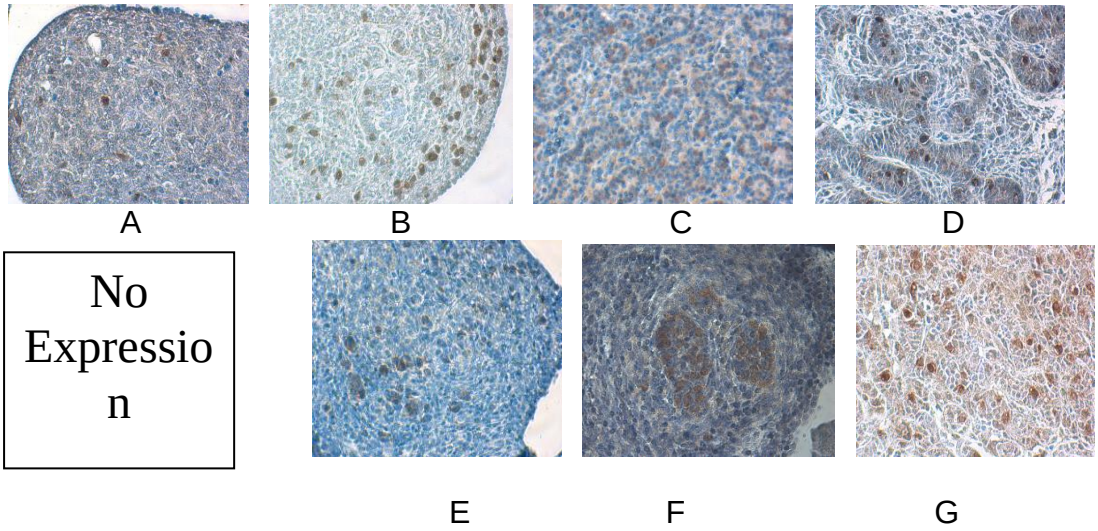


Figure 3.7 Images of vasa and dazl expression in pig embryo by means of immunohistochemistry. Images (X20) were taken with microscope (Olympus BH2) and top row is vasa expression while bottom row is dazl. A, vasa at E28; B, vasa at E31; C, vasa at E42; D vasa at genital ridge, E51. E,F,G are dazl expression images of respectively E31, E42, E51.

3.4 Oct-4 may be expressed in pig adult testis.

After various times detecting of oct-4expression in pig feotal testis and asult testis, no positive cells were detected. However, when detecting CT-1 expression with method immnofluorence using oct4 as positive control, what unexpected is that oct4 was detected in the cord of adult testis, but quantity of the positive cells was really little. According to the location of the positive cells, it was concluded that they were type A spermatogonia.

This seems similar to what happens in mouse adult testis. In mouse adult testis, Oct4 was expressed by none of the different meiotic germ cell types and appeared to be confined to the type A spermatogonia population. Experiments suggested that in embryonic stem and embryonic carcinoma cells, Oct-4 downregulation could be controlled by a two-step mechanism. The first step appears to involve transrepression due to recruitment of nuclear receptors to the promoter containing three direct repeats to which such nuclear factors could bind and the second step could involve modification of the chromatin structure within the Oct-4 upstream region and loss of occupancy of promoter/enhancer binding sites (Pesce et.al, 1997).

3.5. Dazl, Stella, Nanog, Blimp1 are expressed in adult testis.

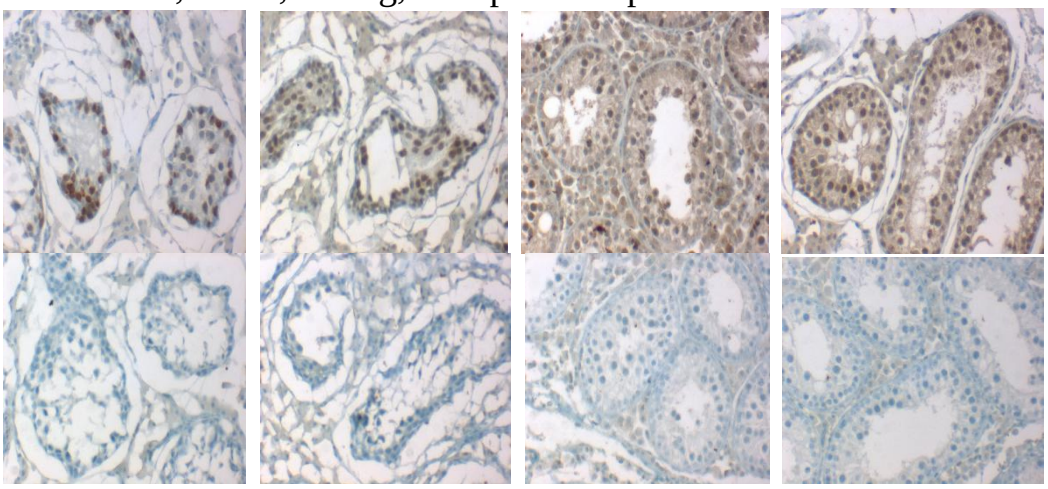


Figure 3.8. Images of dazl, stella, nanog blimp1 expression in pig adult testis. A, Dazl expression in pig adult testis, and negative control (image below)B, stella expression in pig adult testis and negative control C, nanog expression in pig adult testis and negative control, D, Blimp1 expression in pig adult testis and negative control.

3.6. CT-1 expression profile at various embryonic days

3.6.1 E15

As Figure 3.10 shows, several CT-1 positive cells, which are proved as PGCs, are detected at the PGC migrating path by immunofluorescence confocal microscopy, referring to DBA expression which is red fluorescence. Until now, only two makers, oct-4 and ssea-1 can be used for characterizing the porcine PGCs during the migratory stage (day 14-17), this result shows CT-1 may become another novel PGC marker for early embryonic stage.

3.6.2 E22

As the figure below shows, the positive cells (PGCs) for both CT-1 and DBA appeared to be at the gut (Figure3.11 A), and at the area from mesentery to gut (Figure3.11 B) and mesonephros (Figure3.11 C). Compared images of group A, it was shown that CT-1 and DBA are both expressed in the gut. Group B shows CT-1 expression on the way from mesentery to gut but no DBA detected at this location. Mesonephros also has CT-1 expression with no DBA immunostaining.

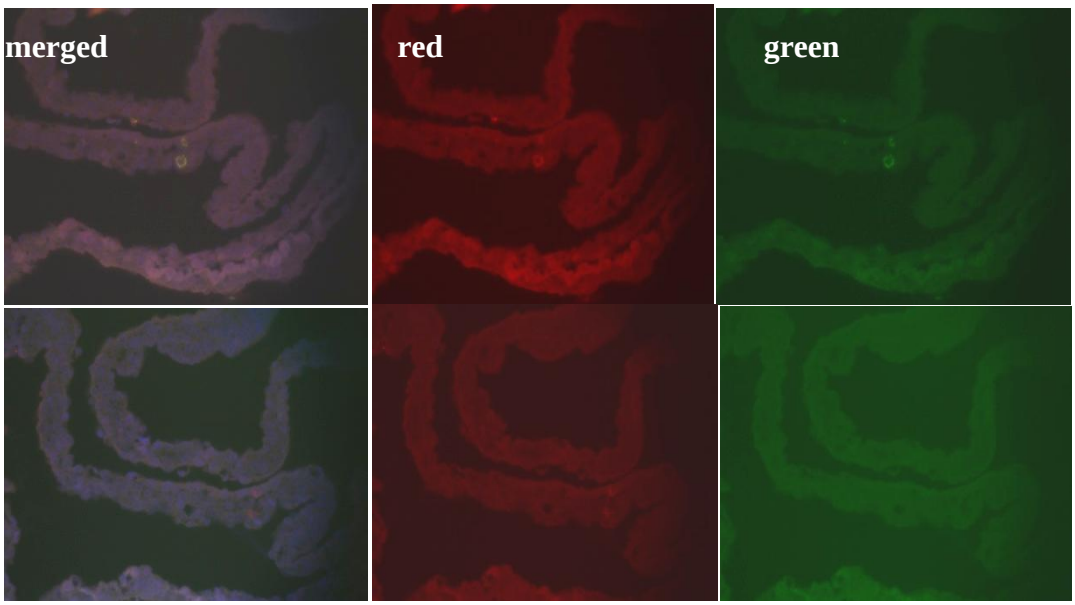


Figure 3.9. Localization of CT-1 by immunofluorescence confocal microscopy at E15. Left column images (X 40) are merged, middle red and right green. The cells detected with green fluoscence are CT-1 and the ones with red fluoscence are lectin (DBA). The images in second row are negative control.

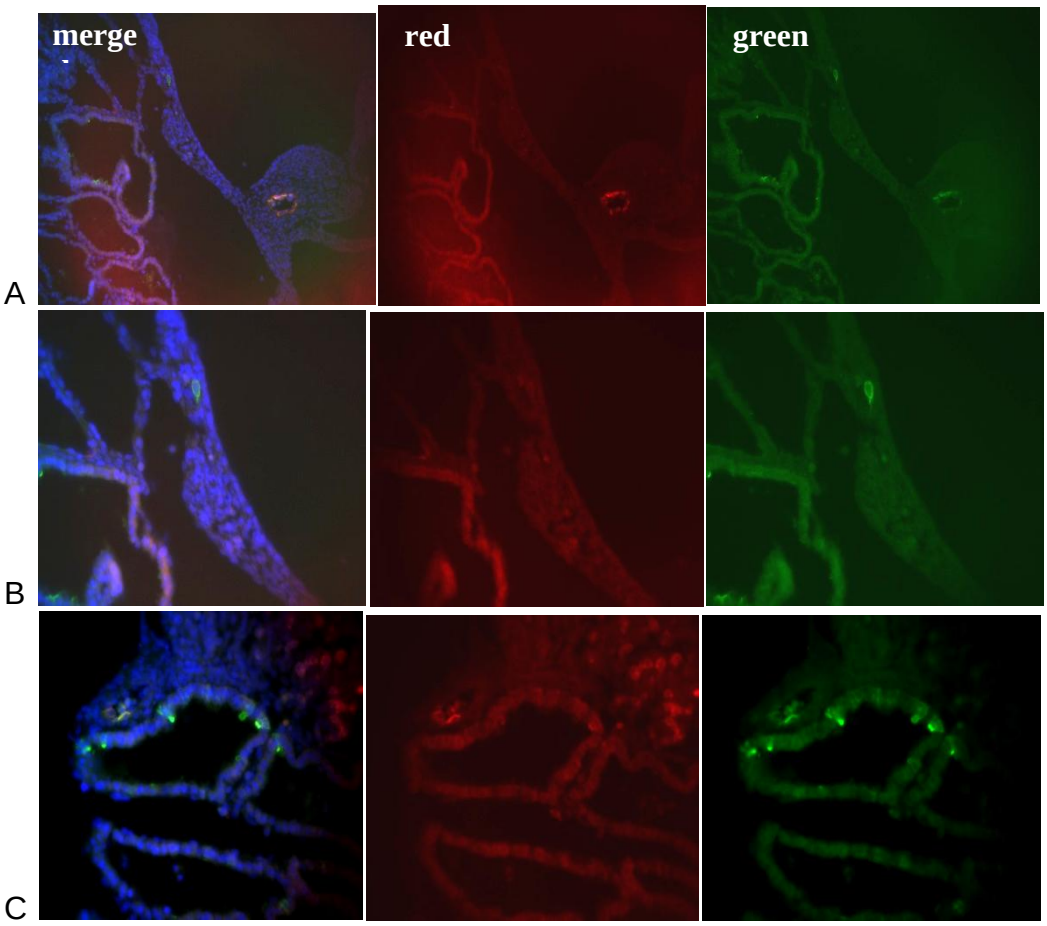


Figure 3.10. Localization of CT-1 by immunofluorescence confocal microscopy at E22. A, gut (20X) B, mesentery to gut (40X) C, mesonephreos (40X)

3.6.3 E28

Figure 3.12 tells us CT-1 and DBA are both highly expressed in the genital ridge (Figure 3.12 A), mesentery (Figure 3.12), and mesonephros (Figure 3.12 C) at E28. The PGCs in genital ridge are round and relatively big. Images of group B proved that CT-! and DBA are also expressed in migrating PGCs and immunofluorescence staining in mesonephros can be used as positive control for the detection of CT-1 expression.

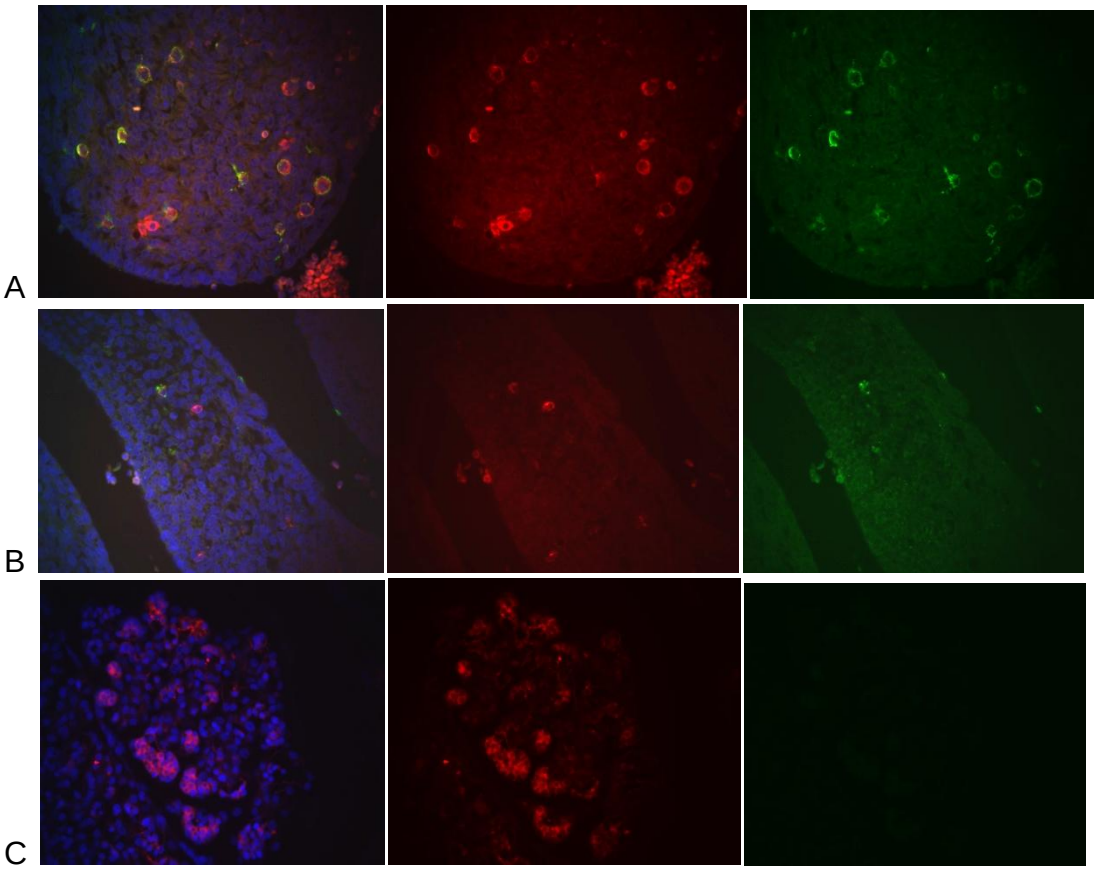


Figure 3.11. Localization of CT-1 by immunofluorescence confocal microscopy at E28. Images (x 40) in group A are pig genital ridge, group B is mesentery and C is mesoephros.

3.6.4 E32

The figure below shows that CT-1 and DBA are localized and concentrated at the embryonic gonad at E32 with the green and red immunofluorescence respectively. At this stage, PGCs are most concentrated at the border part of gonads.

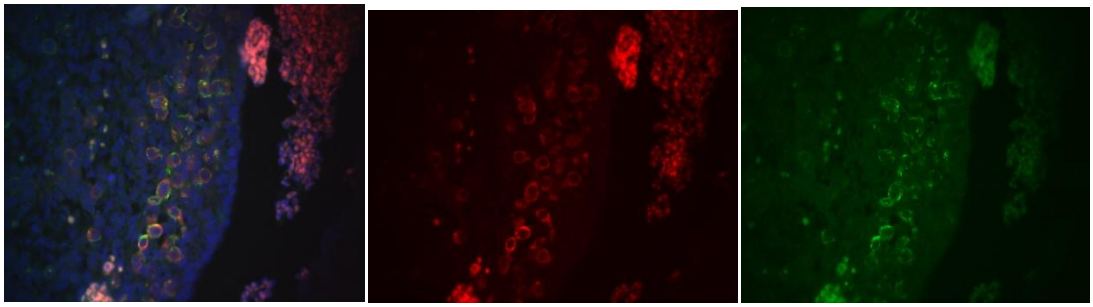


Figure 3.12. Localization of CT-1 by immunofluorescence confocal microscopy at E32. Images are the location of gonads.

3.6.5 E42

Although the background was a little big, it is not tough to say CT-1 was still highly expressed in the embryonic gonad and distributed widely. However, DBA seems no expression according to the middle image of the figure below.

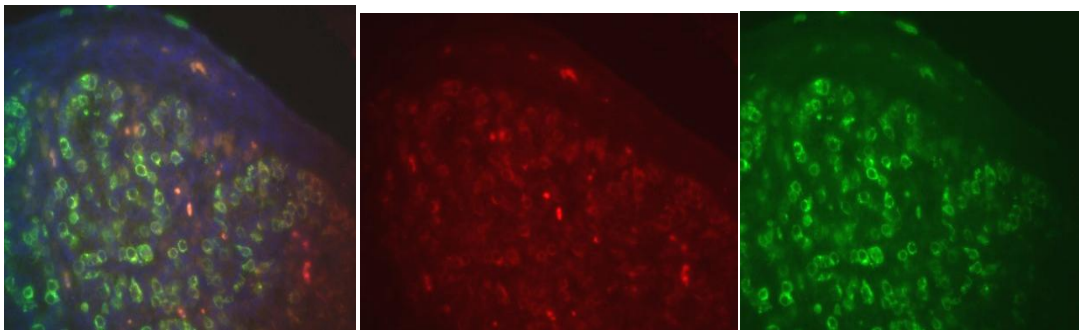


Figure 3.13. Localization of CT-1 by immunofluorescence confocal microscopy at E42. Images (x 20) are the location of gonads.

3.6.6 E51

These paraffin sections are genital ridge sections, which are different from the ones above. Due to the length of the embryo body where we collected these genital ridges, it is concluded the age is day 51. From the images below, it is obvious that CT-1 and DBA were expressed highly and distributed widely in the genital ridge.

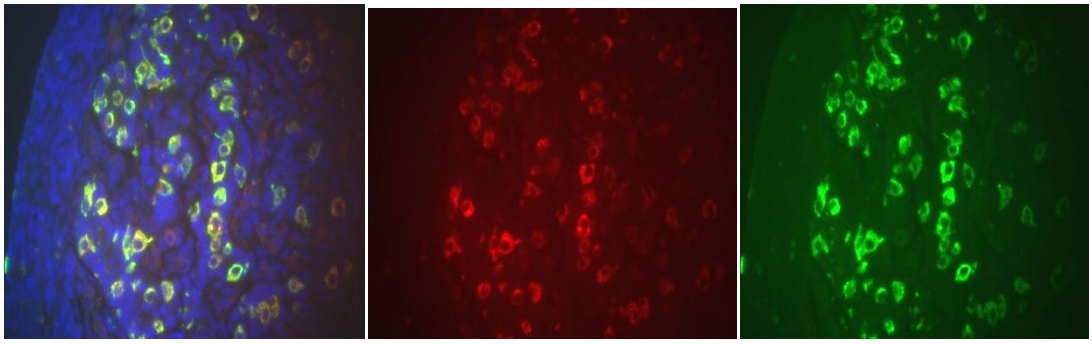


Figure 3.14. Localization of CT-1 by immunofluorescence confocal microscopy at genital ridge.

3.6.7 Fetal ovary

With the same method used for evaluating the embryonic day of genital ridge, we obtain that these fetal ovaries' age are around day 75 to day 80. As what the images below show, primary follicles are beautiful stained with CT-1 and DBA expression.

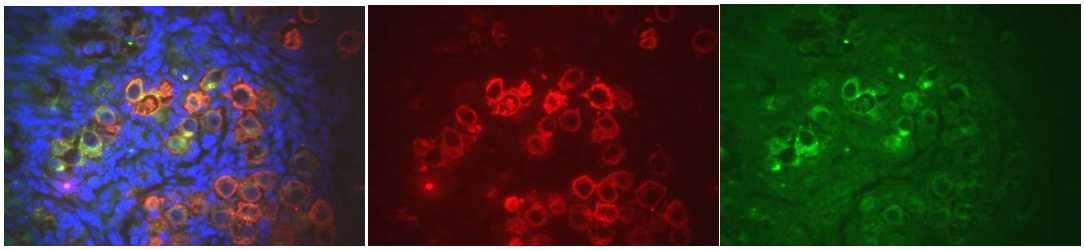


Figure 3.15. Localization of CT-1 by immunofluorescence confocal microscopy at feotal ovaries.

3.6.8 Fetal testis

As the method used for calculate age of genital ridge and feotal ovaries, the age this feotal testis is around embryonic day 51. From the figure below, we can see CT-1 and DBA are highly expressed at inside border of the testis cord.

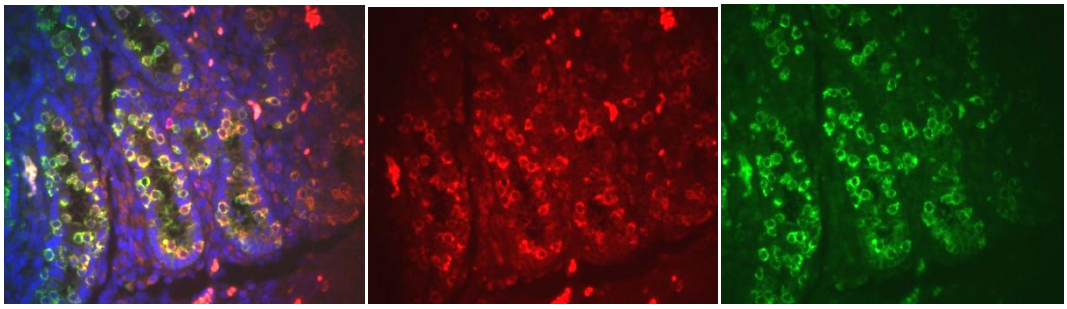


Figure 3.16. Localization of CT-1 by immunofluorescence confocal microscopy at feotal testis (x 40)

3.6.9 Adult testis

As what the figure shows, very few CT-1 positive germ cells are detected in adult testis. According to the location of this germ cell, it is type A spermatogonia, a type of germ cells.

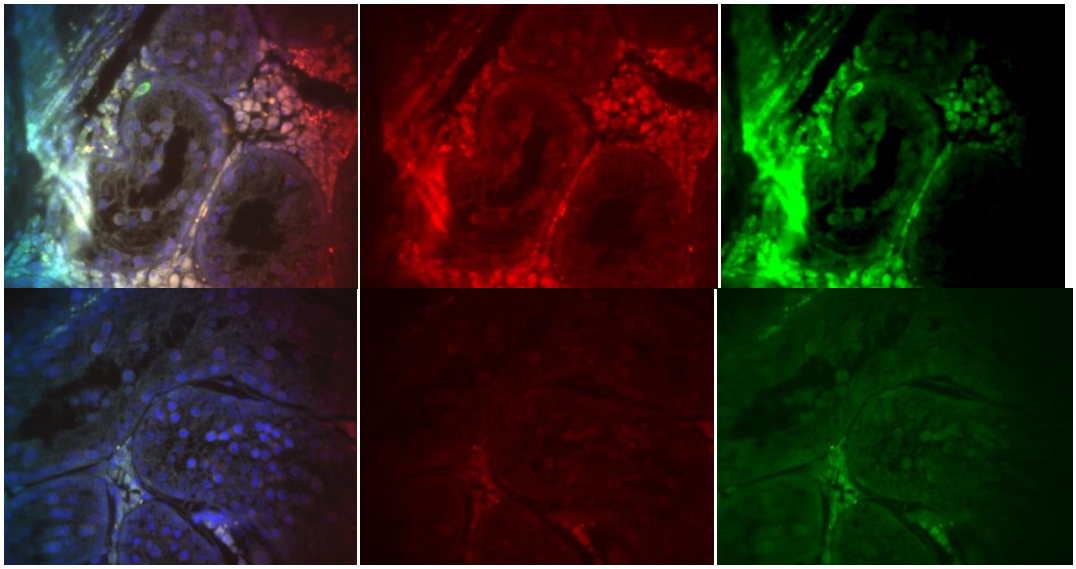


Figure 3.17. Localization of CT-1 by immunofluorescence confocal microscopy at adult testis. Bottom images are negative controls.(X 40)

3.6.10 Adult ovary

No positive cells for CT-1 or DBA were detected in adult ovaries.

Chapter 4: Discussion

4.1 Analysis of germ cell specific gene expression in pig

In this experiment, I have provided some immunological evidence (see Chapter 3) that genes which are known to be involved in mouse germ cell development can also detected in porcine germ cell development. However, the expression pattern is not as the same as the one in mouse. We verify the expression of pluripotency marker Oct4, but similar expression pattern for the germ cell specific markers were not found. At the foundation of the findings on a protein level in the different stages from the developing embryos, Blimp1, stella, as well as dazl, do not seem to be expressed on an equivalent period of time to the mouse during development. This raises the possibility that the mechanism by which the germ cells are specified could be different.

From the immunohistochemistry results that we have obtained, no expression of *stella* in porcine gonad was found before E51 (including E51), but was detected in the feotal ovaries (E75-80), which is quite different gene expression pattern from in mouse. As to the initial date of stella expression, it requires subsequential experiments with the embryo at embryonic days between 52 and 75. Compared with the stella expression pattern in mouse mentioned in Chapter 1, it is expressed really later in pig organism. In mouse, stella is essential for early development, the expression of *Stella* (PGC7) is activated at E7.25 which is equivalent to porcine embryonic days 14, and at E8.5, groups of migrating PGCs were stained exclusively in the developing hindgut, thereafter, it maintains expression in the germ line until about E15.5 in male and E13.5 in female. This suggests that the mechanism of germ cell specification in pig could be different from in mouse.

Also, *vasa* expression in the pig primary follicles implies that it is involved in the germ cell development process in pig embryo. The expression of *vasa* can be detected at E28, E32, E42 and genital ridge stage E51, which indicates that *vasa* always has strong expression in PGCs in the gonad of pig embryo. From table below, it shows the initial date of *dazl* expression detected in pig embryo gonad in on E32 and no expression on E28, which suggests *dazl* is expressed later than vasa in the gonad of

pig embryo. This phenomenon is really different from that in mouse. In mouse, dazl is expressed prior to vasa as a regulation factor. DAZ genes encode a family of conserved RNA-binding proteins which are required for the translational regulation of gene expression and Dazl stimulates translation via the Mvh 3'-UTR. Germ cells of dazl null mice have decreased levels of Mvh (mouse vasa homologue) protein, indicating that dazl regulate Mvh translation in mouse (Reynolds et. al, 2005).

As to Oct4, immunohistochemistry results show its expression in migratory and gonadal PGCs in porcine embryo, which is a strong indication that potential PGCs migrate through mesodermal hindgut, towards the anterior side of embryo. From table 4.1, oct-4 expression is detected in PGCs in pig embryo gonads in from E28 to E51. Apart from these days, it is also highly expressed in feotal ovaries, but only very few positive cells which are considered type A spermatogonia detected in pig adult testis, which is similar to the expression pattern in the mouse adult testis. When detecting new antibody CT-1 using oct4 as positive control, unexpectedly, we discovered there were few oct4 positive cells inside the cord at the border, which are named type A spermatogonia. In mouse adult testis, Oct4 was expressed by none of the different meiotic germ cell types and appeared to be confined to the type A spermatogonia population (Pesce et.al, 1997). Experiments suggested that in embryonic stem and embryonic carcinoma cells, Oct4 downregulation could be controlled by a two-step mechanism. The first step appears to involve transrepression due to recruitment of nuclear receptors to the promoter containing three direct repeats to which such nuclear factors could bind and the second step could involve modification of the chromatin structure within the Oct4 upstream region and loss of occupancy of promoter/enhancer binding sites (Pesce et.al, 1997).

Table 4.1 Summary of gene expression in pig embryo.

	E28	E32	E42	E51
Oct-4	+	+	+	+
Stella	No expression	No expression	No expression	No expression
Blimp1		+		?
Vasa	+	+	+	+
Dazl		+	+	+
Nanog	+	+		+

Note: “+”represents there is expression of that gene, “?” stands for gene expression is unclear.

To complement this work based on an in vivo model the next step would be to take it to an in vitro level. The use of cell lines derived from pig epiblast will help to understand the signalling pathway during early development, together with the possibility to induce germ cells in vitro and confirm the expression pattern throughout this process.

4.2 Mechanism of Sex Determination and Differentiation

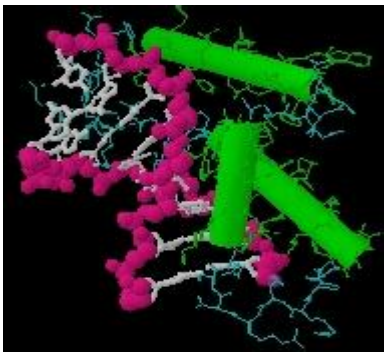
Currently, our understanding of the mechanisms of mamalian sex determination was rather limited. As we all know, karyotype is different between males and females, males are heterogametic (XY) while females are homogametic (XX). Besides, the phenotype is different too, males with testicles and femails with ovaries. However, what decides this phenomenon? In another word, it can be answered with the mechanisms of sex differentiation.

For explaining this question, a special genetic locus present on the Y chromosome was termed “testis – determining factor” (TDF). As a key gene for TDF, sex – determining region Y gene, which is called SRY in humans and Sry in mouse, is a master gene for testes development ans sex differentiation.

Comparing SRY structures of various species, it can be found that porcine SRY is similar to humans’ and bovines’, but different from mouse’s for lacking of the carboxy – terminal activation domain and obtaining amino –terminal domain. What’s more, the porcine SRY genital ridge transcript has a relatively shorter 3' untranslated region (UTR) than in mouse. As mentioned in Chapter 1, SRY gene code for a protein which contains a centrally located "high mobility group" domain (HMG box) with highly conservation among species (Whitfield et al., 1993).

Further studies show that at SRY gene, there are no highly conserved sequence homologies between mammalian species for sequences outside the HMG box lacking of sequence conservation, despite the conservation of HMG box sequences (Whitfield et al., 1993). In male pig embryo, SRY gene is expressed between embryonic days 21 and 26 (E21- E26), and expression disappears on E31 when male testis determination is histologically evident (Daneau et al., 1996). However, mouse as reference, Sry

expression can be detected from Days El0.5 to E11.5 in the undifferentiated genital ridge and expression almost disappeared at E12.5 (Adam Hacker et al., 1995). human genital ridge SRY transcript is expected to be present around the sixth week of gestation, just before histological testicular Referring to table 2.2, E10.5 in mouse may be correspond to about E20 in pig, so it is suggested that porcine genital ridge SRY expression seems relative developmentally and anatomically with Sry expression in the mouse gonadal ridge and is consistent with the fact that for SRY to be TDF (Daneau et al., 1996).



As picture shows at left, SRY binds to other DNA and distort it shape dramatically. So it is possible to alter the properties of the DNA and regulate the expression of a number of genes.

Figure 4.1 SRY (green) binds toDNA (pink) and disorts its shape. (from NCBI)

4.3 Role of CT-1 (β1, 4-N-Acetylgalactosaminyltransferase)

CT-1, also called β1, 4-N-Acetylgalactosaminyltransferase, is a master enzyme in the biosynthesis of complex gangliosides (Kogo Takamiya et al., 1998) for its direct products, GM2 and GD2, which are essential for all the complex gangliosides synthesization. So, mouse for example, if the gene was knockout, there must be lack of complex gangliosides, which are abundantly concentrated in the brain of vertebrates and relative to neural system. However, to our surprise, experiment results show no obvious major abnormalities in the histological architecture of the brain or in gross behavior, but surprisingly, the male mutant mice became sterile and aspermatogenic and got serious defects in the testes (Kogo Takamiya et al., 1998). This suggests CT-1 can directly affect the systhesis of complex gangliosides and indirectly make influence on spermatogenesis in mouse. In the seminiferous tubules of the mutant mice, some multinuclear giant cells and vacuolated Sertoli cells which are abnormal were obsearved and low testosterone level in the serum of mutant mice was tested. Furthermore, testosterone accumulated to a much greater extent in interstitial Leydig cells of the mutant mice than those of wild type. All the results

suggest that complex gangliosides are essential in the transport of testosterone from Leydig cells to the seminiferous tubules (Kogo Takamiya et al., 1998).

At present, paper describing gene CT-1 is quite few and the gene expression profile can be found only in mouse species. For detecting its expression in pig germ cell development, I adopt a novel technique, immunofluoerscence to visualize the location and stages of gene expression, with lectin, a protein expressed in germ cells, for positive control. To our surprise, there seems to be CT-1 expression at pig embryonic days 15, which is really an early stage and belongs to migratory stage (E14 – E17), but further evidence is needed. If there was CT-1 positive cell at E15, it suggests that CT-1 could be used as a primordial germ cell marker for pig early stage germ cell development, apart from oct-4 and ssea-1. Besides, it is also expressed at E22, E28, E32, E42 and E51 in genital ridge, which suggests that it is widely expressed germ cell marker. Through using oct4 (nuleus protein) as positive control, we can confirm that those positive cells are germ cells, which are what we are interested. Foetal ovries, foetal testis and adult testis also have CT-1 positive cells, which are primordial follicles in foetal ovaries, various spermatogonia germ cells in foetal testis and type A spermatogonia in adult testis. As to adult ovaries, there is no fluorescence signal of both CT-1 and lectin (DBA) under the microscope, suggesting CT-1 cannot be expressed in secondary follicles as well as DBA.

4.4 Further study of *stella*

In mouse, germ cell development requires both genetic and epigenetic regulation. However, until today the mechanism of epigenetic reprogramming is not described clearly. Among the germ cell markers studied in my project, stella is the only one which take the epigenetic event, so it will become the objective discussed in the following paragraph.

At molecular level, epigenetic reprogramming represents a range of DNA and chromatin modifications including DNA methylation, histone modifications, remodelling of nucleosomes and higher order chromatin reorganization. The epigenetic profile of germ cells changes dynamically during their development

(Sasaki & Matsui 2008). Seki et al. point out that about E8.0, genome-wide DNA demethylation is occurred with reduction of H3K9 dimethylation and DNA methylation , and around half days later, H3K27 was upregulated, which suggests germ cells are proliferated during the migratory stage.

In mouse, with the number of germ cells increasing, germ cells start to migrate into the developing gonads (see Figure 1.7), and reach around E10.5. Soon afterwards, PGCs suffer a wave of genome-wide DNA demethylation, which is accompanied also by the profound changes in chromatin configuration and erasure of some histone modification marks (Surani et al. 2007). Evidence suggests that the entry of PGCs into the genital ridge may be important for the onset of the reprogramming process (Tam et al. 1994).

PGC7/Stella, a maternal factor essential for early development, protects the DNA methylation state of several imprinted loci and epigenetic asymmetry. Many recent investigations on studying stella gene function show that after fertilization and before 2-cell stage, stella is expressed in preimplantation embryos and functions with the germ cell epigenetic reprogramming in protection the DNA methylation state and genomic imprinting (Nakamura et al., 2007).

4.4 Conclusion

In conclusion, my experimental results show that some gene involved in mouse germ cell development are also expressed during pig germ cell development, although some gene expression patterns are different between these two species. As the first PGC marker in mouse embryo, stella has no expression before E51 including E51 in the pig genital ridge, but was detected in foetal ovaries (E75-80). Oct-4 is not expressed in pig foetal testis and adult testis, whereas, in mouse it has expression in prospermatogonia at least and is expressed in type A spermatogonia of mouse adult testis. Expression of vasa was prior to dazl which is really different from in mouse where dazl is a regulation factor of vasa. Besides, new primary antibody CT-1 was detected in pig genital ridge at various embryonic days and in adult gonads, especially, it is expressed in E15, which is a really early stage, suggesting that it may be a novel PGC marker for pig early germ cell development. It is widely expressed during pig embryonic period, suggesting it is a good germ cell marker during pig germ cell development.

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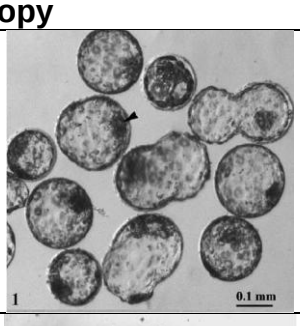
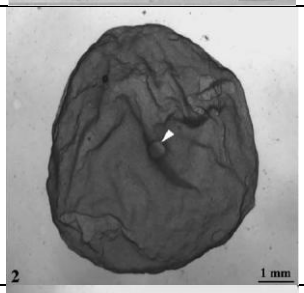
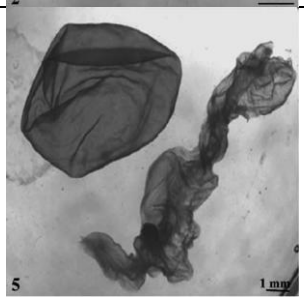
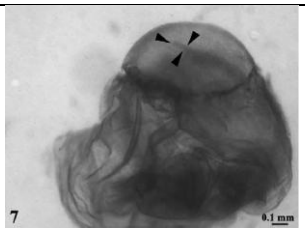
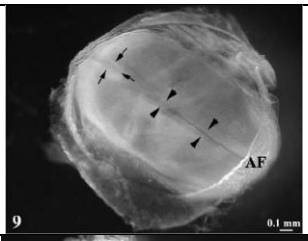
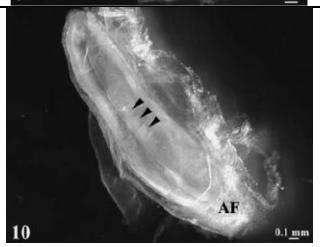
APPENDIX A – STAGES OF PIG EMBRYO

Stage of Pig embryos based on stereomicroscopy	
Expanded hatched blastocyst stage: The spherical conceptus has escaped from the zona pellucida and presents a compact inner cell mass (ICM) at one pole inside a complete trophoblast lining.	
Pre-streak stage 1: The conceptus is still spherical and presents a fully exposed epiblast delineating the embryonic disk at one pole of the blastocyst.	
Pre-streak stage 2: The conceptus presents signs of elongation as it changes from a spherical into an ovoid form. A similar change occurs in the embryonic disk to be transformed from a circular into an oval structure. At one pole of the embryonic disk, a prominent crescent-shaped thickening is visible within the posterior third.	
Primitive streak stage: From the posterior and towards the anterior pole of the embryonic disk, the crescent-shaped thickening appears to have gathered in the midline forming the primitive streak with an anterior node. Amniotic folds appear at the borders of the embryo proper.	
Neural groove stage: A distinct neural groove is observable extending from the anterior towards the posterior pole of the embryo proper, where the primitive streak and node is still visible. The embryo proper is partly enclosed by the amniotic folds.	
Somite stage: In the midline of the embryo proper, mesoderm has thickened to form somites. The embryo proper is partly enclosed by the amniotic folds. At the time when 8–10 pairs of somites have formed, outlines of various organs appear.	

(images and description from from Vejlsted et al. (2006))