

GENE REGULATORY NETWORK AND EPIGENETIC REPROGRAMMING OF PIG PRIMORDIAL GERM CELLS

by

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**Thesis submitted to the University of Nottingham for the
degree of Doctor of Philosophy**

May 2016



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DECLARATION

I hereby declare that this thesis is my own work and that it has not been submitted anywhere for any other degree or award. The work presented herein is my own work and where other sources of information have been used, they have been duly acknowledged.

Haixin Zhang

Abstract

Primordial germ cells (PGC) are the precursors of the gametes. The mechanisms of PGC induction, specification and development are very well characterized in rodents, however recent investigations have demonstrated that the mechanisms of germ cell development differ significantly between mice and humans. Since the knowledge of PGC development in non-rodents is very limited, and early human embryos cannot be accessed it is important to establish a new model for PGC development with relevance to humans. In this thesis, I use pig embryo as a model for investigating PGC development in non-rodent mammals. The expression profile of key transcription factors, epigenetic reprogramming and the role of signalling pathways were investigated during specification and development of pig PGCs. The key findings are: A- Specification of porcine PGC occurs after the onset of gastrulation, requiring BMP4 signalling. B- WNT signalling is required for the generation of precursors competent for germline commitment; however it is downregulated after PGCs are specified. WNT downregulation could be modulated by SOX17, the earliest gene expressed in pig PGCs. C- Epigenetic reprogramming of DNA and histone marks starts in pre-migratory porcine PGC. Furthermore, chromatin dynamics in pig gonadal PGCs resemble that of humans but differs to that of mice. D- The expression profile of transcription factors of porcine PGC is similar to that of humans, but different to mouse PGC.

In conclusion, this study has highlighted critical differences between mice and humans/pigs during germ cell specification. I provide evidence that the pig embryo is a useful model for the study of human development, and future studies will need to be directed to re-evaluate concepts of cell differentiation and early lineage commitment established in mice that may not apply to humans.

Acknowledgement

Firstly, I would like to express my sincere appreciation to my supervisor Dr. Ramiro Alberio for the continuous support of my Ph.D. study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor for my Ph.D. experience.

Besides my advisor, I would like to thank the rest of my supervisors: Dr. Tim Parr and Dr. John Brameld, internal assessor Dr. Simon Welham, external assessor Ian Adams, as well as Prof. Andrew Johnson, Dr. Cinzia Allegrucci, and Dr. Dylan Sweetman, for their insightful comments and encouragement, as well for the questions which inspire me to widen my vision from various perspectives.

My sincere thanks also go to our technicians Klisch Doris, Lydia Kwong, Pat Fisher, Ralph Hourd, Morag Hunter, Leigh Silvester, my fellow lab mates and friends Aida Rodriguez, Adam Hirst, Choulia Mola, Yandy Dai, Rabeea Mohammed, Bayar Zeebaree, Judith Gómez-Martínez, Katherine May, Sarah Withey as well as Miss Katherine Lawson and Sheila Northover in for the stimulating discussions, for the encouragement, for the valuable help, and for all the fun we have had in the last four years. Without their precious support and accompany it would not be possible to complete this project.

Special thanks to my parents who always unconditionally understand, support and encourage me in my life.

Last but not the least, I would like to give my sincere thanks to Chinese Service Center for Scholarly Exchange (CSCSE), Vice Chancellor Scholarship, and Pfizer for their support.

Abbreviation

5fcaC	5-carboxylcytosine
5fC	5-formylcytosine
IAP	Intracisternal A particles
DMR	differentially methylated region
CIS	carcinoma <i>in situ</i>
5-hmC	5-Cytosine hydroxymethylation
5-mC	5-methylcytosine
AVE	anterior visceral endoderm
BLIMP1	B-lymphocyte-induced maturation protein-1
BMP	Bone morphogenetic proteins
DE	Definitive endoderm
dpc	Days post coitum
DVE	distal visceral endoderm
EC	Embryonic carcinoma
EG cell	embryonic germ cell
EMT	epithelial-mesenchymal transition
EPS	Early primitive streak
ESC	Embryonic stem cell
ExE	Extraembryonic ectoderm
ExM	Extraembryonic ectoderm
FGF	Fibroblast growth factor
GLP	Glucagon-like peptide
H3K27me3	histone3 lysine27 trimethylation
H3K9me2	histone3 lysine9 dimethylation
HMG	High mobility group
ICM	Inner cell mass
LIF	Leukemia inhibitory factor
LPS	Late primitive streak
M	mesoderm
MAPK	Mitogen-activated protein kinases
NG	Neural groove
PE	Primitive ectoderm
PGC	primordial germ cell
PGCLC	primordial germ cell like cell
PI3K	phosphoinositide 3-kinase
PS	Primitive streak
Sox	SRY-related HMG box
TE	trophectoderm
TET	Ten-eleven translocation protein
TET-BER	TET mediated base excision repair
TNAP (Akp2)	Tissue non-specific alkaline phosphatase
BER	base excision repair
DNMT	DNA methyltransferase
Xi	inactivated X chromosome

HMT	H3K9 methyltransferase
maGSC	Spermatogonial germ stem cells
iPSC	Induced pluripotent stem cells
LIF	leukaemia inhibitory factor
MEK	mitogen activated protein kinase
GSK3	glycogen synthase kinase3
EpiSC	epiblast stem cells
XIST	X-inactive specific transcript
GCT	Germ cell tumours
IF	immunofluorescence
DAZL	deleted in azospermia-like
EPS	Early primitive streak
NP	Neural plate
NE	Neural ectoderm

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Chapter 1

1. Introduction

Primordial germ cells (PGC) are the precursors of the mature germ line, and are responsible for the transmission of genetic information across generations. Because of their fundamental role in animal development and in ensuring the continuity of species, the mechanisms leading to the formation of this cell type has attracted significant interest among biologists. Two major modes of germ cell specification have been described in metazoans: preformation and epigenesis. Preformation defines the presence of germ cell determinants in the cytosol of oocytes. This mode of germ cell development has been well characterized in *Caenorhabditis elegans*, *Drosophila* (Nakamura and Seydoux 2008), Zebrafish (Raz 2003), and *Xenopus* (Houston and King 2000). However, this deterministic mode of PGC specification is not conserved in vertebrates. Epigenesis, which refers to the inductive mode of germ cell specification, is based on the concept that pluripotent precursor cells are induced gradually as the embryo develops into the different lineages that constitute the make-up of an embryo. The germline is one of the cell types induced under such conditions. Epigenesis, also known as the stem cell model of germ cell development, is conserved in vertebrates and has been best studied in mice, although seminal studies in amphibians (axolotls) and fish (sturgeon) have provided a phylogenetic understanding of the conserved mechanisms of germ cell specification (Johnson, Crother et al. 2003). Furthermore, investigations of the molecular mechanisms of germ cell determination have led to the proposition that liberation from developmental constraints (i.e. germlasm) in the germline can offer evolutionary advantages and results in an accelerated rate of evolution (Johnson and Alberio 2015) (Evans, Wade et al. 2014).

Although epigenesis is the mode of germ cell specification in mammals, our fundamental understanding of PGC development stems from studies in mice. Mouse PGCs are induced from pluripotent epiblast cells and become lineage restricted before the onset of gastrulation. The key PGC inducing signal is BMP4 produced by extraembryonic ectoderm (Surani, Ancelin et al. 2004). Interestingly, the extraembryonic ectoderm is a unique structure developed in certain rodents like mice and rats (Saitou and Yamaji 2010). Thus, one of the key questions in mammalian

germ cell specification is how germ cells are specified in species that lack this extraembryonic structure. Importantly, this is relevant to most mammals, including humans and many domestic species. To begin to address this question, studies in some species have shed some light on the potential differences in the specification mechanisms, based on some key differences in spatiotemporal gene expression and embryo development. For example, the key inducing molecule BMP4 is first detected in the mesoderm of pig (Valdez Magana, Rodriguez et al. 2014) and rabbit (Hopf, Viebahn et al. 2011) embryos, i.e., after the onset of gastrulation. This observation suggests that if BMP4 is also a key molecule inducing germ cell specification in non-rodent mammals, then it must be inducing cells in a mesodermal location rather than the pluripotent epiblast, as described in mice (Ohinata, Ohta et al. 2009). Furthermore, in mice the epiblast develops as a conical structure. Cells in the posterior proximal epiblast are induced to PGCs as a result of their proximity to the extraembryonic ectoderm (ExE). In most non-rodent mammals, the epiblast develops as a flat embryonic disc, and these cells are in contact with the trophectoderm around the time when gastrulation starts. The different development of the epiblast and the temporal shift in the expression of the key inducer (BMP4) raises the question of whether the specification mechanisms are conserved between mammals with different embryology. Furthermore, it is important to note that rodents represent 40% of all mammals, and therefore with the same line of argument presented for species that develop germplasm by Johnson and Alberio, (2015), whereby liberation of a developmental constraint may result in increased speciation, one has to wonder whether rodents (specifically mice and rats) are under a similar evolutionary path, and have changed the PGC development mechanisms as a result of changes in their embryology. During the course of this thesis several landmark studies were published that have in part supported the initial ideas of differences in the developmental mechanisms between mice and other mammals. For instance, studies in pig embryos show that NANOG is always detected in PGC precursors from specification onwards (Wolf, Serup et al. 2011). This is in contrast to findings in mice, which show Nanog protein disappears from PGCs between 7.25-7.5 days post coitum(dpc) (Yamaguchi, Kimura et al. 2005). In a model of human PGC differentiation *in vitro*, it was shown that SOX17, rather than BLIMP1, is a key specifier of the germline (Irie, Weinberger et al. 2015). Furthermore, it was also shown that SOX2 does not play any role during hPGC development, whereas in mice it does (Perrett, Turnpenny et al. 2008). The

expression profile of PRDM14 in mice shows that this gene is key to initiate the epigenetic reprogramming of this lineage, and is considered the first key PGC marker (Yamaji, Seki et al. 2008). In humans, however, PRDM14 does not play any significant role, and is expressed at very low levels in PGCs (Sugawa, Arauzo-Bravo et al. 2015). At the epigenetic level, differences in epigenetic reprogramming have also been reported. For instance tet1/2 mediated DNA demethylation is detected from 8.5-9.5 dpc in mice, i.e. in cells reaching the gonad. In humans, TET1 is highly expressed from early specified 4 week (wk) PGCs (early migratory) until 11wk gonadal PGCs (Guo, Yan et al. 2015), suggesting that active DNA demethylation initiates earlier in humans. This is further supported by in vitro studies which show increases in Tet1 at day 4 after PGC induction, i.e. at pre-migratory stage (Irie, Weinberger et al. 2015). Thus, although studies with human ESC *in vitro* increase our understating of what may be taking place during the earliest stages of PGC development in this species, human embryos younger than 4 weeks cannot be accessed due to ethical constraints. In order to bridge this gap in our knowledge of how PGCs develop, I propose to use the pig as a model species that develops an embryonic disc and that may share developmental mechanisms with humans. This project studied pig PGC specification and development covering aspects of transcriptional regulation, epigenetic reprogramming and signaling pathways. The findings of this study were compared with our current knowledge in mice and human PGCs, and have enabled me to draw some conclusions on the conserved mechanisms of PGC specification.

1.1 Pig embryo development

Development of the post-hatching porcine embryo can be divided into stages as shown in Figure 1.

After the embryo hatches from the zona pellucida, the spherical embryo (E8-9) contains a Raubers' layer that covers the inner cell mass (ICM) (Figure 1 A and Figure 2A) (Vejlsted, Du et al. 2006; Vejlsted, Offenberg et al. 2006). The porcine Rauber's layer is derived from polar trophectoderm (TE), and degenerates at around E10 (Figure 2A). Before the rupture of Rauber's layer, ICM cells polarize as epithelioid cells

(Flechon, Degrouard et al. 2004). This pseudostratified layer of trophoectodermal cells will then be exposed to uterine milieu and is then called epiblast. In contrast, mouse polar trophoctoderm keeps proliferating, and gives rise to the extraembryonic ectoderm and the ectoplacental cone (Gardner, Papaioannou et al. 1973).

During the pre-streak stage I (E10-11) the Rauber layer ruptures and the epiblast is exposed to the uterine cavity (Figure 1 B-D) (Vejlsted, Du et al. 2006). After the loss of the Rauber layer, the embryonic disc expands in diameter until E12-13 and then acquires polarity, and starts to elongate. E12 spherical blastocyst reaches the maximum diameter before the onset of primitive streak formation (Flechon, Degrouard et al. 2004; Vejlsted, Du et al. 2006; Oestrup, Hall et al. 2009).

During the pre-streak stage II (ovoid stage, E9-11), the conceptus initiates elongation and the embryo becomes ovoid (Figure 1 F). The orientation is determined before the non-invasive implantation (Flechon, Degrouard et al. 2004). The early primitive streak is the origin of migrating cells that contribute to extraembryonic mesoderm (Flechon, Degrouard et al. 2004). The extraembryonic mesoderm starts to delaminate from the posterior third of the embryonic disc, visualized as a thickening crescent, before the primitive streak forms (Figure 2 B).

At the primitive streak stage (E11-12), cells begin to converge in the midline to start their epithelial-mesenchymal transition (EMT), contributing to mesoderm and endoderm lineages, along with the formation of amniotic folds (Figure 1 G and H). The remaining epiblast cells will further differentiate into ectoderm or neural ectoderm in the next stage (Vejlsted, Offenberg et al. 2006; Oestrup, Hall et al. 2009).

Neural groove stage occurs at day E13-14 (Figure 1 I) (Oestrup, Hall et al. 2009) and somitic stages (E14-15) are characterized by paraxial mesoderm is forming somites (Vejlsted, Du et al. 2006; Vejlsted, Offenberg et al. 2006) (Figure 1 J).

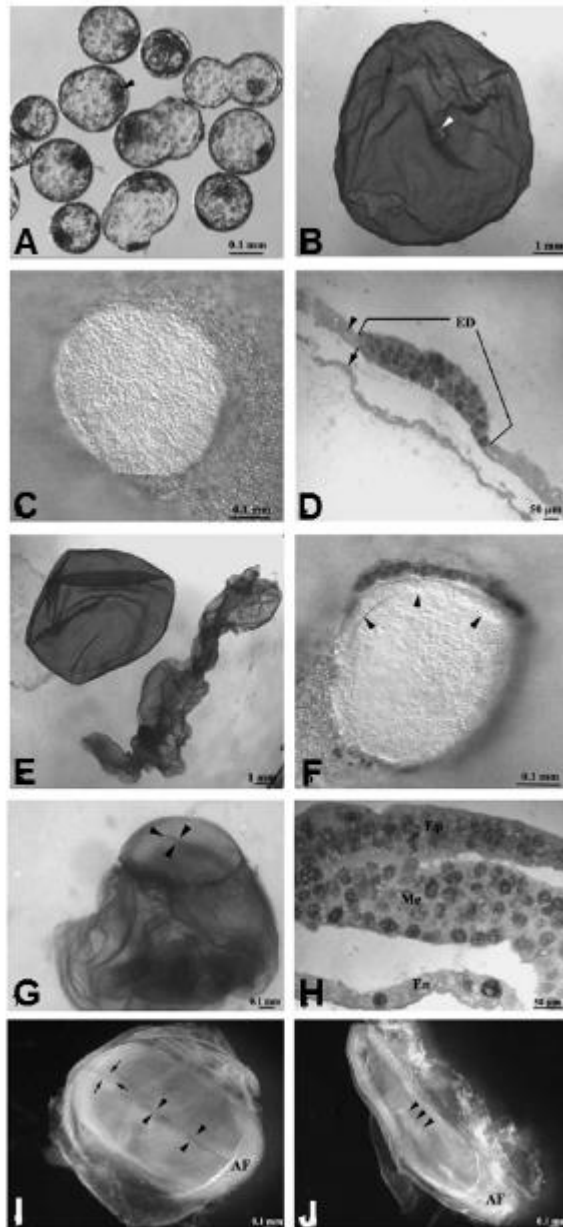


Figure 1. Post hatching porcine embryo stages (Vejlsted, Du et al. 2006).

A. Expanded hatched blastocyst stages. Arrow indicates ICM.

B. PSI stage spherical conceptus. Arrowhead indicates the embryonic disc.

C. PSI stage embryonic disc.

D. Sagittal section of PSI stage embryonic disc. ED, embryonic disc.

E. PSII stage (left) conceptus.

F. PSII stage embryonic disc. Arrowheads indicate the thickening extraembryonic mesoderm at the posterior 1/3 of the disc.

G. Primitive streak stage embryo. Arrowheads indicate the primitive streak.

H. Paraffin sections of primitive streak stage embryo. Me, mesoderm. En, endoderm.

I. Neural groove stage embryo. Arrowheads indicates neural groove. Arrows indicates primitive streak at the caudal 1/4 of embryonic disc. AF, amniotic fold.

J. Somatic stage embryo. Arrowheads indicate the somite.

As shown in Figure 2, the outer cells of pig ICM lose morphology of undifferentiated blastocyst, just before Rauber layer degeneration. After the loss of Rauber layer, the

flattened embryonic disc is exposed. The outer layer of ICM is called the epiblast or primitive ectoderm (PE). Early signs of gastrulation appear when spherical blastocysts reach their maximum expansion before elongation (~E12), no primitive streak has developed. Early primitive streak is the resource of nascent extraembryonic mesoderm. With the appearance of mesoderm, the PE layer is called embryonic ectoderm. Mitotic ectodermal cells majorly locate at the apical anterior region of the embryonic disc. Proliferating cells migrate to the presumptive streak area, where mitotic cells form the primitive streak, through which epithelial-mesenchymal transitions commence (Flechon, Degrouard et al. 2004).

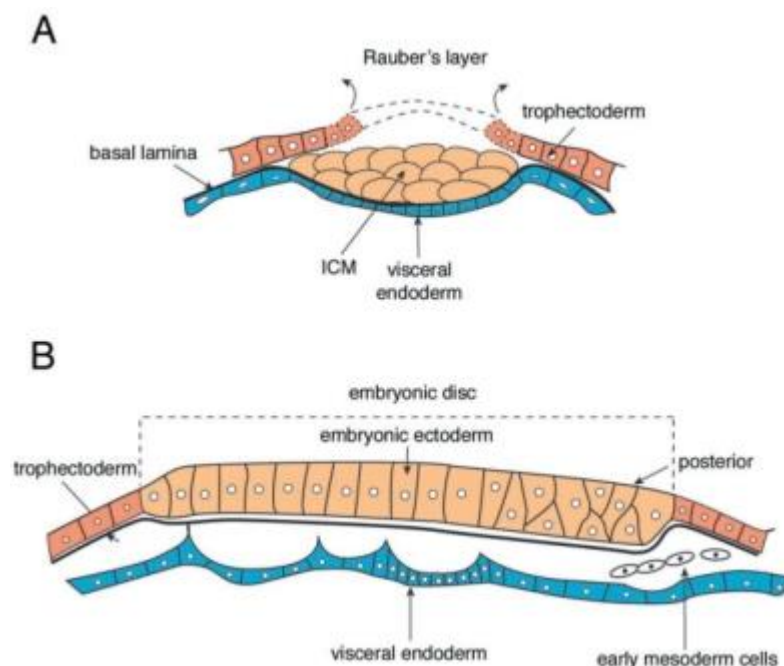


Figure 2. Scheme of porcine prestreak stage embryonic disc (Flechon, Degrouard et al. 2004).

- A. Embryonic disc at the stage when Rauber layer (polar trophectoderm) is degenerated.**
- B. Nascent mesoderm delaminates from the posterior epiblast before the formation of the primitive streak.**

1.2 Mouse embryo development

The molecular decision for the anterior-posterior axis starts from E6 dpc (days post coitum), while morphological changes are not detectable until the onset of gastrulation (~6.75 dpc). During this period, pluripotent epiblast cells converge to form the incipient primitive streak from the posterior proximal epiblast. Through the

developing primitive streak, some epiblast cells perform EMT, ingressing between the epiblast and visceral endoderm (VE) to shape the mesoderm. The mesoderm initiation is under the influence of BMP signaling from the ExE, and nodal-SMAD2/3, as well as canonical WNT signals from the epiblast. Abundant nodal-SMAD2/3 also induces the formation of the primitive streak, particularly from the posterior proximal end (Tam and Loebe 2007).

Generally, the post implantation stage (6.75-8.0 dpc) can be divided into five stages: pre-primitive streak stage, primitive streak stage, neural plate stage, headfold stage and somite stage. The exact day varies slightly, but morphological landmarks identify the stage (as shown in **Figure 3**). Each stage can be further subdivided into several stages. a) At the PS (pre-primitive streak stage, 6.25 dpc), embryonic (e) and extraembryonic ectoderm (ec) are conspicuously separated. b) ES (early primitive streak stage) and MS (mid-primitive streak stage) (6.5-6.75 dpc), mesoderm (m) is visible, and primitive streak is extending anteriorly. c) LS (late primitive streak stage) and OB (no allantoic bud stage) (6.75-7.0 dpc), node (n) is obvious at the distal tip of primitive streak, but no allantoic bud is formed yet. Posterior amniotic fold (paf) features the LS stage and OB is distinguished by the closure of amnion (am) and subsequent formation of exocoelomic cavity (ec). d) EB (early allantoic bud stage) and MB (mid allantoic bud stage) (7.25-7.5 dpc), allantois (al) is shaping at the base of the posterior proximal epiblast. e) EHF (early headfold stage) and LHF (late headfold stage) (E7.5-8.0 dpc), headfold (hf) and foregut (fg) are completely formed. f) Somitic stage (E8.0 dpc and beyond) (Downs and Davies 1993; Kurimoto, Yabuta et al. 2008).

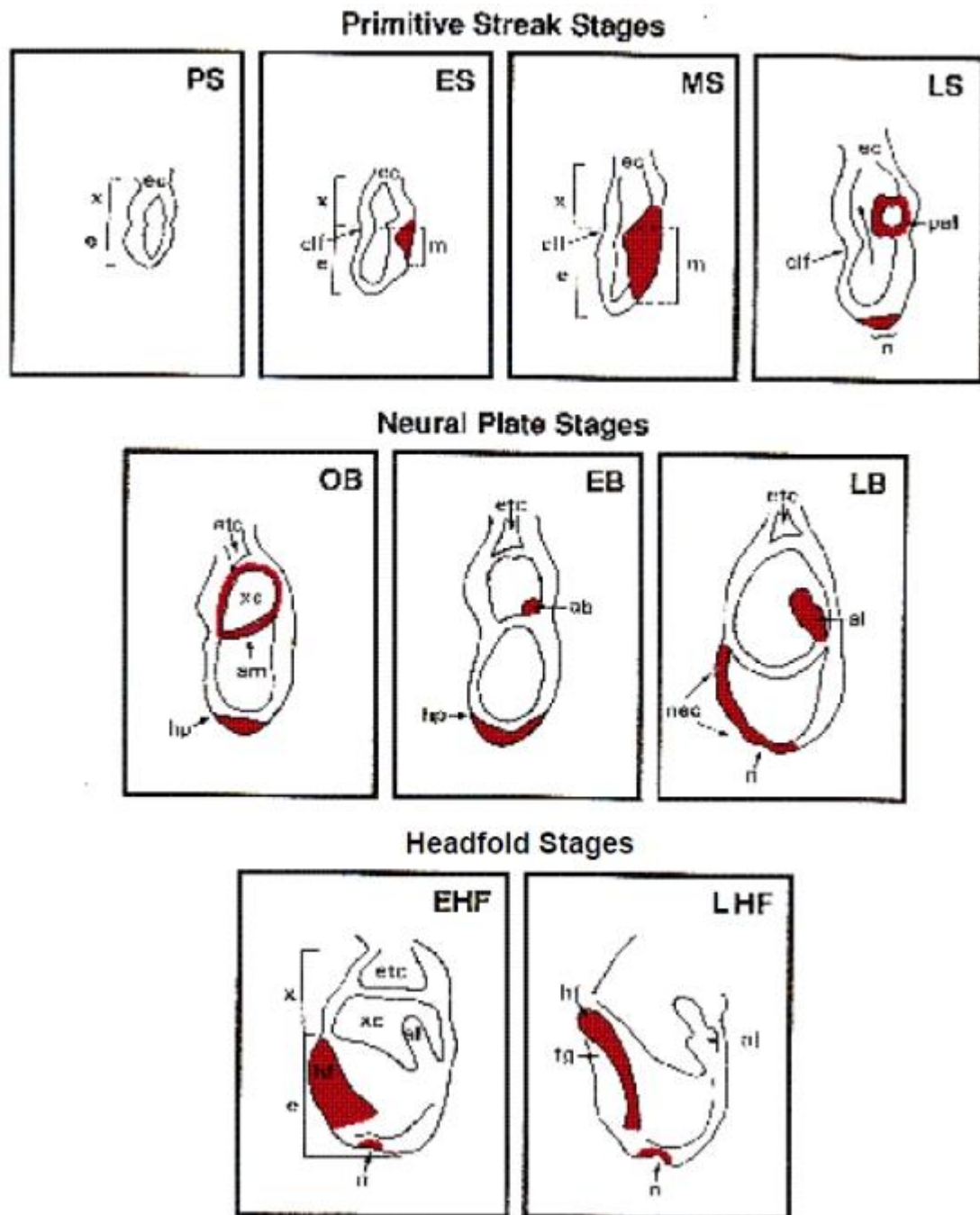


Figure 3. Morphological landmarks of the mouse egg cylinder during gastrulation (Downs and Davies 1993).

PS, pre-primitive streak stage. ES, early primitive streak stage. MS, mid-primitive streak stage. LS, late primitive streak stage. OB, no allantoic bud stage. EB, early allantoic bud stage. LB, late allantoic bud stage. EHF, early head fold stage. LHF, late headfold stage. al, allantois. clf, cranial limiting furrow. e, embryonic compartment of egg cylinder. ec, ectoplacental cone. fg, foregut. hf, headfold. hp, head process. m, mesoderm. n, node. paf, posterior amniotic fold. X, extraembryonic compartment of egg cylinder. xc, extraembryonic ectoderm. nec, neural ectoderm.

1.3 Ontogeny and development of primordial germ cells

1.3.1 Mouse PGC induction and specification

Pre-PGCs develop from pluripotent epiblast, which become lineage restricted following the activation of *Blimp1* at E6.25 (Figure 4). These PGC precursors locate at posterior proximal epiblast from pregastrulation until early streak stages (Lawson and Hage 1994). At the mid-primitive streak stage (~7.25 dpc), a cluster of ~40 lineage restricted PGC cells are segregated from mesoderm cells and then disperse at the base of allantois (Ginsburg, Snow et al. 1990; Lawson and Hage 1994). Migration is sequentially conducted from E8.0 dpc onwards, through hindgut endoderm towards the genital ridges. On arrival at the developing gonads around E10.5 dpc, the gonocytes colonize and initiate gametogenesis.

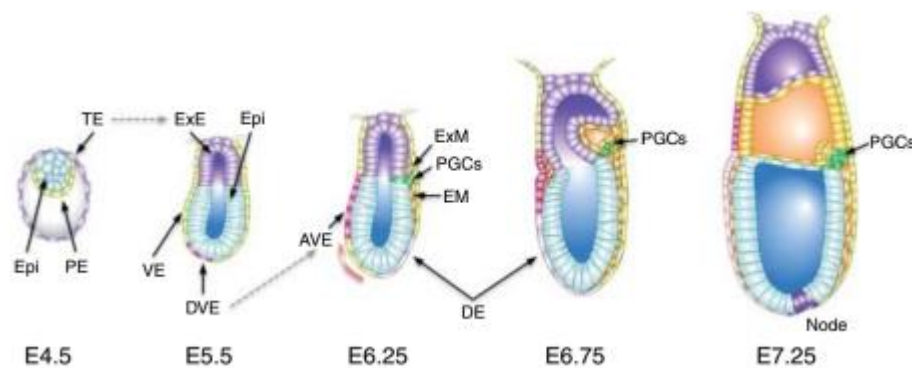


Figure 4. Schematic of mouse embryo during PGC induction and specification (Saitou and Yamaji 2010).

Pre-PGCs are induced at E6.25, where a subpopulation of proximal posterior epiblast cells become lineage restricted. At E6.75 PGC become specified and expand by forming a cluster of ~40 founder by E7.25.

1.3.1.1 Inductive factors

A set of signaling factors contribute to the induction of mouse PGCs from 5.5 dpc, primarily the Bone morphogenetic proteins (BMPs), including *Bmp4*, *Bmp2* and *Bmp8b*.

Bmp4 is produced by in epiblast derived ExM (extraembryonic mesoderm) and ExE (Extraembryonic ectoderm) (Fujiwara, Dunn et al. 2001). The ExE-secreted *Bmp4* induces PGCs specification and allantois development (Lawson, Dunn et al. 1999). *WNT3* and *Nodal* cooperate to ensure the epiblast is responsive to *Bmp4* (Saitou and Yamaji 2010). Meanwhile, *Nodal* specifies DVE (distal visceral endoderm). *BMP8b*

prevents anteriorization of the epiblasts by antagonising the AVE (anterior visceral endoderm). As AVE antagonizes Bmp4 induction, after the embryo turning over at 6.0-6.25 dpi, anteriorized DVE restricts the responsive area to the posterior end of the proximal epiblast (Ohinata, Ohta et al. 2009; Saitou and Yamaji 2010). Bmp4 plays a permissive role for mesodermal factor T to activate key genes, Blimp1 and Prdm14 (Aramaki, Hayashi et al. 2013). In addition, Bmp2 transcribed from 6-6.5 dpc in VE (visceral endoderm) plays an additive role with Bmp4 in PGC specification (Ying and Zhao 2001).

PGC specification is transcriptionally regulated by a germ line specific tripartite network of Blimp1, Prdm14 and Tfap2c (Magnusdottir, Dietmann et al. 2013), and the pluripotency network of Oct4, Sox2 and Nanog. The roles of these transcription factors will be introduced in sections 1.4.1 and 1.4.2

1.3.2 Human PGC specification

Human early embryonic development is similar to many other non-rodent mammals, including pigs and cattle. There are some key embryological differences between non-rodent and rodent mammals with potential impact on our understanding of germ cell specification. Firstly, there is no ExE structure equivalent to that described in mouse, which provides Bmp4 and Bmp8b. Secondly, from E8, the human epiblast and amnioblast cells are separated by the amniotic cavity, which doesn't exist in the mouse embryo (Saitou and Yamaji 2010).

Little is known about the early human germline until the third week, when a cluster of PGCs is detected at the base of allantois and future umbilical cord, on the wall of the yolk sac (Sadler and Langman 2012).

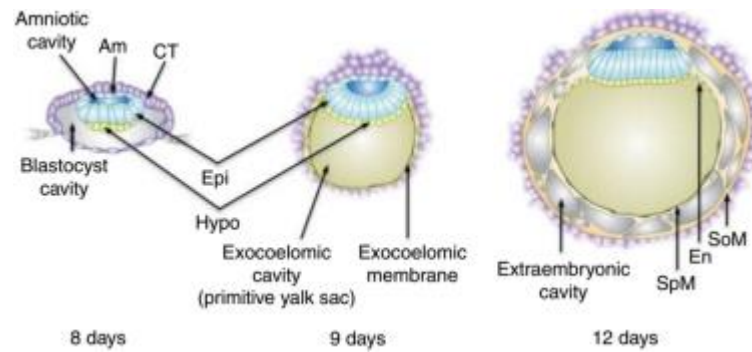


Figure 5. Schematic of early post implantation human embryo (Saitou and Yamaji 2010).

Different from mouse, human embryo doesn't have extraembryonic ectoderm. Human embryo represents basal mammals embryonic disc.

Besides the critical factors specifying mouse PGCs (sections 1.3.1.2 and 1.3.1.3), SOX17 is also reported to be a critical factor for human PGC specification in in vitro differentiation system. In contrast to mouse PGC, SOX17 itself is sufficient to activate BLIMP1, thus it initiates PGC fate from competent hESCs. Interestingly, BLIMP1 suppresses endodermal fate, as well as mesoderm. PRDM14, another important specifier for mPGC, is downregulated during human PGC-like cells (hPGCLC) differentiation. The opposite functions might partially be attributed to divergent proteins between the two species (Irie, Weinberger et al. 2015). However, the in vitro differentiation system might not fully recapitulate in vivo specification system. For this reason, an in vivo model for human PGC specification is required.

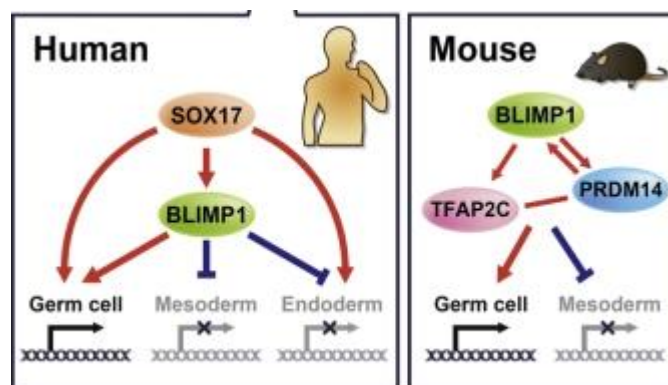


Figure 6. Graph of PGC specification mechanism in human and mouse (Irie, Weinberger et al. 2015).

1.3.3 Pig PGC specification and development

The first observation of pig PGC is in the mesonephric at E18 of gestation. Those PGCs are migrating towards the genital ridges, which are incipient from E24 and

sexually dimorphic by E27 (Black and Erickson 1968). At this stage, seminiferous tubules start to form in male primordia, which make the genital ridges striated. As tunica albuginea develops, testes become more compact and opaque and later vascularized. Meanwhile, the presumptive ovaries appear relatively homogenous and fragile (Leichthammer, Baunack et al. 1990). E40-E50 aged ovaries are predominantly made of oogonia, while oocytes are incipient from E50. Mitotic activity of gonads decreases from E60, accompanied by dominant pachytene phase oocytes until E70, when diplotene oocytes become prevalent. From birth to 20 days post-partum, almost 99% of oocytes arrive at the diplotene phase (Black and Erickson 1968). In terms of number, germinal mitoses, reflected by increasing numbers of germ cells, reaches a peak at 50 dpc. The mitoses rate decreases, but actual numbers accumulate until 90 dpc, when the germ cell number then reduces (Black and Erickson 1968).

1.3.4 PGC migration

From ~7.25 dpc, mouse PGCs begin to migrate. They are observed extending filopodia, with polarized morphology, actively migrating from the posterior primitive streak into the endodermal epithelium. Moving in the hindgut endothelium, PGCs distribute in the border area (Anderson, Copeland et al. 2000). Until 11.5 dpc, when the PGCs reach the genital ridges, they remain highly motile during the whole migration.

Pig PGC migration is roughly similar to mouse, except they tend to migrate individually, whereas mouse PGCs are always observed moving as a cluster (Hyldig, Ostrup et al. 2011).

During migration, PGCs undergo epigenetic reprogramming which will be discussed in more detail in chapter 4.

1.4 Genetic regulation of PGC specification

PGC specification is regulated by important transcription factors related to the germ line (i.e., *Blimp1*, *Tfap2c*, and *Prdm14*), pluripotency (i.e. *Nanog*, *Oct4*, and *Sox2*), or other PGC regulatory genes, such as *Fragilis*, *Stella*, *Sox17*, *Nanos3*, *Akp2*, and *Sda/GM2*. This section describes the function and expression pattern of each

transcription factor in mouse, human, or pig early embryonic development and PGC specification, as well as functions detected in relevant cell culture systems.

1.4.1 The tripartite network of Blimp1, Tfap2C and Prdm14 in PGC specification

For mouse PGC induction and specification, the transcription factors Blimp1 (encoded by *Prdm1*), AP2y (encoded by *Tcfap2c*) and *Prdm14* comprise an interdependent tripartite network (Magnusdottir, Dietmann et al. 2013).

Blimp1 (B-lymphocyte-induced maturation protein-1) is a zinc finger transcriptional repressor that appears to be a critical coordinator of PGC specification (Ohinata, Payer et al. 2005; Vincent, Dunn et al. 2005; Magnusdottir, Dietmann et al. 2013). Blimp1 activation causes lineage restriction of 6 PGC precursors at the posterior proximal epiblast from 6.25 dpc (Ohinata, Payer et al. 2005). During PGC specification, Blimp1 represses homeobox genes such as *Hoxa1* and *Hoxb1*, as well as the epithelial mesenchymal transition (EMT) gene, cell cycle related genes and DNA methylation (Kurimoto, Yabuta et al. 2008). The repression of the mesodermal programme is of particular importance for PGC segregation (Ohinata, Payer et al. 2005). Almost all Blimp1 positive precursors give rise to proper PGCs, marked by the expression of *Stella* (Saitou, Barton et al. 2002; Kurimoto, Yabuta et al. 2008). *Hoxb1* is transcribed in Blimp1 positive PGC precursors at 7.0 dpc, but is repressed in high Blimp1 expressing PGCs from 7.25 dpc and onwards (Kurimoto, Yabuta et al. 2008).

Prdm14, another key transcriptional regulator of the mouse germ cells lineage, is expressed PGC, and ICM cells, playing a key role in pluripotency and initiation of epigenetic reprogramming. In response to the *Bmp4*-*Smad1* pathway, an independent subpopulation of proximal epiblast cells commence transcription of *Prdm14*, that subsequently contribute to the founder PGC as well as Blimp1 populations (Yamaji, Seki et al. 2008). *Prdm14* null mice are infertile but viable (Ma, Swigut et al. 2011). *Prdm14* homozygous mutant PGCs are unable to reacquire *Sox2* expression, while *Nanog* is unaffected. *Prdm14* (-/-) embryos have reduced numbers of PGCs, while the PGC-like cells are able to migrate. *Prdm14* prevents PGC embarking on the somatic program, as *Hoxa1* and *Hoxb1* are upregulated in mutant embryos. However, *Prdm14* mutant PGCs are unable to decrease levels of histone3 lysine9 dimethylation (H3K9me2) at 8.5 dpc, and to increase histone3 lysine27 trimethylation (H3K27me3)

levels from 9.5 dpc. Both H3K9me2 downregulation and H3K27me3 upregulation during 8.5-9.5 dpc are features of mouse migratory PGCs. Histone3 lysine 9 methyltransferase GLP is repressed in PGCs from 7.75 dpc. Prdm14 mutant mouse embryos fail to repress the expression of GLP (Yamaji, Seki et al. 2008). Prdm14 promotes the methylcytosine dioxygenase TET mediated base excision repair (TET-BER) pathway, thus accelerating active DNA demethylation in ESCs. Prdm14 physically interacts with Tet1 and Tet2 proteins with N-terminal domains, and recognizes specific motifs of DNA with C-terminal zinc finger domains. In this way, it enhances recruitment of Tets at target loci. Prdm14 also represses the DNA (cytosine-5) methyltransferases, Dnmt3b and Dnmt3l (Okashita, Kumaki et al. 2014).

Activating enhancer binding protein2 gamma (AP2 γ), encoded by Tcfap2c, is a PGC marker downstream of Prdm1 (Blimp1). It is expressed in mouse PGC through 7.25-12.5 dpc. AP2 γ null mice die around 6.5 dpc, while PGCs are still specified in AP2 γ single allele mutant mice, but are not sustained after 8.0 dpc. Conditionally mutant AP2 γ alleles lead to the reduction of gonad size. Ovaries are observed with significantly less germ cells, while testes show atrophy of seminiferous tubules. Human AP2 γ is transcribed in foetal germ cells through wk 12-37, whereas adult testes lose AP2 γ protein. Conditional knockout of AP2 γ in the TCam-2 human seminoma cell line results in the upregulation of HOXB1 and the mesoderm markers, MYOD1 and HAND1 (Weber, Eckert et al. 2010).

As a tripartite network of germ cells, Blimp1 was found to inhibit proliferation and somatic regulators. Activated independently after PRDM1/Blimp1, PRDM14 plays an important role in epigenetic reprogramming (Magnusdottir, Dietmann et al. 2013). Both PRDM14 and AP2y were reported to preferentially target cell-motility and cytoskeletal organization related genes, and thus participate in germ cell gene activation, PGC migration and somatic fate switch-off. Besides AP2y, Blimp1 directly binds to repress Dnmt3b transcription, which connects the genetic regulation to epigenetic changes (Magnusdottir, Dietmann et al. 2013).

1.4.2 Roles of the pluripotency genes Oct4, Nanog and Sox2 in PGC specification.

As well as the germ line related tripartite network, the pluripotency network also plays a critical role in PGC specification. Nanog, Sox2 and Oct4, three core transcription factors of the pluripotency network, are all involved in mouse PGC specification and development.

Nanog is a member of the homeodomain consisting transcription family. It maintains pluripotency and self-renewal in mouse preimplantation embryos and pluripotent cells, such as embryonic stem (ES) cells, embryonic germ (EG) cells and embryonic carcinoma (EC) cells. The Octamer (Oct) and Sox response elements in the Nanog promoter enable initiation of Nanog transcription. This response element is conserved in mouse, monkey and human genome (Rodda, Chew et al. 2005). *Nanog*-null mice are unable to form epiblast at 5.5 dpc. Mouse Nanog is consistently detected in the epiblast at 6.5-7.5 dpc (Yabuta, Kurimoto et al. 2006). At E7.5, when a cluster of ~40 founder PGCs was established, Nanog is expressed only in pluripotent epiblast, but not in PGC. Until E7.75, Nanog positive (+) PGCs are observed in both the PGC cluster and migratory PGCs (Yamaguchi, Kimura et al. 2005). From 7.75 to 12.5 dpc, Nanog expression levels increase in proliferating and migrating PGCs. Nanog expression in oogonia is repressed from 13.5-14.5 dpc during meiosis, while both the number of Nanog positive cells and Nanog expression levels in spermatogonia are down regulated from 14.5-16.5 dpc during mitotic arrest. No Nanog expression is detectable in either gender of adult mouse (Yamaguchi, Kimura et al. 2005). Unlike in mice, Nanog is not expressed in the pig until the blastocyst is hatched around E9-10, although the transcripts are detected in 4-8 cell stage morulae, blastocyst and epiblast. Nanog is required for epiblast and hypoblast establishment (Wolf, Serup et al. 2011). Pig Nanog is observed throughout the whole of the PGC specification period (Wolf, Serup et al. 2011).

Sox2 is a member of the SRY-related HMG box (Sox) gene family. It is pluripotency-conferring gene that plays an important role in early embryogenesis and PGC specification. In mouse PGCs, Sox2 expression is undetectable or low at 6.75 dpc and gradually accumulates afterwards (Yabuta, Kurimoto et al. 2006). Sox2-null mouse embryos die shortly after blastocyst implantation. Sox2-conditional deletion in mice

significantly decrease the number of PGCs at early specification. During PGC development, Sox2 is required for PGC proliferation (Campolo, Gori et al. 2013). Mouse Sox2 continues to be expressed during specification (Kurimoto, Yabuta et al. 2008), migration and gonadal colonization, while human PGCs do not express SOX2 during the migratory and gonadal stages (Perrett, Turnpenny et al. 2008).

In human ESCs, SOX2 prevents mesodermal differentiation, although the SOX2 knock-down hESC cell line maintains pluripotency with lower efficiency (Wang, Oron et al. 2012).

Octamer-binding transcription factor 4 (Oct4), also known as POU domain class5 transcription factor 1 (Pou5f1), is an essential transcriptional factor regulating pluripotency. Oct4 expression is maintained from induction to germ line establishment in both mice and pigs (Kurimoto, Yabuta et al. 2008). OCT4 is sequentially expressed in epiblast after hatching, mesoderm during gastrulation, presumptive PGC during neurulation, along with gradual downregulation in the posterior-anterior aspect of the ectoderm. In pigs, OCT4 is still detectable during elongation, in extra embryonic tissue until E11 (Wolf, Serup et al. 2011). In mice, Oct4 remains at high expression levels throughout PGC specification (Yabuta, Kurimoto et al. 2006). Conditional Oct4 KO mice show significantly decreased PGC numbers from ~10.5 dpc, suggesting a role in the maintenance of PGCs (Kehler, Tolkunova et al. 2004). In human ESCs, high OCT4 levels induce differentiation of mesoderm under BMP4 treatment, but maintain self-renewal without BMP4 treatment. Low levels of OCT4 stimulate extraembryonic tissue differentiation with BMP4, but result in ectoderm fate in the absence of BMP4 (Wang, Oron et al. 2012).

1.4.3 Other important germ cell lineage-related genes

1.4.3.1 Stella, Nanos3 and Akp2

Transcription factors Stella, Nanos3 and Akp2 are germ cell related genes that are expressed variously in different species and stages.

Stella, also known as Dppa3 or Pgc7, is expressed in nucleus and cytoplasm, involved in chromosomal organization and RNA processing. Stella is expressed in totipotent and pluripotent cells (Saitou, Barton et al. 2002), and is highly detected in mouse

founder PGCs (Saitou, Barton et al. 2002). In early embryogenesis, Stella binds histone H3K9me2 to protect DNA methylation of the maternal genome (Nakamura, Liu et al. 2012).

The Nanos gene family is required for germ cell development via diverse regulatory mechanisms and homologs are found in various species, including mice, humans, *Drosophila*, *C.Elegans* and *Xenopus* PGCs transcribe Nanos3 from 7.25 dpc (Yabuta, Kurimoto et al. 2006). Nanos3 knockout mice have reduced size of gonads and become infertile in both genders (Tsuda, Sasaoka et al. 2003). Within the human ESC differentiation system, Nanos3 is detected in germ cells through spermatogenesis and oogenesis. Nanos3 knock down results in reduced germ cell number and downregulated expression level of genes related to maintenance of pluripotency and meiotic progress (Julaton and Reijo Pera 2011).

Tissue non-specific alkaline phosphatase (TNAP, or Akp2) doesn't specifically mark PGC precursors or PGCs (Yabuta, Kurimoto et al. 2006), but has been used as surface marker of PGCs (Ginsburg, Snow et al. 1990).

1.4.3.2 Brachyury (T)

Brachyury (T) is an important transcription factor, which directly mediates various early embryonic development genes, such as mesoderm lineage related factors like Fgf8, Dusp6, Foxa2 and some muscle and chondrocyte regulators. In addition, T regulates morphogenetic cell movements, epithelial-mesenchymal transition (EMT) and cell-mobility regulators, and even some cancer-related genes (Lolas, Valenzuela et al. 2014). During PGC specification, expression of T is induced by WNT3 signaling, with the presence of inducing signal BMP4. T directly binds to regulatory elements of Blimp1 and Prdm14 promoters, thereby initiating PGC fate (Aramaki, Hayashi et al. 2013). Mouse PGCs are segregated from extraembryonic mesoderm, acquiring a partial mesodermal property, but are prevented from differentiating into mesoderm by the lineage restrictor, Blimp1. PGC precursors maintain consistent levels of posterior mesoderm markers, such as Evx1, T and Fgf8, until E7.25, and then they gradually decrease (Yabuta, Kurimoto et al. 2006). In mouse E7.5 primitive streak, T targets and promotes SOX17, which is an important endoderm gene.

Activated high levels of SOX17 in turn repress T, directly or indirectly (Lolas, Valenzuela et al. 2014).

1.4.3.3 Gene expression differences between mouse and human PGCs

Sox17 (Sry-related HMG box factor 17) belongs to the SoxF family, that includes Sox7 and Sox18. It has been identified as a critical transcription factor for definitive endoderm (DE), particularly the mid and hind gut endoderm (Kanai-Azuma, Kanai et al. 2002; Uemura, Hara et al. 2010), as well as being important in the generation and maintenance of HSC (hematopoietic stem cells) in fetal liver (Clarke, Yzaguirre et al. 2013) and being a transcriptional activator of mouse premeiotic spermatogonia cells (Kanai, Kanai-Azuma et al. 1996).

Mouse PGCs show transient increases in Sox17 expression around 7.25 dpc, which disappears at 7.75 dpc (Yabuta, Kurimoto et al. 2006). SOX17 is detected in human carcinoma *in situ* (CIS) and seminomas, but not in embryonal carcinoma, while Sox2 has the opposite expression pattern (de Jong, Stoop et al. 2008). In hPGCLC (human PGC like cells) cells, SOX17 is identified not only as the earliest marker of hPGCLCs, but also the critical specifier of hPGCLC fate (Irie, Weinberger et al. 2015). SOX2 expression is absent in human fetal gonads, but expressed in the early germ lineage of mice (Perrett, Turnpenny et al. 2008).

Interestingly, point mutations of Sox17 and Sox2 result in a lineage switch between endoderm and pluripotency in mouse ES cells (Julaton and Reijo Pera 2011). The interaction between Sox2 and Oct4 is altered when the chosen amino acid on helix3 is mutated. As the assembly of Oct4 and Sox2 or Sox17 into a complex depends on the sox/oct element, a distinct ternary Sox/Oct4/DNA complex forms when Oct4 swaps its partner. Therefore different germ lineage-related genes will be transcribed (Julaton and Reijo Pera 2011; Aksoy, Jauch et al. 2013).

The mouse monoclonal CT-1 antibody specifically recognizes the carbohydrate structures, Sda glycotope (Neu5Aca2–3(GalNAcb1–4) Galb1–4GlcNAcb-) and GM2 glycotope (Neu5Aca2–3(GalNAcb1–4)Galb1–4Glc. It has been validated for pig, cattle, horse and llama. CT-1 possesses advantages over the traditional early germ cell marker, plant lectin *Dolichos biflorus* agglutinin (DBA) (Takagi, Talbot et al.

1997; Klisch, Contreras et al. 2011), when gametogenesis is considered. Compared with DBA, CT-1 stains specifically PGCs and gonocytes (E22-E75), rather than mesonephris cells (Klisch, Contreras et al. 2011).

1.5 Primordial germ cell signaling pathways

Signaling pathways BMP/Smad1 and canonical WNT/ β -catenin are of particular importance to mouse PGC induction and specification.

Bone Morphogenetic protein (BMP) binds to a receptor and Smad1 is an intracellular signaling molecule, which is non-redundantly required for the commitment of early mouse PGC specification. Smad1-null mouse embryos develop no PGC and short allantois, although early mesoderm induction is not affected. Phosphorylated SMAD proteins actively transduce BMP4 signaling in the proximal posterior epiblast at 6.0-6.5 dpc, where PGCs are specified (Hayashi, Kobayashi et al. 2002). Smad1 is abundant in 6.75 dpc PGCs and gradually decreases afterwards. Smad5, as well as Smad4, a co-SMAD that binds to receptor-regulated Smads, is maintained at constant levels during 6.75-7.75 dpc periods. However, Smad3, a mediator of the TGF β pathway via Smad4 accumulates from 7.25 to 7.75 dpc (Yabuta, Kurimoto et al. 2006).

Canonical WNT/ β -catenin signalling is required for mouse PGC specification. As direct or indirect downstream effector of BMP4 signal, WNT3 activates T, which directly activates Blimp1 and Prdm14, therefore specifies PGCs (Aramaki, Hayashi et al. 2013). During mouse primitive streak formation period, T directly targets Sox17 and other key developmental regulation genes, such as Fgf8, Foxa2, Foxj1, and Dusp6 (Lolas, Valenzuela et al. 2014). The interaction between T, Sox17 and Blimp1 during pig and human PGC specification is further discussed in chapter 1 and chapter 6.

WNT signalling is detected by nuclear localization of β -catenin. With the presence of WNT ligands and natural receptors, cytoplasmic β -catenin translocates to the nucleus, where it interact with Tcf/Lef family of transcription factors to regulate the expression of downstream target genes (Atlasi, Looijenga et al. 2014). *Xenopus* animal cap experiments suggests that Sox17 is direct negative regulator of β -catenin/TCF transcription activity in the WNT signalling pathway (Zorn, Barish et al. 1999). In a

different context, hSOX17-WNT interactions fine-tune the activity of canonical WNT signalling (Sinner, Kordich et al. 2007; Zhang, Glockner et al. 2008). Carcinoma *in situ* (CIS) and seminomas are forms of germ cell tumours that express Sox17 (de Jong, Stoop et al. 2008). Only membrane β -catenin is detected in seminoma cells (TCAM2), which suggests the inactivation of WNT signalling. Consistent with this observation, some downstream target genes are also downregulated, including TCF7L1 and FGF4, compared with embryonal carcinoma cells. Interestingly, Sox17 is highly expressed in seminomas (Korkola, Houldsworth et al. 2006). WNT signalling is also detected in spermatocytes and round spermatids of mouse adult gonads. Sox17 is significantly upregulated, and WNT target gene Lef1 is downregulated in adult testes (Kerr, Young et al. 2014).

The WNT / β -catenin signaling pathway regulates self-renewal and differentiation in many adult stem cells niches, such as small intestine, mammary gland, hair follicle, skin, bone marrow, brain, as well as in cancer cells (Reya and Clevers 2005). In ESC, β -catenin plays the role of guardian of the self-renewal, as β -catenin-null ESCs rapidly exit self-renewal and enter differentiation (Wagner, Xu et al. 2010; Wray, Kalkan et al. 2011). WNT signaling is required at multiple stages of mouse spermatogenesis. Upregulated WNT signaling results in reduced numbers of spermatocytes and spermatids compared to control mice (Kerr, Young et al. 2014). As shown in Figure 7, in the canonical WNT signaling pathway, β -catenin partially associates to the cell membrane, thereby mediating cell-cell adhesion, and partially localizes in the cytosol, where it forms a destruction complex. In the absence of WNT ligands, β -catenin is negatively regulated and therefore mediates ubiquitination and proteolytic degradation. WNT ligands bind to the receptors, Frizzled and LRP6, leading to the activity of the destruction complex to be inhibited, therefore the cytoplasmic β -catenins are stabilized and translocate to the nucleus. Nuclear β -catenins interact with Tcf/Lef family transcription factors, acting as cotranscriptional factors, regulating the expression of downstream WNT target genes (Atlasi, Looijenga et al. 2014).

Plasma membrane β -catenin is highly expressed in mouse gonads at 13.5 dpc (Kimura, Nakamura et al. 2006). Active WNT signaling is typically detected in nuclei of postmitotic germ cells of the adult mouse testis, typically round spermatids from 20

dpc, but not in gonocytes or spermatogonia (Kerr, Young et al. 2014). Aberrant activation of β -catenin shows abnormal G1/S transition of the cell cycle, which leads to the reduction in numbers of gonadal PGC (Kimura, Nakamura et al. 2006).

At 5.5 dpc in mice, the PGC inducing signal, BMP4 from ExE is dose-dependent on Nodal, which directly activates WNT3 in the posterior of epiblast, which in turn maintains robust Nodal expression. The Nodal signal cannot be sustained if there is a lack of WNT3 (Tam and Loebel 2007). To date, there appears to be no reports on the regulation of the WNT/ β -catenin signaling pathway in early PGC in mice or humans. In this thesis, we will investigate the function and dynamics in the pig PGCs.

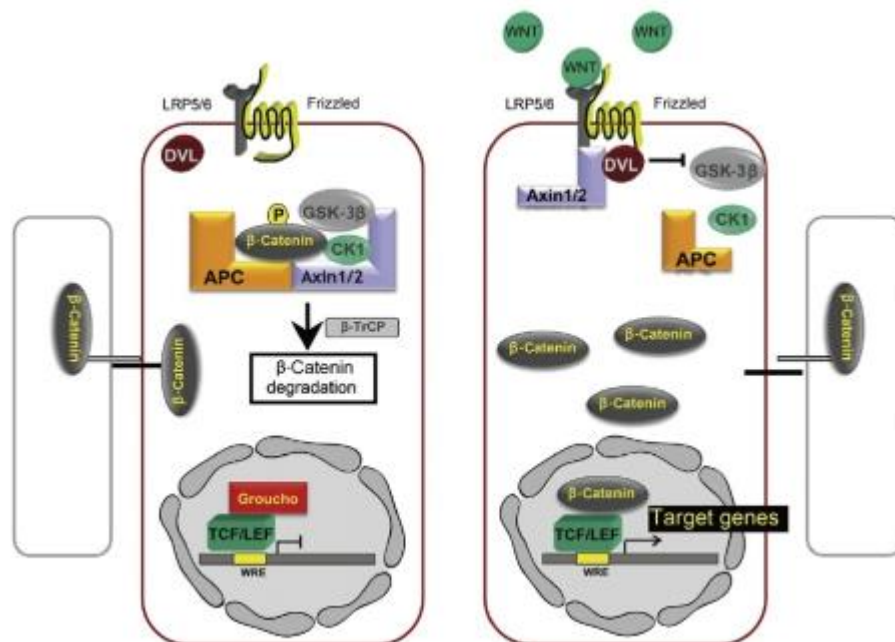


Figure 7. Schematic representation of the canonical WNT/ β -catenin Signalling pathway (Atlasi, Looijenga et al. 2014).

Left half shows the cells in the absence of WNT ligand, where cytosol β -catenin forms destruction complex with APC and Axin scaffolding proteins. β -catenin is down regulated and then ubiquitinated or proteolytically degraded. The right half shows the cells in the presence of WNT ligands, receptors Frizzled and LRPs inhibit the destruction complex. Therefore the cytoplasmic β -catenin is stabilized, and translocate to the nucleus, where it interacts with TCF/LEF transcription factors, regulating the downstream genes of WNT signaling.

1.6 Epigenetic regulation of PGC specification and development

Epigenomic changes influence epigenetic pre-patterning for lineage specification, co-regulating transcription and inheritance of chromatin modifications. Generally,

epigenetic regulation is characterized by DNA methylation, alteration of chromatin configuration and histone modification as shown in Figure 8.

In this section, markers and enzymes related to key epigenetic regulation will be described from aspects of demethylation (5-mC, 5-hmC, Tet1/2, Dnmt1, Uhrf1, Dnmt3) and histone modifiers (H3K9me2, H3K27me3, and H2A.Z).

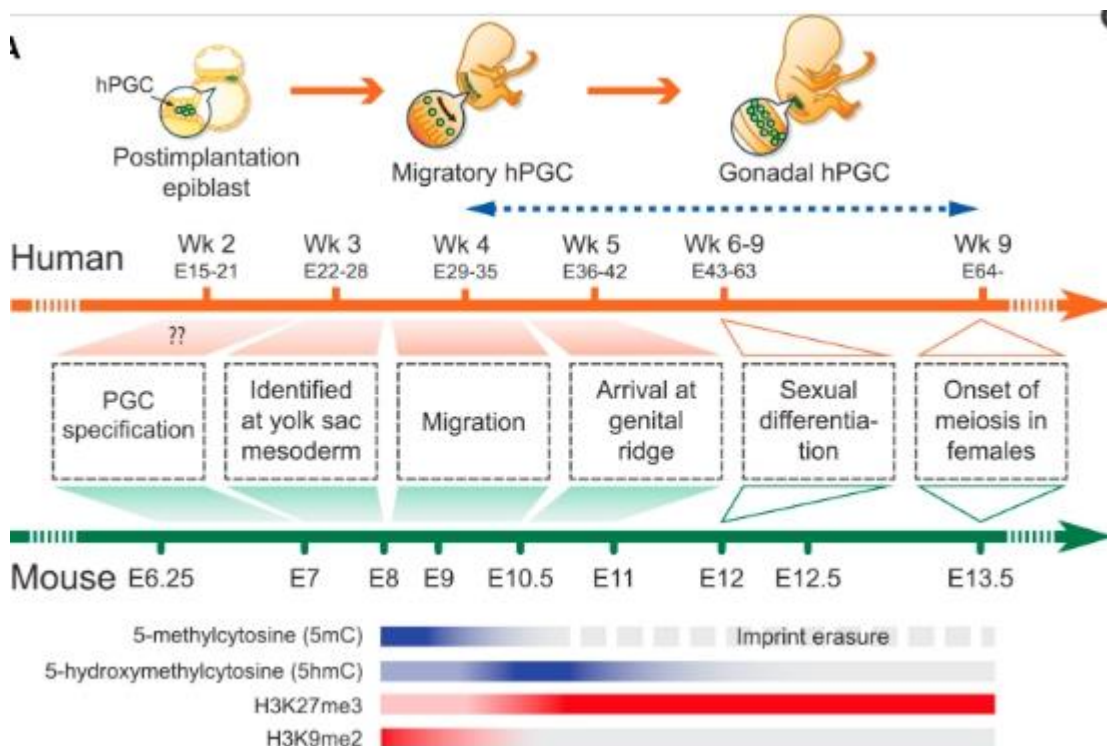


Figure 8. Comparison of epigenetic dynamics between hPGC and mPGC (Tang, Dietmann et al. 2015).

Developmental timelines of hPGC and mPGC are depicted based on the embryological landmarks as labelled in the graph. Epigenetic dynamics of mPGC are depicted as colour bars at the bottom.

1.6.1 Methylation and demethylation in PGCs

Methylation of the fifth carbon of cytosine produces 5mC, which is frequently found in a symmetrical CpG context. CpG islands are commonly observed in promoters, enhancers, and specialized regions of genes, which regulate the expression of corresponding genes. Cytosine hydroxymethylation (5-hmC) is another form of DNA modification, as well as the product of 5-mC oxidization.

In mice, methylation levels at CG dinucleotides are equivalent among epiblast, somatic cells and 6.5 dpc post-implantation embryos. From 6.5 to 16.5 dpc, global DNA de/re-methylation in mouse PGCs has been characterized as occurring in three stages (Yamaguchi, Hong et al. 2013). First, globally passive demethylation occurs in recruiting and migratory PGCs before 9.5 dpc. Second, demethylation is actively sustained from 9.5 to 10.5 dpc. Third, replication-dependent 5-hmC dilution is observed from 11.5 dpc and beyond. The 5-hmC level reaches a nadir in both male and female PGCs at 13.5 dpc (Yamaguchi, Hong et al. 2013). Meanwhile, de novo methylation is onset during this phase (Yamaguchi, Hong et al. 2013). Oogonia sustain hypomethylation during meiotic arrest until 16.5 dpc, while spermatogonia retain methylation marks. Transcription of core pluripotency factors is silenced during oogonia meiotic arrest and spermatogonia mitotic arrest at 13.5 dpc, and later on are replaced by meiosis and germline related network components (Seisenberger, Andrews et al. 2012). The first phase of demethylation involves the global genome, such as CpG islands, introns, exons and promoters. For example, gene promoters of *Nanog* and *Prdm14* are highly demethylated by 9.5 dpc. Exceptions from the first stage are imprinted genes, germline specific genes and CpG islands on the X chromosome, particularly paternal DMRs (differentially methylated regions) will be demethylated during the second phase, while IAPs (intracisternal A particles) are protected (Seisenberger, Andrews et al. 2012).

Ten-eleven translocation protein (Tet) catalyses the oxidization of 5-mC (5-methylcytosine) to 5-hmC (5-hydroxymethylcytosine), then 5fC (5-formylcytosine) and 5fcaC (5-carboxylcytosine). The latter two cytosines can be excised and repaired via the base excision repair (BER) pathway (Surani, Hayashi et al. 2007). Tet1 and Tet2 are enriched in the nuclei of PGCs from 9.5-11.5 dpc, while they peak at 10.5-11.5 dpc. Coincident with Tet accumulation is 5-mC reduction and 5-hmC elevation during 9.5-10.5 dpc. 5-mC is completely depleted at 11.5, while a progressive reduction of 5-hmC takes place from 11.5-13.5 dpc. Some methylation dependent germline specific genes, such as *Dazl*, are initiated from 9.5 dpc and become progressively more prevalent until 11.5 dpc, because their promoters are demethylated during this “Tets function” period. Chromocenter staining by DAPI of 5-hmC shows that heterochromatic satellite regions are affected by 5-hmC conversion (Hackett, Sengupta et al. 2013). *Prdm14* physically interacts with Tet1 and Tet2 proteins with

N-terminal domain, and recognize specific motifs of DNA via their C-terminal zinc finger domains. In this way, Prdm14 enhances recruitment of TETs at target loci (Okashita, Kumaki et al. 2014).

DNA methyltransferase 1 (DNMT1) maintains hemimethylated CpGs on one strand. The expression of Dnmt1 is initiated by cell-cycle dependent transcription factors during the S phase, therefore it is normally detected in mitotic cells (Kishikawa, Murata et al. 2003). Mouse Dnmt1 retains equivalent levels in PGC and somatic neighbours during PGC specification.

Uhrf1, also known as Np95, forms a complex with DNMT1 and then targets hemimethylated CpGs in newly synthesized DNA during cell division, thereby maintaining methylation during mitosis (Bostick, Kim et al. 2007; Surani, Hayashi et al. 2007; Seisenberger, Andrews et al. 2012).

Reduced transcript/expression levels of DNMT3a and DNMT3b, de novo DNA methyltransferases (Okano, Bell et al. 1999), are observed at 7.25 dpc and 8 dpc (Leitch, Tang et al. 2013). Neighbouring somatic cells also experience significant transcriptional suppression at 7.25 dpc (Yabuta, Kurimoto et al. 2006). As a consequence, 5-mC is less abundant at 8 dpc, compared with somatic cells (Leitch, Tang et al. 2013). Dnmt3a plays an indispensable role in methylating imprinted genes in germ cells and Xist genes on the X chromosome. Dnmt3b is pivotal in methylating centromeric minor satellite repeats. Synergy of Dnmt3a and Dnmt3b initiates de novo methylation on Oct4 and Nanog promoters during mouse ES differentiation (Li, Pu et al. 2007). Dnmt3b is downregulated in mouse PGCs after 7.25 dpc and localizes to the cytoplasm (Surani, Hayashi et al. 2007).

IGF2-H19 differentially methylated domains (DMD) and centromeric repeats represent the global methylation status of cells. De novo methylation is established divergently between spermatogonia and oogonia. Mouse spermatogonia acquire new imprints from 14.5 dpc concluding after initiation of meiosis. In contrast, de novo methylation in oogonia initiates postnatally around secondary follicle stage I (50-55 um in diameter), and is completed at antral follicle stage II (65-70 um in diameter). Similarly, porcine PGCs undergo demethylation from E24 to E29-31 in both males

and females after arrival at the genital ridges. From E30, spermatogonia reacquire de novo methylation, while oogonia gradually demethylate from E25 until complete erasure is observed at E42 (Petkov, Reh et al. 2009; Hyldig, Croxall et al. 2011).

1.6.2 Pattern of histone modifiers H3K9me2, H3K27me3, and H2A.Z in PGCs

Demethylation at Lys 9 of histone H3 (H3K9me2) and trimethylation at Lys27 of histone H3 are reversible histone modifications. Maternal genome chromatin contains H3K9me2 (Santos, Peters et al. 2005). H3K9me2 are detected throughout the nucleus, except for nucleoli and pericentric heterochromatin (Rice, Briggs et al. 2003). H3K27me3 is considered as a 'bivalent domain' in many developmental genes, which means that they are poised for transcription. H3K27me3 is involved in the repression of imprinted genes (Kalantry, Mills et al. 2006). It also stabilises inactivated X chromosome (Xi) to protect it from differentiation-induced reactivation (Kalantry, Mills et al. 2006). Both H3K9me2 and H3K27me3 dynamics are landmarks in mouse PGC development.

Histone modification coincides with reprogramming of PGCs during cycle arrest at G2 phase. Mouse H3K27me3 methylation is strong from 8.25 to 10.5 dpc, which is preceded by erasure of H3K9me2 methylation at 7.5-8.75 dpc (Leitch, Tang et al. 2013). Similarly, porcine PGCs show abundant H3K27me3 methylation during E15-21, while H3K9me2 methylation is wiped from E15 to E21, when it is completely erased. Increasing number of E17 PGCs are observed under G2 phase, while E22-E31 PGCs presents normal cell cycle (Hyldig, Croxall et al. 2011). Reactivation of female inactivated X chromosome involves H3K27me3 as well. From 6.5 dpc, the nuclear H3K27me3 foci and some random locations of X chromosome are inactivated. The nuclear H3K27me3 foci gradually dilute during migration from 7 to 13.5 dpc (Leitch, Tang et al. 2013).

Consistent with the reduction of H3K9me2, Ehmt1 (also known as Glp) and Ehmt2 (also known as G9a), essential components to the activity of the H3K9 methyltransferase (HMT) complex, show dramatic decreases in 7.25 dpc or maintains consistent level throughout specification (Yabuta, Kurimoto et al. 2006; Surani,

Hayashi et al. 2007). GLP protein is abrogated from 7.75 dpc (Yamaji, Seki et al. 2008).

At 6.25 dpc, presumptive PGCs suppress expression of somatic genes, although the epiblast cells are permissive for gene transcription, demonstrated by abundant H3K4me2 and rare phosphorylation of RNA polymerase II. In pluripotent stem cells, somatic genes are suppressed by H3K27me3 instead of being transcriptionally silenced (Surani, Hayashi et al. 2007). Prmt5 symmetrically dimethylates arginine3 on histone H2A and/H4 tails (H2A/H4R3me2s). As a result, H2A/H4R3me2s increases at 10.5 dpc (Hajkova, Ancelin et al. 2008). Some members of the polycomb group of genes, such as Ezh, Eed and Suz12, are key components of the PcG complex which is involved in trimethylation of H3K27. They keep constant transcription levels during 6.75-8.25 dpc in both PGCs and somatic neighbouring cells (Yabuta, Kurimoto et al. 2006).

In addition to modifications of Histone 3, mouse PGCs lose linker H1 and replace nucleosomal histone at genital ridges.

1.7 Culture conditions of pluripotent ES, differentiated PGCLC, and seminoma cells.

As shown in Figure 8, different pluripotent cell lines originate from different sources. ES cells come from the inner cell mass (ICM) of preimplanted embryos. Early post-implantation epiblast maintains primed pluripotency, which enables epiblast stem cells (EpiSC) to be derived. Mouse embryonic germ cells (EGC) have been successfully derived from migratory PGCs (Leitch, Nichols et al. 2013). Spermatogonial germ stem cells (maGSC) are derived from adult male gonadal PGCs. Various adult somatic cells have been successfully induced to pluripotent stem cells (iPSC).

In this section, cell lines that share similarity with PGCs, such as PGC-like cells (PGCLC) induced from ES, and the Tcam2 carcinoma cell line will be introduced.

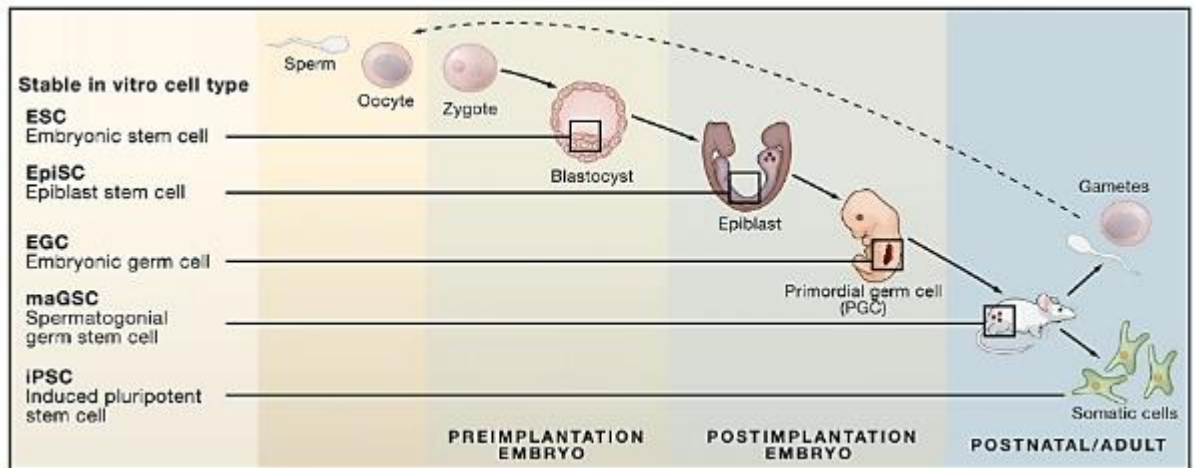


Figure 9. Developmental resources of pluripotent cells (Hanna, Saha et al. 2010).

ESC are derived from the ICM (inner cell mass) of preimplanted blastocysts. **EpiSC** are derived from post implanted pluripotent epiblast. **EGC** are derived from post implanted migratory PGCs. **maGSC** are derived from adult spermatogonia, which make a contribution to gametes in vivo. **iPSC** are derived from somatic cells of adults.

Maintenance of pluripotency in Mouse ES cells require leukaemia inhibitory factor (Lif) and Bmp4, or 2i medium. Lif induces Stat3 phosphorylation, which activates transcription of target genes in ES cells (Niwa, Ogawa et al. 2009). In contrast, human ES cells also need FGF2 as well as these cytokines (Surani, Hayashi et al. 2007). Base on serum medium, 2i medium is optimized for mouse ES cell culture (Ying, Wray et al. 2008), with the 2i indicating the use of inhibitors of mitogen activated protein kinase (Smith, Chan et al.) and glycogen synthase kinase3 (GSK3). Compared with serum-containing medium, the 2i system affects the bivalence of genes. Significantly less H3K27me3 is observed at promoters, along with decreased expression of lineage specific genes and bivalent domains, while the H3K4me3 level is not altered (Chen and Dent 2014). The transcription of *Oct4* is regulated by distal enhancer. The naïve pluripotent mES cells maintains at pre-inactivation X chromosome state and undertaking global reduction in DNA methylation (Gafni, Weinberger et al. 2013). Without 2i/LIF condition, the naïve pluripotent mES cells differentiate into primed pluripotent cells, which are reminiscent of post-implantation epiblast (Gafni, Weinberger et al. 2013).

Unlike the mES, hES in 2i/LIF condition share some molecular similarity with naïve mES, but they possess similar epigenetic properties to epiblast stem cells (EpiSCs) (Gafni, Weinberger et al. 2013). This difference confers different attributes to ESC.

Much effort was needed to optimize the culture conditions to maintain naïve pluripotency in human ES (Koh, Yabuuchi et al.) cells. NHSM (naïve human stem cell medium) has proved effective, with successful conversion of blastocyst to human iPS (hiPS). Pluripotent markers and mature teratomas were generated from NHSM adopted hiPS and hES cell lines. Compared with primed pluripotent hES, naïve pluripotent hES cells are in pre-X inactivation state, absence of H3K27me3 on nuclear foci and X-inactive specific transcript (XIST) transcription on one allele. In NHSM medium, p38i (SB203580, SB202190 or BIRB796), JNKi (SP600125), ERK1/2i (PD0325901) are used to maintain naïve pluripotency. TGFβ1 is supplemented to antagonize the pro-neural differentiation of ERK1/2i. LIF/ STAT3 is required, GSK3βi (CHIR99021), insulin, bFGF, N2 supplement are also added in this medium. To improve viability and growth of cells, ROCKi and PKCi are added for the first 24hrs of passage (Gafni, Weinberger et al. 2013).

NHSM is further developed into 4i medium, containing the 4 inhibitors, GSK3βi (CHIR99021), ERK1/2i (PD0325901), p38i (SB203580) and JNKi (SP600125), which are supplemented with LIF, bFGF and TGFβ1 in serum free knockout DMEM. ROCKi (Y27632) is also used to secure high survival for the first 24 hrs. hES cells in 4i condition form nicely dome-shaped colonies and enable differentiation to hPGCLC on addition of BMP2 and/or BMP4, along with LIF, SCF and EGF (Irie, Weinberger et al. 2015).

To study the mechanisms of PGC specification, human PGC-like cells (hPGCLC) have been induced from hESC. hESCs are cultured in four-inhibitor-containing medium (4i), including GSK3i (CHIR99021), MEKi (PD0325901), p38 MAPKi (SB203580), and JNKi (SP600125), as well as LIF, bFGF, and TGFβ. This medium maintains hESC in a distinct pluripotent state (Gafni, Weinberger et al. 2013), which is similar to early mesoderm environment. If the cells are given induction signals, BMP4, LIF, SCF, and EGF, this leads to differentiation, such that the 4i hESC are induced into hPGCLC. hPGCLC induced by this method share early PGC genes and

pluripotency genes with gonadal hPGCs. This in vitro PGCLC induction system is robust and a valuable tool to further investigate human PGC specification (Irie, Weinberger et al. 2015).

Some multipotent embryonic cancer cells from germ cell tumours (GCTs) can be used to derive teratocarcinoma cells, which are capable of giving rise to three germ layers (Kleinsmith and Pierce 1964) and can even make a contribution to mouse blastocyst (Illmensee and Mintz 1976). This suggests that tumor cells share similarities with ESCs. In testes, GCTs are predominantly diagnosed in relatively young patients, including neonates, infants, and young adults. GCT can be divided into two types, seminomas and non-seminomas. Seminomas resemble undifferentiated primitive germ cells, while the non-seminomas show various degrees of differentiation (Ulbricht 1993; Chaganti and Houldsworth 2000). Some core stem cell genes are expressed in seminomas, such as OCT4 (Looijenga, Stoop et al. 2003) and NANOG (Almstrup, Hoei-Hansen et al. 2004). Hence Seminoma cells are a similar in vivo cell type to PGCs.

1.8 Hypothesis and Aims

The evidence presented above shows that the mechanisms of PGC specification differ between humans and mice. The reasons for the divergence are difficult to elucidate, however it would be important to establish which mechanisms are conserved between mammals. This will help us gain a better understanding of the evolutionary history of speciation in mammals, but also help us identify an alternative animal model to study germ cell biology in vivo, since accessing early human embryos is not possible. Pig and human embryos share many embryological features, such as formation of an embryonic disc, lack of ExE, and delamination of mesoderm before primitive streak formation. Pig mesoderm also produces BMP4 (Valdez Magana, Rodriguez et al. 2014), suggesting that PGC may be induced from a population of mesodermal progenitors. Thus, I hypothesize that pig and human embryos have shared PGC specification mechanisms, which may define the conserved mechanism of PGC specification of mammals.

To test this hypothesis I aim to study three aspects of germline development: 1- establish the genetic regulatory network, 2- epigenetic reprogramming events, and 3-

the signalling pathways involved in PGC specification. These relate to the following aims:

1. Characterize the PGC transcription regulatory network in germ line cells from bilaminar stage (~E10) to gonadal stages (~E70) in both genders, particularly focussing on the PGC specification period (E10-E14). This will involve establishing the expression profile of key candidate genes, including NANOG, SOX17, BLIMP1, OCT4, TFAP2C, T, Sda/GM2, and exploring novel transcriptional regulators using single cell RNA-seq at LPS (~E12.5-13). Key differences in expression of transcriptional factors between pig, human and mouse PGCs should help reveal the mechanism of pig PGC specification.
2. Identify the epigenetic reprogramming events in germ line cells, particularly focussing on the PGC specification period.

DNA methylation-related features, 5-hmC, 5-mC, TET1 and UHRF1 will be studied to investigate DNA demethylation mechanisms in PGCs. This investigation will elucidate whether the epigenetic profile of PGCs is inherited from progenitors or re-established during specification. The demethylation dynamics should also help to explain the mechanisms of PGC reprogramming, which may help in the derivation of embryonic germ (EG) cells.

Histone modifications H3K9me2 and H3K27me3 marks undergo significant remodelling in the mouse germ line. Methylated H3K9 associated with UHRF1 is also linked to the maintenance of DNA methylation (Rothbart, Krajewski et al. 2012). What is the dynamics in pig germ line? Whether the H3K9me2 and H3K27me3 alteration is similar to human or mouse will be studied here.

3. Signalling pathways play a key role in PGC specification. The role of candidate pathways, BMP, WNT and FGF will be studied during PGC specification using IF, single RNA-seq, in vitro PGCLC differentiation from hESC and from pig epiblast cultures.

Chapter2

2. General Material and Methods

2.1 Embryo and tissue collection

On the fourth day after weaning sows were artificially inseminated (AI) twice (12 hours apart). The sows were then slaughtered between Days 10 and 17 after AI, depending on the experiment (see below for experimental details). The reproductive tracks were transported to the laboratory immediately after slaughter.

Embryos between Days 10 and Days 15 were flushed from the uterine horns with 30-40 ml warm PBS (supplemented with 1% FCS). Between D7-25 embryos were recovered per animal and these were transferred into a Petri dish for examination classification and sampling under a stereomicroscope. For Day 10 to Day 12 embryos, spherical (5 mm) to ovoid (5-8 mm) embryos can be manually collected using plastic Pasteur pipettes. More advanced stages (Tubular and filamentous stages) are also found around Day 12 and need to be untangled carefully using needles to avoid damaging the embryo proper. For Days 13-15 embryos, more advanced trophoblast need to be untangled to expose embryos before cutting off the excess tissue (trophoblast). Embryos between Day 16-18 begin to implant and therefore they are retrieved by cutting the uterus longitudinally from the antimesometrial side to expose the embryos, which are normally found between the endometrial folds. Day 30 and beyond fetuses have well developed gonads, which can be dissected prior to dissociation.

After catalogue sizes and stages of all embryos, and washing with DMEM-F12 (HEPES+), they were transferred to either fixative for immunohistochemistry or DMEM-F12 (HEPES+) for transportation at 37°C in a portable incubator. If not dissociate right away, gonads can be kept in 10% complete DMEM at 4°C overnight.

2.2 Embryo processing for immunofluorescence (Koh, Yabuuchi et al.)

Embryos are kept in fixative (4% paraformaldehyde (PFA) in 1xPBS/DEPC treated H₂O) at least overnight (ON) at 4°C and then transferred to methanol at -20°C for long term storage. Fixed embryos were rinsed twice in 1xPBS to remove excessive PFA.

For the less advanced embryos, 2% agarose was used to embed embryos. Agarose blocks were then trimmed and dehydrated with increasing concentrations of ethanol (70%, 90% and 100%) for 2 hours each step. After dehydration, blocks were processed in xylene, hot paraffin wax for 2 hours and then cooled down in metal mould in the desired orientation. Day 14-15 embryos can be dehydrated without agarose embedding, except for shorter processing time for 1 hour per step.

All blocks were cut into 4 μm thick sections and mounted onto superFrost™ plus slides. Sections were air dried overnight and immunohistochemistry was conducted subsequently.

When cryosection is used, fixed embryos are incubated in 30% sucrose/PBS overnight, leaving on rocker or fridge. Before mounting, mark the embryo orientation and location on disposable mould. Place embryos onto marked position and put OCT compound onto embryos. Add isopentane onto glass beaker. Dip glass beaker into liquid nitrogen until white spots appears, which indicates the temperature is ready to freeze embryos. Give sufficient time to let OCT compound to become solid. If blocks are not used immediately, wrap block with parafilm and foil, avoiding evaporation from OCT blocks. Cryosections are cut at 6 μm onto superfrost plus glass slides. Sections are left air dry for 1-2 hours before IF. If not used right away, the slides can be kept in -80°C air tight.

2.3 IF on sections

Sections were selected and dewaxed with xylene for 30 mins and rehydrated with decreasing concentrations of ethanol (100%, 90% and 70%) 15 mins each step and transferred to 1xPBS for 15 mins. Antigen retrieval was then performed with boiling 0.01M Citrate Buffer (PH6.0) for 10 mins. After the citrate buffer cooled down, sections are permeabilized with 1% Triton-100/1xPBS for 15mins. Triton-100 was washed three times for 5 mins each, and blocked with 5% BSA or 10% Donkey serum/5% BSA/PBS for 1-1.5 hours. After blocking, sections were incubated with desired primary antibody overnight at 4°C in humidity chamber. Slides were washed three times with 0.1% Tween-20/1xPBS. Finally slides were incubated with fluorescent-conjugated secondary antibody for 40 minutes. Wash off excessive secondary antibody in dark place after incubation. Mount sections with

FluoroshieldTM with DAPI histology mounting medium (Sigma, F6057) and seal with nail varnish. Slides can be kept at -20 °C until observed.

Whole mount IF

Fixed embryos were washed three times 5 minutes each with 1xPBS to remove fixative. Epitopes were exposed with 0.01M citrate Buffer (pH6.0) in microwave oven boiling for 10 minutes. After cooling down, the embryos were washed three times with 1xPBS for 5 minutes each time. Permeabilization was then performed with 1% Triton-100/1xPBS for 10 minutes. Triton was washed with 1xPBS and epitopes blocked with 5% FBS for 1.5 hours. Whole embryos were then incubated with desired primary antibody at 4°C overnight. After incubation, excessive primary antibody was washed off and pertinent secondary antibody was applied onto embryos to incubate 40 minutes in the dark. Slides were washed and mount embryo with FluoroshieldTM with DAPI histology mounting medium (Sigma, F6057) onto slide. Coverslips were sealed with nail polish and observe after 30minutes or keep at -20 °C.

2.4 Single cell dissociation and FACS sorting for single cell RNA-seq

Dissected gonads are washed in 1%BSA/PBS. The tunica albuginea is removed from male gonads as much as possible in PBS/1%BSA without damaging the tissue. Tissue pieces were collected with TryPLE into Eppendorf tubes. Incubate at 37 °C. Vortex well and pipette every 5-10 mins. Collagenase IV (2mg/ml) can also be used in this step. Dense cell suspension should be prepared in 30-40 mins. Top up to 12 ml with 10% FSC/5%BSA and pass cell suspension through 40 µm mesh. Pass through is centrifuged at 300xg for 5mins and resuspended with 100µl 10% FSC/5%BSA. Apply desirable primary antibody, incubate for 1 hour at 4°C. Wash abundantly antibody with 900µl 1%BSA/PBST, and spin for 5 mins. Resuspend pellet with applicable secondary antibody suspension. Incubate cells on ice and transport to MoFloTM XDP. To achieve high purity, cells can be passed twice. After sorting, centrifuge cells, and freeze as soon as possible at -80°C.

2.5 Pig Gonads collection, PGC dissociation and purification for cell culture

2.5.1 Pig gonads collection and dissociation

Uteri of day 25-70 gestation were collected from local abattoir. Foetuses and gonads were dissected in laboratory. Age of foetus was estimated according to crown-rump length formula. Gonadal germ cells dissociation method refers to buffalo and pig testis germ cell purification reported by (Goel, Reddy et al. 2010; Kim, Cho et al. 2010). Germinal epithelium of dissected gonads was removed. The gonadal tissue was chopped into small pieces with scalpel. Cells were dissociate with 2mg/ml Collagenase IV at 37°C. The ratio of enzyme volume (ul): tissue weight (mg) was kept at 5. For example, use 130 ul enzymes for 26 mg gonad. Tissues were vortexed every 5 minutes, until homogenous single cell mixture was obtained. If clumps remained, they were removed and dissociated with 0.05% trypsin at 37°C for less than 5 minutes. DNase I was added at a ratio of 1µl DNase/0.3 million cells. Lysate was observed under microscope, making sure single cell lysate was obtained. If big clumps remained, they were picked and trypsinize for 5 minutes. The whole process of dissociation should be no longer than 45 minutes. The enzyme was inactivated with 10ml 10%FBS/DMEM and filtered through 40 µm cell strainer three times. Pass-through was centrifuged at 300 xg for 5 minutes.

For the first time of dissociation, cell viability was evaluated by 0.4% trypan blue (pH7.2) using a haemocytometer. Once cell viability for this dissociation method was worked out, this step was skipped for the following trials.

2.5.2 Pig gonadal PGC purification by differential attachment

Resuspend cells were seeded onto 0.1% gelatin coated 60mm petri dishes at the density of 2×10^5 cells /cm². Incubate for 4 hours and transfer floating cells onto a second dish to incubate for 2 hours. The unattached cells will be collected with DMEM/F12 to perform primary culture.

2.5.3 Pig gonadal PGC enrichment by density gradient separation

50% Percoll solution (50ml) is made up with 25ml percoll solution, 1ml 10x PBS, 500µl FBS, 250µl penicillin/streptomycin (0.5%), 250µl phenol red and 19ml H₂O. Iso-osmotic dilution (50ml) is made with 50ml 1xPBS, 500µl FBS and 250µl

penicillin/streptomycin. Prepare different percoll gradients with 50% Percoll solution and iso-osmotic dilution. 2ml of each percoll gradient is applied in 15ml tube. Percoll gradients of 20%-30%-40%-50%, 25%-30%-35%, 25%-30%-40% were tested.

2.5.4 Pig gonadal PGC purification by magnetic beads

Resuspend the pellet with 100µl 10% donkey serum/5%BSA/DPBS in 1.5ml Eppendorf tube. Incubate cell suspension with CT-1 primary antibody for 30 mins at 4°C. Wash unbound antibody three times with ice-cold MACS buffer (DPBS with 0.5%BSA, 2mM EDTA, pH7.2) at the ratio of 1.5ml/10⁷ cells. Centrifuge 10min at 300g. Aspirate supernatant. Resuspend cell pellet in MACS buffer at the ratio of 80µl/10⁷ cells. Donkey anti mouse Alexa 488 is diluted in MACS buffer, meanwhile Rat anti-Mouse IgG MicroBeads (Miltenyi Biotec Cat.130-048-402) is added at 20µl/10⁷ cells. Mix well and incubate for 20min at 4°C. Wash cells with 1.5ml MACS buffer, centrifuge at 300g for 10min. Resuspend cell pellet with 500µl MACS buffer. Rinse MS column with 500µl MACS buffer. Apply cell suspension onto column. Collect unlabelled cells in pass through in Eppendorf tube and wash column three times with 500µl MACS buffer. Remove column from separator and place column on a new collection tube for CT-1 positive cells collection. Apply 1ml MACS buffer onto MS column, flush out labelled cell by firmly pushing the plunger into column. Collected positive and negative cells can be fixed or FACs or IHC.

2.5.5 Slides coating

For centrifuge and immunostaining of dissociated single PGC, TESPA (3-triethoxysilyl-propylamine, C₉H₂₅NO₃Si) is used to coat the cover slips. Before coating, soak coverslips in soap for 3.5 hrs. Wash cover slips under 60°C running water for 1 hr. Dry cover slips in oven at 60°C. Immerse the dried cover slips in 2% TESPA/99% IMS more than 5 sec. Coated cover slips are washed in 99% IMS and then distilled water. Dry coated slides overnight at 37°C. Keep in a box until use.

2.6 Collect and culture of pig gonadal and embryonic PGCs

E17 pig embryos were collected from abattoir, washed three times with 1%FCS/1% Pen-Strep/PBS and transported to the lab in 10% complete medium/HEPES/Fungizone. On arrival of lab, the embryos were washed in 1%FCS/1% Pen-Strep/PBS. In petri dish, posterior fragment of embryos were cut and

the hind gut was pulled off along with part of the curly crump. Leave dissected guts waiting in 10% complete DMEM until all embryos are dissected. Transfer all gut fragment into PBS and chop into three sections, where were then dissociated with 500ul trypsin in 15ml conical tube. Incubate at 37°C for no more than 5 minutes, keep vortexing every 2 minutes. Inactivate trypsinization with 10ml 10% complete DMEM. Centrifuge the cell suspension for 5 min at 1500rpm. Discard supernatant and resuspend the pellet in PGC culture medium (DMEM-F12/FCS/LIF/bFGF). Seed the dissociated cells onto feeder cells at the ratio of two 2cm² wells per starting embryo. The day of collecting and plating PGCs is called Day0.

Different culture media and methods have been tried to culture migratory and gonadal PGCs. First method was referred to Leitch (Leitch, Nichols et al. 2013). E17 pig hind gut was dissected and dissociated with collagenase IV (2mg/ml)(GIBCO, 17104-019). Before embryos were collected, mitomycin C inactivated Sl4-m220 feeders were plated onto gelatinized 12-well plates in STO medium, at the density of 0.2x10⁶ cells / well. MitomycinC treated MEF feeders were plated onto gelatinized 4-well plates in STO medium, at the density of 0.2x10⁶ cells / well. Dissociated gut cells were plated onto feeder cells on Day0. Early morning on Day1, check contamination and top up 500ul PGC culture medium, without shaking plates. Day2, replace 1ml DMEM-F12/FCS/LIF/bFGF with fresh DMEM-F12/FCS/LIF, because bFGF is required in the first 48hrs. On Day3, swap 50% old medium. On Day4, adapt cells with N2B27/2i/LIF medium, and keep feeding cells every other day. Primary cells should be supplied with fresh feeders every 7-10 days before feeders detach and roll over.

Medium: DMEM/F12 (1:1) (GIBCO, 11320-033), NEUROBASALTM Medium (GIBCO, 21103-049), N-2 Supplement (100x) (GIBCO, 17502-048), B-27 Supplement (50x) (GIBCO, 17504-044). Cytokines: Human LIF (PEPROTECH, 300-05), Human SCF (PEPROTECH, 300-07). Inhibitors: PD0325901 (TOCRIS, 4192), CHIR99021 (TOCRIS, 4423).

2.7 Mitomycin C inactivation

MitomycinC (Sigma, M4287) was reconstituted in medium at 10ug/ml as stock. The stock can be stored in the dark at 4°C for up to 6 month. Alternatively, the stock can be kept at -20°C for long-term storage.

Passage MEF or Sl4-m220 cells in flasks at desired densities. Treat cells when 90% confluence is reached. Aspirate medium and replace with mitomycin C stock medium. Incubate cells for 2.5-3 hrs. After treatment, aspirate medium and wash three times with PBS. Detach cells with trypsin/EDTA solution (Gibco, 25300-054) and inactivate with culture medium. Collect all cells into conical tube to centrifuge. Aspirate medium and resuspend cells with cell culture medium. Count cell number and plate desired number of cells into each well. Freeze the rest cells with the same volume of 20% DMSO/FCS in -80°C.

2.8 hESC culture and hPGCLC differentiation

H9 hESC cell line was cultured on MMC (TOCRIS bioscience, 3258) inactivated MEF feeders in 4i medium (Irie, Weinberger et al. 2015). Knockout DMEM was supplemented with 20% knockout serum replacement (KSR, GIBCO, 10828-028), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β -mercaptoethanol (all from GIBCO), 20 ng/ml Recombinant Human LIF (Peprotech, 300-05), 8 ng/ml bFGF (Peprotech, 100-18B), 1 ng/ml TGF- β 1 (Peprotech, 100-21), 3 μ M CHIR99021 (TOCRIS bioscience, 4423), 1 μ M PD0325901 (TOCRIS bioscience, 4192), 5 μ M SB203580 (TOCRIS bioscience, 1202), and 5 μ M SP600125 (TOCRIS bioscience, 1496). TrypLE Express (GIBCO, 12604-021) was used to passage cells every 3-5 days. 10 μ M of ROCK inhibitor (Y-27632, TOCRIS bioscience, 1254) was added for the first 24 hr after each passage.

For PGCLC differentiation, dissociate cells with TrypLE and seed 3,000 cells/well in 200 μ l PGCLC medium into ultra-low cell attachment U-bottom 96-well plates (Corning, 7007). PGCLC medium is composed of Glasgow's MEM (GMEM, GIBCO, 11710-035), 15% KSR, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 100 U/ml Penicillin-0.1 mg/ml Streptomycin, 2 mM L-Glutamine, 1 mM Sodium pyruvate, 500 ng/ml BMP4 (R&D Systems, 314-Bp-01M), 1 μ g/ml human LIF, 100 ng/ml SCF (R&D Systems, 300-07), 50 ng/ml EGF (Peprotech, AF-100-15), and 10 μ M ROCK inhibitor.

2.9 hPGCLC pathway determination

PGCLC differentiation medium was supplemented with desired pathway inhibitors: 2.5 μ M WNTi (IWR-1-endo, Selleckchem, S7086), 10 μ M PI3Ki (LY294002,

Selleckchem, S1105), 100nM FGFRi (PD173074, TOCRIS bioscience, 3044), 1 μ M MAPKi (PD0325901, TOCRIS bioscience, 4192), 5nM EGFRi (Erlotinib, CST, 5083). Aggregated cells are pre-inhibited in desired inhibitors for 30 minutes before PGC induction medium is added. D1 and D4 hPGCLC were sacrificed for IF and quantification.

2.10 Epiblast culture and pathway determination

E11.5 Embryos were flushed out of reproductive track with warm 1%BSA/PBS. All embryos were washed three times in 10%FCS/15mm Hepes/DMEM/F12 and transported in the same medium in incubator at 37°C. In the lab, embryos are washed in PBS and dissected in fume hood. Epiblasts were neatly dissected and pre-incubated in basal supplemented with individual inhibitors, including 2.5 μ M WNTi (IWR-1-endo, Selleckchem, S7086), 100nM BMPi (LDN193189, SIGMA-ALDRICH, SML0559), 100nM FGFRi (PD173074, TOCRIS bioscience, 3044), and 1 μ M MAPKi (PD0325901, TOCRIS bioscience, 4192). 100ml Basal medium is made up with 80.8ml GMEM, 15ml KSR, 1ml NEAA, 1ml Pen/Strep, 1ml L-Glutamine, 1ml Sodium Pyruvate, and 200 μ l β -mercaptoethanol. After pre-inhibition, cytokines were supplemented to the final concentration of 500ng/ml BMP, 100ng/ml EGF, 1000ng/ml hLIF and 100ng/ml SCF. Epiblasts were incubated for 64 hrs and then sacrificed for IF against antibody combinations NANOG/SOX17, NANOG/BLIMP1, NANOG/OCT4, SOX17/TFAP2C, and NANOG/T. 5 combinations were applied rotatory to make sure successive sections were stained with different combinations. All sections were stained and utilized for quantification. Double positive cell numbers in each combination and embryos found with double positive cells were counted manually.

2.11 RNA extraction and reverse transcription

RNA was extracted with NucleoSpin® RNA (MACHEREY-NAGEL, 7409551250). Cells from 1 well of 6 well-plate were collected in 350ul RA1 buffer and kept at -80°C until use. Concentration of RNA elution was tested with Nanodrop 2000c UV/IV Spectrophotometer. RNA extracted from EBs are normally very low, therefore sometimes skip nanodrop to do revers transcription immediately.

If the total amount of RNA is between 50ng and 2ug, Omniscript RT Kit (QIAGEN, 205110) was used for reverse transcription. If the RNA amount is less than 50ng, Sensiscript Reverse Transcription Kit (QIAGEN, 205211) should be used for reverse transcription.

2.12 Primers and qPCR

Table 1. Pig and human qPCR primers used for single cell RNA-seq screening.

Gene	Forward	Reverse	Length	Temperature (°C)	Efficiency (%)
pNanog Ex2-3	TTCCTTCCTCCATG GATCTG	ATCTGCTGGAGGCT GAGGTA	214	60	83.45
pOct43/4	GCAAACGATCAAG CAGTGA	GGTGACAGACACCG AGGGAA	201	60	80.2
pSox2	AAGAGAACCCCAA GATGCACAAC	GCTTGGCCTCGTCG ATGAAC	219	60	80.47
pSox17	TGTAACCTACCCG ACGTCTG	GCTGTTCAAGTGGC AGACAA	183	60	123.8
pGata4	AGATTCTGCATG GACTTGG	CTCAACTGGAAAGG CCTGAG	234	60	71.64
pAP2γ	GGACGGAATGAGA TGGCAA	TCAGGCTGAAATGC GACAAG	166	60	109.7
pGAPDH	GGGCATGAACCAT GAGAAGT	GTCTTCTGGGTGGC AGTGAT	162	60	91.07
pcKit	GATGCCTTCAAGG ATTTGGA	ATGGAATCTGAGGC CTTCCT	182	60	
pNanos3 Ex1-2	AGCTACACCACCC GCAACTC	AGCAGGGAGACGGA GAAGTT	157	60	94.76
pBlimp1	ACACAAATCCAGA GCCATCC	AGTCAGGGGTGAAA TGTTGG	162	60	102.34
hSOX17	TTCGTGTGCAAGCCT GAGAT	TAATATACCGCGGAGC TGCC	119	60	
pPOU5F1	CCTTCGCAAGCCCTC ATTTC	GAAGCTTAGCCAGGTC CGAG	168	60	
hGAPDH	AGGAGCGAGATCCCT CCAAA	TGGTTCACACCCATGA CGAA	178	50	
hβ-ACTIN	ATAGCAACGTACATG GCTGG	ATAGCAACGTACATGG CTGG	144	50	
hNANOG	TCTTCCTACCACCAGG GATGC	CACTGGCAGGAGAATT TGCC	250	60	
hBLIMP1	TGCTTGGAACACGT GTGGG	CAGGGGTGGTCGTTCA CAAT	188	60	
hTFAP2C	GCCGTAATGAACCCC ACTGA	ACCGGCCTCCATTTTTC GAT	184	60	
hcKIT	CCTGAACACCAGCAG TGGAT	CCATTCACGAGCCTGTC GTA	245	55	

Primers were reconstituted with TE buffer to 100uM stock primer and stored at -20°C. Before PCR, dilute primer stock to 10uM working solution with molecular H₂O. Ready MixTM Taq PCR reaction mix with MgCl₂ (SIGMA, P0476-20RXN)

2.13 Agarose gel electrophoresis and gel extraction

GelRedTM Nucleic Acid Gel Stain (BIOTIUM, 41003) was added to 1-1.5% agarose at the ratio of 1:20000. Silica Bead DNA Gel Extraction Kit (Thermo, K0513) and QIAquick Gel Extraction Kit (QIAGEN, 28704) were used to extract DNA from gel electrophoresis.

2.14 Primer efficiency standard curve and qPCR data analysis

For EBs and samples with little abundance of RNA, PCR amplicon was purified as template to do standard curve. 8-10 template dilutions were made. The first dilution concentration is around 10pg/μl. From the second dilution, 10 times dilute the previous dilution. Use no template reaction as negative control. Make triplicates for each concentration. LightCycler® 480 SYBR Green I Master (Roche) was used for qPCR.

Chapter 3

3. Identification of Primordial germ cells during pig embryo development

3.1 Introduction

Mammalian PGCs are induced from pluripotent cells during early embryo development. It is well established that before the onset of gastrulation, mouse PGCs are induced by BMPs primarily secreted by the extraembryonic ectoderm (Lawson, Dunn et al. 1999). In response to this signal, posterior proximal epiblast cells become lineage restricted and are segregated from their somatic neighbours, forming a PGC cluster in the extraembryonic compartment (Saitou and Yamaji 2010). In non-rodent mammals however, no equivalent structure to the extraembryonic ectoderm exists. This raises the question of what is (are) the PGC inducing signal(s) and which tissues produce them. Recent evidence shows that BMP4 is first detected in the nascent mesoderm of the pig embryo (Valdez Magana, Rodriguez et al. 2014), indicating that if BMP4 is the primary inducer of the germline in this species, then the PGC seem to be induced after the onset of gastrulation, in contrast to the findings in mice.

During mouse PGC induction and specification, Blimp1 (encoded by *Prdm1*), AP2y (encoded by *Tcfap2c*) and *Prdm14* comprise an interdependent tripartite group of factors that initiate the developmental program of this lineage (Magnusdottir, Dietmann et al. 2013). *Prdm1*, AP2y and *Prdm14* are detected in E6.5 putative PGCs. Blimp1 was found to inhibit cell proliferation and somatic regulators of transcription. Induced independently after *Prdm1*, *Prdm14* plays an important role in epigenetic reprogramming (Yamaji, Seki et al. 2008). Both *Prdm14* and AP2y were reported to preferentially target cell-motility and cytoskeleton organization genes, thus participating in germ cell specific gene activation, PGC migration, and somatic fate switch-off (Magnusdottir, Dietmann et al. 2013). Besides *Tcfap2c*, Blimp1 directly binds to repress *T* and *Dnmt3b*, which connect genetic regulation to epigenetic changes (Magnusdottir, Dietmann et al. 2013). Blimp1 interacts with diverse chromatin modifiers under various circumstances. For example, Blimp1 forms a complex with G9a, a histone H3 lysine methyltransferase, whereby it mediates the accumulation of methylated H3K9 in human osteosarcoma cell line (Gyory, Wu et al. 2004). Upon B-cell differentiation, H3 deacetylase is recruited by Blimp1 to facilitate

transcriptional repression (Yu, Angelin-Duclos et al. 2000). In mouse germ cells, Blimp1 forms a complex with Prmt5, an arginine histone methyltransferase, thus it demethylates H2A/H4R3me2s (Ancelin, Lange et al. 2006). Human *BLIMP1* is essential for specification and maintenance of hPGCLCs. mPGCLC induced from *BLIMP1*^{-/-} upregulate *OCT4*, *NANOG* and *SOX2*, but fail to maintain *T* (Sasaki and Matsui 2008). Mouse Prdm14 is a key regulator of pluripotency and epigenetic reprogramming in preimplantation embryos, PGCs and mESCs (Nakaki and Saitou 2014). From 6.5 dpc, Prdm14 is specific to the germ line (Nakaki and Saitou 2014). It represses FGF signalling (Grabole, Tischler et al. 2013) and promotes the Tet1 and Tet2 mediated active DNA base excision repair (BER) pathway in mESC and development in mPGCs (Okashita, Kumaki et al. 2014). However, human PRDM14 is reported to be unnecessary for hPGCLC differentiation (Sugawa, Arauzo-Bravo et al. 2015).

In parallel to the activation of this tripartite gene network, the expression of pluripotency genes are carefully regulated during mouse PGC specification. Nanog, Sox2 and Oct4 (*Pou5f1*), three core components of the pluripotency network, are all involved in mouse PGC specification and development. PGCs are induced from E6.5 epiblast, demonstrated by the lineage restriction marker Blimp1 (Saitou and Yamaji 2010). After this event, nascent PGC precursors lose Nanog expression transiently. At E7.5, when ~40 founder PGC cluster are established, Nanog is expressed only in pluripotent epiblast, but not in PGC. After E7.75, Nanog⁺ PGCs are observed in the PGC cluster and later in migratory PGCs (Yamaguchi, Kimura et al. 2005). In contrast, pig NANOG is observed throughout the whole PGC specification period (Wolf, Serup et al. 2011). In the mouse, Sox2 is continuously expressed during specification (Kurimoto, Yabuta et al. 2008; Aksoy, Jauch et al. 2013), migration and gonadal colonization, while in human PGC SOX2 is not detected in migratory and gonadal stages. SOX17 was instead detected in the germ line (Perrett, Turnpenny et al. 2008). Mouse and pig Oct4 are both detected from induction to germ line establishment (Kurimoto, Yabuta et al. 2008). The discrepancies between mice, pig or human pluripotency gene expression in early PGC may underlie key divergent PGC specification mechanisms.

Mouse Brachyury (T), a T-box transcription factor, plays a critical role in PGC specification. WNT3 is activated by BMP4, which provides a PGC specification context. Brachyury is directly downstream of WNT3, and it directly activates germ line determinants *Blimp1* and *Prdm14*, thus induces PGC specification (Aramaki, Hayashi et al. 2013). The function of human *T* in PGC specification is not yet revealed. Under hPGCLC differentiation conditions, *T* is highly expressed in the majority of D1 cells, and drastically downregulated in the majority of D2. BLIMP1+ PGCLCs maintain low *T* expression until at least D4 (Irie, Weinberger et al. 2015).

BRACHYURY (T) is expressed in the mouse primitive streak at 7.5-8.5 dpc, then, in tail buds, and notochord at 12.5 dpc during mouse gastrulation, playing a crucial role in mesoderm formation, posterior mesoderm movement and somite differentiation (Wilkinson, Bhatt et al. 1990; Rivera-Perez and Magnuson 2005). Similar to mouse, pig *T* is first detected in the posterior epiblast of E10-E11 epiblast, later in primitive streak, notochord and nascent mesoderm (Wolf, Serup et al. 2011). Mouse *T* is reported to directly target *Fgf8*, *WNT3a* (positive regulator of WNT pathway) and *Axin2* (negative regulator of WNT pathway), which are conserved with human *T* (Evans, Faial et al. 2012). Under BMP4 and FGF2 stimulation, hESC and mouse epiblast stem cells are induced towards mesoderm, through high levels of *T* and then *CDX2*, another mesoderm regulator (Bernardo, Faial et al. 2011). It was shown that nascent PGCs (7.25 dpc) express *T* mRNA by single cell RNA sequencing (Saitou, Barton et al. 2002). The conservation between mouse and pig Brachyury expression patterns, and the divergent function between mPGC and hPGCLC differentiation are intriguing and warrant further in vivo investigation on its role during pig PGC development.

Sox17 is a critical regulator of definitive endoderm. In mice, *Sox17* initiates endoderm differentiation during early gastrulation and then specifically regulates endoderm expansion (Hara, Kanai-Azuma et al. 2009). *Sox17*-null mouse embryos fail to expand the hindgut, leading to aberrant PGC migration. No defects were observed in PGCs or other structure development by the early somite stage (Hara, Kanai-Azuma et al. 2009). However, *SOX17* is critical for hPGCLC fate (in humans). Not only does it induce PGCLC directly, but *SOX17* precedes the activation of *BLIMP1*, which in turn represses somatic genes (Irie, Weinberger et al. 2015).

Sda/GM2, a monoclonal antibody concentrated from hybridoma supernatant, specifically binds to the trisaccharide motif NeuAc α 2–3(GalNAc β 1–4) Gal β 1-, which is common to both Sda and GM2 glycotopes. Sda/GM2 positive cells were detected in E15-E75 pig PGCs and a subpopulation of seminiferous tubular cells at the basal membrane of pig, cattle, llama and horse (Klisch, Contreras et al. 2011). Because of the PGC specificity, this marker is useful for identification of PGCs and can be used in conjunction with other antibodies. It is not clear, however, when CT-1 reacting epitopes are first detected in pig PGCs prior to E15, and so this will be investigated in this chapter.

DAZL (deleted in azospermia-like) is a germ line specific cytoplasmic RNA binding protein. Overexpression of DAZL increases PGC number in hESC XX and XY cell lines. Upon PGC differentiation, DAZL overexpression induces elongated synaptonemal complex formation in XY hESC line (Kee, Angeles et al. 2009). In mESC, Dazl enhances Tet-1 mediated demethylation and thus promotes reprogramming towards ground pluripotency (Welling, Chen et al. 2015). In nascent mPGCs, Dazl inhibits pluripotency and somatic differentiation, as well as key Caspases. Therefore if Dazl is lost, the expression of pluripotency will be prolonged, as well as apoptosis, thus the teratoma formation can be avoided (Chen, Welling et al. 2014).

The aim of this chapter is to characterize the expression profile of different markers during the specification and development of the porcine germline. Embryos were collected at different intervals between days 10 and 70, and following sections and immunohistochemistry, the profile of gene expression was established for the following genes: pluripotency genes (OCT4, NANOG, SOX2), germ line specific genes (BLIMP1, DAZL and the Sda/GM2 glycotype), and other genes (T, SOX17).

3.2 Material and Methods

E10 (n=6), E12 (n=4), E13(n=12), E14(n=5), E16/17(n=11), E25 (n=4, male and female each), E31(n=3, male and female each), E42(n=6, male and female each), E50 (n=7 male and female each), E60 (n=5 male and female each) and E70 (n=3 male and female each) pig embryos were collected from the abattoir. E10-E14 embryos were flushed out from the uterus with 1% FBS/PBS. E16/E17 implanted embryos were

picked with forceps. From E25 onwards, gonads were dissected from the fetuses and fixed with 4% PFA/PBS. To fix in the right orientation, E10-E14 embryos were embedded in blocks with 2% agarose and dehydrated. E10-E14 agarose blocks and the other stage embryos or gonads were dehydrated with 70%, 90% and 100% ethanol, 2hrs for each step. After dehydration, xylene was used to replace ethanol. Then agarose blocks, embryos or gonads were immersed in hot paraffin to replace the xylene. Finally, they were moulded with hot wax. When the blocks had cooled down, 4 μ m wax sections were made and stained with desired antibodies. Primary antibodies used in this chapter: Nanog (PEPROTECH, 500-P236, 1:300), Brachyury (R&D, AF2085, 1:300), Oct-3/4 (N-19)(SantaCruz, sc-8628, 1:300), AP-2 γ (H-77) (SantaCruz, sc-8977, 1:300), Sox-2(Y-17) (SantaCruz, sc-17320, 1:300), Sox17(R&D, AF1924, 1:300), Blimp1 (eBioscience, 14-5963, 1:50), DAZL (abcam, ab34139, 1:300) and secondary antibodies Donkey anti-Mouse IgG (H+L) Alexa Fluor® 488 conjugate (Life technologies, A-21202), Goat anti-Rabbit IgG (H+L) Alexa Fluor® 555 conjugate (Life technologies, A-21428), Goat anti-Mouse IgG (H+L) Alexa Fluor® 488 conjugate (Life technologies, A-11001), Goat anti-Rabbit IgG (H+L) Alexa Fluor® 488 conjugate (Life technologies, A-21206), Donkey Anti-Goat IgG H&L (Cy3 ®) preadsorbed (abcam, ab6949) and Anti-Rat IgG TRITC (eBioscience, 26-4826-82) diluted at 1:300 concentration were applied accordingly.

3.3 Results

3.3.1 Expression of SOX17, BLIMP1, and NANOG during porcine PGC specification

To investigate the chronology of key genes in nascent pig PGCs, IF (immunofluorescence) was carried on E10-13 embryos. Before the onset of gastrulation, the bilaminar embryonic disc (E10 embryo) does not have an anterior-posterior axis (Figure 10). Immunostaining was performed against Pluripotency and PGC related genes OCT4, NANOG, SOX2, SOX17, and BLIMP1. All of these transcriptional factors should be localized to the nucleus. OCT4 and SOX2 were detected in the epiblast, whereas NANOG localized in epiblast and hypoblast (or primitive endoderm). SOX17 and BLIMP1 were detected in the primitive endoderm. No PGCs are found at this stage.

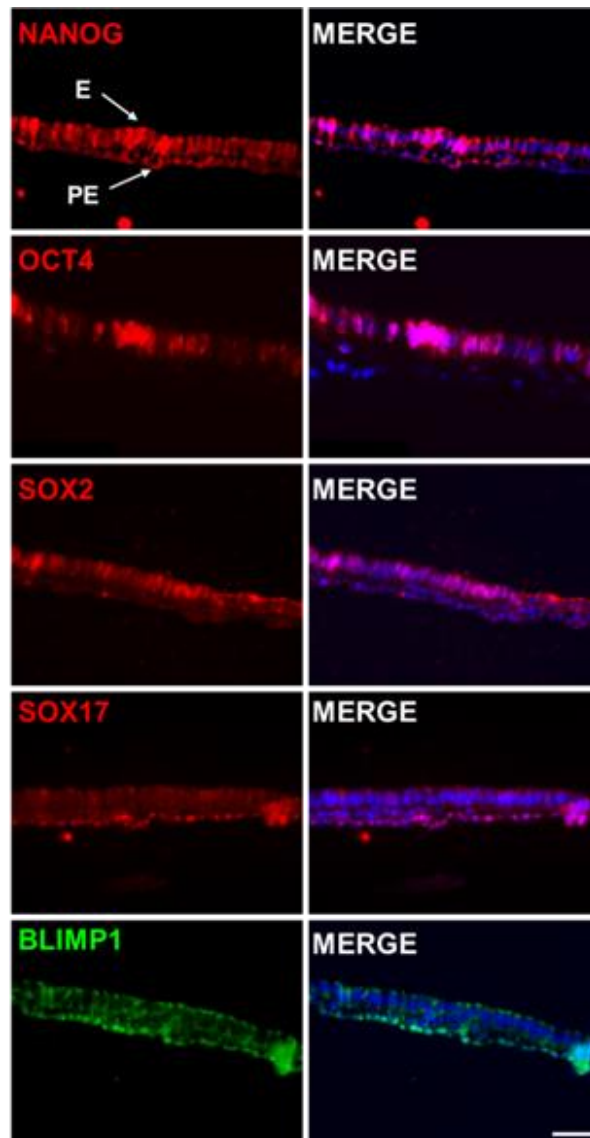


Figure 10. Immunofluorescence of E10 bilaminar embryos.

PGC related genes were detected by IF on bilaminar embryos. NANOG is detected in both epiblast and primitive endoderm. OCT4 and SOX2 are detected in epiblast. SOX17 and BLIMP1 are detected in hypoblast. E, epiblast. PE, primitive endoderm. Scale bar, 10 μ m.

In the Pre-streak stage (~E10.5-11.5) embryos, nascent embryonic mesoderm is visible (Figure 11). The mesoderm will extend towards the anterior end, separating ectoderm and endoderm. OCT4 is detected through the whole ectoderm and nascent mesoderm at this stage. NANOG is detected in all three layers and T is restricted to the posterior ectoderm and mesoderm. SOX17 is only detected in the primitive endoderm (Figure 11).

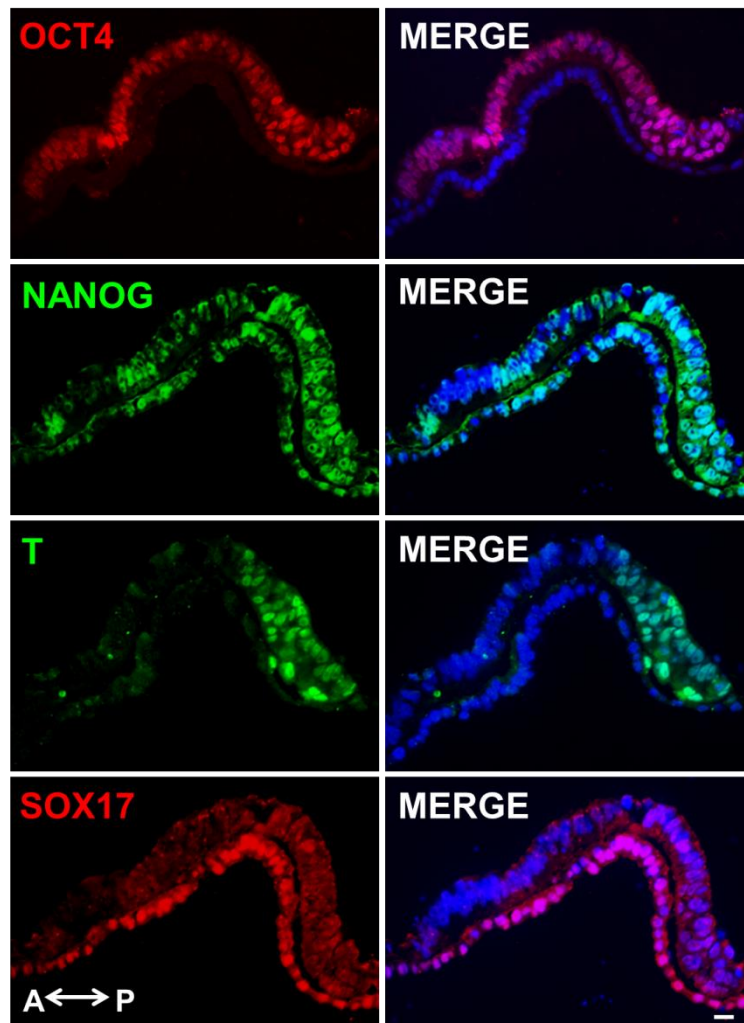


Figure 11. OCT4, NANOG, T and SOX17 expression in pre-streak stage (~E10.5-11.5) embryos.

Sagittal sections of pre-streak embryos stained with antibodies against OCT4, NANOG, T and SOX17. OCT4 was detected in ectoderm/epiblast and nascent mesoderm. NANOG is prevalently detected in the ectoderm and nascent mesoderm, but less intense in the hypoblast. T is restricted to the posterior ectoderm/epiblast and nascent mesoderm. SOX17 marks the primitive endoderm. Double arrow indicates anterior (A) and posterior (P) axis. Scale bar, 10 μ m.

Early PS (~E11.5) is the earliest stage when pig PGC clusters are identified (Figure 12). In these embryos, cells in the primitive endoderm are marked by BLIMP1/SOX17 expression. The PGC cluster localizes at the posterior end of the primitive streak and mesoderm. In the anterior mesoderm, there are several SOX17+/BLIMP1- cells (A-C, arrowheads). Considering the location and the developmental stage, these cells are presumed to be mesoderm cells acquiring definitive endoderm fate, initiating SOX17 expression as a bona fide endoderm marker. Similar transition is observed in 7.0 dpc mouse mesoderm, where endodermal

fated cells are egressing into visceral endoderm (Rivera-Perez and Hadjantonakis 2014).

The nascent pig PGC cluster is made of cells with two expression patterns of BLIMP1 and SOX17. Arrows in Figure 12 (D-F) show five SOX17+/BLIMP1- cells, while the other cells in a more posterior location in the cluster are SOX17+/BLIMP1+. Since this is the earliest observation of PGCs in the pig embryo (based on expression of these two markers), it suggests that SOX17 precedes BLIMP1 expression in PGCs. Also, there are at least 13 SOX17+/BLIMP1+ cells in addition to the 5 SOX17+/BLIMP1+ cells in this section. A total of 62 PGCs were found from 5 sections. Figure 12 is the representative embryo out of 9 embryos.

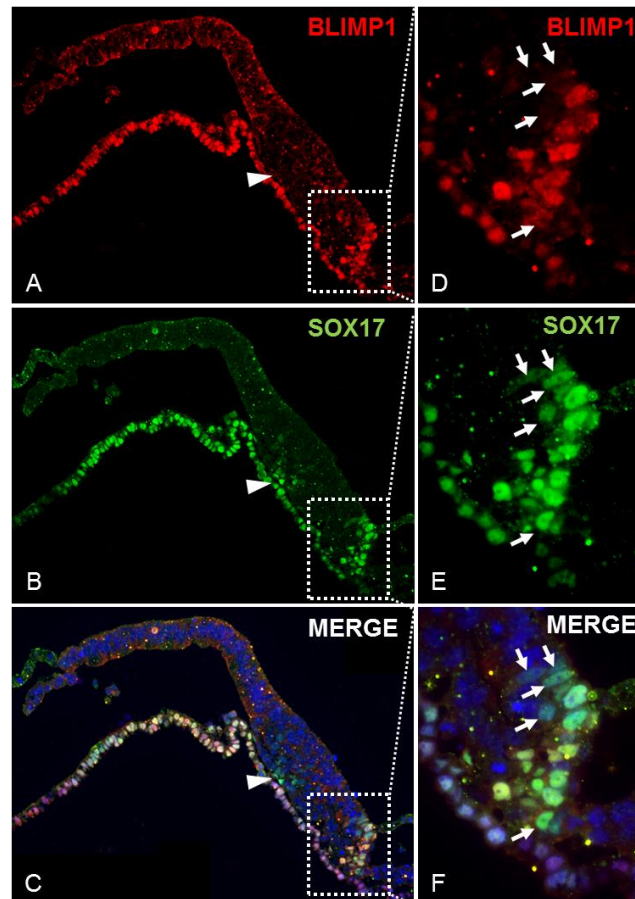


Figure 12. BLIMP1 and SOX17 expression in early primitive streak (EPS) stage PGCs (~E11.5).

A-C, sagittal section of EPS stage embryos. Arrowhead marks the anterior primitive streak area with SOX17+ cells contributing to the DE, which are BLIMP1+/SOX17+. **D-F,** Close-up image of PGC cluster area (dashed line in A-C). Arrows indicate five SOX17+/BLIMP1- cells.

As shown in Figure 13 (A-C), colocalized NANOG-high and SOX17 indicate presumptive PGCs. Nuclear NANOG expression in PGCs is consistent with a previous report (Wolf, Serup et al. 2011). Besides ectodermal cells and PGCs, nuclear NANOG is also detected in endodermal cells. Due to the specificity of NANOG antibody, cytoplasmic background is observed throughout mesoderm. Three SOX17⁺/NANOG⁻/weak cells are detected in the PGC cluster (D-F, indicated by arrowheads). The spatiotemporal localization of these markers suggests that SOX17 activation precedes the upregulation of NANOG in PGC precursors. Consistent with observations in EPS embryos (Figure 13), SOX17⁺ cells in the PGC cluster can be BLIMP1 positive (dashed circles) or negative (Rothbart, Krajewski et al.) as shown in G-I. Based on 11 sections, the size of cluster is estimated of 115 PGCs. 6 PS stage embryos were detected.

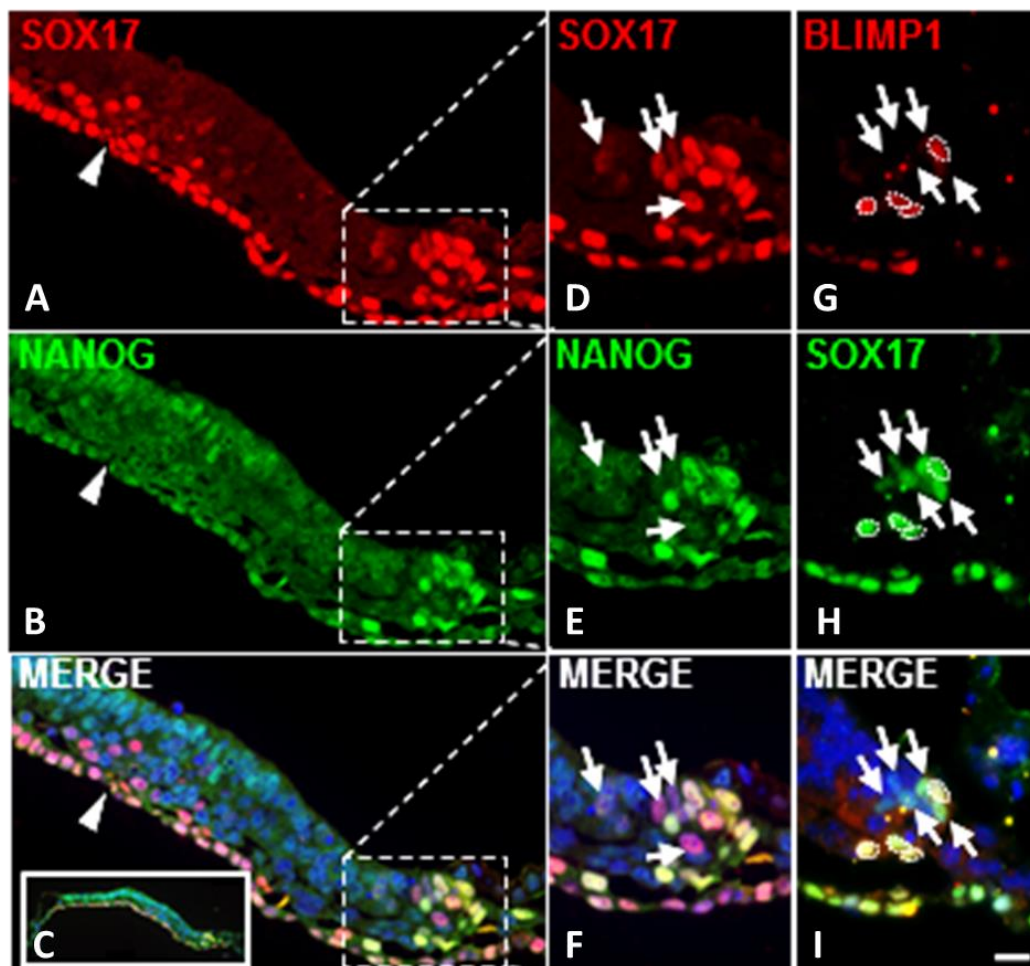


Figure 13. Immunofluorescence of primitive streak (PS) (~E12) PGC cluster.

A-C, Sagittal sections of E12 pig embryo showing the posterior primitive streak. Whole embryo sagittal section is showed at the bottom left of C. Arrowhead marks the anterior primitive streak area with SOX17 cells contributing to the definitive endoderm. The primitive endoderm layer has SOX17+/BLIMP1+ cells. The PGC cluster is found at the posterior end of primitive streak in mesodermal location. Dashed circles indicate three SOX17 weak cells in primitive streak, which could be PGC progenitors. D-F, closed-up images of PGC cluster. Arrowheads indicate SOX17+/NANOG- cells. G-I, serial section of PGC cluster. Arrows indicate SOX17+/BLIMP1- cells. Dashed circles are SOX17+/BLIMP1+ PGC precursors. Scale bar: 10 μ m.

At LPS stage (~E13), Sda/GM2 is detected in PGCs. Due to the orientation, PGCs appear like two clusters. Among BLIMP1+ and NANOG+ cells, there are Sda/GM2+ and Sda/GM2- subpopulations (B and D), which are probably PGCs and PGC precursors respectively (Figure 14).

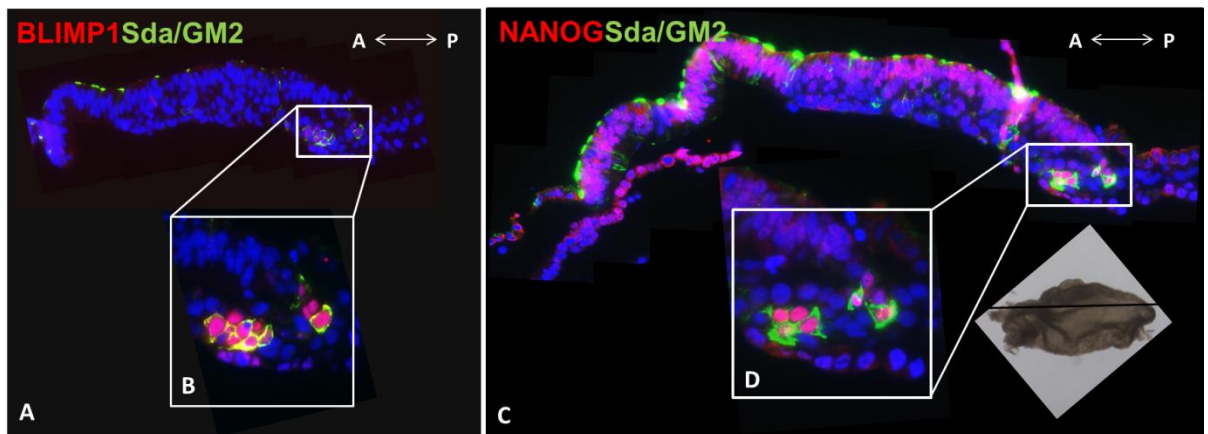


Figure 14. Immunofluorescence on LPS (~E12.5-13) PGCs.

Sagittal section was stained for BLIMP1, NANOG and Sda/GM2 (A and C). The close-up in B and D shows the PGC area. Double arrows indicate anterior (A) and posterior (P) end of the embryo.

At the late primitive streak (LPS) stage (~E13), a distinct PGC cluster is observed at the posterior end of embryonic mesoderm (Figure 15). Within this cluster, BLIMP1, SOX17, Sda/GM2, TFAP2C, and NANOG are activated while T is repressed compared with the surrounding mesoderm and primitive endoderm. T, as a bona fide mesoderm marker, is detected throughout the mesoderm and primitive streak (Figure 15 H, J, and N), which is consistent with previous results (Wolf, Serup et al. 2011). Surrounded by cells with strong T, the PGC appear to have gradually decreasing T levels towards the posterior end of the embryo. BLIMP1+ cells are colocalized with the T low cells (K). Interestingly, BLIMP1+ cells appear to become Sda/GM2+ as they reach the PGC niche (P-R). Some BLIMP1+/Sda/GM2- cells are observed at the proximal end of the cluster where primitive streak just terminates, while almost all

Sda/GM2 signals are clustered to the distal end (M-R). When Sda/GM2 is double stained with T, a narrow crescent of T- or faint/Sda/GM2- cells distinguishes the PGC cluster from T+ in primitive streak and mesoderm (Figure 15 M-O). This crescent coincides with BLIMP1+/Sda/GM2- cells (Figure 15 P-R). SOX17 is heterogeneously expressed in all PGC territory in the mesoderm (Figure 15 C-F) together with TFAP2C+ (Figure 15 G-I). Total number of 221 pre-migratory PGCs and 26 migratory PGCs are estimated based on 13 sections. 8 LPS embryos were counted.

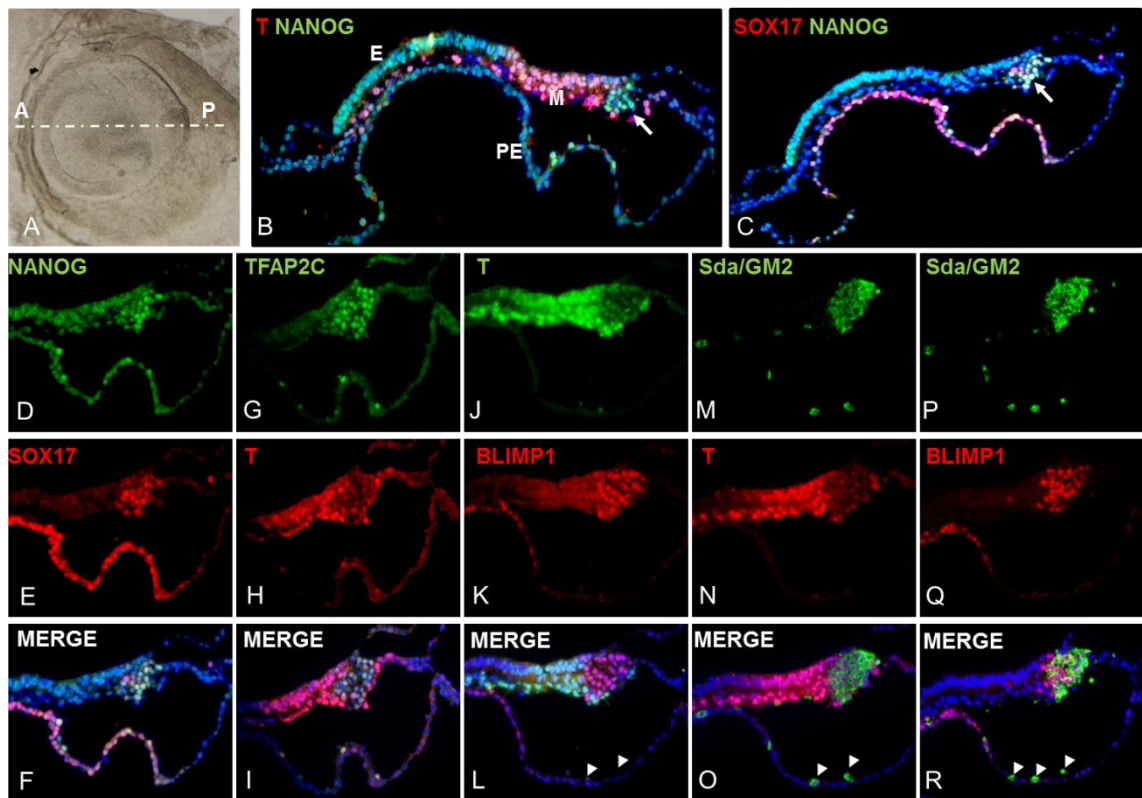


Figure 15. Immunofluorescence of LPS (~E13) pig PGC cluster.

Sections of a late PS stage embryo show a more developed PGC cluster. The PGCs are stained for NANOG, SOX17, TFAP2C, BLIMP1 and Sda/GM2. Note that T staining is reduced in the cluster. A. Dorsal view of LPS stage embryonic disc. B-C. Double staining of LPS stage embryo on sagittal section against T/NANOG and SOX17/NANOG combinations, respectively. Anterior and posterior end of embryo is labelled as A and P. D-R, Double staining of PGC cluster against desired combinations. A, anterior. P, posterior. E: ectoderm, M: mesoderm, PE: primitive endoderm.

At Neural groove stage (~E14), the PGC cluster expands while some PGCs are migrating through the hindgut endoderm (Figure 16 F). Also, there are very few PGCs migrating in other directions towards amnion. Similar to PSI to LPS stages, there are still some NANOG+/Sda/GM2- PGC precursors within the PGC cluster.

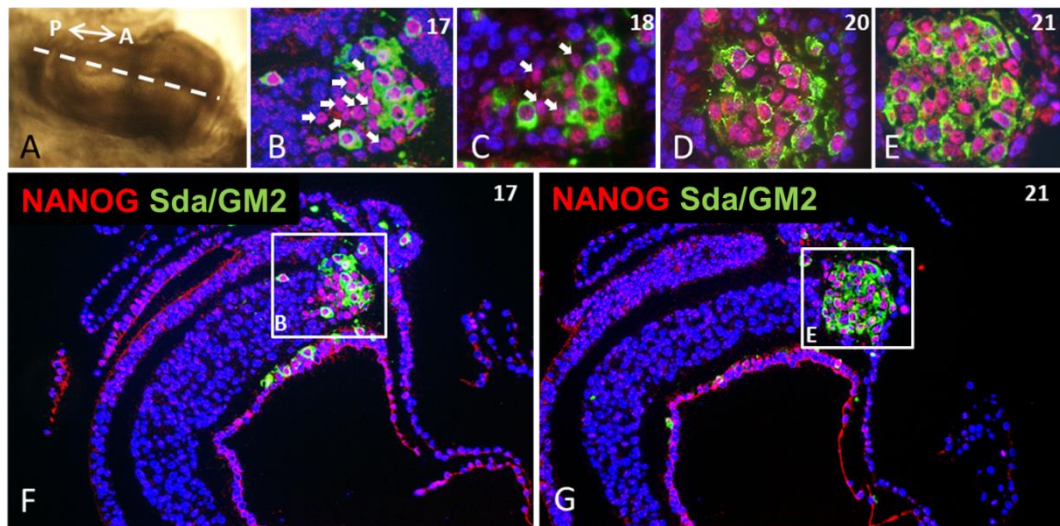


Figure 16. Immunofluorescence on NG (~E13.5) PGC cluster.

A, top view of neural groove stage embryo. **B-E**, Successive close-up images of the PGC cluster. Numbers on the top right indicate the section number in the same embryo. **F** and **G**, Sagittal section of embryo is made alongside the dashed line (**A**) and stained against NANOG/Sda/GM2 (CT-1 antibody). Quadrants **B** and **E** are zoomed in in the upper panel. **F** and **G**, NANOG/Sda/GM2 immunofluorescence on posterior embryo of sagittal sections. **A**, anterior. **P**, posterior.

As embryo development progresses, the PGC cluster expands and the number of migratory PGCs increases. T, BLIMP1, TFAP2C, OCT4, and Sda/GM2 are detected in E14 PGC cluster. PGCs express BLIMP1 and have decreasing levels of T compared to neighbouring cells (Figure 17). This pattern is consistent with LPS (~E13) stage (Figure 15). In contrast to SOX17, SOX2 is still undetectable in Sda/GM2 marked PGCs, while the antibody is validated by the strong expression in neural ectoderm (Figure 22). TFAP2C and OCT4 always co-localize at this stage. OCT4 is prevalently detected in ectoderm, mesoderm, and significantly upregulated in PGCs. TFAP2C is restricted to PGCs. T is detected in primitive streak, posterior mesoderm, and significantly downregulated in PGCs, which are marked by Sda/GM2.

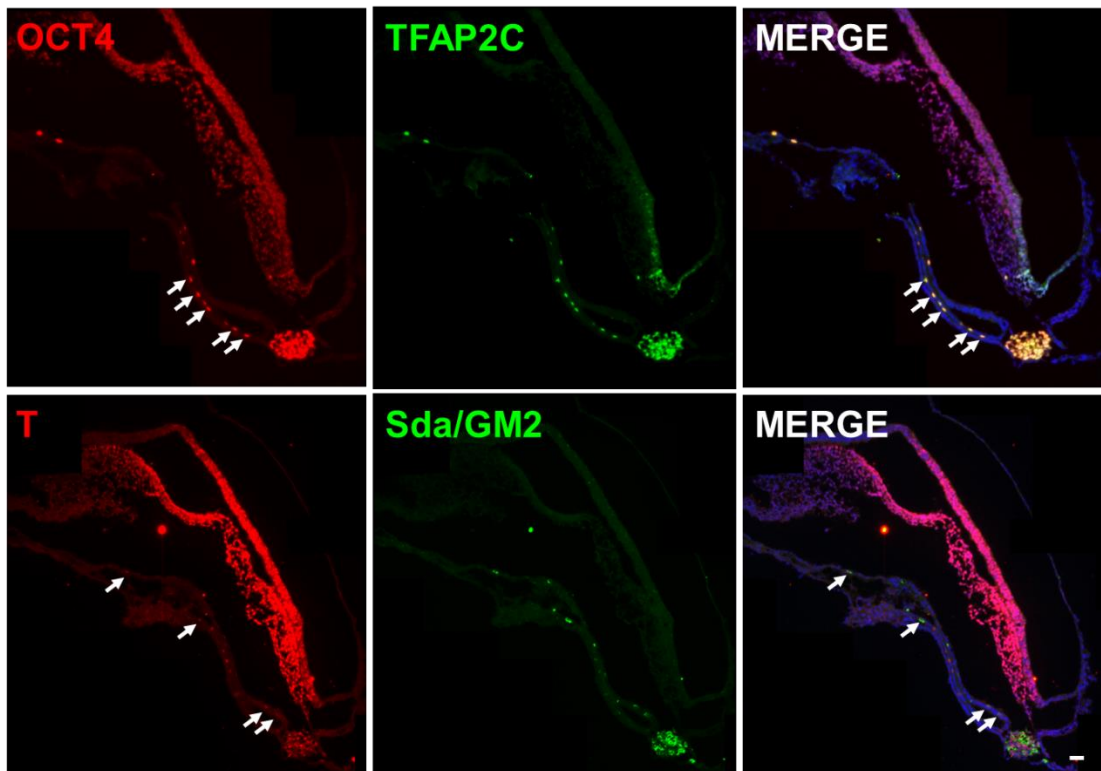


Figure 17. Immunofluorescence on posterior end of 3-somites embryos (~E14).

Sagittal sections of 3-somites embryos are stained against OCT4/TFAP2C, and T/Sda/GM2. A conspicuous PGC cluster is hanging to the posterior end of embryo. Meanwhile some PGCs are migrating towards anterior end via endoderm. Notice that OCT4 stains ectoderm and mesoderm, and significantly upregulated in PGCs. T is detected in primitive streak and posterior mesoderm, and repressed in PGCs. Arrows indicate migratory PGCs. Scale bar, 10µm.

PGC numbers at above stages (Figures 12-17) are quantified and presented in Figure 18. At early PS stage (Figure 12), nascent PGC cluster comprises 62 PGCs. PS stage has 115 PGCs in cluster (Figure 13). From Late PS stage, early migratory PGCs are spotted, as well as PGC cluster (Figure 15). There are 220 pre-migratory PGCs and 26 migratory PGCs. Neural plate (NP) stage embryos (Figure 16) have 201 pre-migratory PGCs and 61 migratory PGCs. There are 259 pre-migratory PGCs and 53 migratory PGCs observed in 3-somite stage embryos (Figure 17). As the PGC numbers are quantified based on the Immunofluorescence images with PGC cluster, there could be more PGCs migrating away from the midline that are not included.

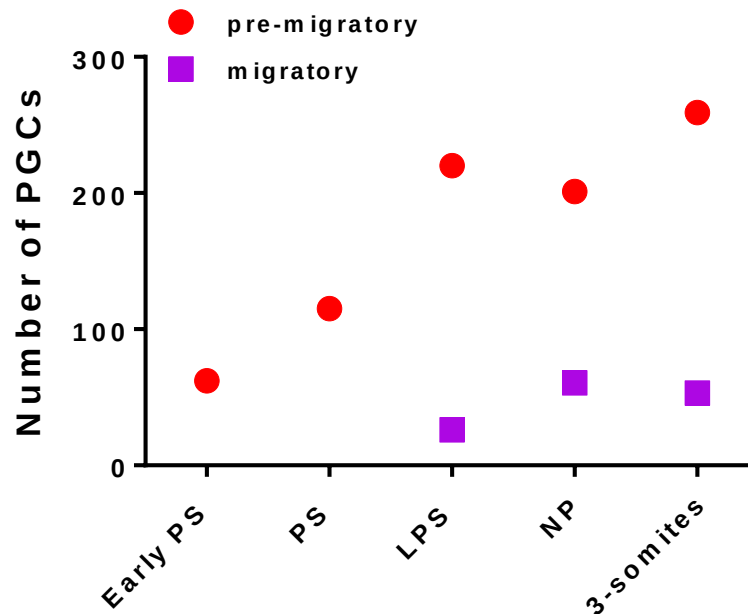


Figure 18. PGC number quantification in early embryonic development.

Numbers of PGCs at early PS stage, PS, late PS, neural plate, and 3-somites stages are presented in the scattered graph. Pre-migratory and migratory PGCs were quantified based on Immunofluorescence images stained against combinations of SOX17, BLIMP1, NANOG, OCT4, TFAP2C, or Sda/GM2. Considering the thickness of sections and average dimension of PGC cells, PGC were counted from every other section made of each embryo. Averages from odd and even numbered sections were averaged and presented in the scatter plot. Migratory PGC numbers are quantified based on the images that have pre-migratory PGCs, therefore this is probably an underestimate. PGCs in more lateral locations to the midline are not included.

3.3.2 Signalling pathways in somite stage embryos

SMAD1 is transducer of BMP4 signal. Activated SMAD1 is detected in VE and E stage embryos by whole-mount immunofluorescence (Figure 19). Pre-streak stage embryo shows no sign of polarity in embryonic disc. Very few nuclear pSMAD1 signals scatter in the embryonic disc. PS stage embryos (Figure 19C and D) show more prevalent mesodermal nuclear pSMAD1, particularly higher in posterior end in the vicinity of the primitive streak (Figure 19D). This distribution pattern partially explains the time window of PGC induction and also intrigues me to directly test the critical role of BMP4 (chapter 6).

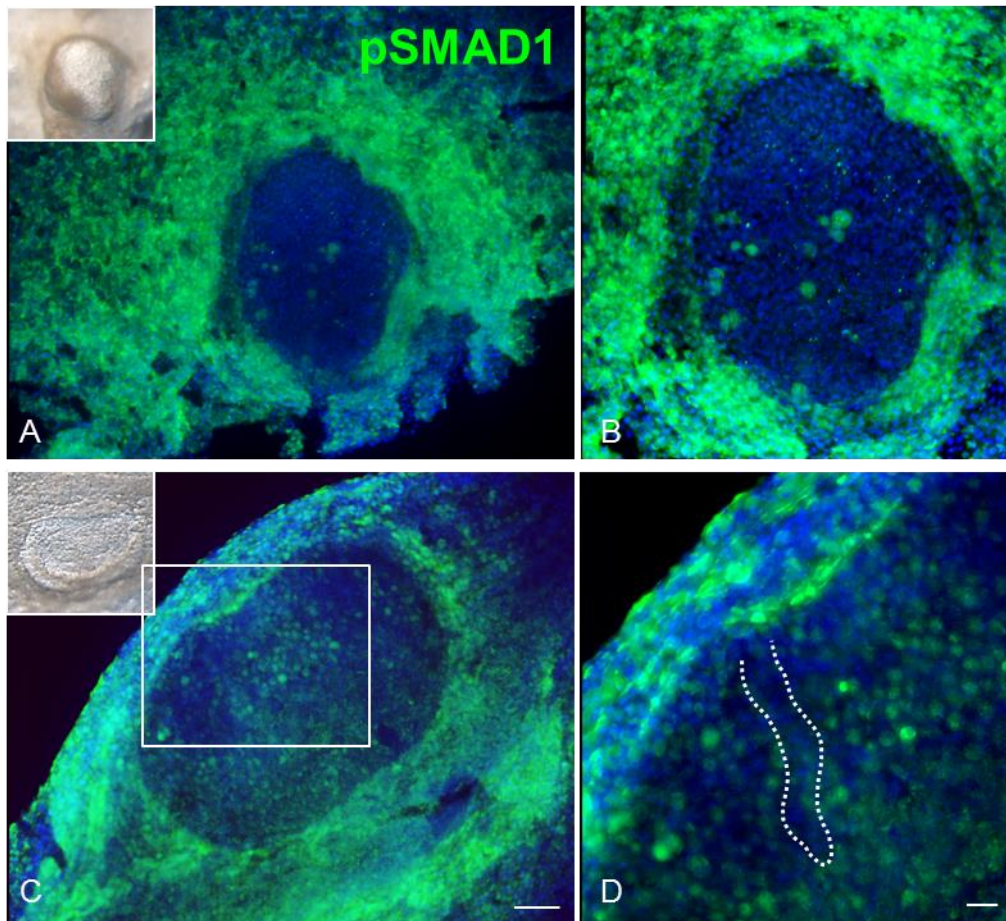


Figure 19. Whole mount immunofluorescence of phospho-SMAD1 on very early (pre-streak) and early (PS) stage embryos.

Phospho-SMAD1 is the transducer of BMP4 signal. A and B, during pre-streak stage (equivalent to very early group in epiblast culture experiment), very few nuclear pSMAD1 scattered in embryonic disk. C and D, primitive streak stage (equivalent to early in experiment) embryos show more prevalent nuclear staining, particularly in posterior end around the primitive streak area (D). Top views of embryonic discs at bright field is inserted at top left of A and C. Scale bar, 100µm.

To gain insight into the active signalling pathways in the cluster of E14 PGCs, MAPK and SMAD2 signalling was investigated using specific phosphor-antibodies against these proteins. p-MAPK was negative in PGCs compared with the pronounced signal in mesoderm and yolk sac (Figure 20A-C and G). From this profile it seems that there is no correlation between p-MAPK levels and BLIMP1+ PGCs at this stage. However, the involvement of p-MAPK cannot be excluded during PGC induction or specification. The function of activated MAPK will be discussed in Chapter 6. In contrast to p-MAPK, pSmad2 was detected within PGC cluster; however no

significant differences were observed between Sda/GM2 positive PGCs and other lineages (Figure 20 D-E).

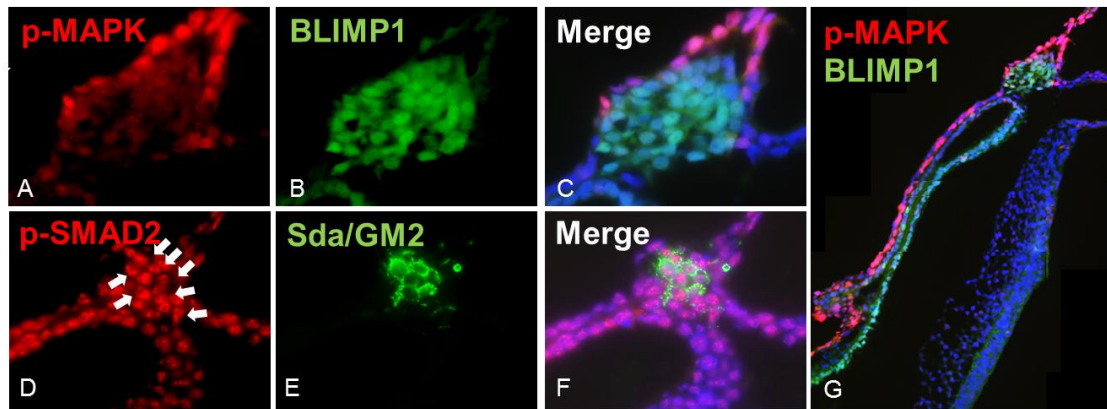


Figure 20. Immunofluorescence of phospho-MAPK and phosphor-SMAD2 on E14 PGC cluster.

p-MAPK and pSMAD2 on E14 PGCs in 3-somite embryos. A-C, closed-up of PGC cluster stained against BLIMP1/p-MAPK on PGC cluster. D-F, double staining of PGC cluster against p-SMAD2/Sda/GM2 antibodies. G, double staining against p-MAPK/BLIMP1 on the posterior end of embryo.

3.3.3 SOX17, instead of SOX2, is expressed in porcine PGCs

Bilaminar embryos (E10) were stained with SOX17 and SOX2 (Figure 21). Sox17 is detected in hypoblast (C) (or primitive endoderm) and Sox2 is detected in epiblast (D).

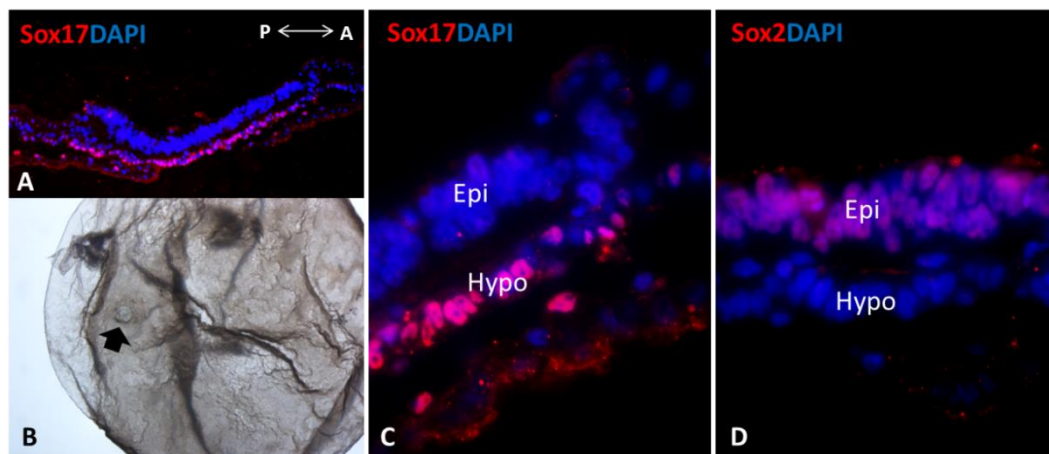


Figure 21. Sox2 and Sox17 immunofluorescence on PSI (~E10-11) pig embryo.

Black arrow in B indicates the embryonic disc. Double arrows indicate anterior (A) and posterior (P) end of sagittal section. Epi, epiblast. Hypo, hypoblast.

From pre-streak stage (~E10.5-11.5), mesoderm emerges in more advanced embryos. At this stage epiblast/ectoderm starts to lose SOX2 signal in the posterior end and maintains expression in the anterior end (Figure 22A). This pattern is reinforced at early PS (~ E11.5-12) stage (Figure 22B).

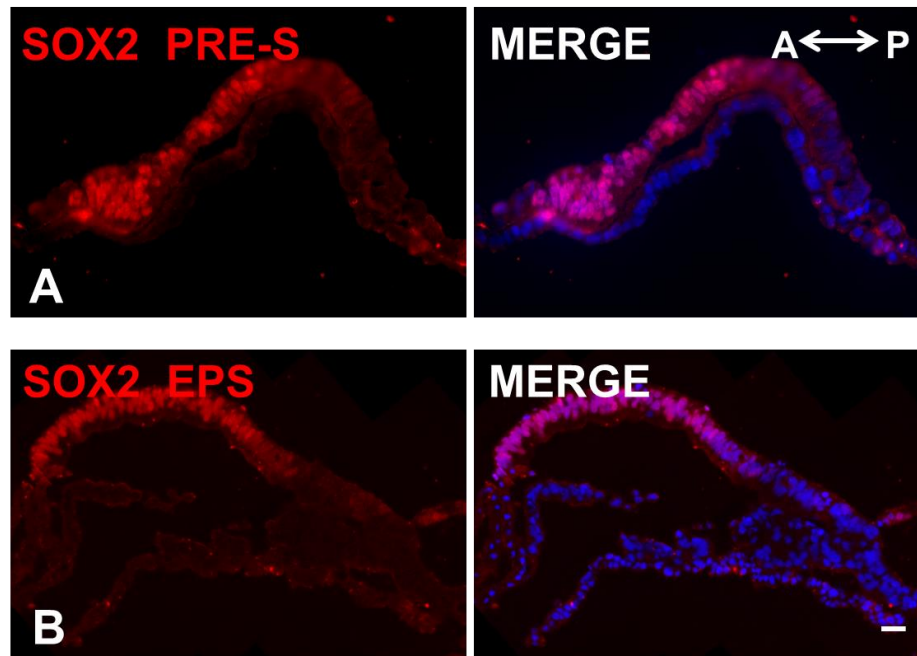


Figure 22. Sox2 immunofluorescence on Pre-streak (~E10.5-11.5) and Early-PS (~E11.5-12) pig embryos.

Sagittal sections of pre-streak and EPS embryos. A, anterior ectoderm SOX2 is detected in pre-streak embryos. B, SOX2 is detected in anterior ectoderm of EPS embryos. Double arrows indicate anterior (A) and posterior (P) end of embryos. Scale bar, 20µm

At NG stage, Sox2 is detected in anterior neural ectoderm (Figure 23B) but not in PGCs (Figure 23C). The absence of Sox2 in PGC is consistent with observations in 3.3.1.

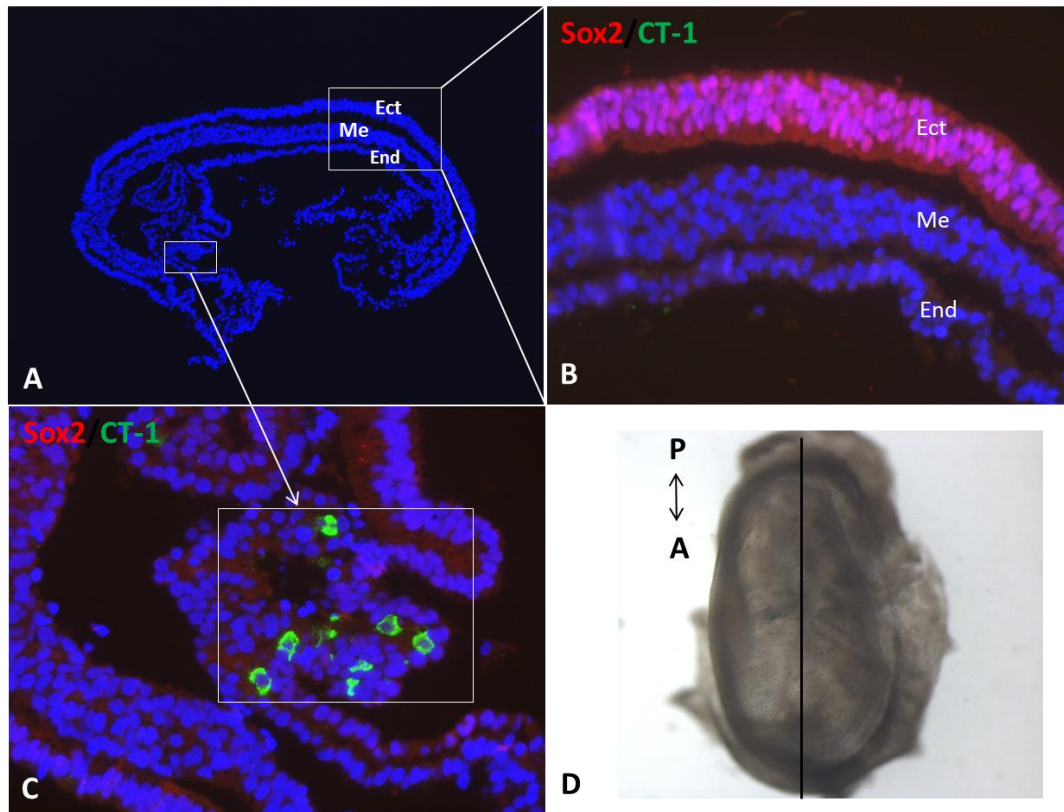


Figure 23. Sox2 immunofluorescence on NG (~E14) pig embryo.

sagittal section of NG stage pig embryo. Neural ectoderm at anterior end of embryo is stained with Sox2 (B). PGCs at posterior end of embryo is Sox2 negative (C). D, top view of NG embryo. Ect, ectoderm. Me, mesoderm. End, endoderm.

3.3.4 Germline markers during PGC migration and gonadal colonization

As indicated by the arrows in Figure 24, PGCs migrate through epithelium and mesenchyme of the hind gut. BLIMP1, TFAP2C, OCT4, NANOG, SOX17 and Sda/GM2 are expressed in migratory PGCs, while SOX2 is absent. SOX2 antibody is validated with the positive neural tube (Figure 24). PGCs show a large nuclear to cytoplasm ratio than somatic cells.

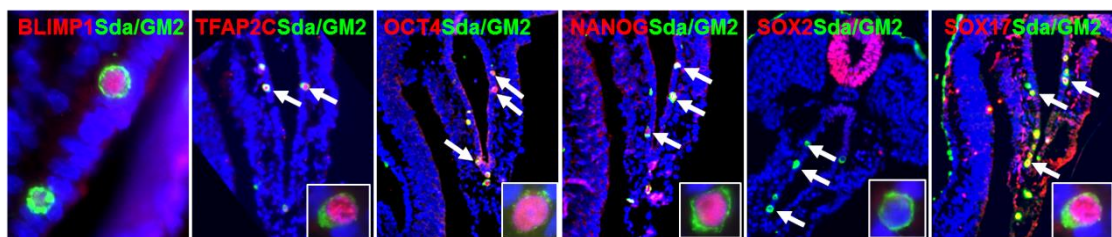


Figure 24. E17 migratory PGC characterization.

Cross sections at the hindgut area of E17 pig embryos. Arrows indicate migratory PGCs. Insets show representative migratory PGCs at higher magnification.

From ~E20, migratory PGCs arrive at the gonadal ridges and the gender can be established visually during dissection from ~E23. E25, E31, E42, E50, E60 and E70 gonads were stained with PGC markers (Figure 25 and Figure 26). CT-1 and DAZL were consistently positive and SOX2 negative through all time points. Blimp1 was positive at E25 and E31, E42 is weak positive or negative and negative from E50 in both genders. TFAP2C is positive at E25 and E31. From E42, female PGCs show cytoplasmic TFAP2C pattern. Only a subgroup of E42 male PGCs are detected with nuclear TFAP2C and this proportion decreases to zero from E50. Similar to TFAP2C, OCT4 is detected in all E25 and E31 gonadal PGCs of both genders and the positive proportion decreases from E42 in male PGCs. In female PGCs, nuclear OCT4 is replaced by cytoplasmic pattern from E42. NANOG shows typical nuclear localization from E25 to E42 in both genders, and is absent in many cells from then on. SOX17 is detected in E25 to E50 PGCs in both genders, with decreasing intensity at E42 and E50. A few of E60 male PGCs remain SOX17 positive, which becomes negative at E70. Female PGCs are SOX17 negative from E60 to E70.

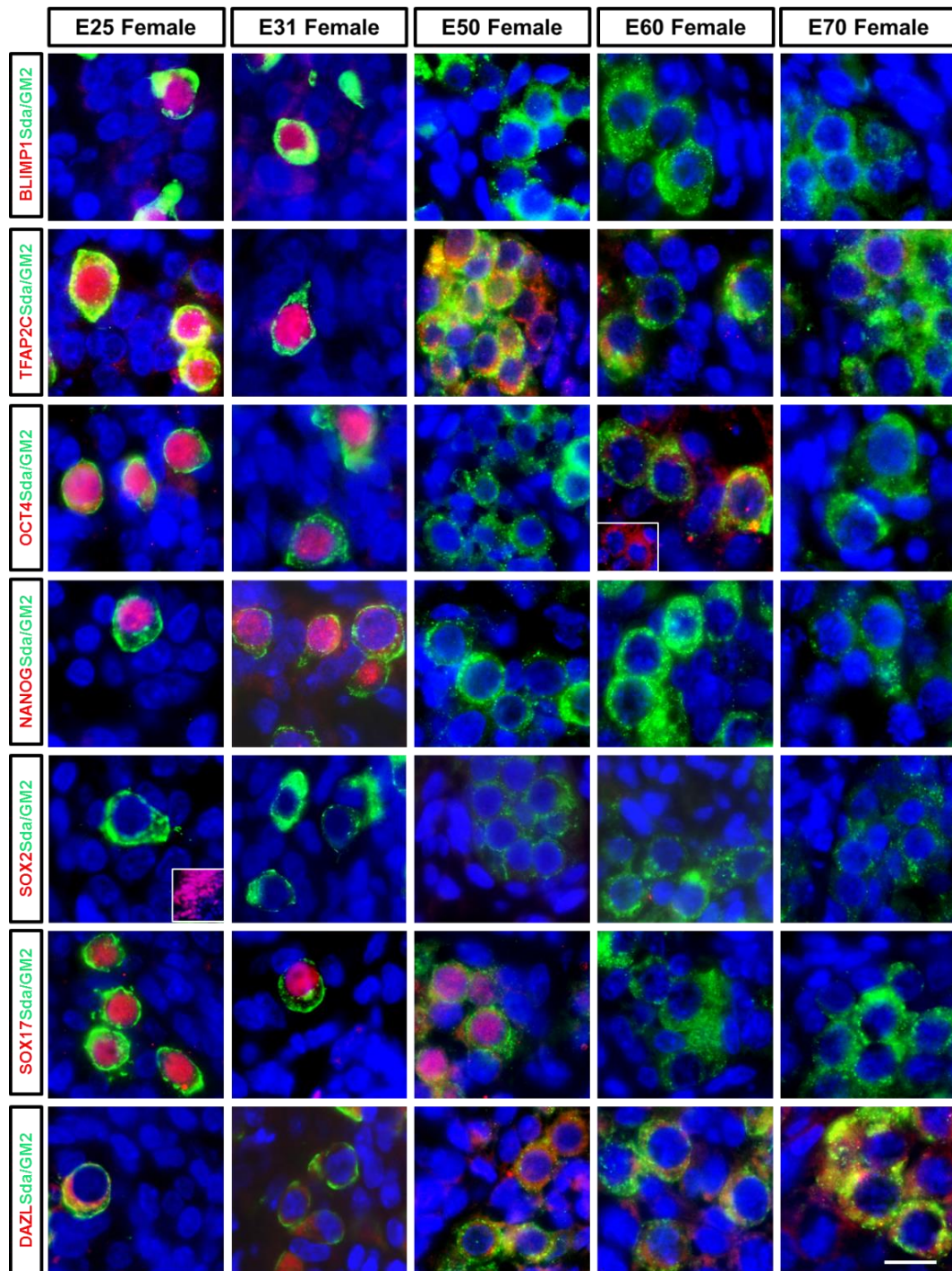


Figure 25. Characterization of E25-E70 female gonadal PGCs.

Female gonadal PGCs were stained with the panel of PGC markers, including BLIMP1, TFAP2C, OCT4, NANOG, SOX17, DAZL, Sda/GM2 and SOX2. Neural tube is shown at right bottom of E25 image as positive control of SOX2.

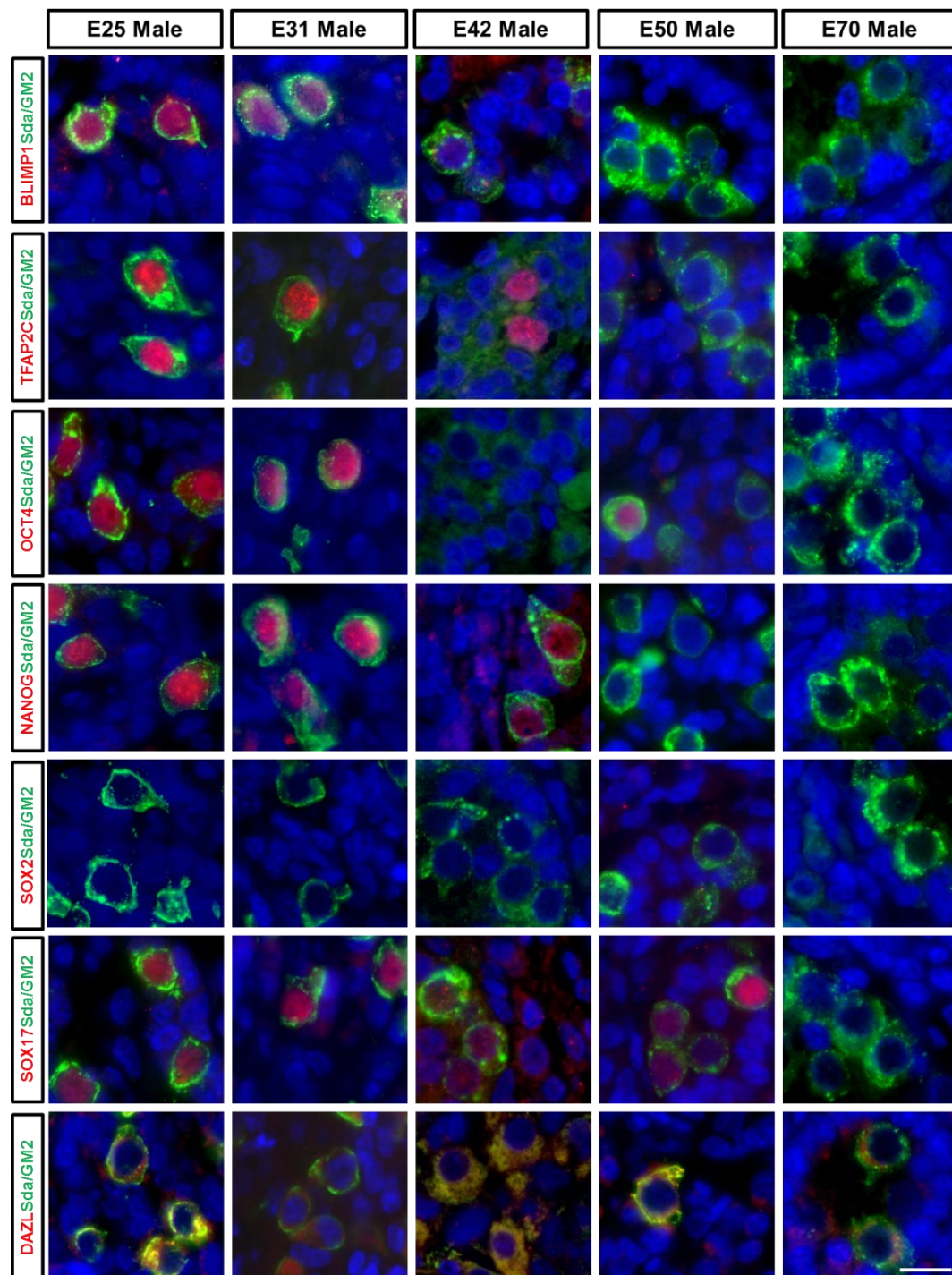


Figure 26. Characterization E25-E70 male gonadal PGCs.

Male gonadal PGCs were stained with the panel of PGC markers, including BLIMP1, TFAP2C, OCT4, NANOG, SOX17, DAZL, Sda/GM2 and SOX2.

3.4 Discussion

Immunofluorescence was carried out on E10-E14 (nascent PGCs), E16/E17 (migratory PGCs) and E25-E70 (gonadal PGCs) to characterize the gene expression profile of the pig germ line. This characterization provided three major findings: First,

because no BLIMP1 or SOX17 were found in the bilaminar epiblast, it is assumed that presumptive PGCs arise from mesodermal progenitors in the posterior streak region. These cells are identified by the expression of SOX17 preceding the activation of BLIMP1 and the reactivation/upregulation of NANOG. BLIMP1 is detected in a subpopulation of Nanog-high positive cells. Second, SOX2 is absent in the porcine germ line from the stage of SOX17+/BLIMP1+/NANOG strong cells and onwards. Third, BRACHYURY (T) is repressed in SOX17+/BLIMP1+/NANOG strong cells.

3.4.1 NANOG, SOX17 and BLIMP1 expression pattern in PGCs

SOX17, Sry-related HMG box gene, is required for definitive endoderm (DE) maintenance and differentiation, although it may be dispensable for DE progenitor formation during gastrulation (Kanai-Azuma, Kanai et al. 2002). By regulating morphological development of hind gut, SOX17 plays a critical role in directing mouse PGC migration (Hara, Kanai-Azuma et al. 2009). Also, constitutive expression of SOX17 in hESC induces a DE gene expression profile and propensity towards hepatic and pancreatic differentiation. When injected into immunosuppressed mice, the teratomas formed have mesendodermal derivatives (Seguin, Draper et al. 2008). SOX17 and BLIMP1 (or PRDM1) have been conventionally used to identify endoderm cells. However, here we show that both are early markers of the pig germline. When cultured in the presence of BMP4, human LIF, SCF and EGF, the hESC differentiates into hPGCLC, expressing SOX17, BLIMP1 and then TFAP2C chronologically (Irie, Weinberger et al. 2015). When hESC are cultured in the presence of Activin A and FGF, differentiated cells present transient expression patterns of high SOX17/high PRDM1/low T, which was claimed to be proof of endoderm fate (Bernardo, Faial et al. 2011), but this is also in line with our porcine PGC gene expression profile. The potential of SOX17 to drive PGC or endoderm specification points to a context dependent interpretation of transcription factor effects in cells with multipotent properties in the primitive streak. In mouse airway submucosal gland progenitor cells, Sox17 directly regulates WNT/ β -catenin-dependent transcription of Lef1 promoter (Liu, Luo et al. 2010). This suggests that there may be a relationship between SOX17 and WNT signalling pathway during PGC specification. To date, the mechanism of SOX17 activation in PGCs remains elusive, however later in this thesis this issue will be revisited. In addition, since

SOX17 is not exclusive to PGC it brings difficulties in identifying early PGCs during *in vitro* differentiation, since SOX17/BLIMP1 positive cells could be PGC precursors, PGCs or endodermal cells. To solve this problem, more markers should be included to confirm PGC identity or exclude endodermal fate. For example, the PGC markers TFAP2C, Sda/GM2, or endoderm markers Gata4 or Gata6 can complement the characterization of the SOX17/BLIMP1 population as PGCs or other cell types (Artus, Piliszek et al. 2011).

Pig NANOG is detected in epiblast and primitive endoderm from bilaminar stage (~E10) (Figure 10), which is consistent with a previous report (Wolf, Serup et al. 2011). At the PS stage (~E12), the expression of NANOG is suppressed in posterior primitive streak and mesoderm cells, including potential PGC progenitors (Figure 13), and then upregulated in PGCs. This is similar to the Nanog- pattern in 6.5-7.5 dpc mouse PGCs (Yamaguchi, Kimura et al. 2005). Nanog alone is sufficient to induce mPGCLC from mEpiLC by binding to enhancers of Blimp1 and Prdm14 (Murakami, Gunesdogan et al. 2016). It mimics the 6.5-7.5 dpc Nanog+ mouse epiblasts and Nanog- PGC pattern. However, Nanog is not essential to make PGCs in mice, although the conditional Nanog KD significantly decreases PGC number by 12.5 dpc (Yamaguchi, Kurimoto et al. 2009). However, pig PGCs are specified from mesoderm progenitors, in which NANOG is suppressed. The D1 expression pattern in the 4i *in vitro* PGC differentiation system resembles this context (Irie, Weinberger et al. 2015). The mechanism of pig NANOG in PGC specification is intriguing and worth further investigation.

3.4.2 SOX2 is absent in porcine germ line

In contrast to SOX17, SOX2 is not expressed in pig PGC at any stage of their development. The absence of SOX2 protein in migratory and gonadal PGC is in line with that reported in human PGCs (Perrett, Turnpenny et al. 2008), and low level of transcripts compared with human EGCs, iPSCs, ECCs and ESCs (Pashai, Hao et al. 2012). In contrast, mouse Sox2 is expressed from E6.75 nascent PGC (Kurimoto, Yabuta et al. 2008) until E13.5 ovary and E15.5 testis (Western, Maldonado-Saldivia et al. 2005). This discrepancy of SOX2 expression between mouse and human/pig could be a reason for the failure of pluripotent EG cell conversion from pig or human PGCs when using similar culture conditions as used for mouse EGC cultures.

Concomitant with pig and human SOX2 loss is the elevation of SOX17. Does SOX17 take over the role of SOX2 as consequence? A model has been proposed whereby SOX2 and SOX17 compete with each other to form a complex with OCT4, and thus switch on different genes, therefore determining cell fate. SOX2-OCT4 complex initiates pluripotent gene expression while SOX17-OCT4 activates endodermal genes (Aksoy, Jauch et al. 2013). Due to the almost identical DNA binding structure between SOX2 and SOX17, point-mutant Sox2 has similar morphology to Sox17 overexpression mES cell line and point-mutant Sox17 cell line resembles Sox2 overexpression mES cell line in morphology (Jauch, Aksoy et al. 2011). It is possible that SOX17 expression initiates in response to signals, rather than particular germ fate. This germ layer signal pool terminates SOX2 expression, while SOX17 takes over the role of SOX2. A recent report shows that the function of mouse Sox2 is context dependent. In mESC context, Sox2 and Nanog promote pluripotency, while Sox2 prevents mPGCLC induction via Nanog in mEpiLC condition (Murakami, Gunesdogan et al. 2016). This might explain the absent SOX2 in human and pig germ line, as the progenitors are primed pluripotent cells in nascent mesodermal location.

3.4.3 Expression of T in porcine PGCs

The low T expression in BLIMP1+/SOX17+PGCs is reminiscent of mouse primitive streak, where T targets and promotes Sox17, and the activated Sox17 in turn represses T (Lolas, Valenzuela et al. 2014). Pig primitive streak and mesoderm has high expression levels of T (Figure 14), which is consistent with previous report (Wolf, Serup et al. 2011). It is possible that high levels of T in PGC progenitors activates Sox17, which then downregulates T in specified PGCs. Another relevant report is that in mouse, T directly binds to regulatory elements of Blimp1 and therefore activates its expression (Aramaki, Hayashi et al. 2013) in a BMP4 independent manner. Also, Blimp1 DNA was reported to bind directly to T in PGCs (Magnusdottir, Dietmann et al. 2013). This reciprocal binding pattern may explain the downregulation of T observed as a mutually exclusive expression of Blimp1 in PGCs. By In situ hybridization, T RNA was detected in both mouse extra-embryonic ectoderm and proximal posterior epiblast at 6.0 dpc mouse embryo. T protein was only detected at proximal posterior epiblast by 6.5 dpc (Rivera-Perez and Magnuson 2005), which is equivalent to ~E11 pig embryonic disc. Since the promoter of T is a direct target of

the WNT signalling pathway (Rivera-Perez and Magnuson 2005), it is possible that repressed T levels in porcine PGC is correlated with WNT signalling. In addition, BMP4 inducing signal is produced by mouse extraembryonic ectoderm and pig mesoderm. The concurrence of mouse T and BMP4 in extraembryonic ectoderm during PGC induction are equivalent with pig mesoderm expression of these two genes (Valdez Magana, Rodriguez et al. 2014). Although BMP4 doesn't directly activate T (Aramaki, Hayashi et al. 2013) the existence of BMP4 is indispensable for PGCs induction and specification. When mouse epiblast cells become lineage restricted at 6.5 dpc, the extraembryonic ectoderm T becomes discernible, leaving only proximal posterior epiblast positive, which is similar to our results showing that PGC niche (mesoderm and primitive streak) is positive of T at pre-streak stage (~E10.5-11.5) (Figure 11), although it is slightly later than the mouse counterpart. By single-cell sequencing, mouse T RNA was detected abundant in PGCs from E6.5 to E7.5, followed by a sudden decrease immediately after (Magnusdottir, Dietmann et al. 2013). To corroborate this result, spherical stage bilaminar embryos are required to perform IHC.

3.4.4 Expression profile of PGC related cells

A schematic summary of the pig pre-migratory PGC expression profile is shown in Figure 27. NANOG, SOX17, BLIMP1, OCT4, and TFAP2C, marks PGC precursor and pre-migratory/ early migratory PGCs. Sda/GM2 is a mature PGC marker that is expressed in PGCs that express SOX17 and BLIMP1. TFAP2C, OCT4 and SOX2 are typically expressed in anterior ectoderm. NANOG is expressed in both mesoderm and anterior ectoderm.

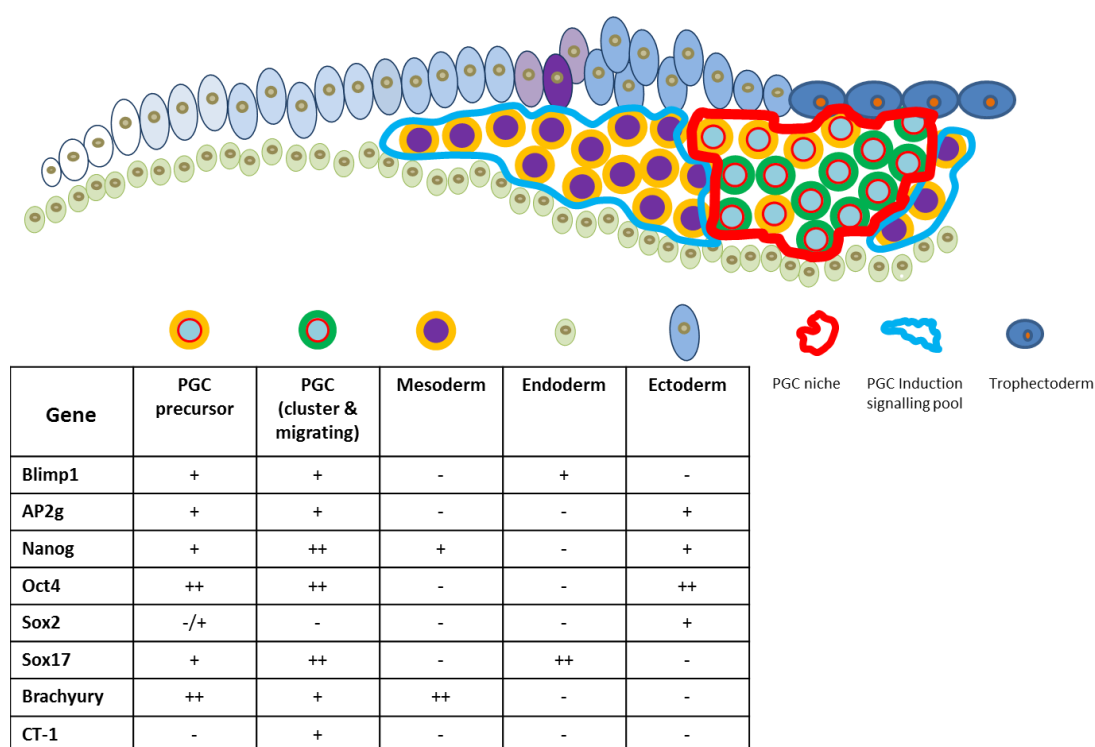


Figure 27. Schematic model of pig nascent PGC characterization (~E13).

Pig PGCs are specified from EPS stage (~E11.5). During expansion of PGC cluster, some ‘mature’ PGCs are on their migratory path. From E16-20, almost all PGCs are detected in the hindgut. From E25, PGCs colonize the gonads. A brief summary of germ line related gene expression profile is shown in Figure 28. nuclear NANOG is maintained in epiblast, and downregulated in nascent mesodermal PGC progenitors. Due to antibody specificity issue, cytoplasmic background is frequently observed. SOX17 is activated in potential PGC precursors, which then activates BLIMP1. OCT4, TFAP2C, Sda/GM2 is only after BLIMP1 is activated. DAZL is detected from E25 to E70 gonadal PGCs. Sda/GM2 is detectable until at least E75 in both genders (Klisch, Contreras et al. 2011). SOX2 is absent in germ line. From E42, NANOG is down regulated in a cell-to-cell manner. Varying levels of NANOG are detected both in male and female PGCs until E60, when NANOG is no longer expressed. After E50, nuclear OCT4, TFAP2C and SOX17 are downregulated and extinguished from E60 (Figure 28). The specificity of some antibodies, such as NANOG and BLIMP1 are not as good as other antibodies. It requires discrimination of cytoplasmic background from nuclear signals.

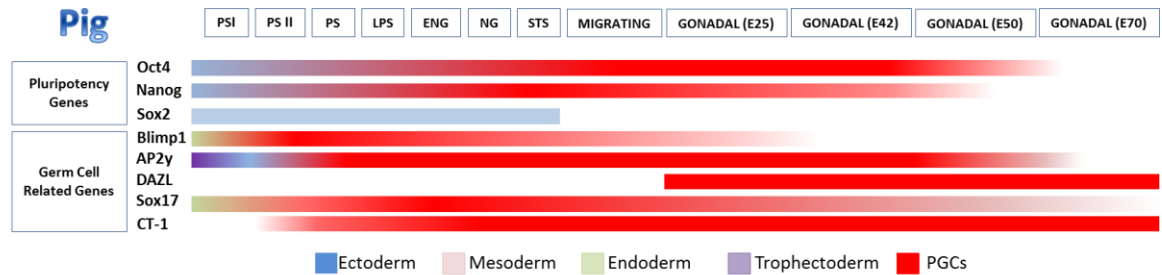


Figure 28. Expression profile of germ line related genes.

Expression profile of selected pluripotency genes OCT4, NANOG, and SOX2, and germ cell related genes BLIMP1, AP2 γ , DAZL, SOX17, and Sda/GM2 are represented in the graph. Different embryonic stages are labelled at the top. Localization of expressed proteins are represented in different colours.

An interesting observation is the cytoplasmic localization of transcription factors OCT4, NANOG and TFAP2C. Cytoplasmic OCT4 was also well reported in human 11-16.5 week old testes and 9.5-14 week old ovaries (Atlasi, Mowla et al. 2008; Gkoutela, Li et al. 2013). Perinuclear and cytoplasmic NANOG are detected in germ cells of postnatal testis as well as some Sertoli and interstitial cells (Goel, Fujihara et al. 2008). In addition, cytoplasmic NANOG is also detected in human stromal cells, which is argued they promote stromal distribution in cervical cancer (Gu, Liu et al. 2012). In cultured human prostate cancer cells, Nanog is sometimes observed in the cytoplasm (Jeter, Badeaux et al. 2009). A recent study suggests that pluripotent transcription factors are partially regulated by a nuclear-pore-coordinated network, which takes part in the pluripotency and differentiation decision during embryogenesis (Yang, Cai et al. 2014). The function of cytoplasmic expression of OCT4, Nanog and TFAP2C is elusive and there is no data showing the potential in normal embryology.

Another question remained unclear is that when does lineage restriction occur for pig PGCs. Due to the technique limits, IF against selected transcriptional factors only characterize the expression profile, but does not explain when and how the PGCs are lineage restricted. Cre-LoxP recombination technique is one potential resolution to address this question.

3.5.5 Signalling pathways involved in porcine PGC specification

When induced by BMP and FGF signals, mesodermal fate was identified by mesoderm and mesoendoderm markers including T. Successful induction of

multipotential mesendodermal progenitors from mESC requires sustained WNT signalling pathway (Bakre, Hoi et al. 2007). This condition is similar to the *in vivo* mesodermal environment surrounding the PGC niche when the cells are undergoing specification to different germ layers. During PGC specification, mesoderm provides BMP4 (Valdez Magana, Rodriguez et al. 2014). pSMAD1, transducer of BMP4, is upregulated in posterior end of epiblast, particularly enriched around primitive streak at PS stage (Figure 19), which is consistent with a previous report (Valdez Magana, Rodriguez et al. 2014). However, it cannot be excluded that phospho-SMAD1 could also reflect other BMPs. pSMAD2 is detected throughout the whole embryo, including PGCs at 3-somite stage (Figure 20). It is incorrect to exclude the function of activated SMAD2 in PGC specification, although it is not exclusive to PGC. In addition, the phospho-SMAD2 could also reflect the activated TGF β /Activin/Nodal pathway (Derynck and Zhang 2003). This suggests that BMP4 is activated and involved in porcine PGC specification, as mouse *Bmp4*. This is the first evidence that BMP4 participates in PGC specification in non-rodent mammals. Functional experiments in chapter 6 will complement the observations reported in this chapter. pMAPK is detected in epiblast, hypoblast, and TE (Valdez Magana, Rodriguez et al. 2014), which means FGF signalling is activated prior pig PGC specification. At 3-somite stage, pre-migratory PGCs shows pMAPK- pattern (Figure 20). However, the absence of pMAPK doesn't necessarily prove that pMAPK is not required, as it could be too late for the PGC specification window. pMAPK is still an interesting pathway, and the role during PGC specification will be investigated in chapter 6. Mouse WNT is detected in $\sim$ E5.5 epiblast (Liu, Wakamiya et al. 1999) and is prerequisite for BMP4 induction of PGC specification (Aramaki, Hayashi et al. 2013). Due to technical limitations with the b-catenin antibody, I don't report on WNT signalling by IF in this chapter, but the function of WNT will be studied in chapter 6.

Chapter4

4. Epigenetic Dynamics in Porcine PGC Development

4.1 Introduction

Epigenetic reprogramming in germ cells is thought to be part of the essential events leading to the formation of new gametes, with implications for the transmission of epimutations to subsequent generations. Epigenetic reprogramming follows a well characterized sequence in the developmental program of mouse PGC. DNA demethylation and histone marks reprogramming are two major aspects of epigenetic regulation described in mouse and human germline.

Methylation on the 5-position of cytosine on CpG dinucleotide is one of the key epigenetic modifications. It is associated with parental imprints, gene expression regulation, and genomic structure (Bird 2002). Mouse PGCs shows enriched 5-hmC during 9.5-10.5 dpc, when the global imprint erasure takes place. Tet1 and Tet2, which actively oxidise 5-mC to 5-hmC, reaches peak mRNA levels at 8 dpc and 10.5-11.5 dpc. The nuclear proteins are highly detected around 9.5-11.5 dpc (Hackett, Sengupta et al. 2013). A passive cell replication-dependent 5-hmC dilution follows the stage, and 5-hmC is almost erased during 11.5-12.5 dpc. UHRF1 recruits DNMT1 on replication foci to maintain DNA methylation (Liu, Gao et al. 2013). *Uhrf1*, *Dnmt3a* and *Dnmt3b* is downregulated after E/MB stage (7.25-7.5 dpc) although *Dnmt1* is constantly high in germ line (Kurimoto, Yabuta et al. 2008). In D4 hPGCLC induced from hES cells, UHRF1 and DNMT3A are not detected as well (Irie, Weinberger et al. 2015). This stage resembles the nascent hPGCs, which are featured by SOX17, BLIMP1, and TFAP2C expression. Enriched TET1 and 5-hmC in D4 hPGCLCs (Irie, Weinberger et al. 2015) suggests that nascent PGCs actively demethylate DNA, while the maintenance and *de novo* methylation are repressed. The DNA demethylation status in D4 hPGCLC is equivalent to 8 dpc mouse PGCs (Irie, Weinberger et al. 2015). This suggests that hPGCs might perform DNA demethylation earlier than mouse does. Human PGCs are negative for 5mc in W7-17 testis and W6-19 ovaries. While W7-8 common progenitor male and female PGCs are detected with lower 5-hmC level than somatic cells. Signal of 5-hmC is under detection in W13.5-16 testis, and retained in a subpopulation of W17 male PGCs. From W11 to W19, ovaries show heterogeneous 5-hmC signal in some of PGCs

(Gkountela, Li et al. 2013). UHRF1 and DNMT3A are constantly repressed in human germ line from W4.5-W10 in both genders (Gkountela, Zhang et al. 2015).

Histone modification is epigenetic regulator of chromatin, affecting the contact not only between various histones, but also between histone and DNA. Histone code influence the chromatin environment, therefor regulate replication, transcription, DNA repair, and chromosome condensation. H3K9 and H3K27 methylations are associated with repressive chromatin structure (Turner 2002). Constitutive heterochromatin is characterised by H3K9me3. Similar to H3K9me3, H3K9me2 is bind by HP1, which recruits SUV39 methyltransferase, therefor methylates H3K9 on surrounding nucleosomes with unmodified H3 (Rothbart, Krajewski et al. 2012). Methylated H3K9 is also required for the DNA methylation maintenance by mitotic chromatin association of UHRF1 to DNMT1 (Rothbart, Krajewski et al. 2012). H3K27me3 marks facultative heterochromatin on Xi location in mammalian female cells (Trojer and Reinberg 2007) through DNA replication (Hansen, Bracken et al. 2008). In gonadal stage, human testis show similar level of H3K27me3 to somatic cells during W7-10.5, and the level drops at W11, which is the endpoint of the common progenitor stage. Testis maintain undetected levels of H3K27me3 until W17, almost 38% PGCs retained similar or lower level than somatic cells. In the ovary, over half of W6-8.5 PGCs maintain similar level of H3K27me3 as somatic neighbours. Beyond W8.5, female PGCs maintain H3K27me3 negative up to at least W18 (Gkountela, Li et al. 2013). The loss of H3K27me3 in mouse and human germline is also considered to be critical progress for the activation of mature germ line program, as well as DNA demethylation (Gkountela, Zhang et al. 2015).

LB stage (7.25 dpc) mouse PGCs start to show signs of H3K9me2 reduction (Seki, Yamaji et al. 2007). The trend is increasingly significant in a cell-to-cell manner until 8.75 dpc, when almost all PGCs show low H3K9me2 level. Enzymes responsible for direct H3K9me2 demethylation contains JumonjiC (JmjC) domain. *Jmjd1a* (*Jmjd2a*), for example, demethylase for H3K9me2, is detected but not exclusive to PGCs during this period (Seki, Yamaji et al. 2007). This observation suggests that there is additional mechanism demethylate H3K9. From 9.5 to 12.5 dpc, the mouse PGCs are constantly deprived of H3K9me2 (Seki, Hayashi et al. 2005). From 14.5 dpc to 18.5 dpc, H3K9me2 maintains low in male gonadal cell until birth (Deguchi, Nagamatsu et

al. 2013). In juvenile testis, the level of H3K9me2 is low in undifferentiated spermatogonia and upregulated in differentiated spermatogonia. The intensity of H3K9me2 is then downregulated in pachytene of meiosis of spermatocytes (Deguchi, Nagamatsu et al. 2013). Different to mouse, human W4-W9 post-migratory PGCs remains detectable but lower level of H3K9me2 than somatic cells (Tang, Dietmann et al. 2015).

While H3K9me2 is downregulated, H3K27me3 is upregulated from 8.25 dpc in a cell-by-cell manner until 10 dpc (Seki, Yamaji et al. 2007). This substantial upregulation is maintained at least until 12.5 dpc (Seki, Hayashi et al. 2005). Similarly, porcine PGCs acquire H3K27me3 during migration around E15-21 (Hyldig, Croxall et al. 2011). Human mitotic PGCs (W7-10 female and W10-11 male) show very low levels of H3K27me3, even the Xi enrichment disappears in female PGCs (Guo, Yan et al. 2015). The upregulation of H3K27me3 in mPGC is contrary to the pattern observed in human gonadal hPGC. W4 hPGCs show 2.5 fold higher intensity of H3K27me3, which is then gradually downregulated to half as much of somatic neighbours at W9, and maintained the level to at least W12 (Gkoutela, Li et al. 2013; Tang, Dietmann et al. 2015). The reactivation of Xi, marked by H3K27me3 is observed at W7 ovary (Tang, Dietmann et al. 2015).

In general, the dynamic of epigenetic markers are DNA replication dependent, except for H2A.Z, which can be incorporated into histone out of cell cycle (Hajkova, Ancelin et al. 2008). Mouse H2A.Z shows abrupt peak at 10.5 dpc, and disappear at 11.5-12.5 dpc (Hajkova, Ancelin et al. 2008). Human gonadal PGCs are negative for H2A.Z at W11. This trend is maintained until W17, when H2A.Z is retained in a few of PGCs in both genders (Gkoutela, Li et al. 2013).

Overall, the DNA demethylation and histone modification events in gonadal PGCs are different between human and mouse. There are a few reports about porcine gonadal PGC showing more similar trends to hPGC, however this is limited to early gonadal stages. . However more recently, in vitro hPGCLC differentiation system have provided evidence that hPGCs undergo DNA demethylation earlier than mPGCs. Whether this really happens in vivo will be presented in this chapter based on the data on the pig embryo. IF will be performed on E12-E70 embryos and foetal gonads, to

characterize the pattern of 5-mC, 5-hmC, TET1, UHRF1, H3K9me2, H3K27me3, and H2A.Z. The expression profile of 5 cytosine methylation in PGC progenitors and nascent PGCs will help establish the ontogeny of porcine PGCs. Later expression profiles of epigenetic landmarks will be valuable evidence for species comparisons and to establish the similarities between human and pig PGCs, and to determine the discrepancies to mouse PGC development.

4.2 Material and Methods

Pig embryos at E12, E14, E16/17, E25, E31, E42, E50, E60 and E70 were collected and fixed with PFA. 4µm wax sections or 8µm cryosections were made and stained with desired antibodies. Primary antibodies 5-mC (Eurogentec, B1-MECY-0100, 1:700), 5-hmC (Active Motif, 39791, 1:700), Tet1 (GeneTex, GT1462, 1:500), Dnmt3a (SantaCruz, sc-20703, 1:500), Uhrf1 (Active Motif, 61341, 1:500), H3K9me2 (abcam, ab1220, 1:700), H3K27me2 (Active Motif, 39157, 1:700), H2A.Z (Active Motif, 39113, 1:1500) and secondary antibodies Donkey anti-Mouse IgG (H+L) Alexa Fluor® 488 conjugate (Life technologies, A-21202), Goat anti-Rabbit IgG (H+L) Alexa Fluor® 555 conjugate (Life technologies, A-21428), Goat anti-Mouse IgG (H+L) Alexa Fluor® 488 conjugate (Life technologies, A-11001), Goat anti-Rabbit IgG (H+L) Alexa Fluor® 488 conjugate (Life technologies, A-21206), Donkey Anti-Goat IgG H&L (Cy3 ®) preadsorbed (abcam, ab6949) at 1:500 concentration are applied accordingly.

4.3 Results

4.3.1 DNA Demethylation initiates in pre-migratory PGCs

Strong signal for 5-mC and 5-hmC are detected in the ectoderm and nascent mesoderm of E11 embryos, whereas a relatively low immunofluorescent signal is found in the primitive endoderm (Figure 29). In E13 PGC cluster and early migratory PGCs, high 5-hmC level is detected compared with mesoderm and endoderm (Figure 31D-I), although 5-mC level is maintained similar to endoderm and mesoderm (Figure 30). These observations were confirmed by staining parallel sections to the cluster region, where 5-hmC is homogeneously detected in mesoderm (Figure 31 A-C). E14 pre-migratory and nascent migratory PGCs maintain high 5-hmC levels (Figure 34). Thus, porcine premigratory PGCs show visible sign of active demethylation, as shown by 5-hmC immunofluorescence, whereas equivalent 8.0 dpc nascent mouse

PGCs were detected with similar intensity of 5-mC on perinuclear foci with surrounding somatic cells (Seki, Hayashi et al. 2005). Mouse 9.5 dpc PGCs shows low level of 5-hmC (Yamaguchi, Hong et al. 2013). This is similar to D4 hPGCLC, which shows significant increase in 5-hmC (Irie, Weinberger et al. 2015).

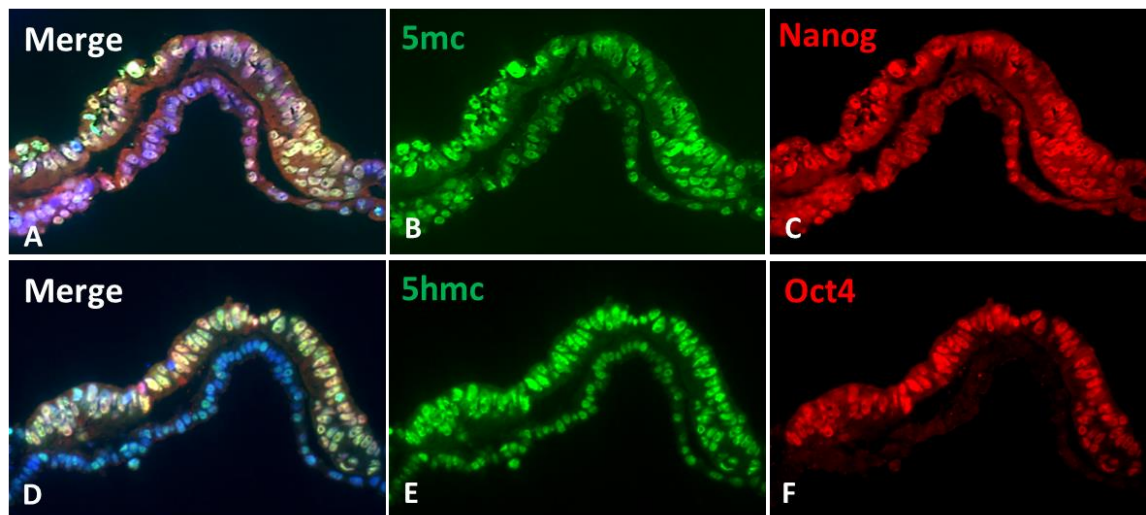


Figure 29. 5-mC and 5-hmC expression in pre-streak embryo (~E12).

Nascent mesoderm appears from posterior end of embryonic disc. No primitive streak has developed.

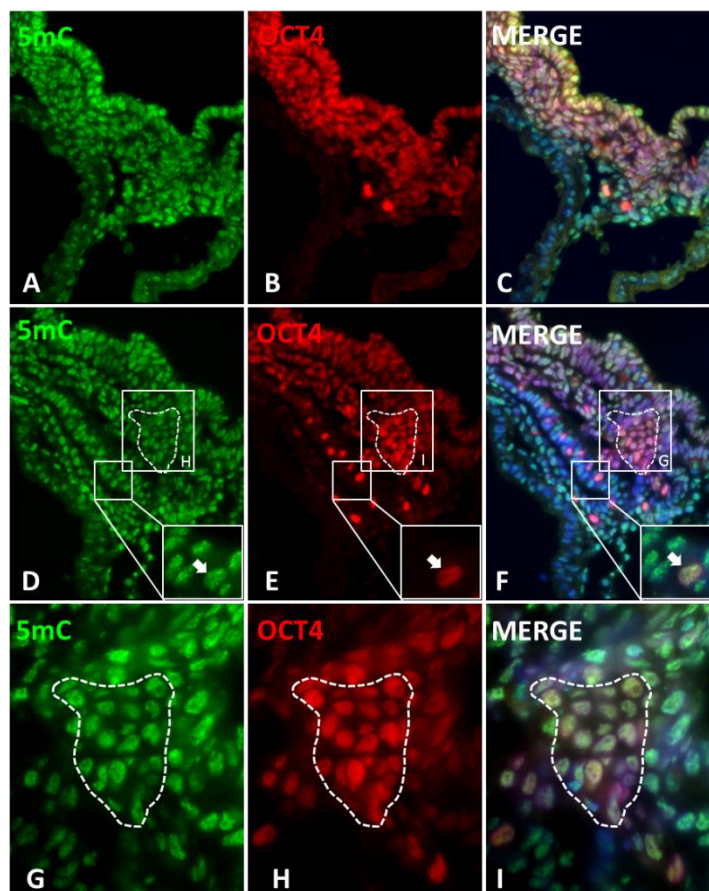


Figure 30. 5-mC expression profile in E13 PGCs.

A-C, parallel section to the PGC cluster (no cluster present in this section). D-F, posterior end of E13 PGC cluster. PGC clusters are zoomed in in G-I respectively. PGC cluster are circled with dashed line.

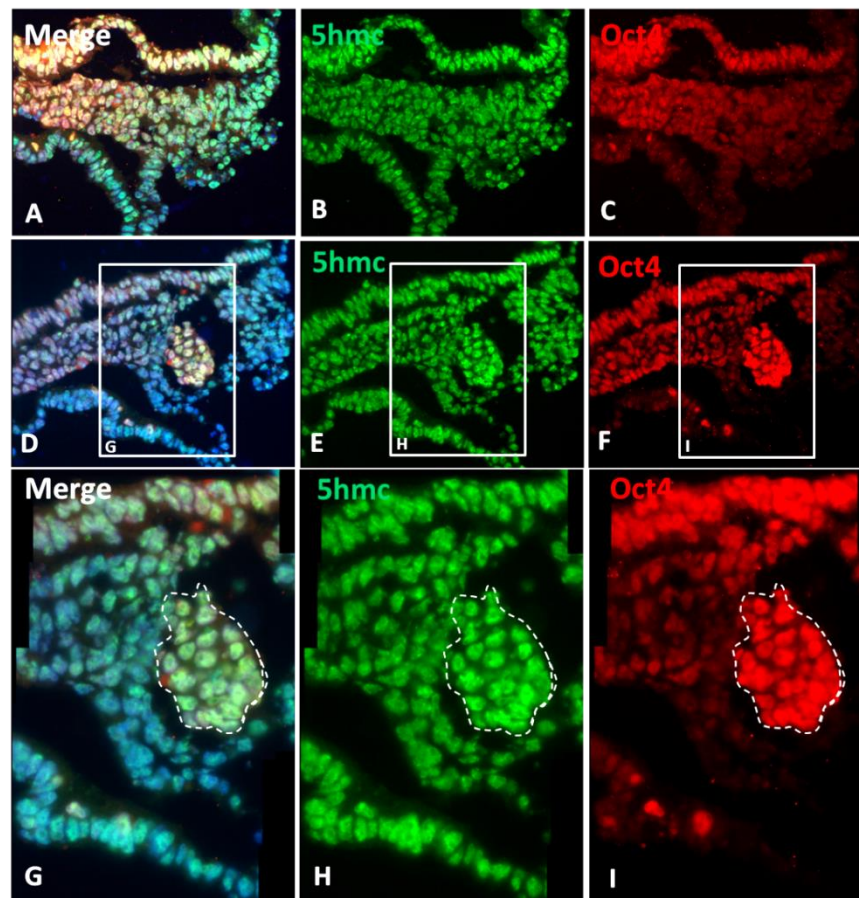


Figure 31.Immunofluorescence of 5-hmC in E13 PGC cluster.

A-C, parallel section of PGC clusters. D-F, posterior end of E13 PGC cluster. PGC clusters are zoomed in in G-I respectively. PGC cluster are circled with dashed line.

To understand the mechanism of 5-hmC accumulation in PGCs, DNA demethylation related enzymes TET1, UHRF1 and DNMT3A were studied. Unfortunately, no stable DNMT3A staining was achieved. Tet1 was detected in the PGC cluster and in migratory PGCs (Figure 32A to I). The ectoderm is low/negative and the mesoderm is negative for Tet1 (J-L). Uhrf1 is absent in PGCs and detectable in all three somatic germ layers (Figure 33). The persistent high level of 5-hmC in PGCs from the transition of posterior streak mesodermal cells (E11) to specified PGCs (E13) suggest the intriguing possibility that PGCs may retain the epigenetic profile of pluripotent progenitors, whereas somatic cells lose tet1 expression and consequently 5-hmC level as differentiation proceeds. The maintenance of tet1 expression, combined with the loss in UHRF1, the cofactor that contributes to recruit Dnmt1 to newly synthesized

DNA, suggests the PGCs initiate the wave of DNA demethylation using a combination of active and passive mechanisms of DNA demethylation.

As the result, E14 PGC cluster significantly upregulates 5-hmC level (Figure 34).

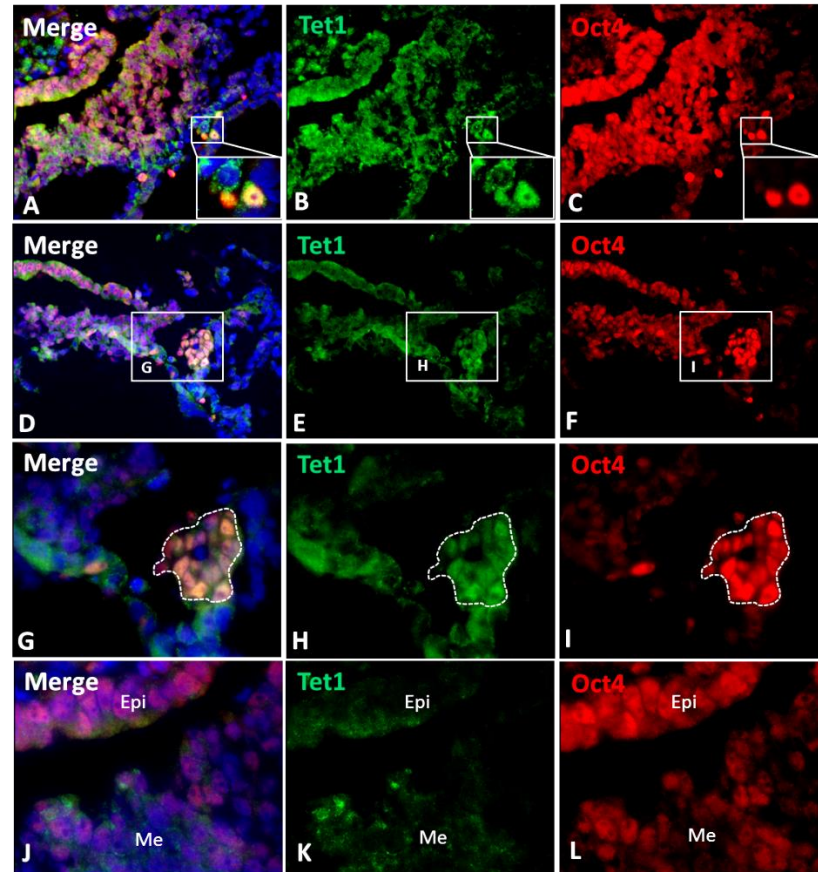


Figure 32. Tet1 expression in E13 embryo.

A-C, parallel section of PGC clusters. D-F, posterior end of E13 PGC cluster. PGC clusters are zoomed in in G-I respectively. Representative migratory PGCs are closed up in squares. PGC cluster are circled with dashed line.

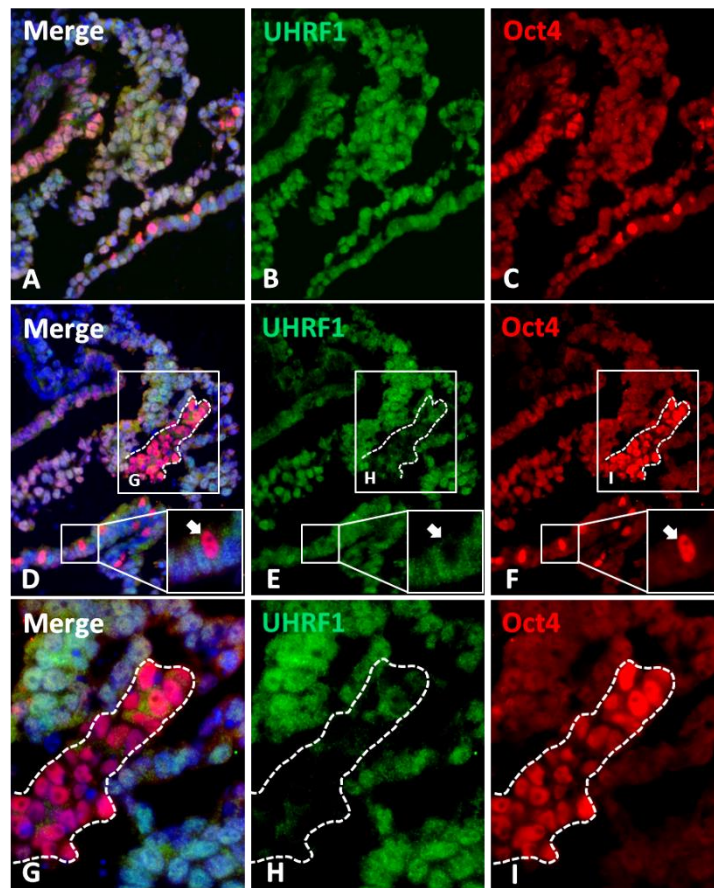


Figure 33. *Uhrf1* expression in E13 embryo.

A-C, parallel section of PGC clusters. D-F, posterior end of E13 PGC cluster. PGC clusters are zoomed in in G-I respectively. Arrows indicate migratory PGC. Representative migratory PGCs are closed up in squares. PGC cluster are circled with dashed line.

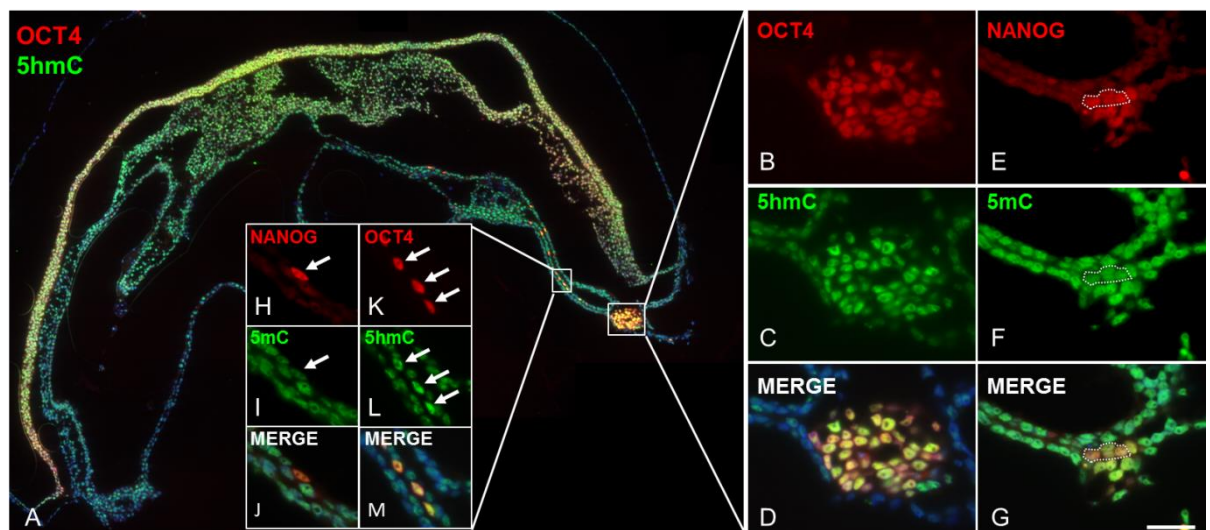


Figure 34. Immunofluorescence of 5-mC and 5-hmC in E14 PGCs.

A, Immunofluorescence against OCT4/5-hmC on E14 embryo sagittal sections. B-G, 5-hmC/OCT4 or NANOG/5-mC staining on PGC clusters. Four PGCs stained with

suppressed 5-mC are indicated in dashed circles. H-M, 5-mC/NANOG or 5-hmC/OCT4 staining on migratory PGCs. Arrows indicate migratory PGCs.

4.3.2 Histone modification fluctuations in pre-migratory PGCs

E13 pre-migratory and nascent migratory PGCs show significantly reduced H3K9me2 than somatic cells (Figure 35 A-C). Parallel sections to the PGC cluster show H3K9me2 homogenously staining cells in the mesoderm. This observation is consistent with H3K9me2 reduction in E8.0 pre-migratory reported in mouse PGCs (Seki, Hayashi et al. 2005).

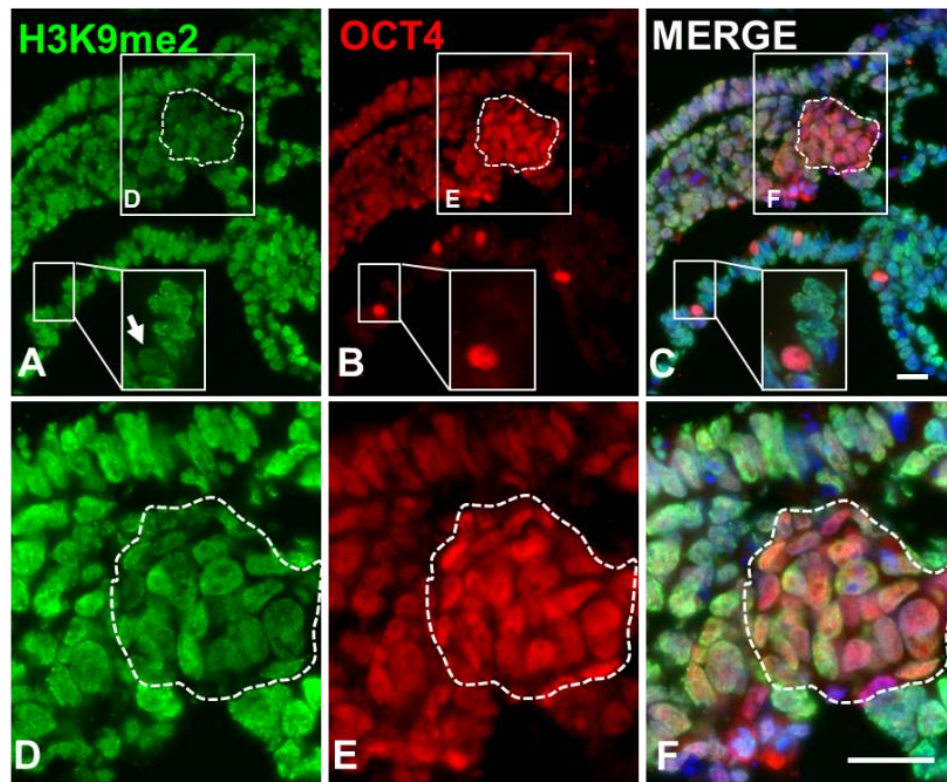


Figure 35. H3K9me2 expression profile in E13 PGCs.

A-C, posterior end of E13 PGC cluster. PGC clusters are zoomed in in D-F respectively. Representative migratory PGCs are closed up in squares. Arrows indicate migratory PGCs. Dashed circle shows the PGC cluster. G, quantification of H3K9me2 intensity on 21 PGCs and 22 somatic cells. $T < 0.0001$.

Before PGC specification, E11 embryos present high endodermal, medium ectodermal and low mesodermal H2A.Z. In E13 embryos the levels of H2A.Z were not different between somatic cells and PGCs (Figure 36).

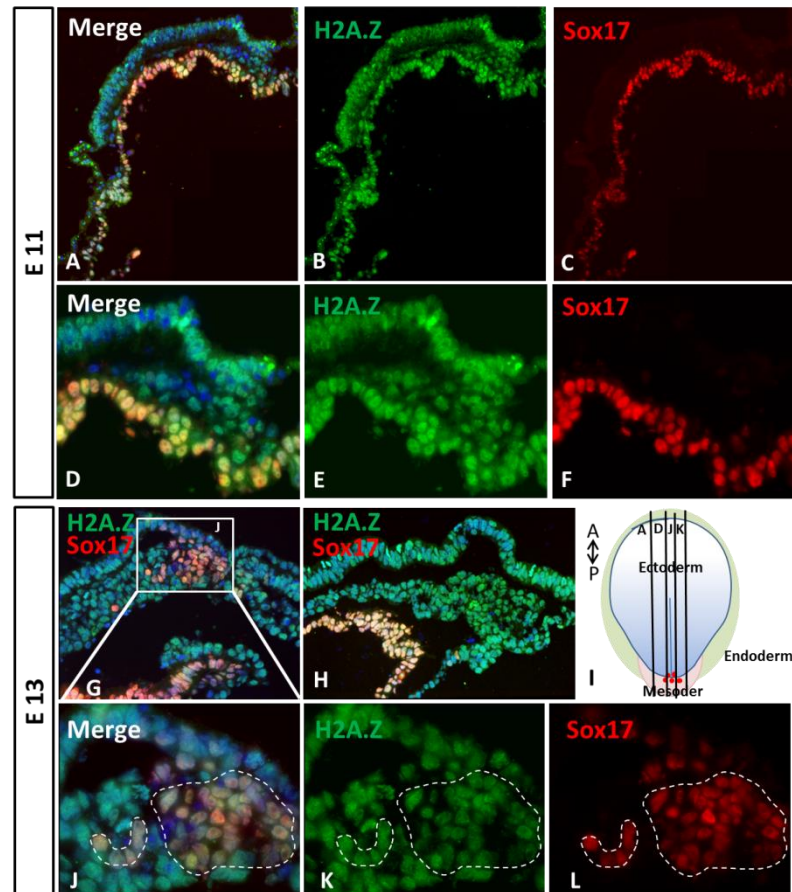


Figure 36. Immunofluorescence of H2A.Z in E11 and E13 embryos.

4.3.3 Epigenetic markers in migratory PGCs

Next, the levels of DNA methylation modifying molecules were studied in migratory PGCs in E16-17 embryos. OCT4 was double stained to identify migrating PGCs in endoderm. Low levels 5-mC and increased 5-hmC were observed in PGCs, which is consistent with the presence of TET1 and absence of UHRF1 in these cells (Figure 37). Dnmt3a is less intense than in somatic cells. H3K9me2 was low or negative in PGCs, while somatic cells were stained homogenously. E16 PGC presents different H3K27me3 pattern, compared with surrounding somatic cells. PGC shows obvious heterogeneous intensity, while the somatic cells have a uniform signal, with few bright dots, which possibly are the repressed regions on euchromatic regions. H2A.Z staining in PGCs was similar to that of somatic cells (Figure 37).

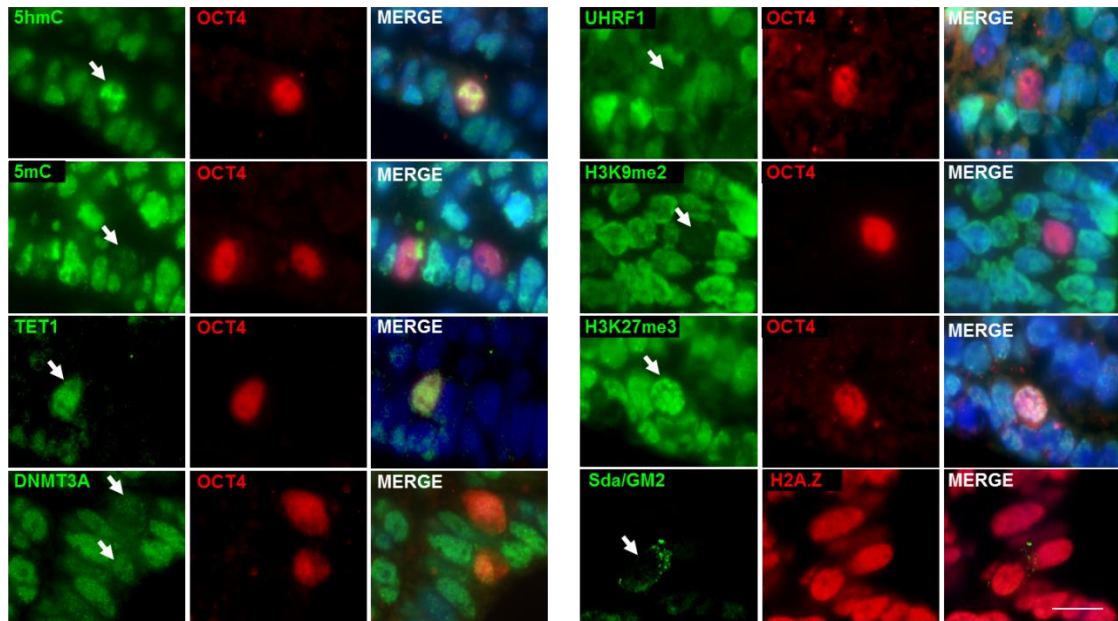


Figure 37. Key epigenetic markers in migratory E16/17 PGCs.

Arrows indicate migratory PGCs. OCT4 or Sda/GM2 were used as PGC markers. Scale bar, 10 μ m.

4.3.4 Hydroxylation of 5-mC to 5-hmC and methyl transferase Tet1 dynamics in gonadal PGCs

To depict the methylation pattern of germ line in late migratory and gonadal PGCs, 5-mC and 5-hmC IF was carried out in gonadal tissues collected between E25 to E70. From E25 and onwards, porcine gonadal PGCs show low or negative 5-mC and 5-hmC level in both genders (Figure 38 and Figure 39). This is consistent with the increased 5-hmC and Tet1 staining detectable in E16 migrating PGCs (Figure 37), suggesting that when PGCs reach the gonad they have undergone significant TET-mediated DNA demethylation (Figure 40). This observation is different from mouse gonadal PGCs, where Tet1/2 actively oxidise certain loci, generating 5-hmC peak at 11.5 dpi (Kohli and Zhang 2013). However, porcine migratory and gonadal PGC 5-mC and 5-hmC pattern is similar to that of wk4-wk9 hPGCs and 9.5-13.5 dpc mPGCs (Tang, Dietmann et al. 2015).

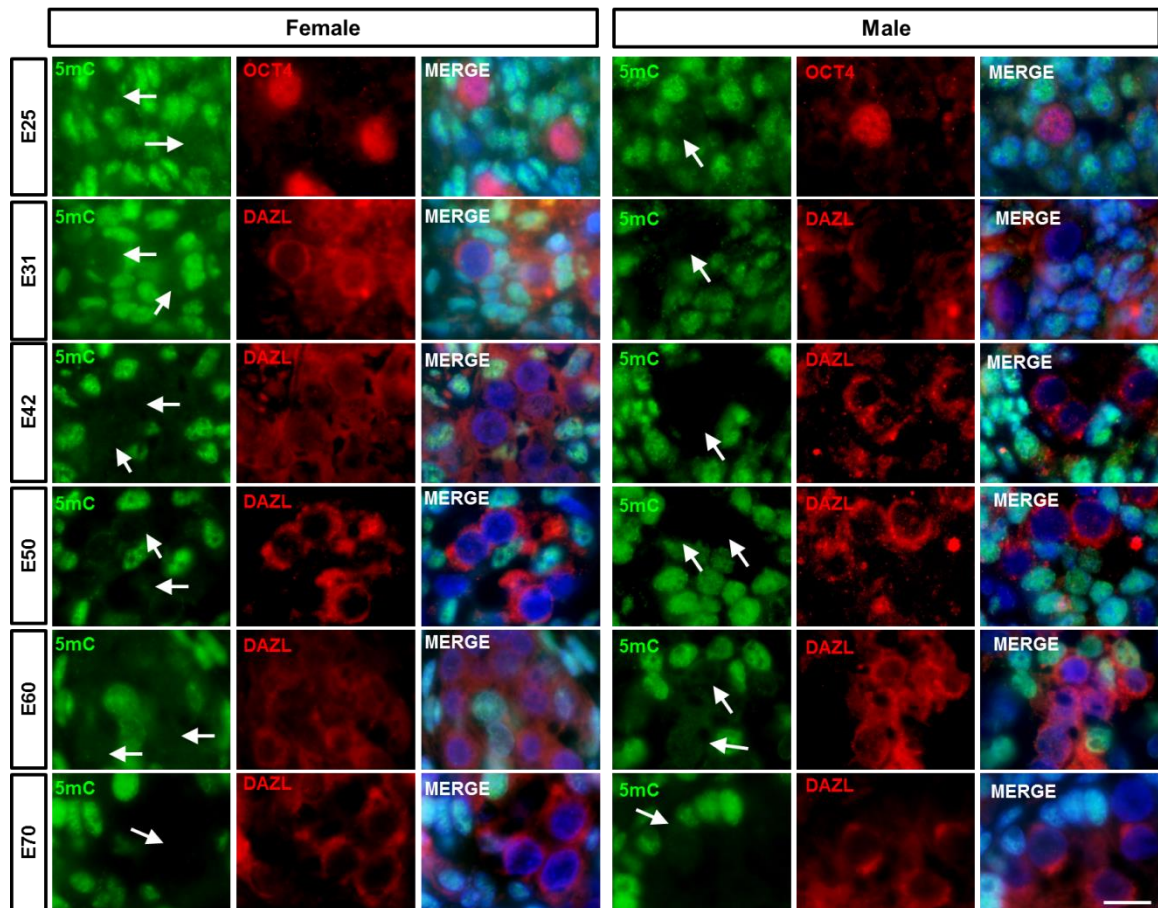


Figure 38. 5-mC expression profile in E25-E70 gonadal PGCs.

OCT4 and DAZL were used as PGC markers. Gonadal PGCs are indicated by arrows. Scale bar, 10µm.

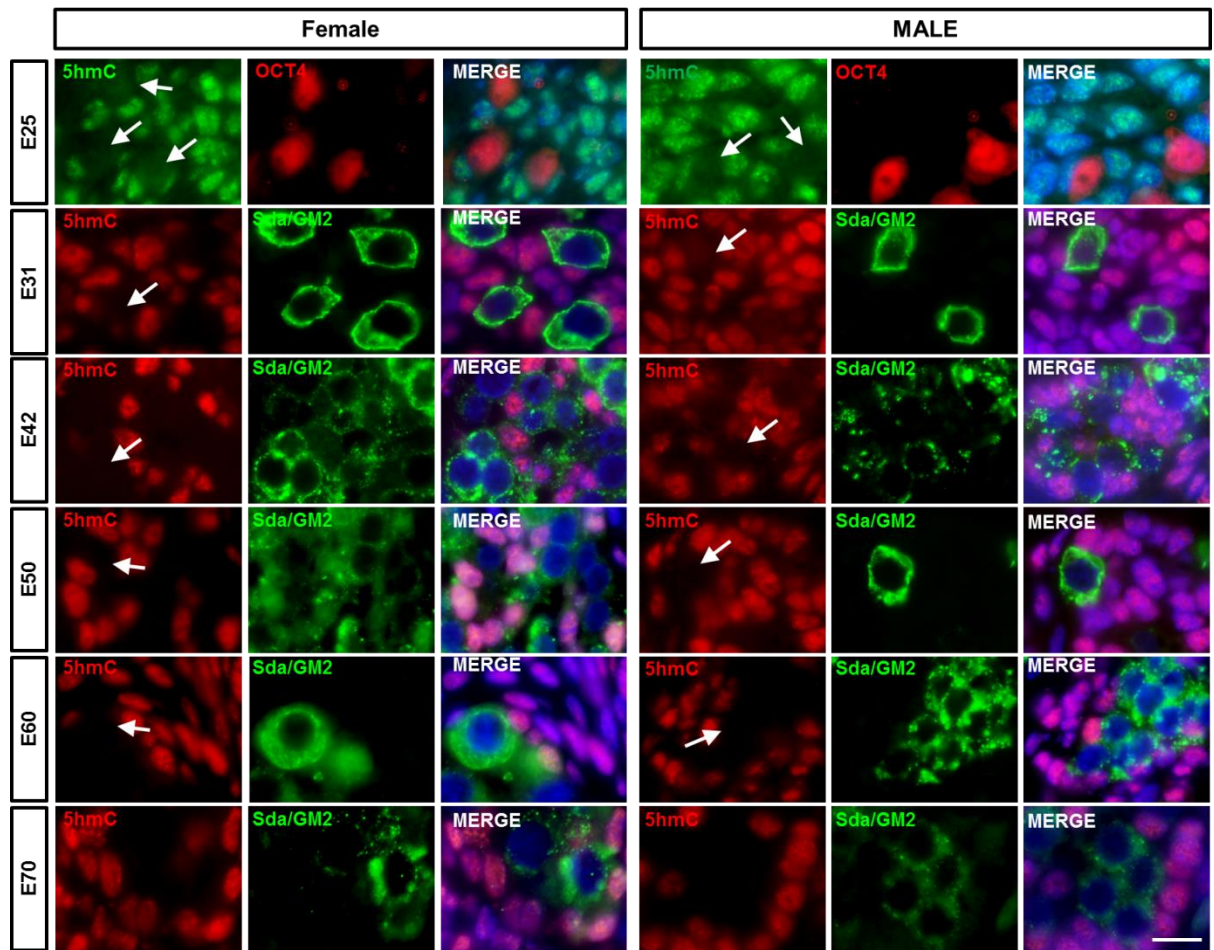


Figure 39. 5-hmC expression profile in E25-E70 gonadal PGCs.

OCT4 and Sda/GM2 were used as PGC markers. Gonadal PGCs are indicated by arrows. Scale bar, 10 μ m.

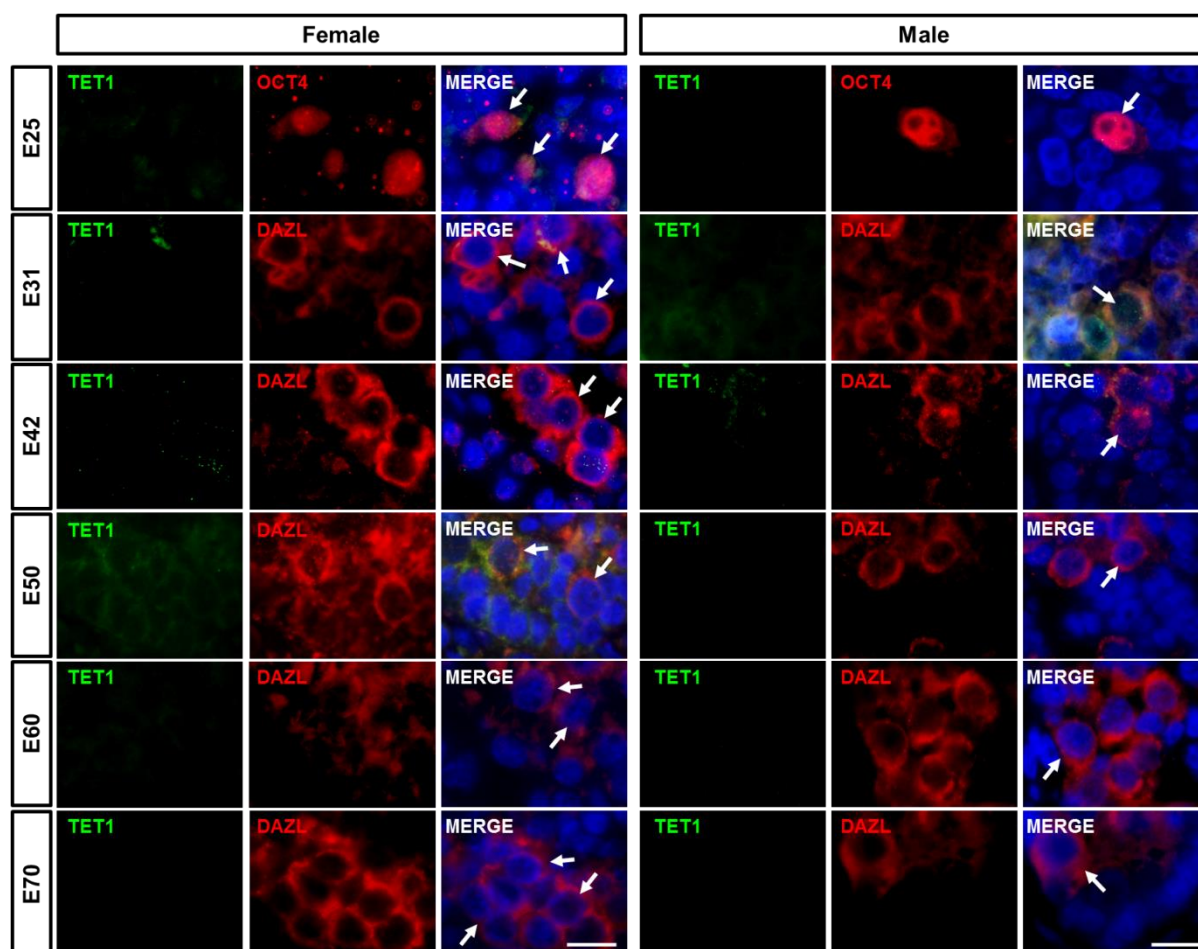


Figure 40. TET1 expression profile in E25-E70 gonadal PGCs.

OCT4 and DAZL are double stained as PGC markers. Gonadal PGCs are indicated by arrows. Scale bar, 10 μ m.

4.3.5 UHRF1 (NP95) and De novo methyltransferase DNMT3a Expression profile in Gonadal PGCs

To investigate the maintenance and de novo DNA methylation mechanisms, Uhrf1 and Dnmt3a were studied in germline. UHRF1 forms a complex with Dnmt1 to target replication foci maintaining CpG methylation (Liu, Gao et al. 2013). UHRF1 association with methylated H3K9 is also required for the maintenance of DNA methylation (Rothbart, Krajewski et al. 2012). Dnmt3a catalyses *de novo* methylation of DNA during early development (Okano, Bell et al. 1999).

Porcine Uhrf1 is negative in migrating and gonadal PGCs (Figure 41). Similarly, Uhrf1 is devoid in mouse PGC throughout 9.5-10.5 dpc (Kagiwada, Kurimoto et al.

2013) and hPGC from wk5.5 to wk16 (Gkountela, Zhang et al. 2015; Tang, Dietmann et al. 2015).

DNMT3A is absent in gonadal pPGCs (Figure 42), similar to observations in mouse and human PGC. Mouse *Dnmt3a/3b* are repressed upon PGC specification (Kurimoto, Yabuta et al. 2008) and maintained at low level until E13.5 dpc (Kagiwada, Kurimoto et al. 2013). hPGC have low DNMT3a and no DNMT3b level from wk5.5 to wk14 in both genders (Kagiwada, Kurimoto et al. 2013; Gkountela, Zhang et al. 2015).

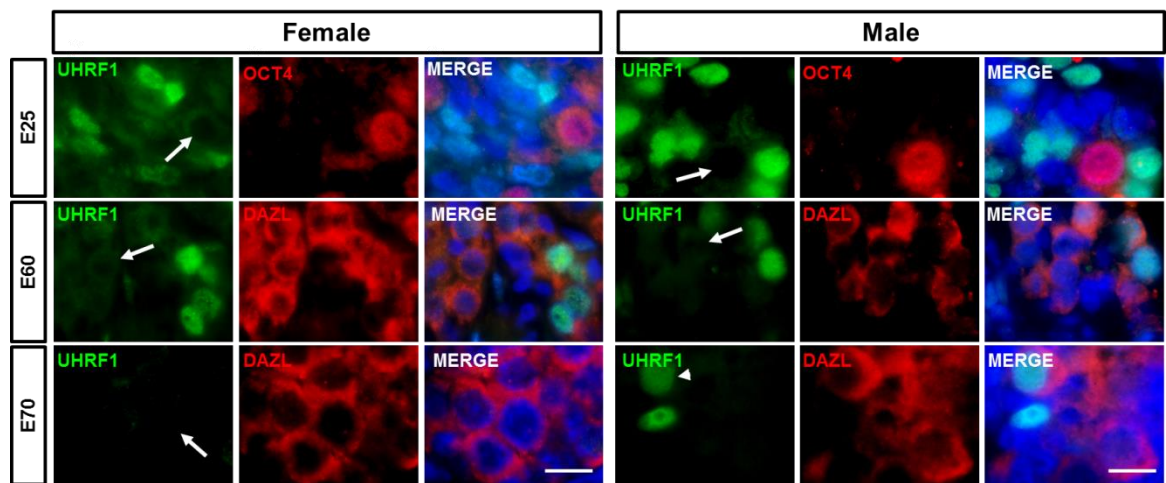


Figure 41. UHRF1 expression profile in E25-E70 gonadal PGCs.

OCT4 and DAZL markers for gonadal PGCs, which are indicated by arrows. Scale bar, 10µm.

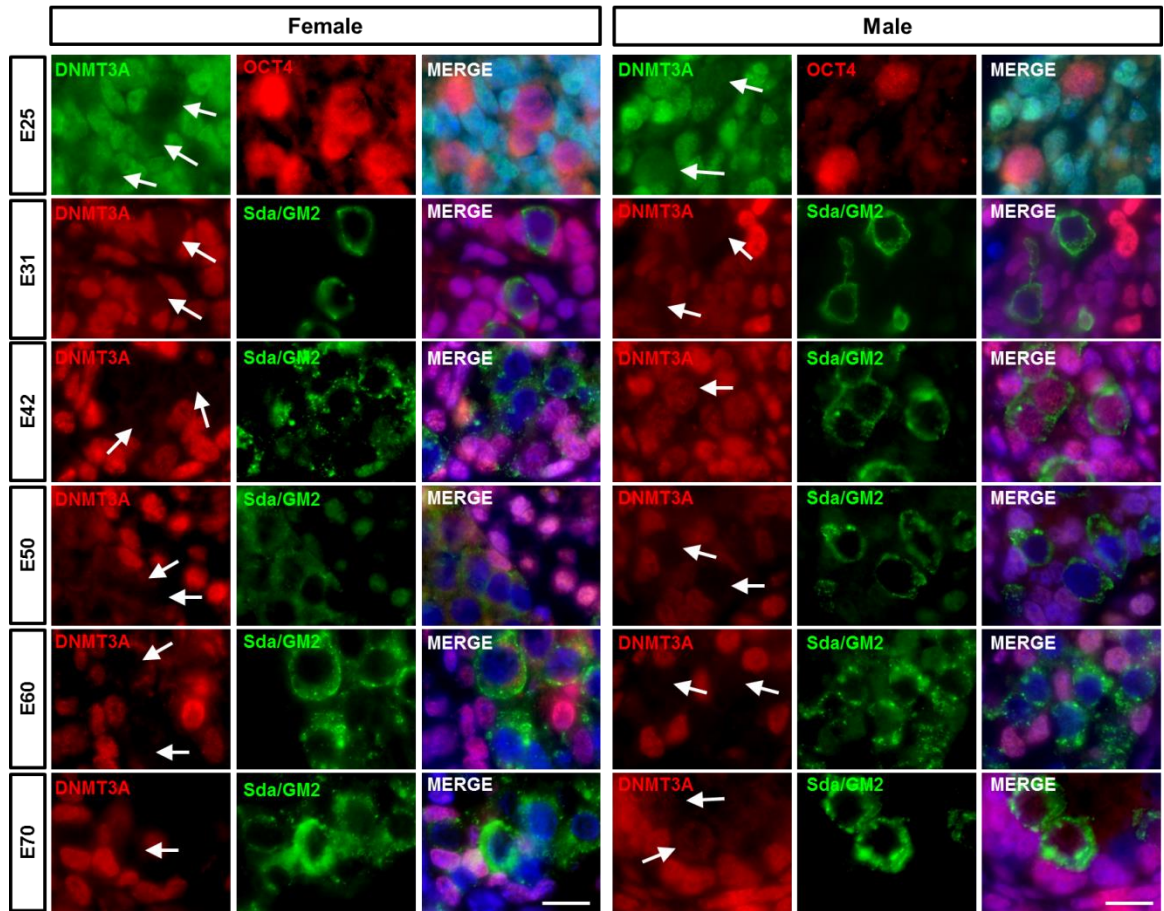


Figure 42. DNMT3A expression profile in E25-E70 gonadal PGCs.

OCT4 and Sda/GM2 were double stained to identify gonadal PGCs, which are indicated by arrows. Scale bar, 10µm.

4.3.6 Histone modification dynamics of H3K9me2, H3K27me3 and H2A.Z expression profile in gonadal PGCs

Finally the profile of H3K9me2, H3K27me3 and H2A.Z were investigated in gonadal stage PGCs. H3K9me2 maintained similar or lower level than somatic cells in both genders (Figure 43). There seem to be two waves of H3K9me2 intensity: 1) between E16-E31, a decline of H3K9me2 to half to total absence, compared with soma, 2) between E42-E60, H3K9me2 is restored at low level than the somatic neighbours, and then by E70 it is absent again.

In E16 PGCs H3K9me2 is homogenous with negative nuclei, while in E42-E60 the signal appears as dotty. The reduction is similar to the description in hPGC showing declining intensity than soma during wk4 to wk10, whereas the mark is retained in wk14 hPGCs in both genders (Yamaji, Seki et al. 2008; Tang, Dietmann et al. 2015).

However, there is no data beyond that stage showing whether signal is maintained or reduced like pPGC does. In mice H3K9me2 is depleted in PGC after 10.5 dpc.

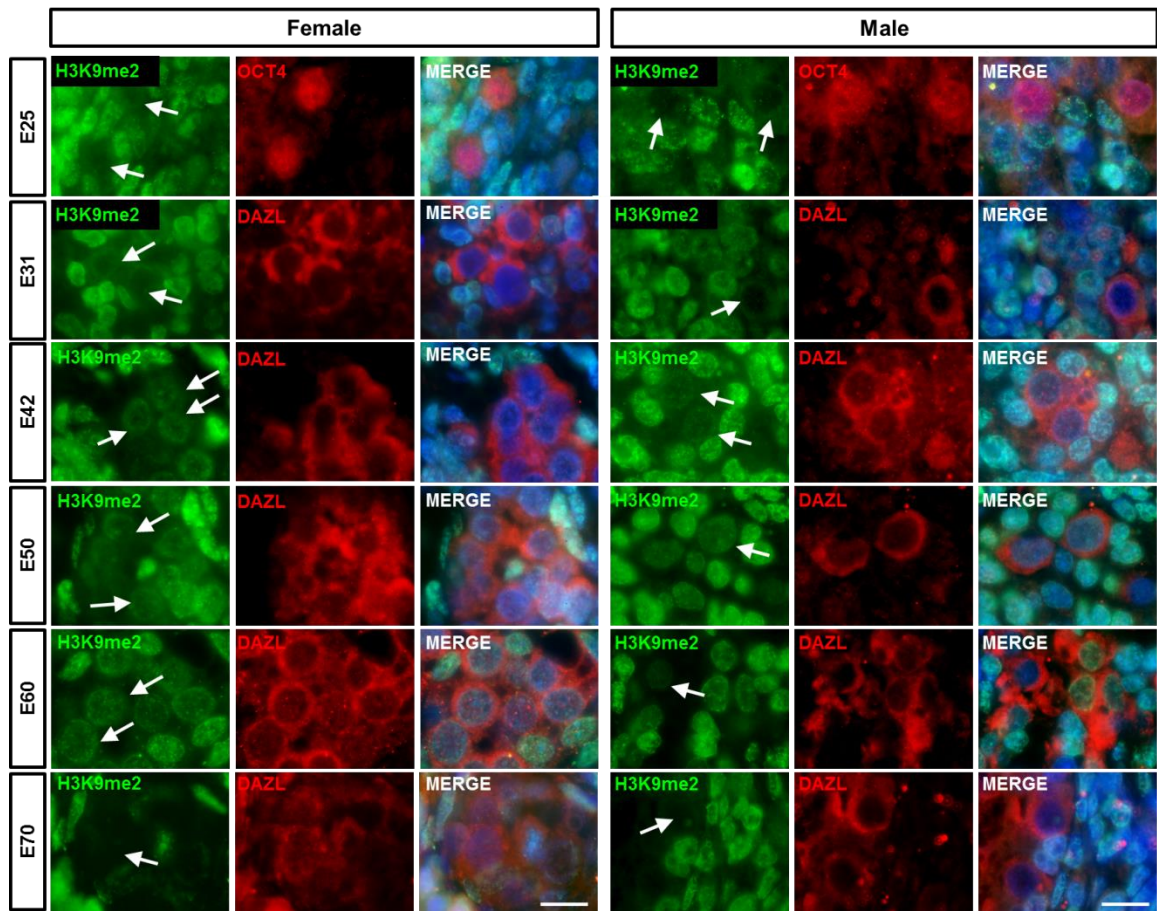


Figure 43. H3K9me2 expression profile in E25-E70 gonadal PGCs.

OCT4 and DAZL were double stained to identify gonadal PGCs, which are indicated by arrows. Scale bar, 10µm.

E31 female PGCs distinguish themselves from somatic cells by absence of inactivated X chromosome, demonstrated by bright single big dot in H3K27me3 (Figure 44). Besides, both germ cell and somatic cells have homogenous signal and negative nucleoli. In addition, tiny dotted pattern occupies some cells, this are repressed regions on euchromatic arms. The overall H3K27me3 signal in PGC is similar or relatively lower than somatic cells. E42 female PGC the signal declines, and is retained at very low level until E50, with general uniform signal and scattered bright dots on euchromatic arms. E60-70 female PGCs are undertaking zygotene I and pachytene I (Black and Erickson 1968), showing weak signal outlining the dividing chromosomes. Meanwhile, the somatic cells of female gonad are featured by the big intense territory of inactivated X chromosome. Male PGC shows similar pattern but continuously

declining signal intensity from E25 to E70. Granular H3K27me3 signal prevails among the whole nuclear area. This expression pattern is reminiscent of that in hPGC that enriched signal in migrating PGC and progressive decline to half intensity of neighbouring soma until wk9 (Tang, Dietmann et al. 2015). Both pig and human PGCs show conspicuous discrepancy on H3K27me3 pattern to mouse, which increases from 8 dpc and is maintained at high level until 13.5 dpc (Peters, Kubicek et al. 2003). Mouse PGCs lose H3K27me3 during 11.5-12.5 dpc and H2A.Z permanently after 11.5 dpc. During common hPGC progenitor stage, H3K27me3 is enriched in testes (wk7-wk10.5), while it is negative in half oogonia cells from wk6-wk8.5 and depleted onwards. From the endpoint of common genitor stage (wk11), spermatogonia is devoid of H3K27me3 until wk17, when 38% Oct4+/VASA+ cells retained H3K27me3 (Gkoutela, Li et al. 2013).

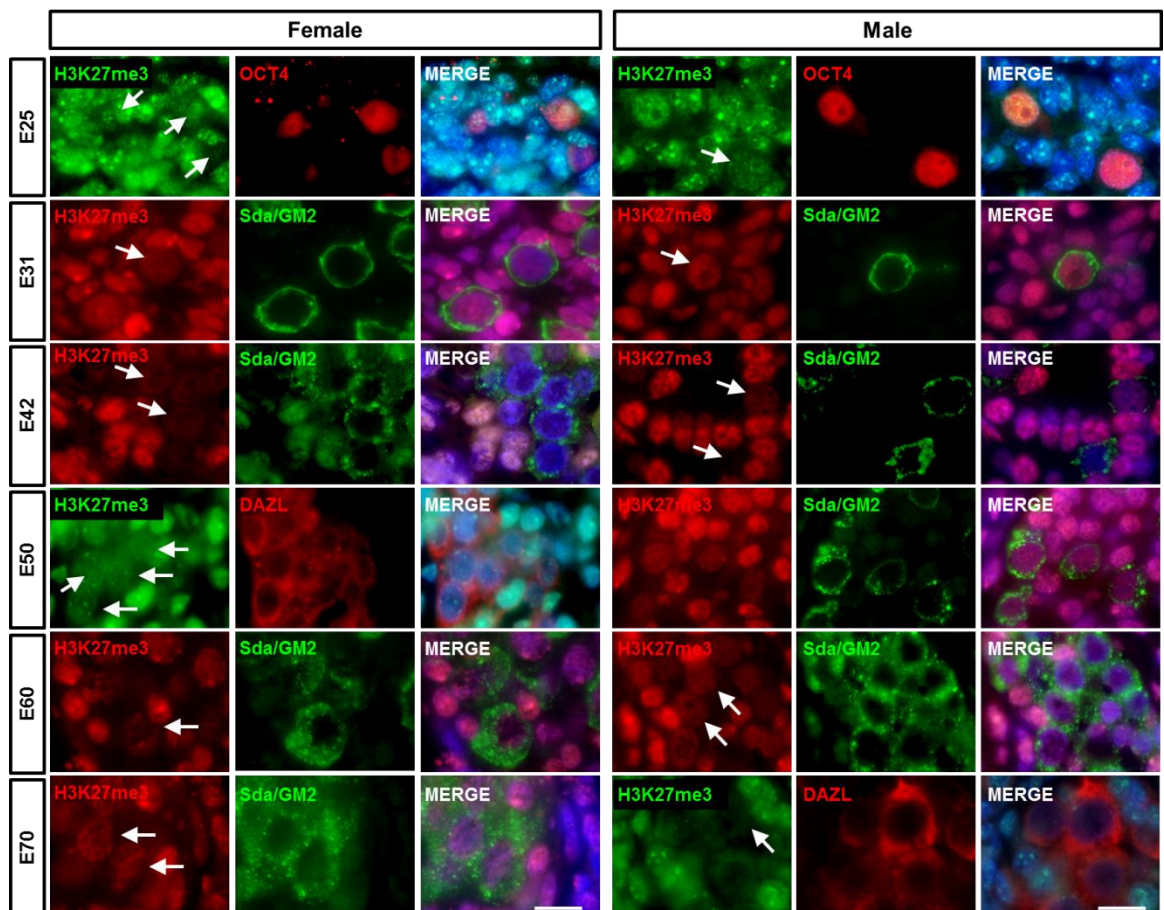


Figure 44. H3K27me3 expression profile in E25-E70 gonadal PGCs.

OCT4, DAZL and Sda/GM2 were double stained to identify gonadal PGCs, which are indicated by arrows. Scale bar, 10µm.

From E25 to E70, H2A.Z is continuously maintained at similar or lower level in PGCs, compared with somatic cells. This contrasts to the findings in mouse PGC, which show high intensity at 10.5 dpc which drastically declines until it disappears by 11.5-12.5 dpc. Meanwhile, H2A.Z remains constant in somatic cells (Hajkova, Ancelin et al. 2008). Similar to H3K27me3 profile, hPGC is enriched with H2A.Z in wk7-9 spermatogonia and wk7.5 oogonia, and devoid from wk11 until wk17, when it's retained in testis (Gkountela, Li et al. 2013). E25 and E31 spermatogonia are homogenous; while E50 and E60 H2A.Z display dots and territories, and E70 presents clear pachytene and zygotene morphology (Figure 45).

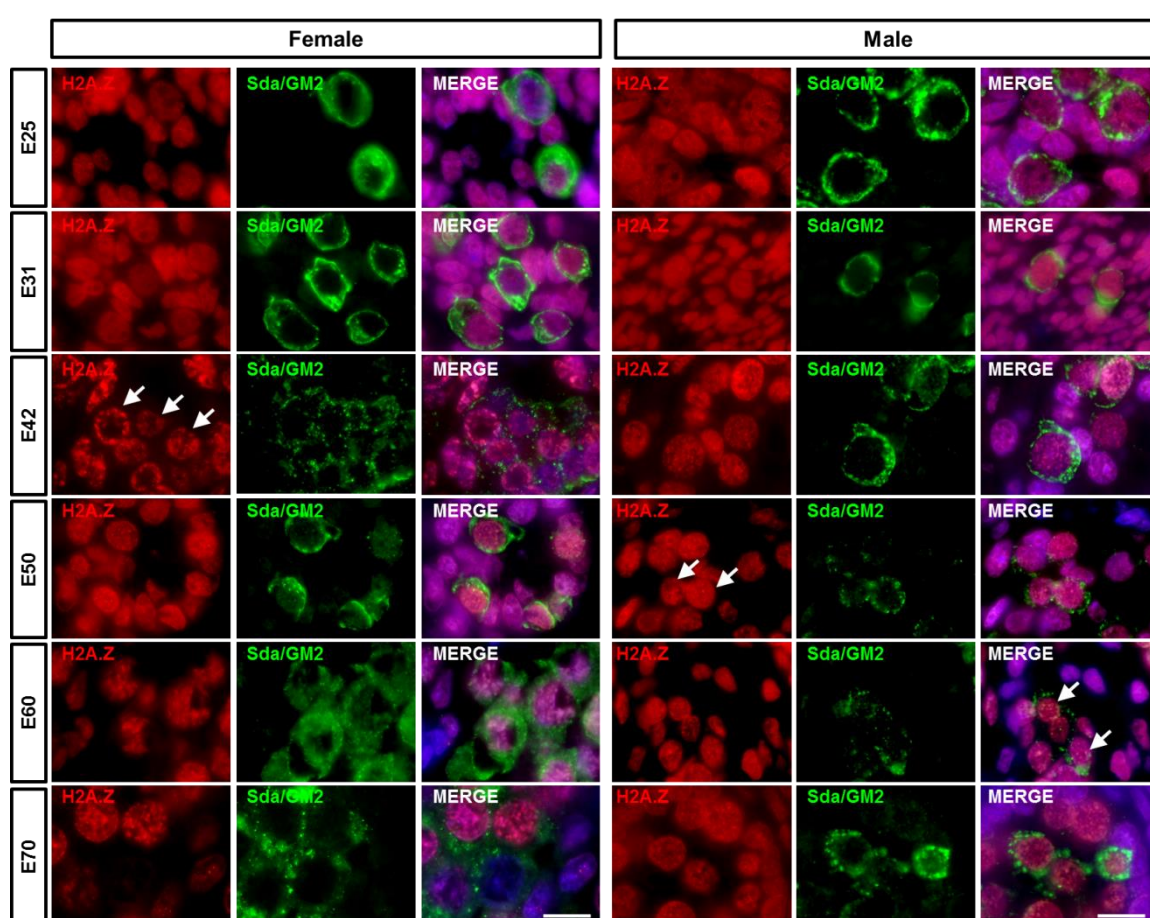


Figure 45. H2A.Z expression profile in E25-E70 gonadal PGCs.

Sda/GM2 is double stained to identify gonadal PGCs, which are indicated by arrows. Scale bar, 10µm.

The dynamic changes of the above mentioned epigenetic marks are summarized in Figure 46. 5-mC and 5-hmC are maintained high from PGC progenitors. TET1 and DNMT3a are expressed in nascent PGCs. As result, 5-mC level is declining and 5-hmC is upregulated in nascent PGCs. Both 5-mC and 5-hmC keep decreasing during PGC expansion, as TET1 or TET2 carry on oxidation, generating further product 5fC

and 5caC. 5-mC is significantly suppressed in migratory PGCs and negative from gonadal stages, while 5-hmC is devoid at gonadal stage. Expression of DNMT3A gradually decreases until sex determination in both genders. Female gonadal PGCs are deprived of DNMT3A, while male gonadal PGCs restore its expression around E70. UHRF1 is undetected all the time from early specification in female, but start to be detected in male E70 gonads.

H3K9me2 is slightly repressed in premigratory PGCs, and disappears between E16-31 and is maintained at low level between E42-60 until becoming negative from E70 in both genders. H3K27me3 is upregulated in migratory PGCs, but is very low in later stages. H2A.Z shows no visible differences to somatic cells in the pig germ line.

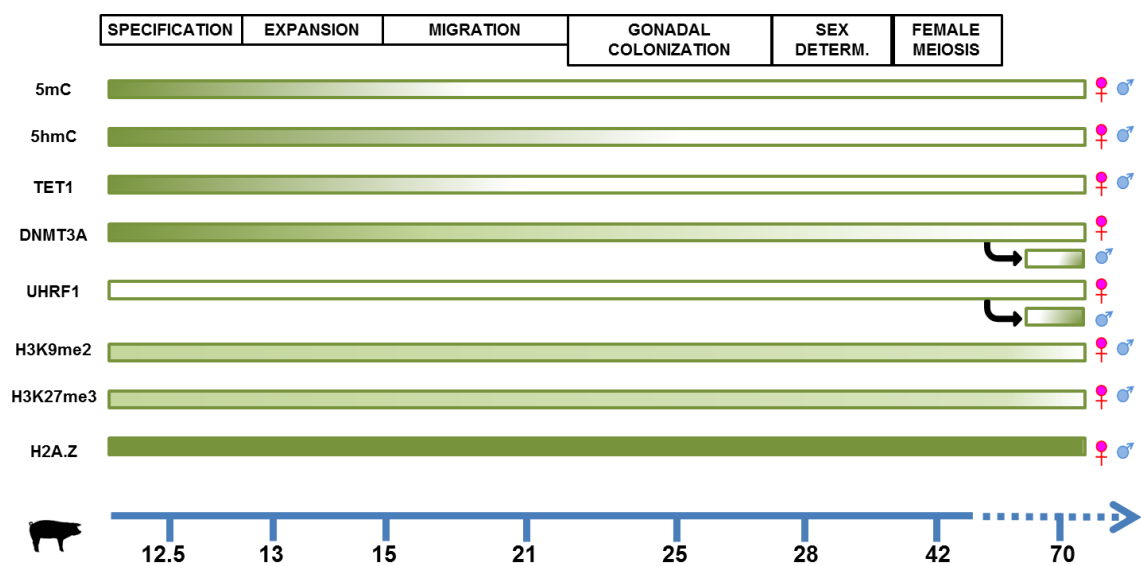


Figure 46. Summary of key epigenetic markers in porcine germ line.

Dynamics of demethylation related markers 5-mC, 5-hmC, TET1, DNMT3A and UHRF1, histone modification H3K9me2 and H3K27me3, and Histone variants H2A.Z in porcine germ line are summarized in this figure. The time window covers from ~E12 PGC precursors to E70 gonadal PGCs. Boxes at the top of graph labels the developmental stage of PGCs. Discrepancies and similarities between male and female PGCs are annotated. Timeline is presented at the bottom in days.

4.4 Discussion

In this chapter, epigenetic landmarks are detected by immunostaining in E13-70 pig germline. To compare this profile with mouse and human germline patterns, pig germline epigenetic events were summarized and discussed (Figure 46). E13 PGCs show higher 5-hmC intensity in PGCs than somatic neighbours. E14-17, developing PGCs show reduced levels of DNA methylation (5-mC), which was consistent with

low levels of Dnmt3a and UHRF, and increased levels of 5-hmC and Tet-1. In gonadal PGCs, 5-mC and 5-hmC are both very low/absent in male and female PGC. H3K9me2 is absent/low in migratory PGCs and increases in gonadal PGC until E70, and it maintains lower than in somatic cells. H3K27me3 is detected in migratory PGCs and decreases during gonadal PGCs development, remaining lower intensity than somatic cells. Histone H2A.Z is detected in nascent and migratory PGCs, and the signal increases in late gonadal stages. This systematic characterization of porcine PGCs identified important differences in the program of germ cell development compared to mice, and similar patterns of gene expression and epigenetic marks to those reported for humans.

Before PGCs are specified, the bilaminar embryo shows high levels of 5-hmC in the epiblasts and hypoblast cells. After PGCs are specified, however, only the PGC cluster show strong 5-hmC signal compared to the neighbouring somatic cells. The staining profiles suggest that PGCs may inherit the 5-hmC profile from the progenitors. Once the PGC program is initiated the wave of DNA demethylation is further reinforced by the downregulation of DNMT3A, and UHRF1, a cofactor that recruits DNMT1 to the newly synthesised DNA strand.

One limitation of IF is that it provides a genome wide perspective of the chromatin but does not provide specific information on loci of interest such as for example gene promoters, imprinted loci, or repetitive DNA sequences. Bisulfite conversion is one potential high resolution methods to study specific sequences, although it cannot distinguish between 5-mC and 5-hmC. New approaches incorporating modification to bisulfite protocol enable the distinction between these two states of cytosine methylation.

Tet1 (Ten-eleven translocation methylcytosine dioxygenase 1) and Tet2 hydroxylases catalyse the 5-mC (5 methylcytosine) to 5-hmC (5-hydroxymethylcytosine) conversion. Pig pre-migratory PGCs show higher levels of 5-hmC compared to their somatic neighbours. Porcine migratory and gonadal PGC 5-mC and 5-hmC pattern is similar to that of wk4-9 hPGCs and 9.5-13.5 dpc mPGCs (Tang, Dietmann et al. 2015). TET1 and TET2 are enriched in hPGCs at wk4 with transiently enriched 5-hmC and then TET1 remains in female PGCs from wk5 to wk9 (Tang, Dietmann et

al. 2015). In mouse PGC, 5-mC level declines from 9.5-10.5 dpc and diminishes from 11.5 dpc and onwards. Consistently with the reduction in DNA methylation mark, 5-hmC appears asynchronously in PGC from 9.5-10.5 dpc, and then gradually decreases from 11.5 dpc. The initiation of 5-hmC from 9.5-11.5 dpc is consistent with the presence of Tet1 and Tet2, while Tet3 is undetectable. Tet1 and Tet2 reach the peak abundance between 10.5-11.5 dpc, following relatively lower levels between 9.5-10.5 dpc, although still more abundant in PGC than neighbouring somatic cells (Hackett, Sengupta et al. 2013). Mouse Tet2 transcripts are detected by RNA-seq. The upstream promoter of Tet1 contains a cluster of TF binding sites interacting with pluripotency genes (Ficz, Branco et al. 2011). Tet1 is highly expressed and drastically decreases upon differentiation of mESC (Koh, Yabuuchi et al. 2011). This evidence suggests a correlation between mouse Tet1 and pluripotency. The temporary appearance of Tet1 in early migrating PGCs may partially initiate the pluripotency of early mPGCs. However, pPGCs and hPGCs do not re-establish pluripotency in the germ line, i.e. SOX2 is not expressed. This could be a difference in interaction between epigenetic and transcription regulation among pig, human, and mouse PGCs.

Mouse Prdm14 promotes Tet mediated base excision repair (BER) pathway in mESC (Okashita, Kumaki et al. 2014). However, the human PRDM14 doesn't play as essential role to germline as the mouse version (Sugawa, Arauzo-Bravo et al. 2015), although the protein is detectable in developing hPGCs (Gkountela, Zhang et al. 2015). Also, as mouse Prdm14 is largely involved in epigenetic reprogramming of mouse PGC development (Magnusdottir, Dietmann et al. 2013; Nakaki and Saitou 2014), including global DNA demethylation and H3K9me2 demethylation (Yamaji, Seki et al. 2008). The functional counterpart in human and porcine requires further investigation. Preliminary tests using a validated antibody did not provide consistent results in the pig samples, thus it is not possible to conclude whether this factor plays a role during pig PGC development. Further discussion on PRDM14 will be presented in the next chapter.

In parallel with active hydroxymethylation by Tet1 and Tet2, cell-division dependent passive dilution of CpG methylation is another mechanism contributing to DNA demethylation in the germline. In mouse, the passive demethylation takes place between E7.25 and E9 dpc (phase1). The repression of de novo methyltransferase

Dnmt3A/3B/3L (8.5-13.5 dpc) and methylation maintenance component Uhrf1 (9.5-13.5 dpc) causes the transient loss of active methylation, although Dnmt1 expression is high during 9.5-13.5 dpc (Kagiwada, Kurimoto et al. 2013). This wave of replication dependent reprogramming affects the bulk genomic DNA, leaving exceptional loci, including some germline specific genes. Protected 5-mC and 5-hmC will be directly oxidized in the forthcoming phase2 (9.5-12 dpc) by Tet1/2 (Wu and Zhang 2014). In this study I show that pig Dnmt3a is detected in nascent PGCs, as well as in somatic cells. Then, Dnmt3a declines in migratory PGCs and is completely absent in gonadal PGCs. The lack of Dnmt3a and Uhrf1 indicates impaired de novo methylation and maintenance mechanism, respectively, during migration and gonadal colonization.

Uhrf1 recruits Dnmt1 to replication foci, which is marked by PCNA (proliferating cell nuclear antigen) at mid-late S phase (Kagiwada, Kurimoto et al. 2013). Uhrf1 shows homogenous distribution in nuclei out of S phase. Within mid-late S phase, Uhrf1 is characterized by the conspicuous nuclear localization. This subnuclear pattern is colocalized with Dnmt1, PCNA and dense DAPI peri-centromeric heterochromatin staining. During 10.5-13.5 dpc, only less than 30% mPGCs are detected with Dnmt1 at replicating heterochromatic foci (Kagiwada, Kurimoto et al. 2013). Between 10.5-11.5 dpc the proportion is even lower, with only 10%-20% positive cells. Pig Uhrf1 is depleted from PGCs from E13 to gonadal stages. Similarly, human Uhrf1 is detected in gonadal PGCs (Gkoutela, Zhang et al. 2015). Even in faint E16 pPGCs, there is no typical mid-late S phase density observed, which is consistent with the G2 arrest during migration, when almost half cells were under G2 phase, while only a quarter PGCs were at S phase (Hyldig, Croxall et al. 2011). There is very rare chance to capture the mid-late S replication foci. Taken together, human and pig demethylation don't present distinct phases as mouse PGCs do. It is likely that combined passive and active demethylation occur during the same period of time. However, due to the lack of direct evidence, it is difficult to give conclusive explanation.

mPGCs undertake cell-by-cell progressive reduction of H3K9me2 and H3K27me3 during G2 arrest, when RNA polymerase II dependent RNA transcription is temporarily repressed until the onset of H3K27me3 (Seki, Yamaji et al. 2007). This is similar to E16-E25 pPGC, except for different starting levels of various H3 lysine

modifications. H3K9me2 preferentially marks repressed gene/regions on euchromatic regions, and less prevalent is centromeric heterochromatin of interphase nuclei (Peters, Kubicek et al. 2003). pPGCs show lower H3K9me2 levels than somatic cells. Equivalent 8.0 dpc mPGCs present similar or lower level of H3K9me2 compared with somatic cells. H3K9me2 intensity at 9.5-12.5 dpc is very low, although it progressively increases (Seki, Hayashi et al. 2005). In hPGC low level of H3K9me2 are detected in wk5-9, is depleted at wk10, and then is restored at wk14 (Gkountela, Zhang et al. 2015). The current study shows that similar two waves of H3K9me2 are observed in pPGCs. The abrupt increase in pPGCs during E42-E60 and E70 drop correlates with the cell proliferation and meiotic dividing, respectively. During E9.5-11.5, mPGC are rapidly proliferating while Glp is actively repressed, resulting passive histone demethylation (Ohno, Nakayama et al. 2013). Constantly low H3K9me2 level in male mPGC is attributed to the absence of GLP, although G9a is significantly upregulated (Deguchi, Nagamatsu et al. 2013). Mouse H3K9me2 is maintained low in undifferentiated spermatogonia and is upregulated in differentiated spermatogonia, then downregulated during meiosis in pachytene spermatocytes (Deguchi, Nagamatsu et al. 2013).

H3K27me3 typically associates with facultative heterochromatin (Xi) and broadly disperses through euchromatin (Peters, Kubicek et al. 2003). mPGC shows rapidly elevating H3K27me3 levels during 8.5-9.0 dpc, and then maintain high levels until 12 dpc (Seki, Hayashi et al. 2005). The heterogeneity of H3K27me3 level along the cell cycle also indicate different phase of cell cleavage of PGCs. H3K27me3 display highest level at S phase and the number of high-H3K27me3 PGC is in proportion with the rate of S phase PGCs (Kagiwada, Kurimoto et al. 2013). Similarly, E16-31 pPGCs, are detected with declining S phase proportion (25.26% at E17 to 6% in male and 3% in female at E31) and H3K27me3 intensity (Hyldig, Croxall et al. 2011). Consistently, wk7-10 mitotic gonadal hPGCs have low H3K27me3 level in both genders, except for significant dot on Xi (Guo, Yan et al. 2015). The hypomethylation status of H3K9me2 and H3K27me3 suggests less repressed gene/region on euchromatin arm and active X chromosome of oogonia, which is followed by the normal meiosis, when the imprints are erased in PGCs (Hyldig, Croxall et al. 2011).

Similar to H3K27me3 profile, hPGC are enriched with H2A.Z in wk7-9 spermatogonia and wk7.5 oogonia, and devoid from wk11 until wk17, when is retained in the testis (Gkountela, Li et al. 2013). A subpopulation of mPGCs are depleted of H2A.Z from 11.5 dpc (Hajkova, Ancelin et al. 2008). This is different from pPGC, in which H2A.Z is continuously observed with similar expression level as somatic cells from premigratory PGC cluster.

During G2 arrest, a subpopulation of more advanced 11.5 dpc mouse PGCs lose H1 and H2A.Z in replication-independent manner (Hajkova, Ancelin et al. 2008). This depletion is preceded by the loss of 5-mC and H3 modifications, such as H3K27me3, H3K9me3 and H3K9ac. In contrast, another study showed that the chromatin state is constant throughout 9.5-12.5 dpc in mPGCs from aspects of chromocentres localization of linker histone H1 and H3K27me3 pattern. This discrepancy was reasoned for divergent methods of PGC collection (Kagiwada, Kurimoto et al. 2013). In mESC, panH2A.Z and acH2A.Z distribution largely correlated with H3K4me3 at active promoters, and H3K27me3 at gene inactive regions. H2A.Z is critical to the modulation of nucleosome occupancy and chromatin accessibility at enhancers, and therefore required for self-renewal and pluripotency of mESCs. Besides, it facilitates Oct4 to target key pluripotency genes in ESC, such as Klf4. Interestingly, H2A.Z also facilitates differentiation of mESCs (Hu, Cui et al. 2013). It is also suggested that temporary of H2A.Z is predictive for 5-hmC fluctuation, not in causal manner. Therefore, the H2A.Z is correlated with gene expression depending on the context (Yu, Xiao et al. 2013). Unlike canonical histones, the variant H2A.Z can be incorporated into the chromatin outside of S phase (Hajkova, Ancelin et al. 2008). Pig PGCs maintain H2A.Z level, which might permit the expression of pluripotency genes such as OCT4, on the partially repressed euchromatin.

The observations reported in this chapter show that epigenetic changes occur gradually during porcine germ line reprogramming compared to the reports in the mouse. All selected key epigenetic marks show continuously changing trends over many days, instead of abrupt changes reported in mouse PGCs. Overall, human gonadal PGCs show similar gradual fluctuations to those observed in pig PGCs.

Chapter 5

5. Single cell transcriptome analysis of pig PGCs

5.1 Introduction

To complement the findings in the previous chapters, it was decided that a more comprehensive analysis of the gene expression profile of pig PGCs could be determined by analysis of the transcriptome of early pig PGCs. New advances in single cell transcriptomics have enabled the analysis of small cell populations obtained from in vivo samples (Shapiro, Biezuner et al. 2013). The advantage of performing this analysis from in vivo pig PGCs is that they will provide for the first time a description of the gene regulatory network of this lineage in a non-rodent species. Studies of the transcriptome in human PGCs report the status of cells collected at wk 4-5, which is equivalent to migratory PGCs (~E18-20 in the pig). To bridge the gap in our knowledge, this chapter reports the analysis of the transcriptome of ~E13 pig PGCs. To achieve this aim, RNA seq. was performed in single cells isolated from posterior end of ~E13 porcine embryos. A combination of PGCs (n=6) and neighbouring somatic cells (n=60) was analysed and differentially expressed genes were identified. The transcriptome of PGC was further compared to hPGCL cells obtained in vitro, the TCAM2 cells line (an early human PGC tumour cell line) and human gonadal PGCs, reported previously by Irie et al.(Irie, Weinberger et al. 2015).

5.2 Materials and Methods

5.2.1 Embryo dissociation and RNA-seq

E13 embryos (30 embryos from two biological replicates) were collected as shown in Figure 47. A triangular area which contains PGC cluster was cut at the posterior end of embryo. Dissociated cells were loaded onto the C1 (Fluidigm system) for cDNA preparation of 66 single cells. After reverse transcription, qPCR was performed prior to sequencing, with *Oct4*, *Nanog*, *Blimp1*, *Sox2* and housekeeping *GAPDH*, to screen the 66 single cells. Cells with high expression of these genes plus other cells were picked for sequencing. Finally, 6 out of 66 cells were detected as *bona fide* PGCs based on the expression of different markers (see below in Figure 47 more details on how the cells were clustered).

Excess of cDNA was used for additional qPCR for *NANOS3*, *TFAP2C*, *SOX17*, *cKIT*, and *GATA4*. All above genes were chosen according to IF profile of E13 pig embryos. Expression profiles of these genes helped determine the identity of the single cells.

Excess cells from dissociated clusters were stained with Oct4 and Sda/GM-2 to confirm that PGC were present in the preparations. A total of 258 cells were quantified, in which 14.7% (38 cells) were Sda/GM-2⁺/OCT-4^{high}, 29% (75 cells) were Sda/GM-2⁺/OCT-4 weak or partial nuclear OCT4, and 56% (145 cells) were Sda/GM-2⁻/OCT4⁻. In addition the cell diameter was measured. Sda/GM-2⁺/Oct4⁺ high group: 12.9±2.26 µm, Sda/GM-2⁺/Oct4 low or negative group: 13.4±2.45 µm, and negative group was 12.76±2.87 µm. No significant differences were found in the diameters of the different cell types.

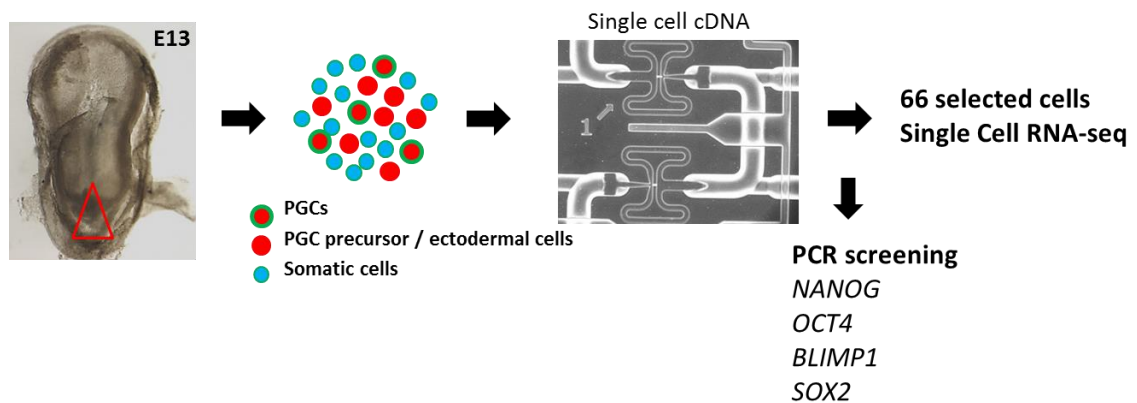


Figure 47. Workflow of single cells RNA seq.

Triangular pieces at the posterior end of ~E12.5-13 porcine embryos were dissected and dissociated using Trypsin. The pool of single cells consists of PGCs, PGC precursors and other somatic cells (indicated by different colors). Dissociated cells were then loaded to Fluidigm C1 to generate single cell cDNA library. After qPCR screening, 66 selected single cells were subjected to Illumina RNA-seq.

5.2.2 RNA-seq exploratory analysis of pig PGCs and comparison with somatic cells

RNA-seq was carried out on PCR selected single cells. 6 PGCs and 60 somatic cells were sequenced. Normalized reads were presented in RPKM. All samples had at least more than 4.5 million reads. When unsupervised hierarchical clustering was applied, 4 PGCs were clustered together, while the other two fall in different clusters. This suggests that the transcriptomic variances between some PGCs might be greater than that between PGCs and neighbouring somatic cells. To assess the overall relationship between cells, supervised hierarchical clustering using a panel of markers of different

germ layers was used to categorize the cells (Figure 48). The different colours at the left hand side indicate genes belonging to different lineages: germ cells, pluripotency, mesoderm, endoderm and ectoderm. The dendrogram at the top shows the clustering of all single cells based on the selected panel of genes. The 6 PGCs clustered together on the left (blue cells). All PGCs share high expression of germ cell related genes (NANOS3, AP2 γ (TFAP2C), and KIT), the pluripotency genes (OCT4 (POU5F1), NANOG, and GDF3), low expression of endoderm GATA3 and variable levels of SOX17 (Table 1 and

Table 2). PRDM14 and SOX2 are very low or negative in all cells and PRDM1 varies at low RPKM. NANOS3 is exclusively expressed in PGCs, which is consistent with the report in hPGCLC and gonadal hPGCs (Irie, Weinberger et al. 2015). Based on the clustering obtained with the selected panel of genes, an analysis of the whole transcriptome was carried out to find genes with similar expression profiles as key genes such as NANOS3. Genes with similar expression profile, i.e. high expression in PGCs and very low/no expression in other cells include: GPR50, SASH3, CRSP3, C9orf152, TMEM163, HUMMLC2B, APBB1IP, FBP1, APCS, PLAUI, SERPINI1, ISL1, GIP, PDPN, CXCL13, SPTLC2, ETV1, HERC5, G3PD, TGFB2, MCUR1, MAFB, AGA, DND1, GPD1L, SLC7A3, CD9, STRADB and PARP1. Most of them are not well studied in the context of PGCs; however it was interesting to see that DND1, a well-known PGC marker is also part of this group.

Apart from PGC branch, the rest are somatic cells which fall into two clusters. Group 1 cells (red cells) are clustered closer to PGCs. Upregulation of selected mesodermal genes, such as *TBX3*, *TCF7L1* and *CDX4*; make them appear like mesodermal cells. However, within this group 4 closest cells to PGCs also share high expression of *TFAP2C*, *NANOG* and *OCT4*. Interestingly, these 4 closer cells have relatively lower *T* level than the other group 1 cells, except for 2 PGC (P1.N706_N503 and P1.N701_N504). Could they be PGC precursors in transition to PGC? Or they are differentiating towards mesoderm cells? In chapter 6, I'll test the origin of PGC from mesoendoderm progenitors.

The other cluster (green group 2) on the somatic branch has a more endodermal identity. Selected endodermal genes, like *GATA6*, *ANXA4* and *TACSTD1* are highly expressed in the majority of them.

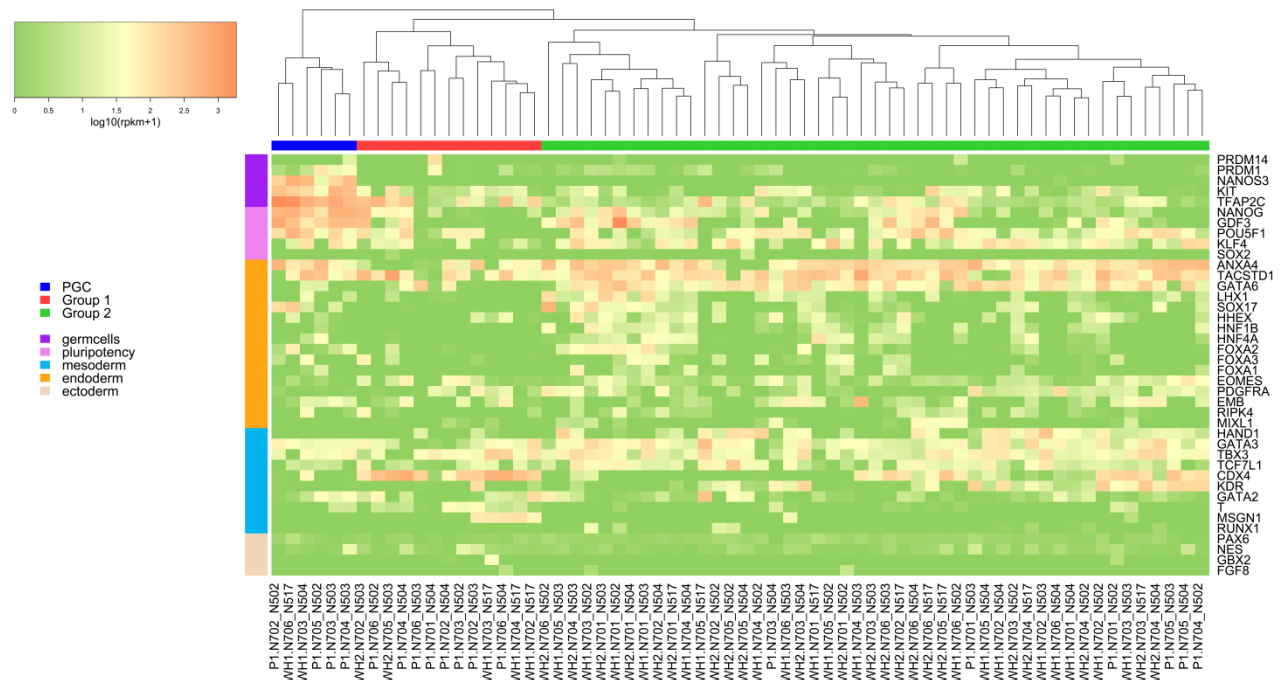


Figure 48. Heatmap of UHC with selected genes related to germ cell, pluripotency, mesoderm and endoderm.

Gene names are labelled at the right side of heatmap. Colour key of heat map is showed at the top left. Different cell clusters and cell types are indicated in different colours.

The mRNA level of genes varies in PGCs. Table2 shows examples of PGC related genes. 2 out of 6 PGCs have no *SOX17* expression, 2 have ~10 RPKMs, while the other 2 PGCs has over 10 times mRNA level over the 2 medium PGCs. Interestingly, the lowest *SOX17* level coincided with highest *PRDM1* level, and the PGCs with the highest *SOX17* level shows lowest *PRDM1* level. Low and high *TFAP2C* level are positively correlated with *SOX17* levels. *NANOS3* is expressed in all PGCs without obvious pattern. Importantly, no somatic cells express *NANOS3*. *Prdm14* is only expressed in 1 *SOX17*- PGC.

Table 2. Transcription level of *SOX17*, *PRDM1*, *PRDM14*, *NANOS3* and *TFAP2C* in 6 single PGCs. Expression levels are shown in RPKM (normalised counts).

GENE_ID	P1.N702_N502	P1.N703_N503	P1.N704_N503	P1.N705_N502	WH1.N703-N504	WH1.N706-N517
006256 <i>SOX17</i>	121	12	0	0	10	236
004369 <i>PRDM1</i>	2	23	36	98	5	1
006195 <i>PRDM14</i>	0	0	14	0	0	0
013759 <i>NANOS3</i>	169	147	359	22	412	607
007499 <i>TFAP2C</i>	1815	688	395	397	626	1523

Due to the divergent gene expression profile in group1 cells, a more detailed comparison was performed between PGC and two subgroups of group1 cells on whole transcriptome (Figure 49). Distinctive expression profiles between PGCs and both subgroups of group1 cells were observed. Compared with PGCs, there are more upregulated genes in sub1 (566) (A) than sub2 (208) (B). Besides, less downregulated genes are plotted from sub1 (152) (C) than sub2 (584) (D). This suggests that although the PGCs and sub1 cells in group1 are clustered close to each other, they are distinctive populations.

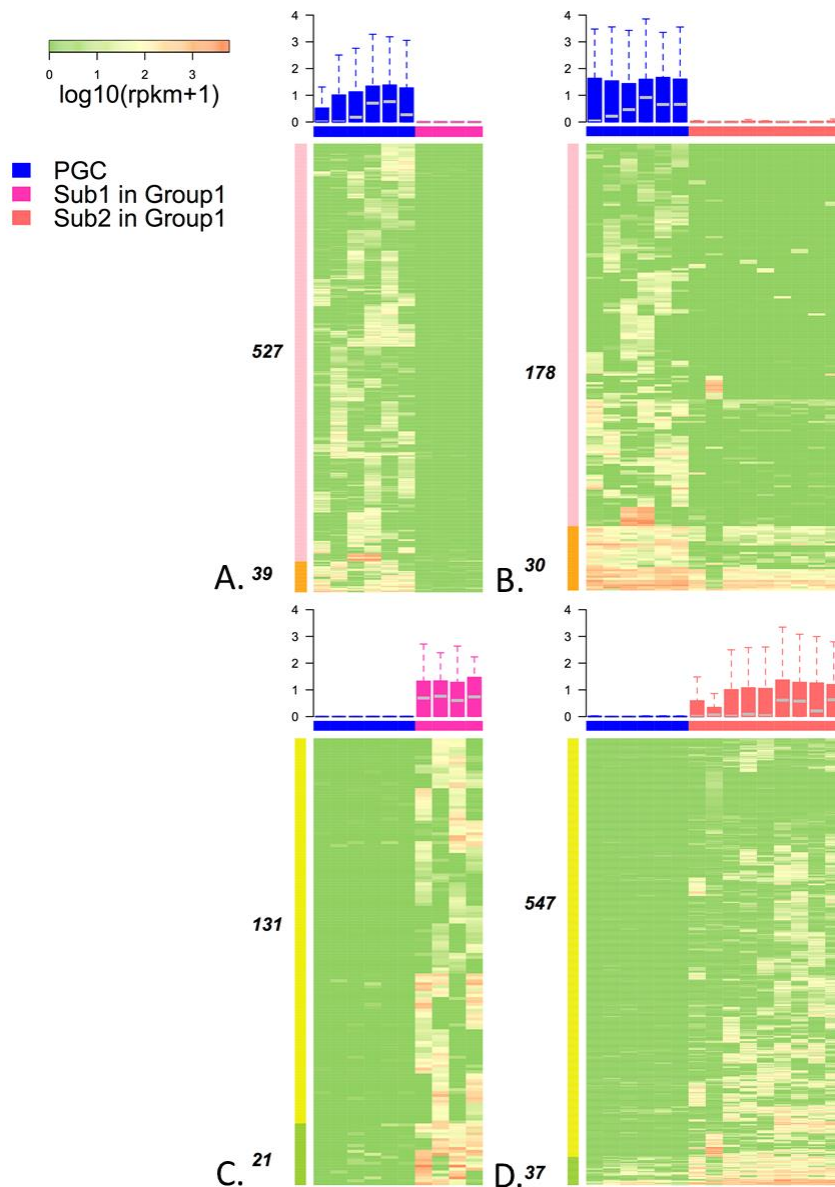


Figure 49. Heatmap and expression level of DE genes between PGCs and group1 cells.

A. Upregulated genes in PGCs compared with 4 closely clustered group1 cells. **B.** Upregulated genes in PGCs compared with 9 further clustered group1 cells. **C.** Downregulated genes in PGCs compared with 4 closely clustered group1 cells. **D.** Downregulated genes in PGCs compared with 9 further clustered group1 cells. Boxplots on top of heat maps show the over expression level of corresponding cells. Unit of boxplot is $\log_{10}(\text{rpkm}+1)$. Numbers of DE genes are indicated at left of each heat map. Colour key is shown at top left.

Next, differential gene expression (DE) analysis was performed between the three groups of cells (Figure 50). Overall the number of downregulated genes is larger and the number of upregulated genes is smaller in PGC compared to the other groups. This observation is consistent with the fact that PGCs express multiple genes (such as BLIMP1, NANOS3, DND1, etc.) that have specific role in inhibiting transcription and translation (Hayashi, de Sousa Lopes et al. 2007).

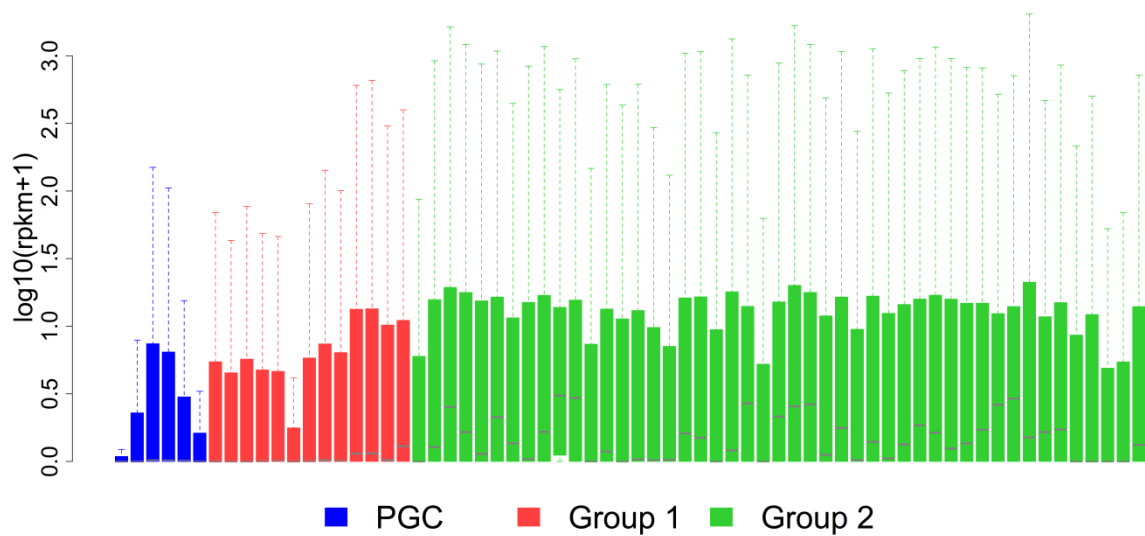


Figure 50. Global DE genes in pig PGCs and somatic cells.

RPKM are expressed as $\log_{10}(\text{RPKM}+1)$ values. The ratio of upregulated/downregulated genes of each cell is plotted as one bar. Blue, PGCs. Red, group1 somatic cells. Green group2 somatic cells. Median of global gene expression level is showed in black bar.

The three way Venn diagram in Figure 51 shows uniquely and commonly expressed DE genes in PGCs, group1 and group2 cells. Only the DE genes that have human orthologues are plotted. There are 568 genes uniquely expressed in PGCs, 243 shared with group1 and 439 with group, respectively. 3443 genes shared among all groups. The gene and expression level in all categories are listed in appendix I.

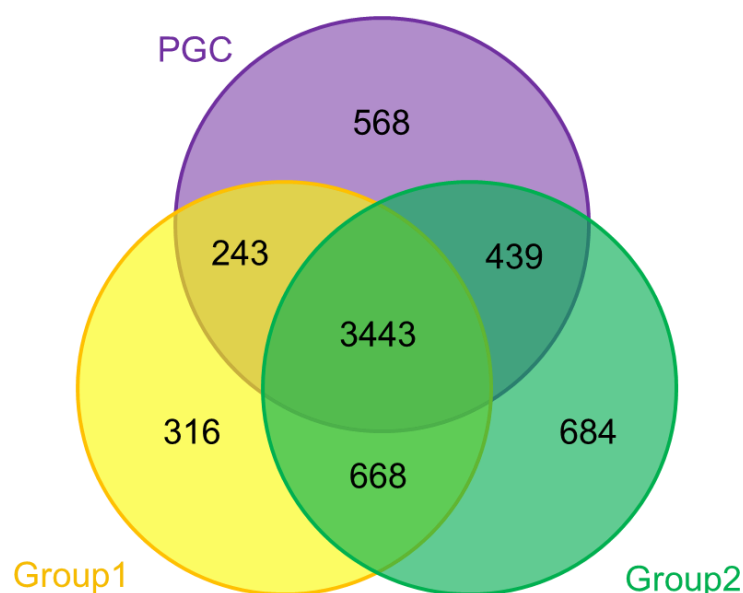


Figure 51. Venn diagram of uniquely or commonly expressed DE genes in PGC, group1 and group2 cells.

Numbers in each category is the number of pig genes that has human orthologue. List of gene name and expression level is shown in appendix I.

The 3-way Venn diagram in Figure 52 shows the number of differentially expressed genes between PGCs and somatic cells. In the 2-fold upregulation diagram (A), there are 242 genes specifically upregulated in PGCs, 344 specific in group1 cells and 19 shared between the two groups. In 2-fold downregulation diagram (B), 921 genes specifically downregulated in PGCs, 749 specific in group1 cells and 150 shared between the two cell types. See appendix II for the list of genes and expression in each categories.

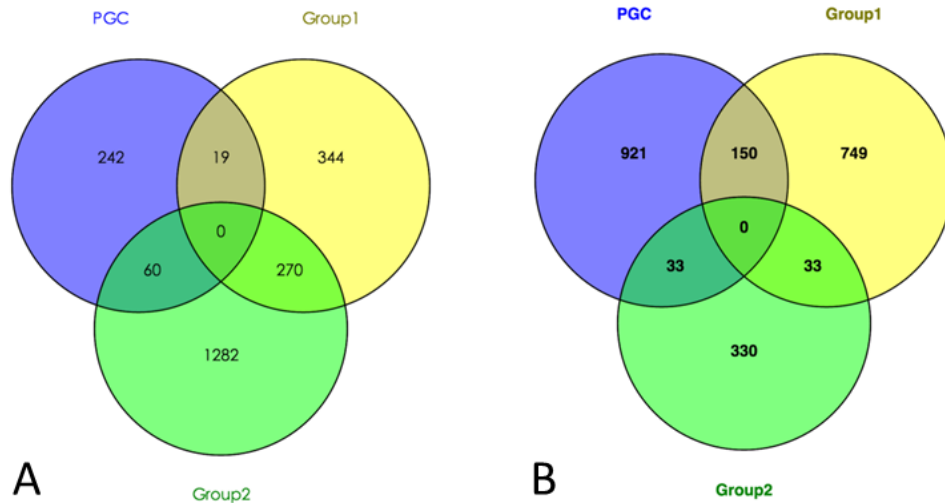


Figure 52. Venn diagram illustrates DE genes in PGC, group1 and group2 somatic cells.

A. 2-fold up regulated genes. **B.** 2-fold down regulated genes. Numbers of genes are labelled in sets. Purple, PGC. Yellow, group1 cells. Green, group2 cells.

5.2.3 Analysis of Gene Ontology

GO analysis was performed using DAVID bioinformatics tool. Biological processes were studied in the different groups of up- and down- regulated genes (Figure 53 and Figure 54). A comparison of PGC with group1 cells shows that significantly upregulated terms include mRNA translation, oogenesis, oogenesis stage, Kit signalling pathway, stem cell differentiation, positive regulation of JAK-STAT cascade, signal transduction by phosphorylation, double-strand break repair, DNA repair, regulation of translational initiation, and G1/S transition of mitotic cell cycle (Figure 53 A). Besides the above terms, PGC GO terms enriched over group2 cells (green cells) include mesodermal cell fate commitment (Figure 54 A).

Compared with group1 and group2, commonly downregulated terms in PGC include canonical WNT signalling pathway, BMP signalling pathway, positive regulation of ERK1/2 cascade, DNA methylation, negative regulation of transcription from RNA polymerase II promotor, and post-embryonic body morphogenesis (Figure 53 B and Figure 54 B). Besides, GO terms downregulated PGC over group1 also include somitogenesis, mesodermal cell fate determination, embryo development, histone H3K27 methylation, negative regulation of transcription regulatory region DNA binding, protein phosphorylation, negative regulation of cell proliferation, protein

folding, chaperone mediated protein folding, maintenance of location in cells, and various differentiation related processes (Figure 53 B). Downregulated GO terms in PGCs against group2 also include cell fate commitment, hippo signalling, and regulation of protein kinase activity (Figure 54 B). All DE GO terms are listed in appendix III.

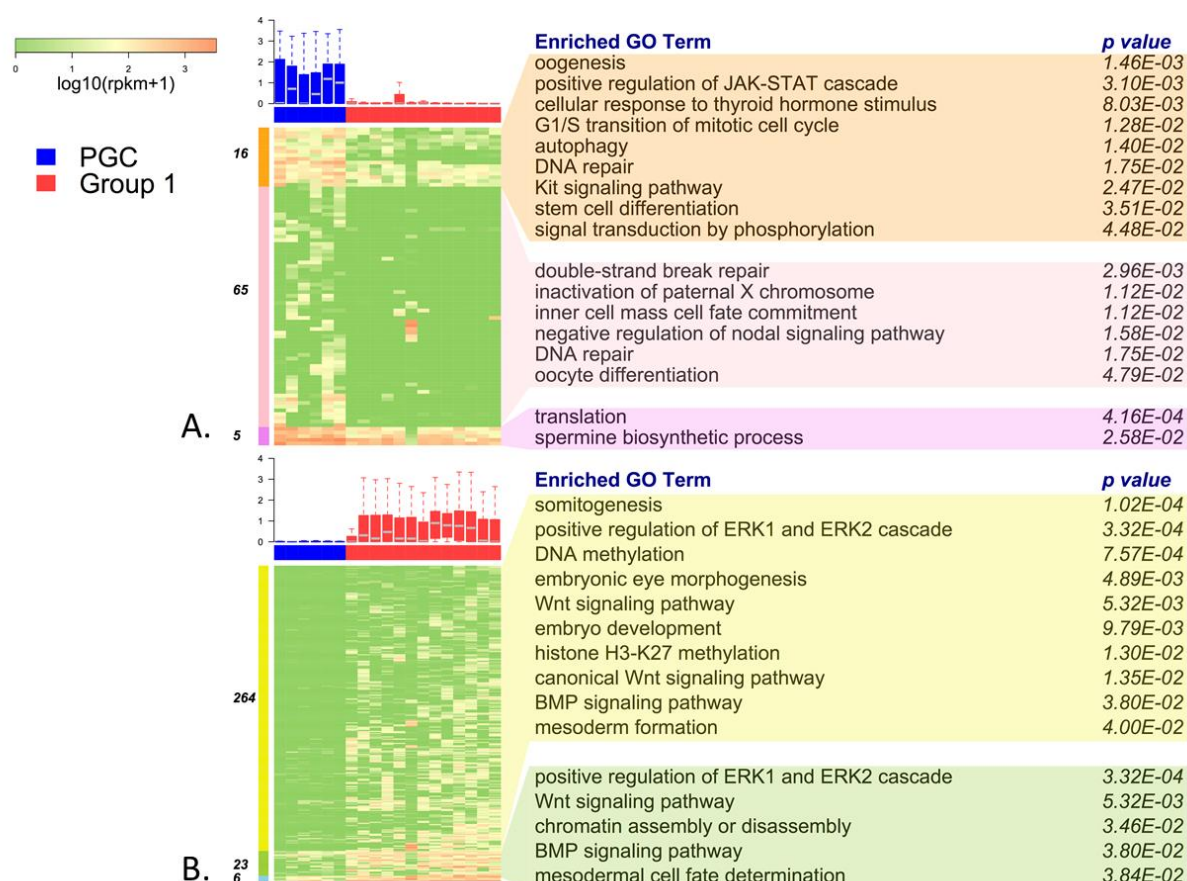


Figure 53. Gene ontology (GO) terms analysis of DE genes between PGCs and group1 cells.

A. Upregulated genes and selected GO terms in PGCs over group1 cells. B. Downregulated genes and GO terms in PGCs over group1 cells. Heatmap of DE genes between PGC and group1 cells are plotted at the left. On top of heat maps are the overall expression levels of individual cells. Unit of boxplots are $\log_{10}(\text{rpkm}+1)$. Numbers of DE genes at the labelled at the left side of heat map. GO term and corresponding P values within each gene clusters are annotated at the right side. Colour key of heatmap is shown at the top left of figure. Blue, PGC. Red, group1 cells. Numbers on the left hand indicate the number of genes in each coloured group, belonging to different terms.

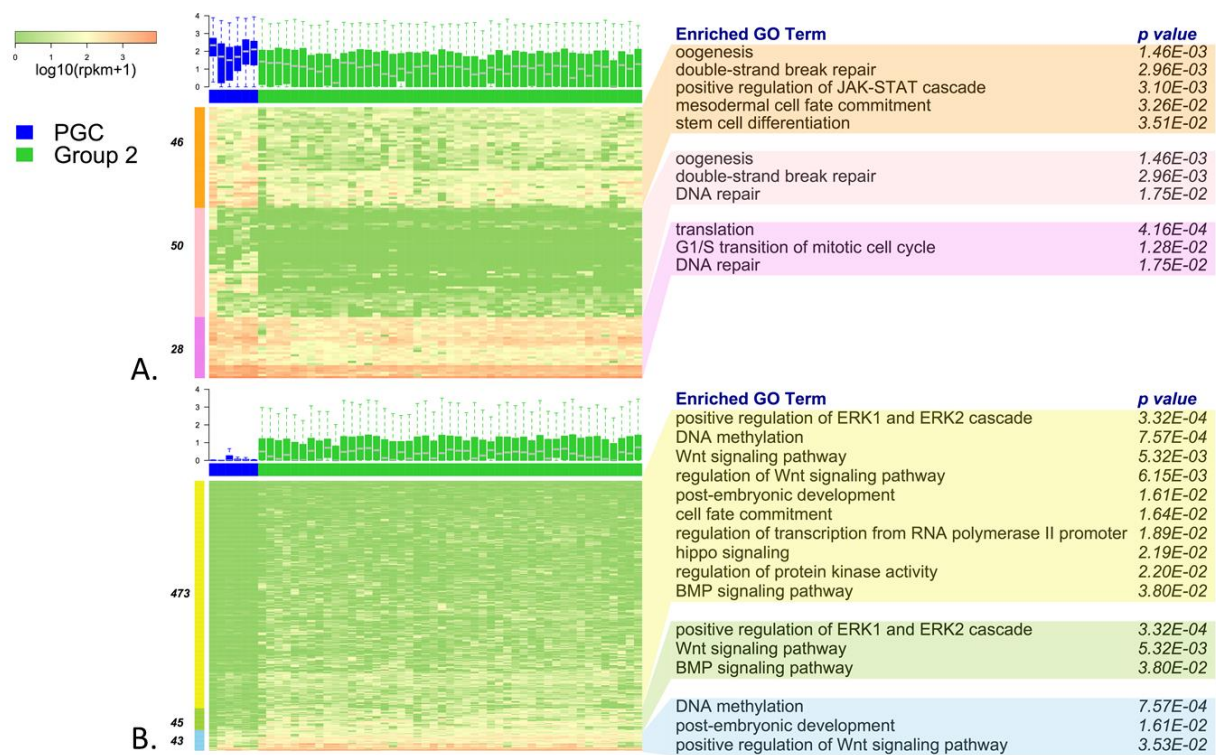


Figure 54. Gene ontology (GO) terms analysis of DE genes between PGCs and group2 somatic cells.

A. Upregulated genes and corresponding GO terms in PGCs over group2 cells. B. Downregulated genes and GO terms in PGCs over group2 cells. Heatmap of DE genes between PGC and group2 cells are plotted at the left. On top of heat maps are the overall expression levels of individual cells. Unit of boxplots are log10 (rpkm+1). Numbers of DE genes at the labelled at the left side of heat map. GO term and corresponding P values within each gene clusters are annotated at the right side. Blue, PGC. Green, group2 cells. Numbers on the left hand indicate the number of genes in each coloured group, belonging to different terms.

5.2.4 RNA-seq exploratory analysis of signalling pathways in pig PGCs.

To gain a better understanding of the PGC signals operating in early pig PGCs we analysed three key signalling pathways represented by the DE genes in the three groups of cells. We selected WNT, FGF, TGFβ signalling based on evidence that these pathways play key roles during germ cell development in mice (Chuva de Sousa Lopes, van den Driesche et al. 2005; Takeuchi, Molyneaux et al. 2005; Ohinata, Ohta et al. 2009). Heatmaps representing the profile of genes for each pathway are presented below. The list of genes in each pathway was retrieved from the KEGG database.

WNT pathway related genes were clustered in four groups (group A, B, C and D), based on differential expression patterns (Figure 55). Overall expression level of group A type genes is low in all cells. Group B genes are significantly downregulated in

PGCs, compared with group1 ($P=0.002$) and group2 ($p=0.003$). Downregulated genes include *WNT5a*, *WNT8A*, *LEF1*, *CCND1*, *PSEN1*, *CDH2*, *TCF7L1*, *SDC1*, *TCF7L2*, *ZRANB1*, *TBL1XR1*, *UBE2B*, *KDM6A*, *CTNND1*, *STK11*, *SMARCA4*, and *CAV1*. Group C genes show significant difference between group1 and group2 genes ($P=0.001$) but not with PGCs ($p=0.052$). Overall level of group D is high in all groups of cells.

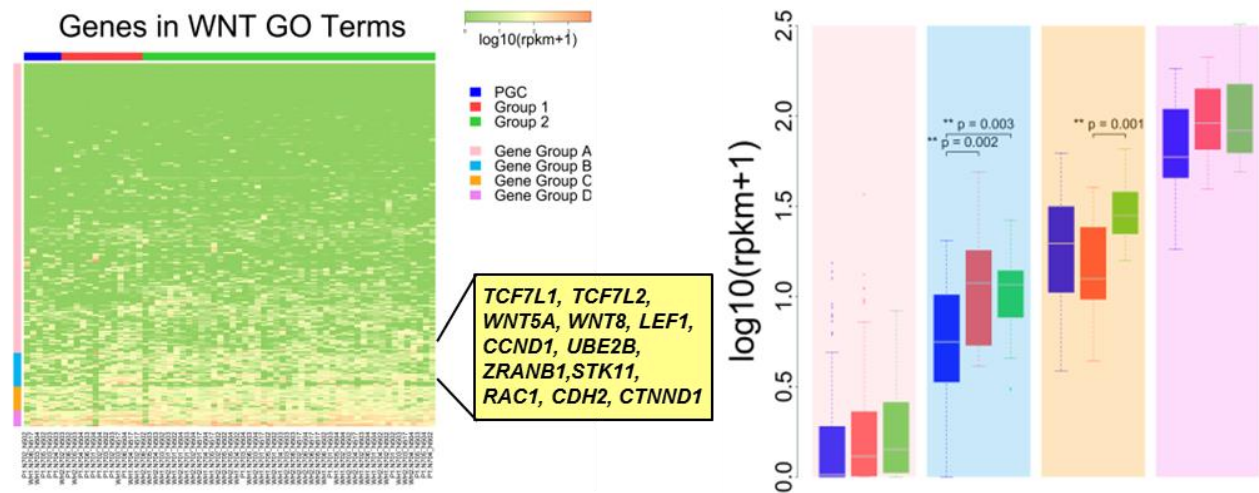


Figure 55. Heatmap and expression level of WNT pathway related genes.

A. Heatmap of WNT related genes in all cells. Genes form 4 clusters. **B.** Expression levels of 4 clustered genes in three cell types. Unit of expression level is plotted in log10 (rpkm+1). p values and significance are labelled on top of boxplots. Colour key is shown at the top right of figure. Different cell types and gene clusters are indicated in different colours.

When FGF pathway related genes was analysed, two main clustered of genes were obtained (Figure 56). One shows very low expression level. The other cluster of genes are higher overall but not differentially expressed between PGCs and somatic cells.

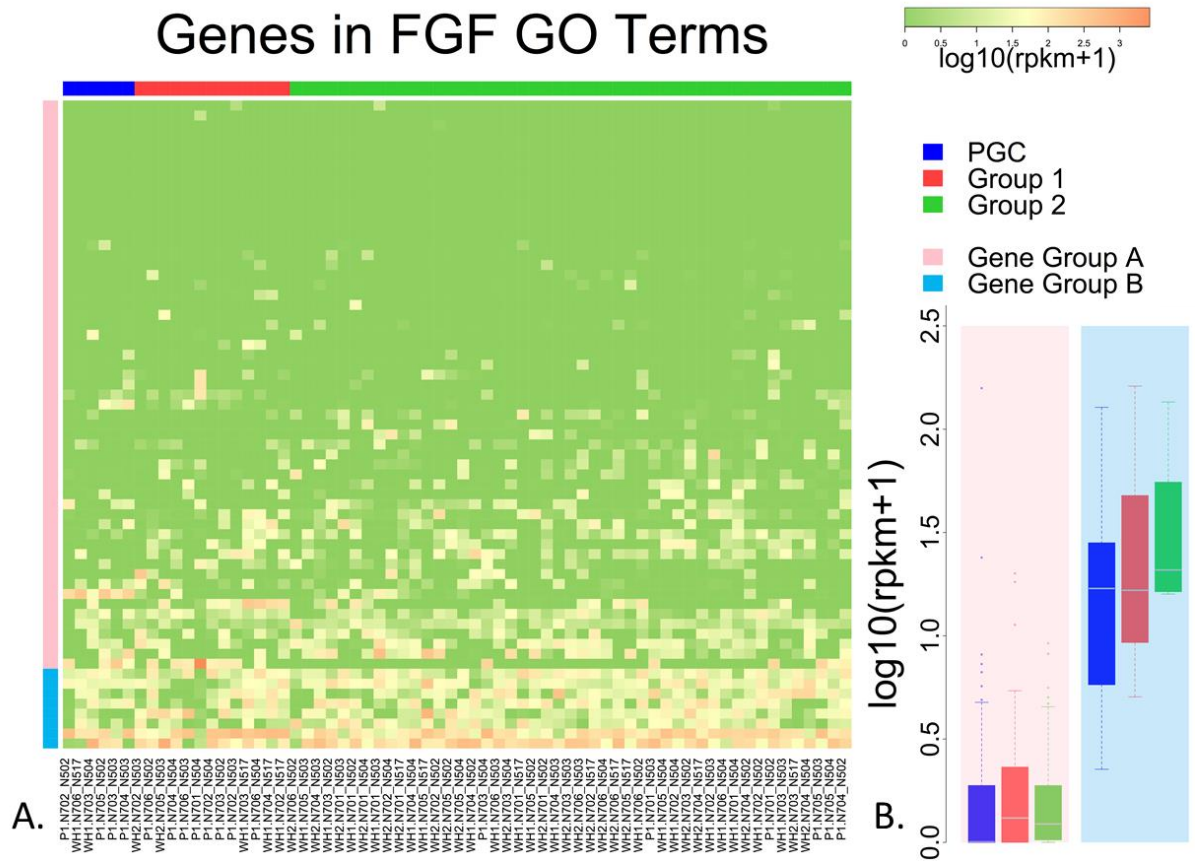


Figure 56. Heatmap and expression level of FGF pathway related genes.

A. Heatmap of FGF related genes in all cells. Genes fall in 2 clusters. B. Expression levels of 2 clusters of genes in three cell types. Unit of expression level is plotted in $\log_{10}(\text{rpkm}+1)$. Both clusters are not significantly expressed. Colour key is shown at the top right of figure. Different cell types and gene clusters are indicated in different colours.

The heatmap of TGF β -related genes fall into four clusters (Figure 57). Group A genes show low expression pattern in all cells. Group B genes are significantly downregulated in PGCs, compared with group2 cells, although the overall expression level is not high. These genes include *SOX9*, *SMAD2*, *SMAD6*, *SMAD7*, *MAP3K1*, *SKIL*, *LIMS1*, *ING2*, *C-JUN*, *COL4A2*, *GLG1*, *FBLN1* and *TAB1*. Group C and D genes are not differentially expressed between cell types. Downregulation of *SMAD2*, *SMAD6* and *SMAD7* suggests that TGF β signalling in PGCs is regulated by more than one mechanism or pathways (Labbe, Silvestri et al. 1998; Jung, Lee et al. 2013).

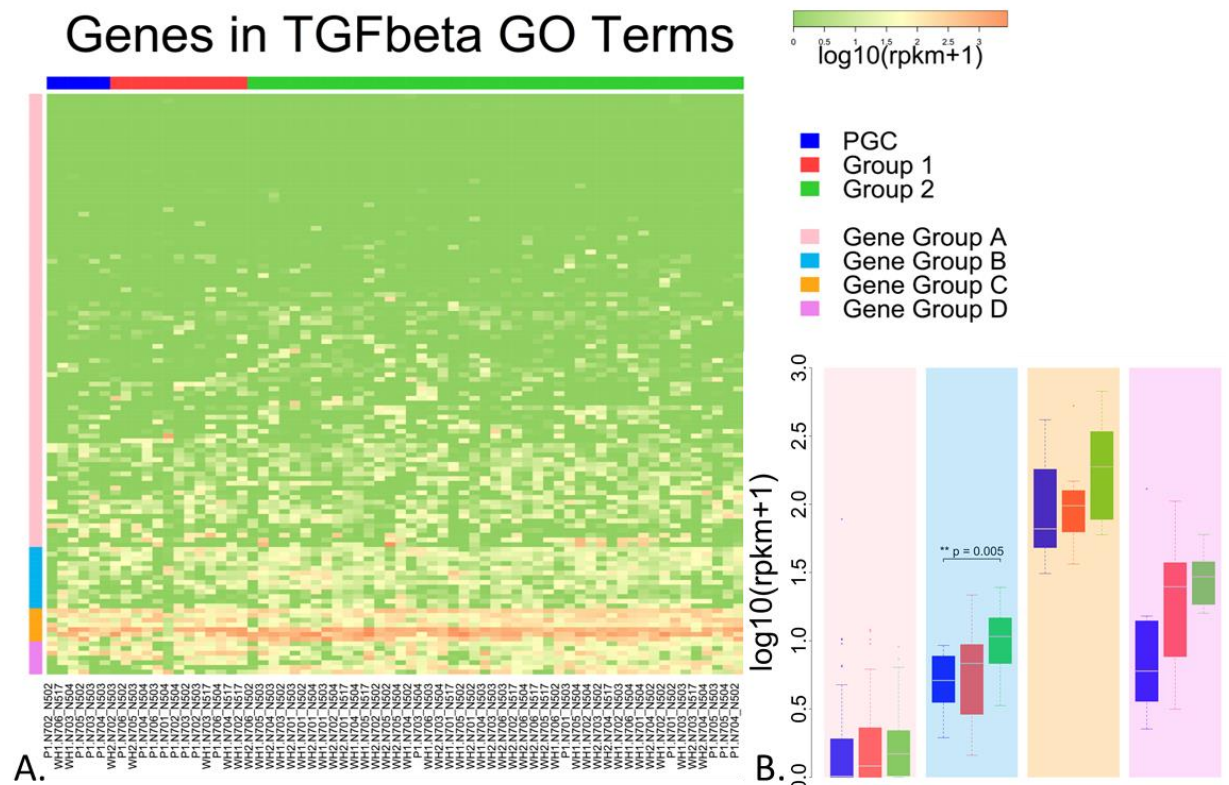


Figure 57. Heatmap and expression level of TGFβ pathway related gene expression.

A. Heatmap of FGF related genes in all cells. Genes fall in 4 clusters. **B.** Expression levels of 2 clusters of genes in three cell types. Unit of expression level is plotted in log10 (rpkm+1). Significantly DE cluster are labelled with P value on top of boxplots. Colour key is shown at the top right of figure. Different cell types and gene clusters are indicated in different colours.

Next, specific genes involved in epigenetic regulation was analysed as a mean to provide independent validation of the IF data described in chapter 4. RNA-seq shows that *Tet1* is upregulated in 4 out of 6 PGCs and is low or negative in the majority of somatic cells. The overall mRNA level is higher in PGC than somatic cells (Figure 58). This is in line with the observation in E13 PGC cluster IF (Chapter 4). Due to the lack of reliable antibody against pig *TET2*, we didn't have IF result to compare with. But the 4 out of 6 *Tet2* positive PGCs is consistent with the observations that 5-hmC is increased in these cells at this stage. *De novo* methyltransferase *DNMT3B* is low or negative in 4 out of 6 PGCs, and highly expressed in somatic cells, again consistent with a reduced level of 5-mC in pre-migratory PGCs.

UHRF1 and *EHMT2* are negative in all 6 PGCs. The negative *UHRF1* confirms the IF result (Chapter 4) of E13 PGC. *EHMT2* (also known as *G9a*) is the functional H3K9

methyltransferase working in the heteromeric complex G9a-GLP. The low level of *EHMT2* in PGCs suggests that H3K9me2 will be decreased in subsequent stages, as shown in E16 PGCs (Chapter 4).

EZH2 (enhancer of zeste 2) catalyses repressive H3K27me3 on Xi chromosome (Seki, Hayashi et al. 2005). The low level expression of *EZH2* in PGCs is consistent with the moderate presence of H3K27me3 in these cells (Chapter4 Figure 37).

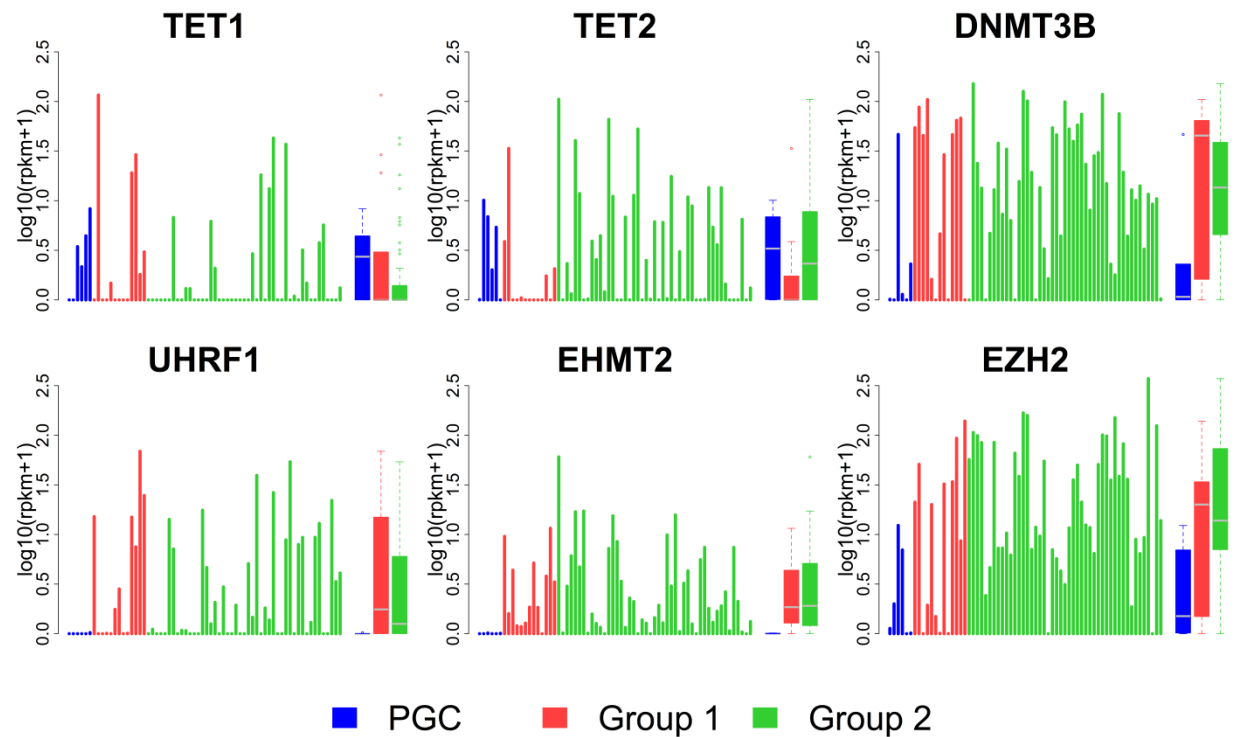


Figure 58. Transcriptional level of DE epigenetic reprogramming related genes TET1, TET2, DNMT3B, UHRF1, EHMT2 AND EZH2.

Bar graph shows gene expression level of each single cells in $\log_{10}(\text{rpkm}+1)$. Overall expression level of three cell types are box-plotted at the right side of each bar graph. Median of all quantified cells is shown in each bar. Blue, PGCs. Red, group1 cells. Group2, group2 cells.

Other histone modifiers shown not to play a significant role in mouse PGCs (Seki, Yamaji et al. 2007) were also analysed. Histone demethylases *KDM1A* (H3K4me and H3K9me), *KDM3A* (H3K9me), *KDM6A* (H3K27me2 and h3K27me3), and *JMJD1C* (H3K9me), are heterogeneously expressed in PGCs and their levels are not significantly different between different cell types (Jung, Lee et al. 2013). This observation is similar to the mouse 7.25-7.5 dpc PGC mRNA detection (Seki, Yamaji et al. 2007).

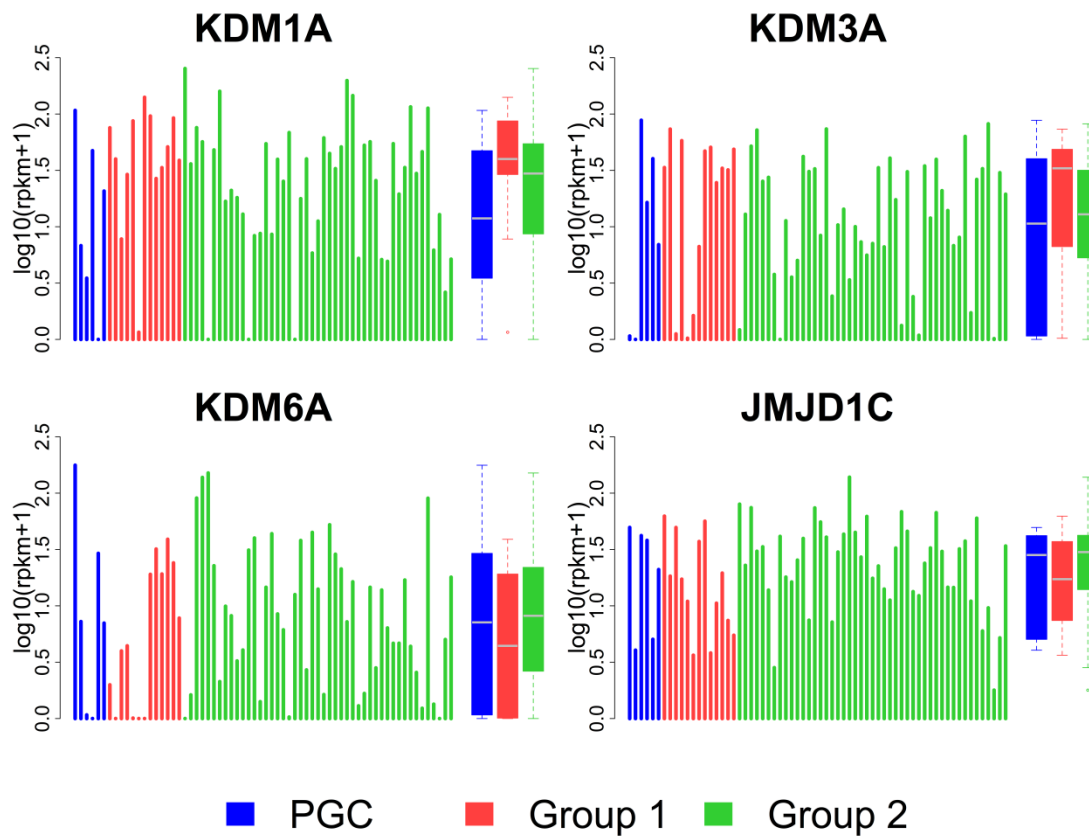


Figure 59. Expression levels of maintained histone modifiers, Kdm1a, Kdm3a, Kdm6a and Jmjd1c.

Bar graph shows the mRNA level in each cell in log10 (rpkm+1). Overall expression level of cell types are shown in the boxplots at the right of each bar graph. Different cell types are indicated in different colours.

5.2.5 RNA-seq exploratory analysis of potential signalling pathways in pig PGCs

To understand whether the pig PGC specification transcriptional network is conserved in mammals, particularly with human, a comparison of differentially expressed genes of pig PGC vs. hPGC, hPGCLC and TCam-2 cells was performed. The human dataset was obtained from the recently published paper by Irie et al., 2015. Two-fold up- and down-regulated pig orthologues were plotted in the Venn diagram (Figure 60). As shown in A, there are 15 upregulated genes shared among all cell types or cell lines, including *PRDM1*, *NANOG*, *NANOGP8*, *KIT*, *RTN3*, *SLC25A5*, *EEF1G*, *PARP1*, *PDPN*, *HENMT1* and *PIM2*. These genes have high median RPKM in pig PGCs. In addition, *PRDM14*, *C9orf135*, *ANGPTL4*, *RYR1*, *PYCARD* and *PYDC1*, although

they are listed in the PGCs upregulated genes, the range of their median values is between 0 and 0.027.

Besides, there are 55 genes commonly downregulated in all four cell types. Interestingly, downstream target of canonical WNT *LEF1*, as well as *GATA6*, are shared by all of them. The commonly downregulated genes suggests that the inhibition of WNT signalling pathway is a conserved mechanism of PGC generation. 5 upregulated (*RPL35A*, *MKRN1*, *NANOS3*, *MYL6* and *RFFL* from high to low median in RPKM) and 26 downregulated (such as *TCF7L1*, *MACF1*, *RASA3* and *FST*.) genes are shared among pig PGCs, hPGC and hPGCLC. 6 upregulated (*STMN1*, *UGP2*, *NCBP2*, *FAM20A*, *DDX28*, and *MAAL2*) and 17 downregulated (such as *SRI*, *CDCA7L* and *NEK9*) genes shared among pig PGCs, hPGCLC and TCam-2 cells.

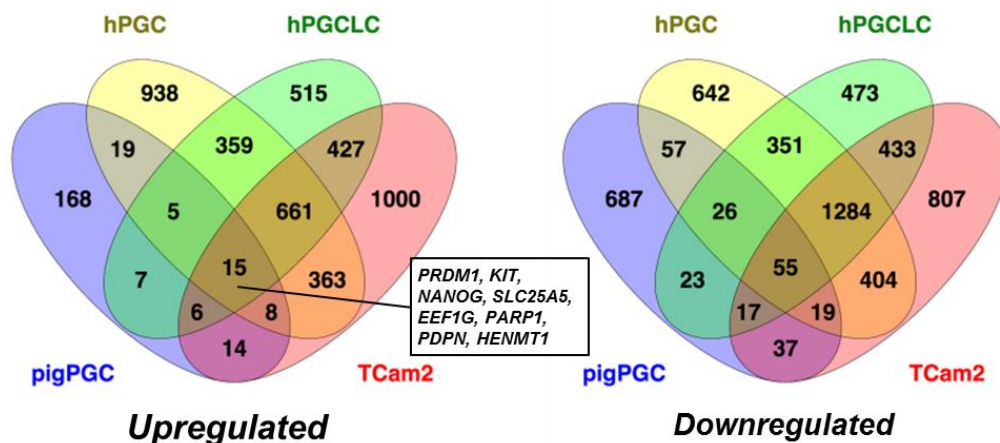


Figure 60. Venn diagram of DE genes among pig PGC, gonadal hPGC, hPGCLC and human TCam-2.

A. 2 fold downregulated orthologues. B. 2 fold upregulated orthologues. Plotted genes are orthologues of DE pig PGCs, gonadal hPGC, hPGCLC, and TCam2. Numbers of orthologues are labelled in corresponding sets.

5.3 Discussion

In this chapter, early porcine PGCs are characterized by single cell RNA-seq. 6 single PGCs and 60 somatic cells were isolated and sequenced from pool of 30 embryos. From IF result, we understand that pig PGCs are not synchronous, even in nascent premigratory cluster there are differences in expression profiles of different proteins. This is observed both at the levels of transcription factors and epigenetic marks. It seems that each individual PGC represents a distinct development stage. When

hierarchical clustering based on selected genes was performed three clusters were identified as falling under two branches (Figure 48). Six PGCs clustered under one branch and two types of somatic cells clustered under the other branch. As judged by the gene expression profile, 13 mesodermal like cells fall in one cluster and the other 47 endodermal cells are on the other cluster. The proportion of each cell type is consistent with the type of tissue obtained from the embryo and the location of PGC at this stage. Two out of 6 PGCs were T^+ , and 8 out of 13 group1 somatic cells were T^+ . On the other branch, 3 out of 47 group2 somatic cells had low levels of T , while the others are T^- . The distribution of positive/negative T in PGCs and mesodermal cells suggests a developmental relationship between the two cell types. Besides T , mesodermal gene *GATA3* is expressed in all 6 PGCs, and 9 somatic cells. Another mesodermal gene *GATA2* is expressed in 3 PGCs and 6 somatic cells. *CDX4* is highly expressed in 12 mesodermal cells (red cells), some endodermal cells (green cells), but not in PGCs (blue cells). The gene list (Appendix I) of PGC unique genes and DE genes between group1 genes would help dissect the transcriptional regulation mechanism of PGC specification (Figure 49).

Mouse T plays critical role in PGC specification (Aramaki, Hayashi et al. 2013). Considering the expression pattern of porcine T in PGCs, mesoderm and primitive streak, whether T regulates porcine PGC specification intrigues us to explore in chapter 6.

In our RNA-seq analysis, *SOX17* mRNA is not highly expressed in all PGCs, in contrast to the IF (shown in Chapter 3). Only 2 of them show high expression level, 2 are of medium level and 2 are of low/negative. This shows the advantage of single cells RNA-seq. analysis, which in contrast to pooled samples, has enabled me to identify fluctuations in *SOX17* mRNA as a potential mechanism regulating the levels of this protein in a cell. Future studies should establish whether this is a functional observation or a potential artefact of single cell sequencing. Nevertheless, the discrepancy of *SOX17* expression between PGCs might also suggest their different stages of development. Within 6 PGCs, the low/negative *SOX17* PGCs could be nascent PGCs, whereas high *SOX17* could represent specified PGCs. Another possibility is the transient fluctuation of gene transcription in individual cells. As shown in Table 1, the 2 PGCs that have highest *SOX17* level show the lowest *BLIMP1*

level and highest *TFAP2C* level. The negative correlation between *SOX17* and *BLIMP1* is interesting. Positive correlation between *SOX17* and *TFAP2C* is consistent with the idea that *SOX17*⁺ PGC precursors will become PGCs marked by *TFAP2C*. 2 to 4 most closely clustered mesodermal cells share expression profile of *TFAP2C*, *NANOG*, *GDF3*, *OCT4*, *TACSTD1*, and *GATA3*. Are they being induced to become PGCs? Or they are fated to differentiate to mesodermal cells and losing above genes? The key difference is PGC uniquely expressed *NANOS3*. The identity and mechanism is unknown.

The negative/low *SOX2* and *PRDM14* mRNA level is consistent with the IF results and a human report (de Jong, Stoop et al. 2008; Sugawa, Arauzo-Bravo et al. 2015), but contrasts findings in mouse PGC (Yamaji, Seki et al. 2008; Nakaki and Saitou 2014).

mRNA of DNA methylation and histone modifying enzymes validates and explains the IF of epigenetic marks. High 5-hmC level and low 5-mC level coincides with low/negative *TET1*, *TET2*, *UHRF1* and *DNMT3B* in PGCs. *TET1* and *TET2* actively convert 5-mC to 5-hmC. *UHRF1* recruits *DNMT1* on hemimethylated DNA in a replication dependent manner, maintaining 5-mC level. De novo methyltransferase *Dnmt3b* is down regulated in nascent PGCs. *Dnmt3a* and *Dnmt3b* synergistically methylate *OCT4* and *Nanog* promoters during mouse embryonic development and ES cell differentiation (Li, Pu et al. 2007). This partially explains the coincidence of repressed *DNMT3B* (Figure 58) and upregulation of *NANOG* and *OCT4* (Figure 48) in PGCs.

Homogeneous reduced expression of *EHMT2* in E13 PGCs is consistent with the observation that H3K9me2 begins to decrease at E13 reaching lower levels in E16 PGCs (Figure 37). Downregulation of *EZH2* in E13 PGCs suggests that H3K27me3 is not upregulated at this stage. Indeed, IF for this mark did not show different profile to somatic cells (not shown). In migratory PGCs H3K27me3 is upregulated (Figure 37).

The analysis of other H3K9me demethylases *KDM1A*, *KDM3A* and *JMJD1C* showed no changes in PGCs (Figure 59). Similarly, *Kdm1a* (also known as *LSD1* or *AOX2*), *Kdm3a* (also known as *Jmjd1a* or *Jhdm2a*), and *Jmjd1c*, are also detected but not

exclusively in 7.25-7.75 dpc mouse PGCs (Seki, Yamaji et al. 2007). Other demethylases of H3K9me2, such as *Jmjd1b* (also known as *Jhdm2b* or *KDM4A* in human), *Jmjd2a* (or *Jhdm3a* or *KDM4A* in human), *Jmjd2b* (*KDM4B* in human), *Jmjd2c* (also known as *Gasc1* or *KDM4C* in human), and *KIAA1718* (*KDM7* in human) show heterogeneous and not PGC specific expression pattern (Seki, Yamaji et al. 2007). Kdm6a (also known as *UTX*, or *MGC141941*) is a histone demethylases for H3K27me2 and H3K27me3. KDM6A in porcine and mouse PGCs (Figure 59) are heterogeneously expressed at stable level, compared with somatic cells. Another demethylase of H3K27me3, *Jmjd3* (also known as *KIAA0346* or *KDM6B* in human), is very low in 7.25 dpc PGCs and upregulated in both PGC and somatic cells at 7.75 dpc (Seki, Yamaji et al. 2007). This observation suggests that additional mechanism is required for the upregulation of H3K27me3 in PGCs.

GO term analysis on DE genes shows that PGC upregulate Kit signalling pathway and signal transduction by phosphorylation, compared with group1 cells. Positive regulation of JAK-STAT cascade, oogenesis, stem cell differentiation and G1/S transition of mitotic cells are commonly upregulated compared with both somatic groups. Kit encodes c-KIT, the transcripts detected in porcine PGCs (Figure 53 and Figure 48). JAK-STAT cascade signal guides PGCs migration through mesoderm during gastrulation by promoting cellular protrusion (Richardson and Lehmann 2010). E13 PGCs are specified and prepared to migrate. The upregulated JAK-STAT signalling and oogenesis GO terms are indicative of the developmental program of these precursors.

WNT signalling, BMP signalling, DNA methylation and regulation of translation GO terms are commonly downregulated in PGC. WNT and BMP signalling are important for PGC specification, as well as somatic differentiation. The reduction in these signalling pathways shortly after specification suggests that PGCs need to block these pathways to prevent differentiation into somatic lineages. In human colorectal cancer cell lines, SOX17 forms complex with β -Catenin and suppresses its activity (Zhang, Glockner et al. 2008). In mouse airway submucosal gland progenitor cells, SOX17 directly binds to four sites of the LEF1 promoter; therefore suppressing WNT3A/ β -catenin mediated induction (Liu, Luo et al. 2010). The interaction between SOX17 and WNT effectors, such as LEF1, would be an interesting angle to dissect the

function of SOX17 in PGC specification. Functional experiment will be carried out in Chapter 6. Also it provides a possible mechanism of WNT downregulation in PGCs.

WNT signalling pathway is important in various embryonic development processes. Cytoplasmic β -catenin transduces WNT signals into the nucleus. Absence of cytoplasmic β -catenin causes degradation by the proteasome, while in the presence of WNT ligand β -catenin associates with LEF/TCF family. LEF/TCF are a family of transcription factors, including LEF-1, TCF-1,-3, and -4 (Seidensticker and Behrens 2000). SOX17 is reported to be antagonists of WNT signalling while DNA binding to SOX17 is unnecessary. By directly binding to both TCF and β -Catenin, SOX17 antagonize the WNT signalling (Sinner, Kordich et al. 2007). SOX family proteins have low DNA binding affinity and thus require interaction with other TFs containing domains such as POU, zinc finger, homeodomain, and bHLH, to function (Kamachi, Uchikawa et al. 2000). For example, sequences carboxyl to the HMG box of SOX17 protein contacts with β -catenin (Zorn, Barish et al. 1999). In the same context, WNT signalling also regulates the expression of SOX family genes (Kormish, Sinner et al. 2010). The RNA-seq data shows that PGCs significantly downregulate WNT related genes *WNT5A*, *WNT8A*, *LEF-1*, *TCF7L1*, *TCF7L2*, *ZRANB1*, *RAC1*, and *STK11*. This indicates that WNT pathway is downregulated in nascent PGCs (Figure 55). *WNT5A* and *WNT8A* are members of WNT gene family. *LEF-1* is critical transcriptional activator of canonical WNT signalling transduction. *TCF7L1*, also known as *TCF3*, is an inhibitor of self-renewal. It is required in mESC in transition from pluripotent cells to mesoderm lineage specification in WNT/ β -catenin dependent manner (Hoffman, Wu et al. 2013). In hESC, WNT signalling promotes somatic lineage differentiation (Davidson, Adams et al. 2012). Suppression of *TCF7L1* in PGCs suggests that mesodermal fate is shut down in PGCs, which is consistent with the GO term of DE genes between PGCs and group1 cells (Figure 49). *ZRANB1* (also known as *Trabid*) is positive regulator of WNT signalling in mammalian cells, which is required for LEF/TCF complex mediated transcription (Tran, Hamada et al. 2008). In canonical WNT signalling, *RAC1* is required for the nuclear accumulation of β -catenin (Wu, Tu et al. 2008). *STK11* (also known as *LKB1*) is associated with cancer-relevant cell phenotypes, such as cell migration, invasion, metabolism and polarity (Alessi, Sakamoto et al. 2006; Shah, Sharpless et al. 2008). WNT target genes *Bmp4*, *FDR*,

Nkd1, Axin2, and Lgr5 are upregulated in STK11 deficient melanoma metastasis (Liu, Monahan et al. 2012).

Further indirect support for WNT signalling downregulation in PGCs is the reduced expression of some of its targets. One such target is CCND1 (Cyclin D1), which is sharply downregulated in PGC, suggesting that PGCs slow down the cell cycle prior to migration. This is consistent with the reduced speed of proliferation from 5-7h to 16h reported for mouse PGCs (McLaren and Lawson 2005). In human colon cancer cells, SOX17 suppresses activated β -catenin and reduced Cyclin-D1 (Zhang, Glockner et al. 2008). Furthermore, considering that UHRF1 is negative in E13 PGCs (Chapter4 Figure 33), it is possible that porcine PGCs slow down division shortly after specification. If this mechanism is operating in early PGC then one could ask how the number of PGCs is determined: by multiplication of early PGCs or by continuous induction of pluripotent mesodermal cells.

Taken all above reasons into consideration, WNT inhibition plays a key role in PGC specification. The function of WNT will be investigated in Chapter 6.

Some genes in TGF β pathway is also down regulated in PGCs, such as *SMAD2* (Figure 57). Phosphorylated *SMAD2/3* transduce NODAL/ACTIVIN signal via transmembrane receptors (type I and II). *SMAD4* in turn translocate activated *SMAD* complex into nucleus, where activate downstream genes (Schier 2003) regulating mesoendoderm development (Schier and Shen 2000), left-right axis (Bamford, Roessler et al. 2000), anterior-posterior axis (Brennan, Lu et al. 2001) and other vertebrate development related processes. *Smad2* is required for Nodal activation and male PGC differentiation (Wu, Fukuda et al. 2015). During mouse anterior-posterior axis forming, Nodal activates MAPK signalling, which in turn reinforces nodal signalling via p-*Smad2* (Clements, Pernaute et al. 2011). *Smad2* mutant mouse male PGCs fail to express meiotic markers at 17.5 dpc, but it does not affect pluripotent gene expression in the germ line (Wu, Fukuda et al. 2015). Taken the downregulated *SMAD2* mRNA level in E13 PGCs (Figure 57) and prevalent expression of p*SMAD2* in E13 somite stage embryos (Figure 20), I propose that porcine PGCs down regulates *SMAD2* during PGC specification as part of somatic inhibition, although the protein is still detected in these cells.

Venn diagram of DE orthologues between porcine PGC, gonadal hPGCs, human PGCLC and TCam-2 (Figure 60) suggests that some transcriptional regulation of porcine PGC is conserved between these species or cell types. Upregulation of PRDM1 (BLIMP1), *NANOG* and *KIT* is consistent with previous study (Gkoutela, Li et al. 2013). *NANOGP8* is one paralog of human *NANOG*. It encodes full-length *NANOG* protein and is expressed in most human cancer cell lines, particularly pluripotent cells. *NANOGP8* is active in inducing reprogramming in human and murine fibroblast cells as *NANOG1* does (Palla, Piazzolla et al. 2014). This might explain why this gene is also found in TCam-2 cell line. *PRDM14* is not essential in the human germ line (Sugawa, Arauzo-Bravo et al. 2015). Downregulation of *GATA6* and *HOXA3* are examples of commonly suppressed somatic genes in pig and human PGCs.

Comparison between pig PGC, human PGC, PGCLC, and TCam-2 cell line explored shared genes in PGC specification transcription regulatory network. Mouse embryology is unique in mammals. Even some rodents evolved embryonic disc (Johnson and Alberio 2015).

Chapter6

6 Determination of PGC inducing signals in the pig

6.1 Introduction

6.1.1 Signalling and pathway requirement for PGC specification in mouse and human

Mouse BMP4 is secreted by the extraembryonic ectoderm (ExE) before gastrulation (~5.5 dpi) and then upregulated in extraembryonic mesoderm (ExM), particularly between MS to LS stage (6.5-7.0 dpi). The extraembryonic ectoderm is required for PGC induction, size of founder PGC cluster, and allantois formation (Lawson, Dunn et al. 1999). BMP4 produced by the extraembryonic mesoderm is critical for PGC survival and allantois development and localization (Fujiwara, Dunn et al. 2001). Culture of isolated mouse epiblast with recombinant human BMP4 induces PGC in dose a dependent manner (Hayashi, Kobayashi et al. 2002). As well as Bmp4, Bmp8b is produced in ExE and Bmp2 in visceral endoderm (VE) before gastrulation to induce PGC precursors and ExM in proximal epiblast (Fujiwara, Dunn et al. 2001). Bmp4 and Bmp2 have additive effects and Bmp4 and Bmp8b have synergistic effect in PGC specification (Ying, Qi et al. 2001; Ying and Zhao 2001). Recent evidence in human shows that for the transition from hiMeLC to hPGCLC, BMP4 plays a critical role in PGC specification via ALK2/3 receptors (activating receptor-like kinase). This function can be replaced by BMP2 at identical concentration (Sasaki, Yokobayashi et al. 2015). In the pig embryo BMP4 and BMP2 are detected in nascent extraembryonic mesoderm and mesoderm respectively from PSII stage. In response to the paracrine effect, Smad1/5/8 is phosphorylated in the neighbouring TE (Valdez Magana, Rodriguez et al. 2014).

Smad1 plays indispensable role in mouse PGC specification as a transducer of BMP4. Even fully phosphorylated Smad5 and Smad8 are not able to rescue the defect caused by Smad1 knockout (Hayashi, Kobayashi et al. 2002). Smad5 is expressed in proximal epiblast from pre- or early PS stage mouse embryos. As well as Smad1, Smad5 is required by the proximal epiblast to response ExE inductive signals and by ExM cells to localize PGCs. The number of PGC is drastically reduced in Smad5 mutant embryos (Chang and Matzuk 2001), although the effect is still milder,

compared with Bmp4 and Bmp8b mutant mouse embryos. Phosphorylated Smad1 and Smad5 complex combine with Smad4, to activate Blimp1 and Prdm14 in different subpopulations of presumptive PGCs (Arnold and Robertson 2009).

WNT is required for mouse PGC specification. As direct or indirect downstream effector of BMP4 signal, WNT3 activates T, which directly activates Blimp1 and Prdm14, therefore specify PGCs (Aramaki, Hayashi et al. 2013). However, an hPGCLC in vitro differentiation model revealed that SOX17 is key specifier of human PGCs, and indirectly activates and collaborates with BLIMP1 in hPGC specification (Irie, Weinberger et al. 2015; Tang, Dietmann et al. 2015). During mouse primitive streak formation period, T directly targets Sox17 and other key developmental regulation genes, such as Fgf8, Foxa2, Foxj1, and Dusp6 (Lolas, Valenzuela et al. 2014). The chronological sequence of T, Sox17 and Blimp1 during pig and human PGC specification is further investigated in this chapter.

Apart from above mentioned pivotal role in mouse PGC and human PGCLC specification, Canonical WNT/ β -catenin is required in many other cell fate decision processes, including mesoderm, endoderm, seminoma, embryonic carcinoma. TypeII germ cell cancer (GCC) derives from carcinoma in situ (CIS), which derives into undifferentiated seminoma and diversely differentiated non-seminoma (Oosterhuis and Looijenga 2005). Seminomas are similar to CIS and PGCs (Kristensen, Skakkebaek et al. 2013), in terms of their DNA hypomethylation status (Wermann, Stoop et al. 2010; Nettersheim, Heukamp et al. 2013), and key gene expression profile, such as BLIMP1, cKIT, TFAP2C (Biermann, Heukamp et al. 2007), SOX17 (de Jong, Stoop et al. 2008), NANOG and OCT4 (Gillis, Stoop et al. 2011). In this study, we use human seminoma cell line Tcam-2, as well as porcine epiblast, to simulate PGC specification with or without WNT signalling. The expression profile of PGC related key genes will be detected. Meanwhile, targets of WNT signalling

SOX17 antagonizes canonical WNT/ β -catenin signalling in various contexts. Xenopus Sox17 (XSox17) physically interacts with the Armadillo repeats of β -catenin with its C-terminal of HMG box. In Xenopus animal cap experiment, XSox17 represses WNT/ β -catenin activated genes, meanwhile initiates endodermal gene expression (Zorn, Barish et al. 1999).

SOX17 is also considered to be candidate tumour suppressor associated with WNT downregulation. In human colorectal cancer (HCC) cells, DNA hyper methylation on SOX17 promoter region results in accumulation of nuclear β -catenin. While restored SOX17 expression downregulate canonical WNT signalling, meanwhile the growth of HCC is hampered (Jia, Yang et al. 2010). In human lung cancer cells, SOX17 inhibits WNT signalling pathway, as well as the cancer cell proliferation. Hypermethylation on SOX17 promoter suppresses the expression of SOX17; consequentially inhibit the antagonism function on canonical WNT downregulation. Therefore the epigenetic changes on SOX17 expression is associated with the occurrence of lung cancer (Yin, Jia et al. 2012). Consider above facts, it is possible that SOX17 represses tumour progress during early embryo development, via the suppression of WNT antagonism.

Within germ cell tumours (GCT) seminomas resemble undifferentiated primitive germ cells, while embryonal carcinoma (EC) cells show embryonal-like differentiation. SOX17 is upregulated in seminoma cells, compared with embryonal carcinoma (EC) cells (Sperger, Chen et al. 2003). Consistently, WNT is inactivated in seminomas, where activated WNT signalling is required for the response to differentiation signal, rather than maintenance of pluripotency (Sperger, Chen et al. 2003).

In gonadal mouse PGCs, WNT is inactivated in early spermatocytes. Activated WNT is required in postmitotic germ cells. Only spermatocytes and round spermatids are detected with nuclear β -catenin. Early aberrant activation of WNT/ β -catenin pathway results in hampered spermatogenesis. SOX17 is expressed in spermatogonia, where WNT target Lef1 is downregulated (Kerr, Young et al. 2014).

So far, there is no direct study of WNT regulation in early PGC. Evidence from mouse in vitro differentiation studies show that WNT inhibition upon BMP induction prevents PGC specification (Murakami, Gunesdogan et al. 2016). Since SOX17 is expressed in hPGC precursors (previously shown in Chapter 3), and some WNT signalling related genes are downregulated in transcription level (previously shown in Chapter 5), this suggests that WNT/ β -catenin signalling is downregulated in early hPGCs. What is the function of WNT/ β -catenin signalling in PGC specification? In this chapter, porcine epiblast, hPGCLC differentiated in vitro, and human Tcam-2

seminoma cell line are used to investigate the important signalling pathways in PGC specification, particularly focus on canonical WNT/ β -catenin. This thesis proposes for the first time that canonical WNT inhibition is required for PGC specification.

FGF2 also sustains Nanog in hESC and regulates BMP4-induced mesoderm differentiation via MEK-ERK pathway (Yu, Pan et al. 2011). FGF is required for mouse PGC migration (Takeuchi, Molyneaux et al. 2005). Axolotl PGC are induced by Brachyury in the presence of BMP4, and are maintained by MAPK signalling (Chatfield, O'Reilly et al. 2014). Axolotl animal caps treated with MAPK inhibitor are deficient in mesoderm development and lose PGC completely (Chatfield, O'Reilly et al. 2014). In mouse in vitro mesodermal differentiation system, inhibited ERK signalling promotes PGCLC differentiation, meanwhile suppresses mesodermal differentiation (Kimura, Kaga et al. 2014). From PSII stage, p-MAPK is detected in pig hypoblast and epiblast (Valdez Magana, Rodriguez et al. 2014). Whether MAPK is required in hPGC specification will be studied in this chapter.

Steel factor (SF) is critical for survival and migration, defines the migrating pathway to some extent. It is detected along the migratory track to genital ridges from 8 dpc. The midline of gut undertakes reduction of SF at 9.5-10.5 dpc. This reduction causes apoptosis to the PGCs that fail to migrate laterally to genital ridges. The receptor, c-Kit maintains expression throughout the whole development process of PGC (Leitch, Tang et al. 2013).

The aim of this chapter is to establish the roles played by key signalling pathways during the induction of PGCs in the pig. The study will focus on BMP, FGF/MAPK, and WNT pathways.

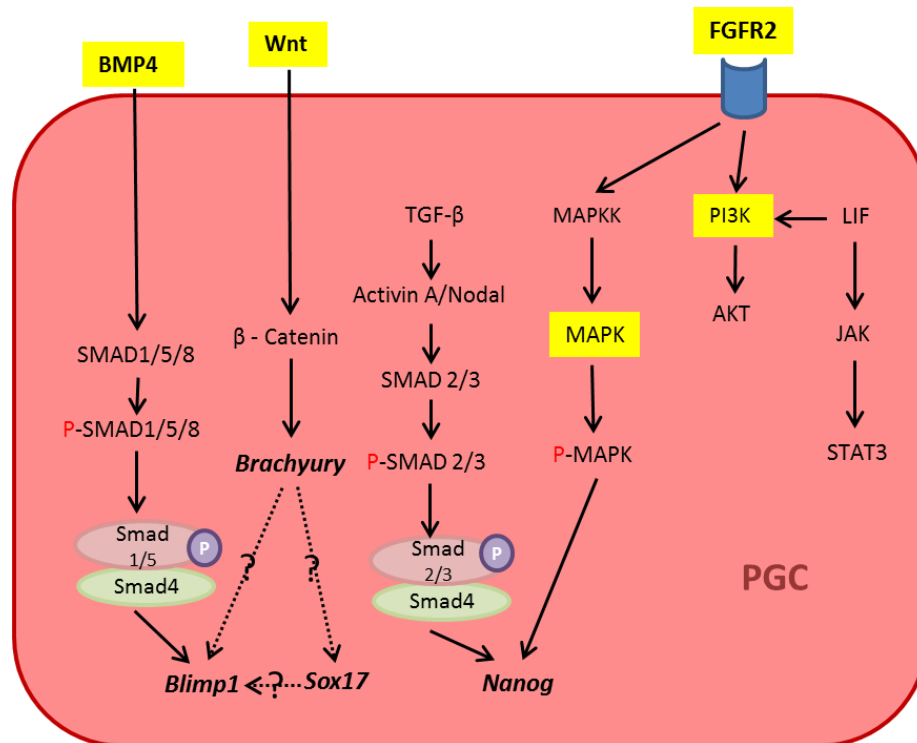


Figure 61. Candidate pathways involved in pig and human PGC specification.

pSMAD1 is signalling transducer of key PGC inducing factor BMP4, which induces BLIMP1. WNT induces mesoderm specification through β -CATENIN pathway. BRACHYURY (T) is activated in response. NANOG is maintained by NODAL signalling pathway, as well as activated p-MAPK. Membrane FGFR2 transduces the FGF signalling, which activates p-MAPK. Another downstream of FGF signalling is PI3K. PGCs also require LIF for maintenance. Arrows indicate signalling pathways. Dashed arrows indicate the proposed relationship of BRACHYURY, SOX17 and BLIMP1.

6.1.2 PGC induction from pluripotent cells of mouse and human pluripotent cells.

Table 3. Methods and Conditions of PGC culture and PGCLC Induction.

Species	Stage of embryo development	Methods	Culture supplement	Identification methods	Chimera ?	Reference
Mouse	E6.5 Blimp1-mVenus (BV) and stella-ECFP (SC)	Epiblast-EpiSCs-EGC like; Co-culture PGC with gonadal cells; Cut gonads into small pieces and put in DMEM + 15%FCS + 2mM L-glutamine + penicillin / streptomycin; meanwhile, another group of gonads are trypsinised and mixed with FACS sorted 3000 stella-GFP ⁺ PGCs; centrifuge, transfer cells to a drop of medium on petri dish. Shake cells till aggregate, transfer cells on transwell inserts. Change medium every day.	Epiblast culture: MEFs & chemically defined medium; Several passages, change to N2B27+20%KSR+2ng/ml recombinant human activin A+12ng/ml bFGF; Chemical defined medium: 50%IMDM + 50%F12 NUT-MIX + 7ug/ml insulin + 5ug/ml transferrin + 450uM monothioglycerol + 5mg/ml bovine serum albumin fraction V; (Brons et al., 2007) EpiSC differentiation: BMP4 (500ng/ml) + epiblast culture medium, wash in 24h; change to Noggin (250ng/ml) + chordin (1.25ug/ml) + activinA + bFGF; FACS cells 48hs after BMP4; Stella + EpiSCs are seeded on inactive SI4-m220 feeder, with DMEM + 15%FCS + NAA + 2mM L-glutamine + penicillin/streptomycin + 1mMsodium pyruvate + 50uM 2-mercaptoethanol + 1000U/ml LIF + 25ng/ml bFGF; 5-6 days later, trypsinise colonies and plate on MEF; After derivation, bFGF is unnecessary ;	RT-PCR	No	(Hayashi and Surani 2009)
Mouse & Rat	Mouse: E8.5-E12.5 Rat: E10 Early morning (late head fold/early somite stage)	Trypsinise posterior fragment, centrifuge and resuspend in FCS-LIF medium+ bFGF(25ng/ml), 2cm ² wells per starting embryo; culture in FCS-LIF; 10-14days later, pick colonies from SI4-m220 and replate on MEF feeder; Derivate in serum-free N2B27+2i-LIF;	Isolation: SI4-m220 feeder & FCS-LIF medium+ bFGF(25ng/ml); D3, replace 50% with FCS-LIF (without bFGF) and pick colonies after 10-14 days; Change medium every 24-48 with FCS-LIF; Derivation: MEF & 2i-LIF Picked colonies are replated on gelatin-coated plates without feeders & DMEM-F12+15% FCS+LIF (1200U/ml) + 0.1% MEM non-essential amino acids + 4mM glutamate+2mM sodium pyruvate + 0.1% mM 2-mecaptanethanol E3.5blastocyst-ESC: N2B27+2i+LIF; MEF feeder; E5.75epiblast-EpiSC: N2B27+activinA (2ng/ml) + bFGF (12ng/ml) + 20%KSR; passage every 3 days with 1mg/ml collagenase IV ESC/iPSC-EpiLC: N2B27 + activinA (20ng/ml) + bFGF (12ng/ml) + 1%KSR; human plasma fibronectin (16.7ug/ml) coated 12well-plate; EpiLC-PGCLC: floating condition: GMEM + 15% KSR (GK15) + 0.1mM NEAA + 1mM sodium pyruvate + 0.1mM 2-mercaptoethanol + 100U/ml penicillin + 0.1mg/ml streptomycin + 2mM L-	Colony forming assay; AP stain; immunostaining; RT-PCR; In vitro differentiation	yes	(Leitch, Nichols et al. 2013)
Mouse	E3.5	ESC-EpiLCs-PGCLCs; iPSCs-EpiLCs-PGCLCs	E3.5blastocyst-ESC: N2B27+2i+LIF; MEF feeder; E5.75epiblast-EpiSC: N2B27+activinA (2ng/ml) + bFGF (12ng/ml) + 20%KSR; passage every 3 days with 1mg/ml collagenase IV ESC/iPSC-EpiLC: N2B27 + activinA (20ng/ml) + bFGF (12ng/ml) + 1%KSR; human plasma fibronectin (16.7ug/ml) coated 12well-plate; EpiLC-PGCLC: floating condition: GMEM + 15% KSR (GK15) + 0.1mM NEAA + 1mM sodium pyruvate + 0.1mM 2-mercaptoethanol + 100U/ml penicillin + 0.1mg/ml streptomycin + 2mM L-	Immunostaining; Q-PCR; Cell number;	No	(Hayashi, Ohta et al. 2011)

			glutamin + BMP4 (500ng/ml) + LIF(1000u/ml) + SCF(100ng/ml) + BMP8b (500ng/ml) +EGF(ng/ml)			
Mouse	E3.5	ESCs-EB-iPGC; ESC derivation: isolate blastocyst, culture in ESC medium + PD98059 for 4 days; EB formation: deplete MEF, plate single cell suspension on tissue culture dishes 2X5mins. 300cells in 20ul/drop on lid of petri dish, with 5ml PBS in bottom, culture for 6 days without LIF, add 3.5ml PBS on day 3 of differentiation. iPGC sorting: culture iPGCs in iPGC sorting medium, change every day for 5 days; AP analysis. EGC derivation: culture iPGC for 5 days with EGC derivation medium, then passage in LIF-only media	ESC medium: Mitomycin C inactivated MEFs, Knockout DMEM + 15% defined lot-tested FBS (Hyclone Lot #ATJ33070) + 1xNEAA+ 1 X Pen/Strep + 1 X L-Glutamine + 55mM β-mercaptoethanol + 1000U/ml LIF Differentiate ESC cells: ESC medium - LIF+ 10uM retinoic acid, four days, don't change; iPGC sorting medium: ESC medium + 15 ng/ml bFGF2 + 30 ng/ml SCF + 2 mM retinoic acid; EGC derivation medium: LIF+SCF+bFGF2+retinoic acid	AP; FACS; Immunostaining; RT-PCR; single-cell real time PCR; microarray; Southern blot; bisulfate sequencing	No	(Vincent, Li et al. 2011)
Pig	E24-E25	Primary culture: Dissect PGCs from uteri and genital ridges; trypsinise 20mins with 0.02% EDTA solution. Seed PGCs on STO feeder; 4-7days later, pick colonies and disaggregate with 0.25% trypsin-EDTA for 10-15mins, then replate on 4-well multidish; Further subculture: wash and 0.25% EDTA trypsinise colonies for 10-15min every 4-7 days. Centrifuge at 800xg for 5min; 39°C; 5%CO₂	STO cell feeder, inactivated by 10μg/ml mitomycin C for 2h. 15%FBS DMEM + 1mM L-glutamine + 0.1M MEM nonessential amino acids+ 10μM 2-mercaptoethanol + 100U/ml penicillin + 0.5ml/ml streptomycin Isolation medium with porcine LIF; EG maintenance medium without porcine LIF ; 39°C; 5%CO₂	AP chemistry before PGC seeding and EG colonies passage; Karyotypic analysis: passage 8-12 EG colonies; In Vitro differentiation;	Yes P7-15 EG cells, contribute to three germ layers;	(Shim, Gutierrez-Adan et al. 1997)
Pig	E26	Primary culture: washed genital ridges in 0.02% EDTA solution and Disaggregate tissue with 0.25% EDTA-trypsin for 15mins at 37°C. Seed PGCs on STO feeder; 7 days later, pick colonies and disaggregate with 0.25% trypsin-EDTA for 10-15mins, then replate on fresh feeder; passage on 1:2-1:8	STO cell feeder, inactivated by 10μg/ml mitomycin C for 2h. DMEM+7.5%FBS+7.5% knockout serum Replacement+ 1mM L-glutamine + 1% MEM nonessential amino acids+ 10μM 2-mercaptoethanol + 100U/ml penicillin + 0.5ml/ml streptomycin Isolation medium with porcine LIF; EG maintenance medium without porcine LIF ; 39°C; 5%CO₂	AP Methylation	No For methylation	(Petkov, Reh et al. 2009)
Pig	E20-E21 E17-18(failed)	Primary culture: medial parts of mesonephros, including genital ridge; Trypsinise; seed on STO feeders in EGC culture medium; P1, purify colonies by disaggregate with 1mg/ml CollagenaseIV for 10mins at 37°C, filter suspensions 3times through 23μm polyester mesh; tightly packed colonies are washed of with PBS, trypsinise and split on fresh feeders; Colonies of the following passages are split together with feeder cells by trypsin, at ratio 1:2-1:3 every 4-5 days	EGC culture medium: AQ media + 17%KSR + nonessential amino acids + penicillin-streptomycin + 10ng/ml hLIF + 10ng /ml hSCF + 3ng/ml hbFGF;	Morphology: normal within first P30, proliferation slows down and stopped till P35; AP staining Immunocytochemistry; In vitro differentiation; Teratoma formation	No	(Petkov, Marks et al. 2011)

Human	5-9 week Gonadal ridge & mesentery	Incubate tissues in 0.05% trypsin-0.5mMEDTA or 0.25% trypsin at 37°C, 10min, or 0.01% hyaluronidase type V+ 0.1% collagenase type IV+0.002% DNase typeI at 37°C for 2hr; Plate on γ-radiation inactivated STO feeders, in primary culture medium	Primary culture: DMEM + 15% FBS + 0.1mM NAA + 0.1mM 2-mercaptoethanol + 2mM glutamine + 1mM sodium pyruvate + 100U/ml penicillin + 100ug/ml streptomycin + 1000U/ml hrLIF + 1ng/ml hrbFGF + 10uM forskolin; 5% or 8% CO ₂ , 95% humidity; Passage every 7 days, disaggregate with 0.05% trypsin/0.53mM EDTA or 0.25% trypsin at 37°C, 5-10min.	AP staining; In vitro differentiation; Immunohistochemistry; karyotype	No	(Shamblott, Axelman et al. 1998)
Human	hESC P8-P45	hESC lines-EB(BMP+/-); hFGSC; EB-on hFGSCs; gonads hESC lines are kept in hESC lines medium; passage every 7 days; EB are differentiated on the 4 th day of passage, disaggregate with 1mg/ml collagenase type IV for 45min at 37°C. Dissociated colonies, plate overnight in ultralow attachment 6-well plates, mTeSR1 medium+10nM ROCK inhibitor (HA-1077). 24hs later, change differentiation medium, replace every 3days. Adherent differentiation: coated with GFRM/G (growth factor reduced matrigel), in differentiation medium, change every 2 days. Differentiation on FGSCs (human fetal gonadal stromal cells): differentiation medium, change every 2 days.	hESC lines medium: MEF; DMEM-F12 + 20% knockout serum + 1%NEAA + 1mM L-glutamine + 0.1mM β-mercaptoethanol + 10ng/ml bFGF; EB differentiation medium: EMEM/F12+20FBS+0.1mM NEAA+0.1mM β-mercaptoethanol + 1mM L-glutamine Adhere differentiation:	Immunofluorescence; FACS; single – cell real-time RT- PCR; cKIT ⁺ , TRA-1-81, OCT4, VASA, NANOS2, NANOS3	No	(Gkoutela, Li et al. 2013)
Human	6-8week fetal gonad and mesenteries	Dissociate Gonadal ridges and mesenteries with 0.25% trypsin for 5min, 37°C EB	Primary culture: DMEM + 15% FBS + 0.1mM NAA + 0.1mM 2-mercaptoethanol + 2mM glutamine + 1mM sodium pyruvate + 100U/ml penicillin + 100ug/ml streptomycin + 1000U/ml hrLIF + 1ng/ml hrbFGF + 10uM forskolin; 5% CO ₂ , 95% humidity; start form from 3-5days; about 80-100 cell EG-like colonies forms formed around day10	P15 hEG: AP high; strong SSEA-1, SSEA-4 and Oct4 and; weakly SSEA-3; Normal Karyotype PCR: Oct4+ EB Immunostaining: Endoderm+:cytokeratin; α-1-fetoprotein; cytokeratin 7. Mesoderm+: Desmin; Muscle actin; Ectoderm+: S-100	No	(Liu, Liu et al. 2004)
Human	hESC NANOS3-mCherry knockin reporter	hESC (in 4i)-PGCLC (EB)	4i: 20 ng/ml Recombinant Human LIF, 8 ng/ml bFGF, 1 ng/ml TGF-β1, 3 μM CHIR99021, 1 μM PD0325901, 5 μM SB203580, 5 μM SP600125. Knockout DMEM, 20% KSR, 2 mM L-glutamine, 0.1 mM NEAA, 0.1 mM β-mercaptoethanol.	qPCR: NANOS3, BLIMP1, TFAP2C, STELLA, TNAP, KIT, OCT4, NANOG, SOX17. ICC:	No	(Irie, Weinberger et al. 2015; Tang, Dietmann et

			Passage cells with TrypLE Express. 10 μ M ROCKi for the first 24 hr. hESC preculture 2 days: bFGF, TGF β , 1%KSR hPGCLC differentiation: 500 ng/ml BMP4, 1 μ g/ml human LIF, 100 ng/ml SCF, 50 ng/ml EGF, and 10 μ M ROCKi, Glasgow's MEM, 15% KSR, 0.1 mM NEAA, 0.1 mM β -mercaptoethanol, 100 U/ml Penicillin-0.1 mg/ml Streptomycin, 2 mM L-Glutamine, 1 mM Sodium pyruvate 2000-4000 cells/EB	CD38, NANOG, OCT4, BLIMP1, TFAP2C, PRDM14, SOX17 Compared with gonadal hPGCS	al. 2015)
Human	hiPSCs BLIMP1-2A-tdTomato (BT) and TFAP2C-2A-EGFP (AG) alleles	hiPSCs-iMeLCs-hPGCLCs	hiPSC medium: feeder-free, defined condition with bFGF with rLN511E8 hiMeLC induction: Activin A and WNTi (CHIR99021), GK15 2 days on fibronectin-coated plate hPGCLC induction: GMEM, 15% knockout KSR, BMP4, SCF, LIF, EGF, ROCKi, 3000 cells/EB	AGBT reporters qPCR OCT4, NANOG, BLIMP1, TFAP2C, SOX17, NANOS3, KLF4	No (Sasaki, Yokobayashi et al. 2015)

6.2 Materials and Methods

For epiblast culture, pig embryos are flushed from reproductive tracks 11 days after insemination. Epiblasts were neatly dissected and cultured individually in desired medium and pre-incubated with inhibitors in basal medium for 30 minutes before cytokines were added. Inhibitors were 10 μ M WNTi (IWR-1-endo, Sigma), 100nM BMPi (LDN193189, SIGMA-ALDRICH, SML0559), 100nM FGFRi (PD173074, TOCRIS bioscience, 3044), and 1 μ M MAPKi (PD0325901, TOCRIS bioscience, 4192), supplemented in basal medium. 100ml basal medium was made up with 80.8ml GMEM, 15ml KSR, 1ml NEAA, 1ml Pen/Strep, 1ml L-Glutamine, 1ml Sodium Pyruvate, and 200 μ l β -mercaptoethanol. After pre-inhibition, cytokines were supplemented to the final concentration of 500ng/ml BMP4, 100ng/ml EGF, 1000ng/ml hLIF and 100ng/ml SCF. Epiblasts were incubated for 64 hrs and then sacrificed for IF against antibody combinations NANOG/SOX17, NANOG/BLIMP1, NANOG/OCT4, SOX17/TFAP2C, and NANOG/T. 5 combinations were applied rotatory to make sure successive sections were stained with different combinations. All sections were stained and utilized for quantification. Double positive cell numbers in each combination and embryos found with double positive cells were counted manually.

6.3 Results

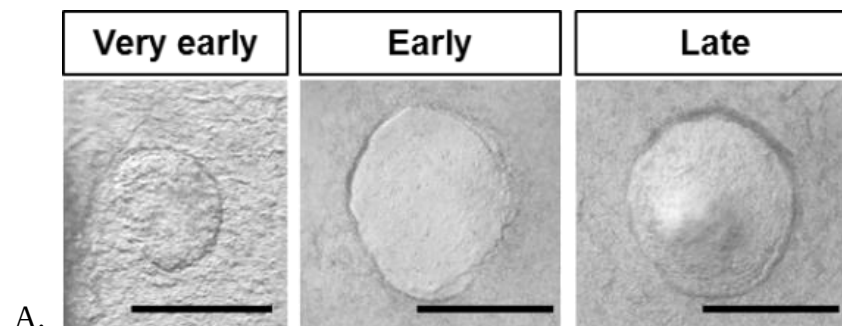
6.3.1 In vivo determination of PGC specification pathways

E10.5-E11.5 pig embryos were flushed from reproductive tracks and transported in 10% FBS/DMEM-F12 at 37C in a portable incubator. Epiblasts were dissected and placed individually in PGC differentiation medium supplemented with WNTi, FGFRi or BMPi (Figure 62). Inhibitors were applied 30 mins before transferring to PGC induction medium. To determine the time window of PGC recruitment, epiblasts were divided into three groups according to the development stage. Very early group (E10-10.5) embryos belong to bilaminar stage. Early group (~ E10.5-11), present a small nascent mesoderm, but no primitive streak. Late group (~E11-11.5), signs of primitive streak appear. After 64 hours treatment, all epiblasts were fixed in 4% PFA for 15 minutes, mounted in OCT, and sectioned (4 μ M) using a cryostat. IF with combinations of OCT4, NANOG, BLIMP1, SOX17, TFAP2C and T were performed

in serial sections. According to IF result from pig embryos (Chapter 3), colocalized OCT4, NANOG, BLIMP1 and SOX17 identifies PGC precursors or nascent PGCs. Colocalization of AP2 γ marks slightly advanced PGC status.

Figure 63 shows the phenotype of epiblast before and after culture. As shown in A, epiblasts were catalogued in three stages. Very early (VE) embryos are bilaminar (~ E10.5). Early (E) (~ E10.5-E11) embryos have incipient mesoderm from posterior end of disc. Late (L) embryos are slightly more advanced with embryonic disc starts to curve.

As cells were individually cultured, there is no interference between each epiblast. Heterogeneous phenotype within one group reflects the starting stage of the epiblast and corresponding PGC specification ability. One epiblast from the BMP/EGF/SCF/LIF control group is bigger with hollow structure, while the others are small and solid. WNTi treated epiblasts are slightly larger and uniformly solid. FGFRi treat epiblasts are bigger balloon like monolayer balls. Some even floated from bottom of dishes (Figure 62 B).



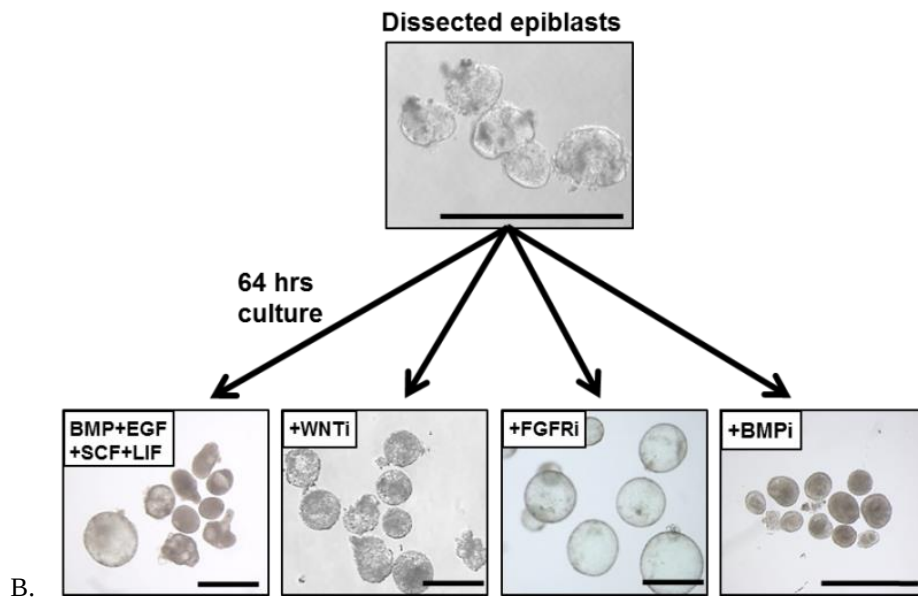


Figure 62. Epiblast stages and phenotype in different treatments.

A, Top view of embryonic discs at VE (very early), E (early) and L (late) stages. VE embryos are bilaminar. E embryos have nascent embryonic mesoderm. L embryos are slightly more advanced. **B.** Phenotype of dissected epiblasts following the culture under different conditions. Scale bar A: 1mm, B: 0.5 mm.

Figure 63 shows examples of early stage control group epiblast IF. Two phenotypes were observed at this stage. The majority were solid EB-looking epiblasts. Positive areas are zoomed in squares. There are also 1 out of 8 embryos formed primitive streak like structures, which are Nanog⁺/Oct4⁺/Sox17⁻/Blimp1⁻. PS structure is surrounded by mesodermal-like cells, which shows T⁺/NANOG⁺ and heterogeneous OCT4⁺/SOX17⁺. All OCT4⁺/SOX17⁺ are subpopulation of NANOG⁺ cells. Outside of the mesodermal domain is endodermal looking monolayer cells, which are OCT4⁻/T⁻, NANOG⁺/⁻ and SOX17⁺/⁻.

Very early group and late group have less responsive embryos, within which less positive cells are stained. All positive embryos are solid EB-like.

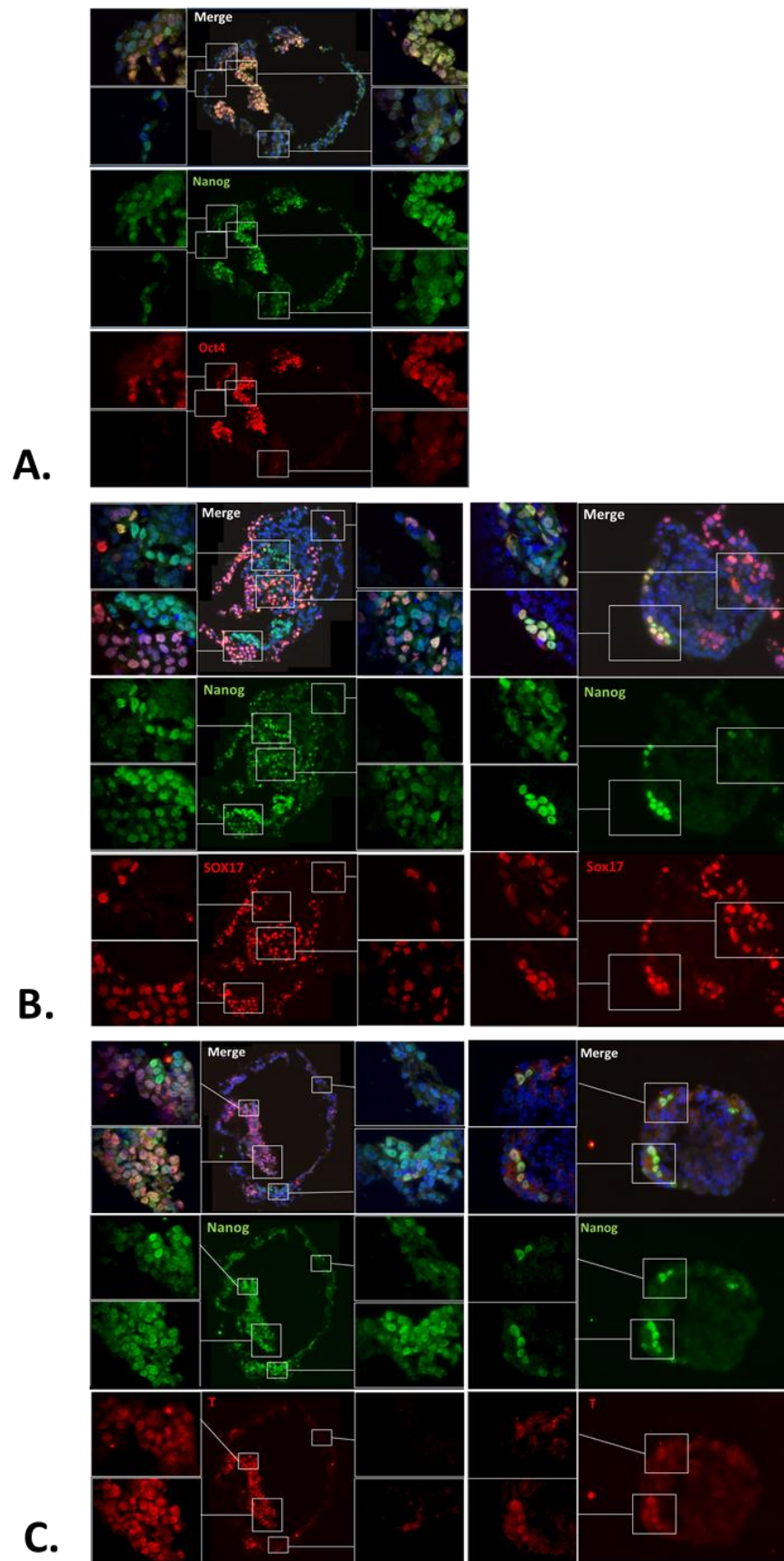


Figure 63. IF of early stage epiblast culture in control group.

Positive areas are zoomed in as squares.

To understand PGC inducing time window and efficiency, all positive embryos and cells were quantified and plotted in Figure 64. Percentage of positive embryos is plotted in bar charts (Figure 64, top). All three stages of epiblast were capable of initiating PGC precursor marker NANOG/OCT4, NANOG/SOX17 and NANOG/BLIMP1, although early group shows higher efficiency. PGC marker SOX17/TCFAP2C embryos are observed in VE and E group, but not in L group. Numbers of double positive cells numbers within each embryo were plotted in the scattered chart (bottom). Although VE group has similar percentage of NANOG/OCT4, NANOG/SOX17 and NANOG/BLIMP1 as E stage, the number of positive cell is significantly less. Two positive embryos in VE group have 1 positive cell for NANOG/SOX17 and SOX17/TCFAP2G. Effect of stage E against VE and L is significant ($P=0.005$), and VE is not significantly differs from L stage ($P=0.236$).

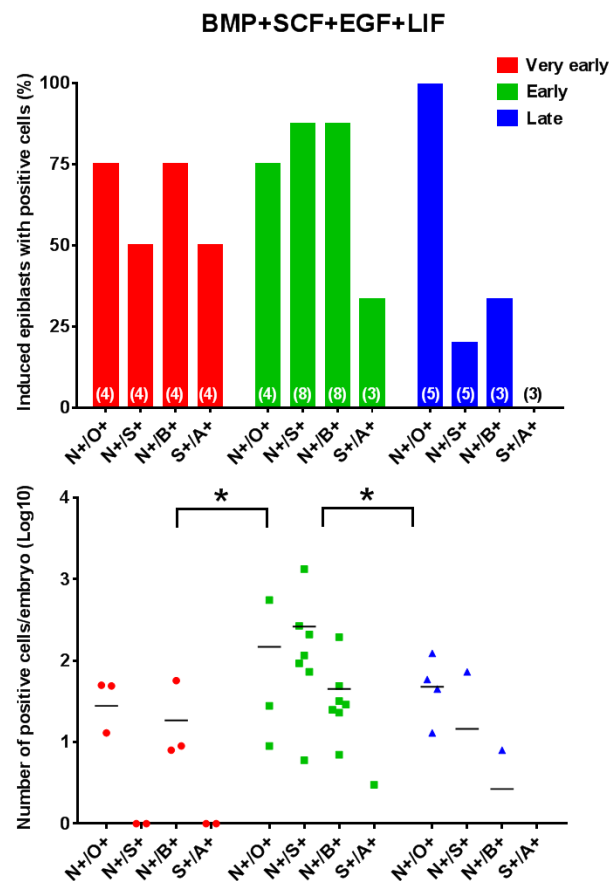


Figure 64. Quantification of positive cells in control group cultured epiblast.

Top, percentage of cultured epiblasts with positive cells. Number of cultured embryos are labelled at the bottom of each bar. Bottom, positive cell number in each embryo. Median of each group is shown in black bars. Effect of E stage is significant against VE

and L stages ($P=0.005$), while VE and L is not significant ($P=0.236$). N, Nanog. O, OCT4. S, SOX17. B, BLIMP1. A, AP2 γ (TFAP2C).

WNTi treated epiblast presents obvious lower PGC competence (Figure 65). Particularly in VE group, only 17 NANOG/OCT4 positive cells found in all cultured epiblasts all stained markers. E stage still maintains the highest efficiency; however the number of positive cells is much lower. For instance, the highest positive number of NANOG/OCT4 drops from 556 (control) to 72 (WNTi), and NANOG/SOX17 drops from 1332 (control) to 16 (WNTi). Highest NANOG/BLIMP1 positive number maintains from 341 (control) to 195 (WNTi). L stage epiblasts also respond to WNTi culture medium, 173, 48 and 33 NANOG/OCT4 positive cells were found out of 4 epiblasts. 13 and 2 NANOG/OCT4 positive cells out of 4 epiblasts and 18 NANOG/BLIMP1 cells out of 3 embryos were detected.

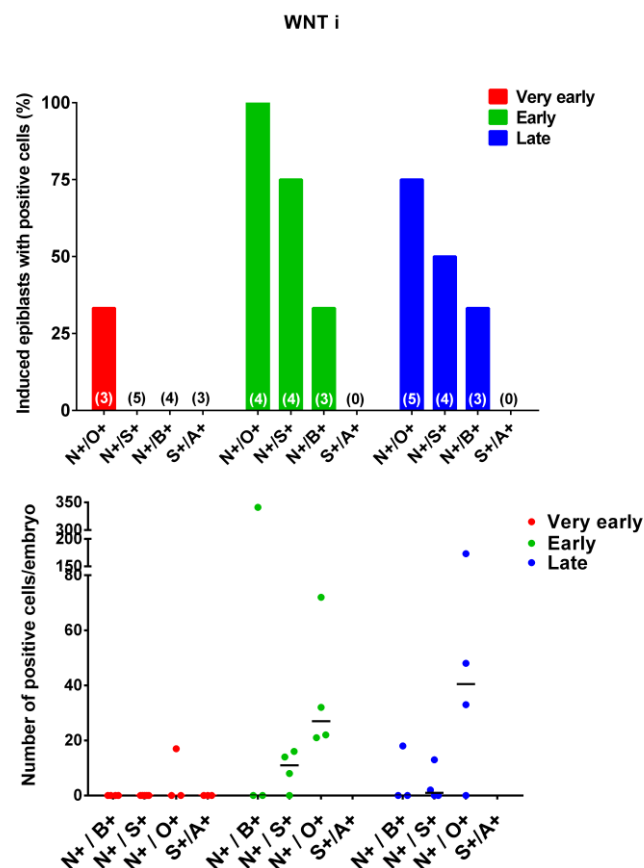


Figure 65. Quantification of positive cells in WNTi treated epiblasts.

Top, percentage of cultured epiblasts with positive cells. Number of cultured embryos are labelled at the bottom of each bar. Bottom, positive cell number in each embryo. Median of each group is shown in black bars. N, Nanog. O, OCT4. S, SOX17. B, BLIMP1. A, TFAP2G.

Figure 66 shows examples of positive cells quantified. For the epiblasts that developed endodermal periphery and mesodermal inner cells, only mesodermal domain cells were quantified.

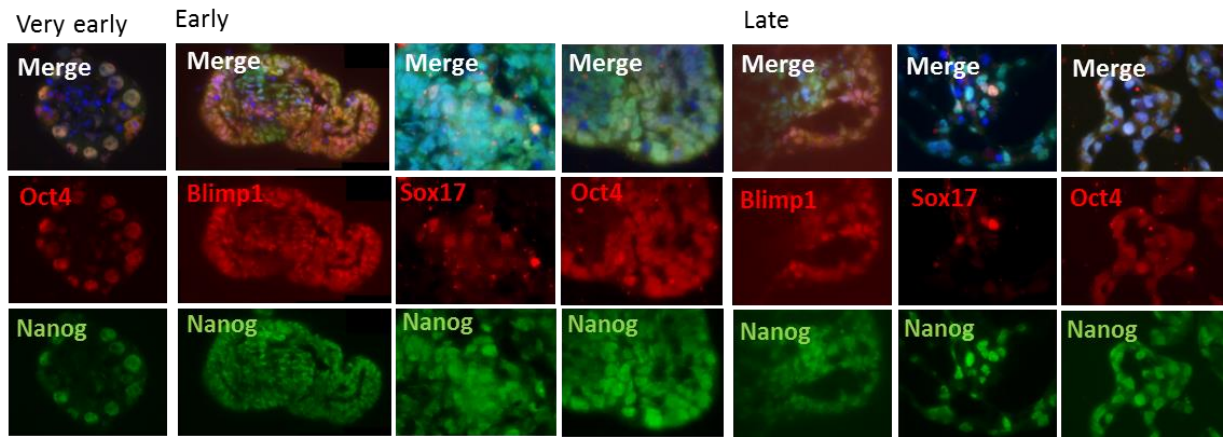


Figure 66. IF of WNTi treat epiblast from VE, E and L stages.

As shown in Figure 67, FGFRi clearly blocks PGC differentiation. In both VE and E stages, almost no positive cells for any PGC markers, except for 3 NANOG/BLIMP1 positive cells at E stage. When MAPK is blocked at VE stage, 153, 49 and 64 NANOG/OCT4 positive cells, 42 NANOG/SOX17 positive cells and 4 NANOG/BLIMP1 cells were observed. No SOX17/TFAP2G positive cells in 4 embryos.

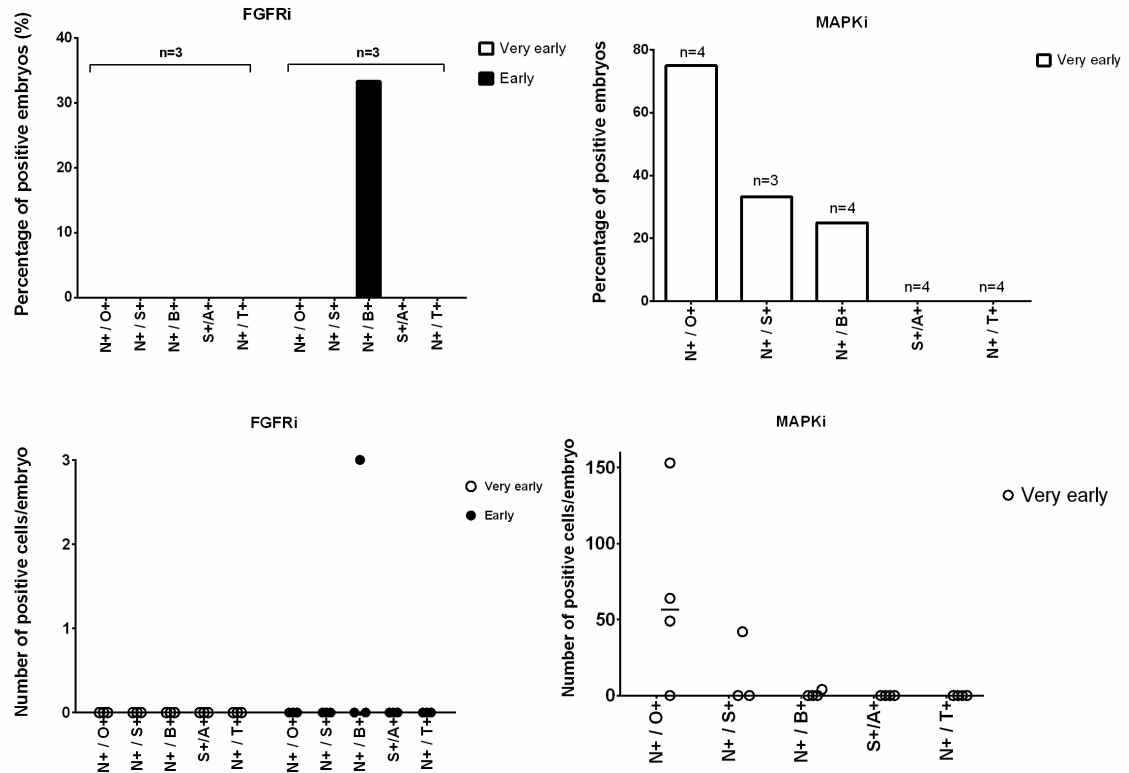


Figure 67. Quantification of positive cells in FGFRi and MAPKi treated epiblast.

N=3 FGFRi treated embryos were subjected for each combination on both VE and E stages. Number of MAPKi embryos is labelled on top of each bar. N, Nanog. O, OCT4. S, SOX17. B, BLIMP1. A, TFAP2G.

6.3.2 Characterization of EBs during PGC differentiation

Next, a similar treatment to the one carried out with epiblasts was applied to H9 hESC cell line cultured in 4i medium before PGC differentiation. Upon differentiation, 3000 cells were seeded per well in PGC media. EBs were collected at 6h, 12h, 1D, 2D, 3D and 4D and IF was performed on 6µm cryosections.

H9 cells adapted to 4i media grow as colonies and have clear boundaries (Figure 68). Pluripotency markers NANOG, OCT4 and SOX2 are maintained (Figure 69). EBs are not formed before 6h, when cells aggregate as flat sheets at the bottom 96-well plate. Size of the EB expands in the following 3 days. BLIMP1, SOX17, NANOS3 are significantly upregulated in D4 EBs. OCT4, NANOG, TFAP2C are maintained during PGC differentiation (Figure 69).

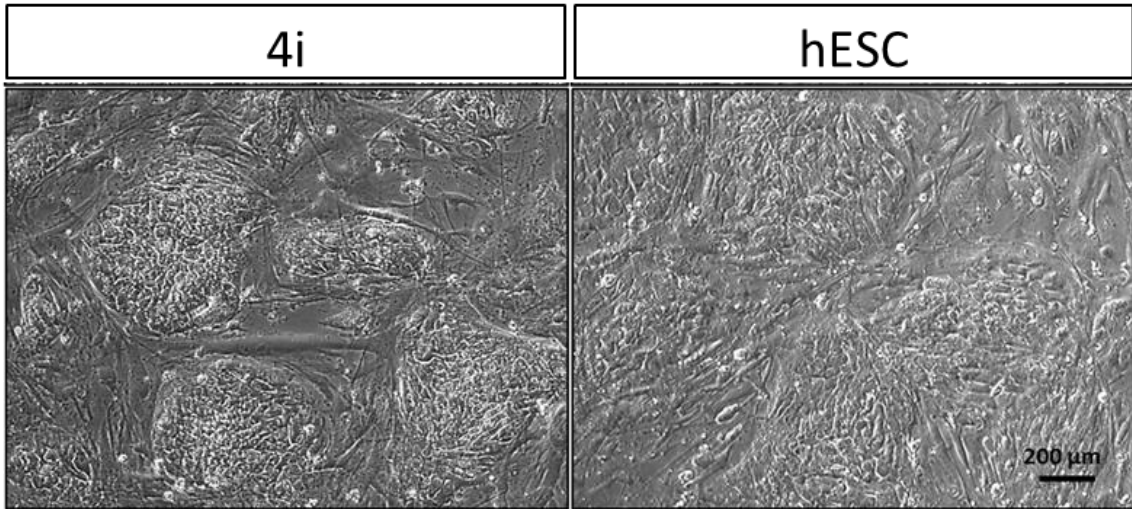


Figure 68. 4i medium adoption on H9 hESC cell line. Scale bar, 200 μm.

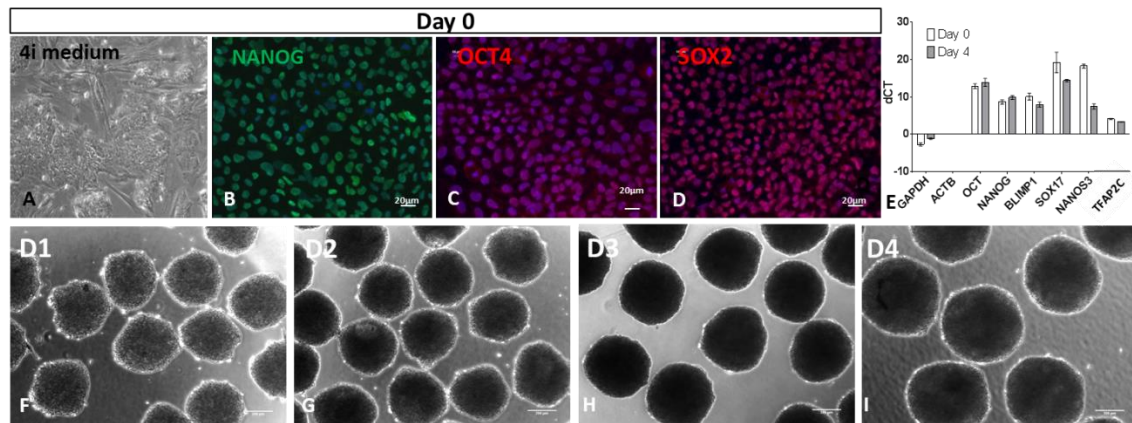


Figure 69. H9 cell line characterization and EB differentiation morphology.

A-D, bright field and IF of pluripotency markers NANOG, OCT4 and SOX2 at H9 cells in 4i medium. E, qPCR dCT value of PGC related genes at D0 and D4 of PGC differentiation. F-I, phenotype of D1-D4 EBs in PGC differentiation. Scale bar, 100 μm.

To investigate the chronology and expression pattern of PGC related genes, particularly BLIMP1, SOX17 AND NANOG, 6h, 12h and 24 EBs were sectioned and stained in various combinations.

For the first 12 hrs of differentiation, NANOG and OCT4 are maintained in every cell (Figure 70 and Figure 71). Until 24 hrs, high NANOG is restricted to a subpopulation of cells. T is not activated until 12 hrs after differentiation. Nanog is downregulated in T+ cells. After 24 hrs, Nanog+ and T+ cells present mutually exclusive patterns (Figure 70). Nanog is always colocalized with Oct4 (Figure 71).

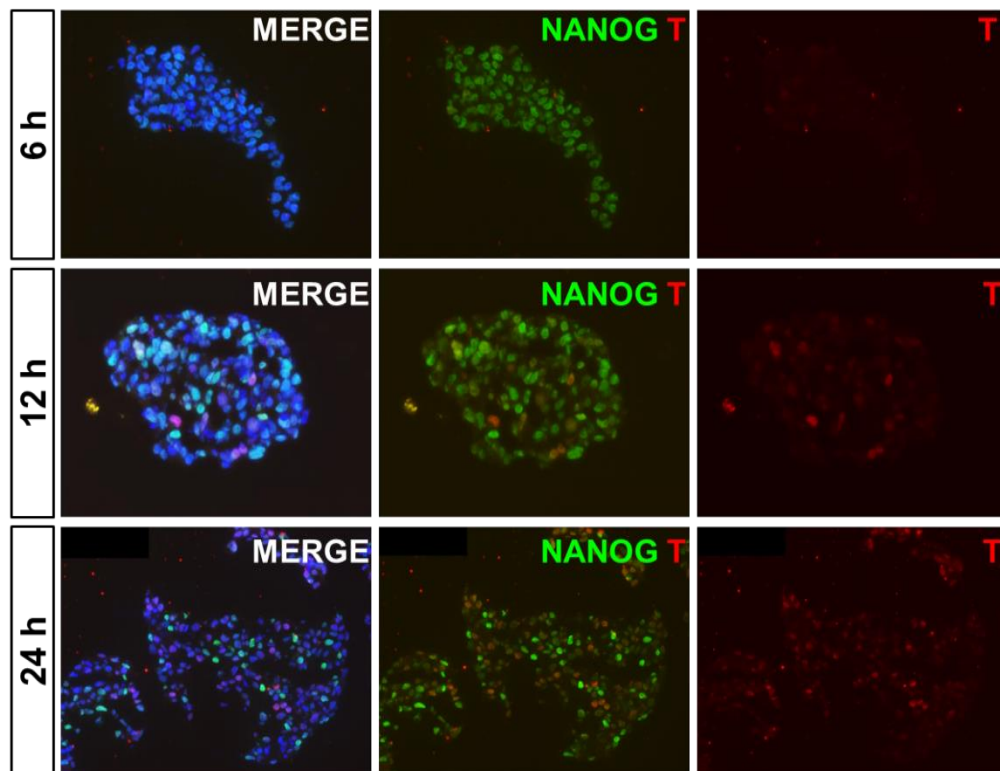


Figure 70. NANOG and T expression profile at 6h, 12h and 24h during PGC differentiation. H9 hESC cell line was cultured in 4i medium before differentiate.

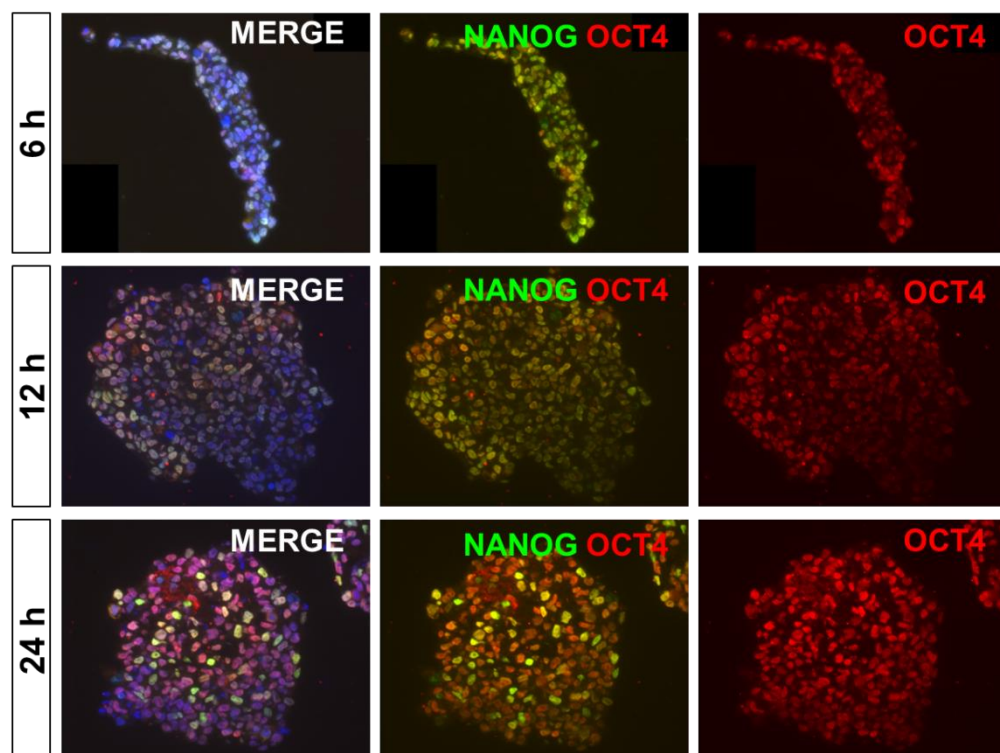


Figure 71. NANOG and OCT4 expression profile at 6h, 12h and 24h during PGC differentiation.

From 12h, Sox17 starts to be expressed in a few Nanog⁺ cells. At 24h, Sox17 expression increases in number and intensity. Nanog localizes with Sox17 cells, although expression level varies (Figure 72).

TFAP2C is detected in a subpopulation of Sox17+ cells from 24h (Figure 73).

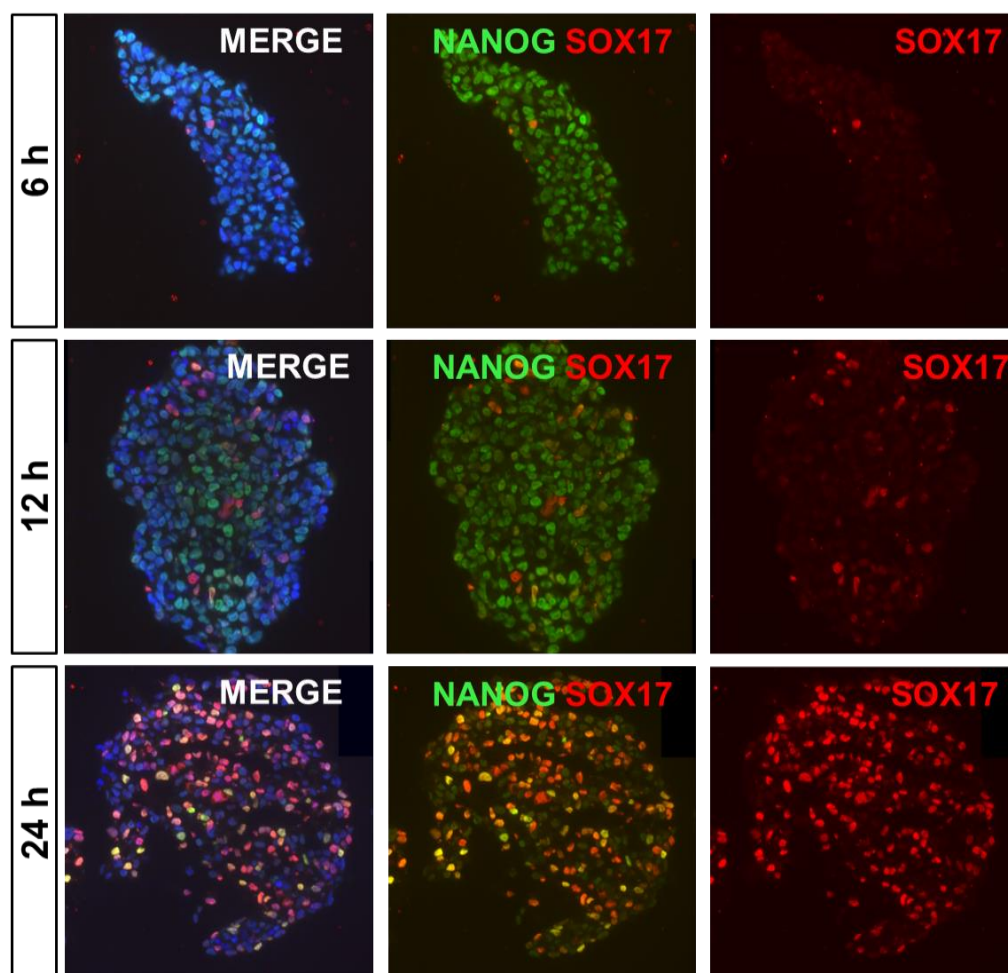


Figure 72. NANOG and SOX17 expression at 6h, 12h and 24h during PGC differentiation.

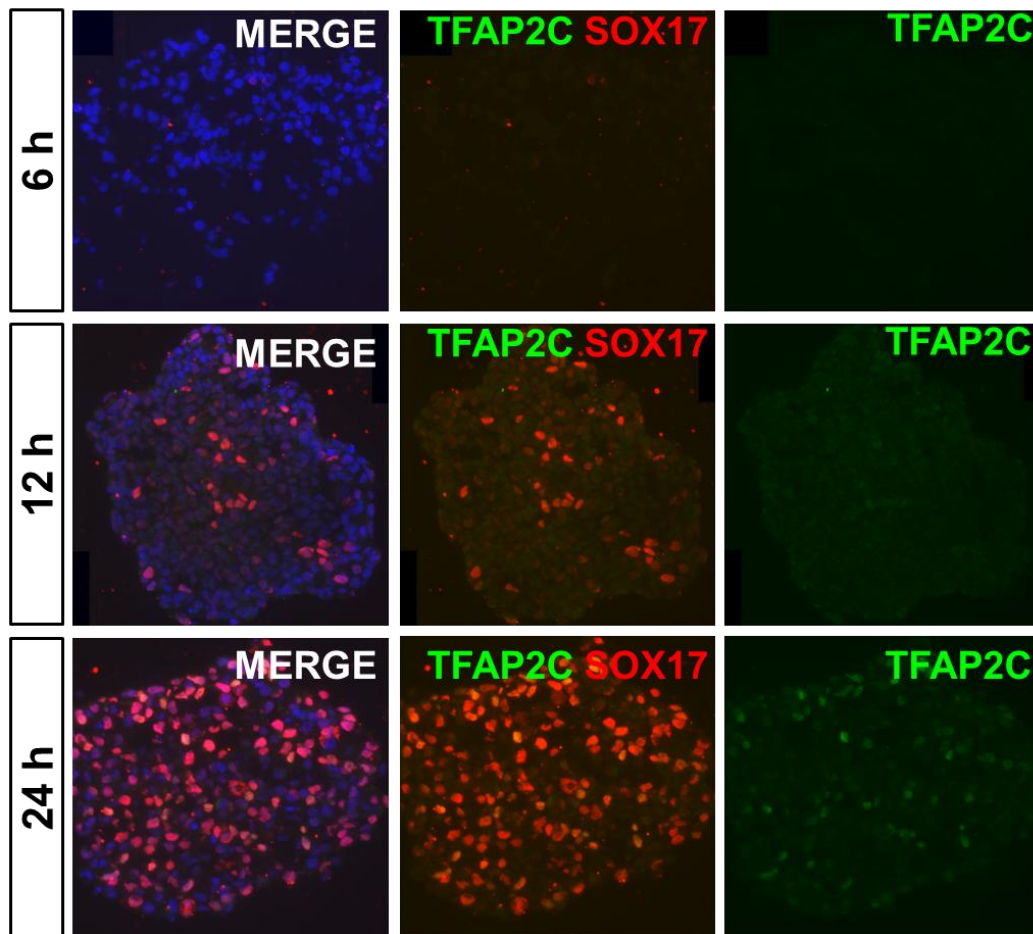


Figure 73. TFAP2C and SOX17 expression at 6h, 12h and 24h during PGC differentiation.

Stained EBs were in turn quantified and plotted in Figure 74. At 6h, 96% of total cells are NANOG⁺ only cells, 90% are NANOG⁺/OCT4⁺, 3.83% are NANOG⁺/SOX17⁺, 6.06% are NANOG⁺/T⁺, and no BLIMP1⁺ or TFAP2G⁺ initiated. At 12h, percentage of NANOG⁺/OCT4⁺ cell maintains high at 92.79%, NANOG⁺/SOX17⁺ increases to 15.73%, NANOG⁺/T⁺ to 32.91% , BLIMP1⁺ and TFAP2C⁺ remains 0%. From 12h to 24h, proportion of NANOG⁺/SOX17⁺ goes up to 60.23%, NANOG⁺/OCT4⁺ declines to 78.07%, incipient NANOG⁺/BLIMP1⁺ at 13.287%, TFAP2C⁺/SOX17⁺ 60.12%, and NANOG⁺/T⁺ 69.84%. There are still 30.18% NANOG⁺/SOX17⁻ cells, 1.8% NANOG⁺/OCT4⁻ cells, 71.33% NANOG⁺/BLIMP⁻ cells, 21.99% NANOG⁺/T⁻ cells in 24h EBs. No NANOG⁻/SOX17⁺, NANOG⁻/BLIMP⁺ and 11% NANOG⁻/OCT4⁺, 3.50% NANOG⁻/T⁺ cells were detected. 8.84% TFAP2G⁺/SOX17⁻ cells are probably differentiated other cell types.

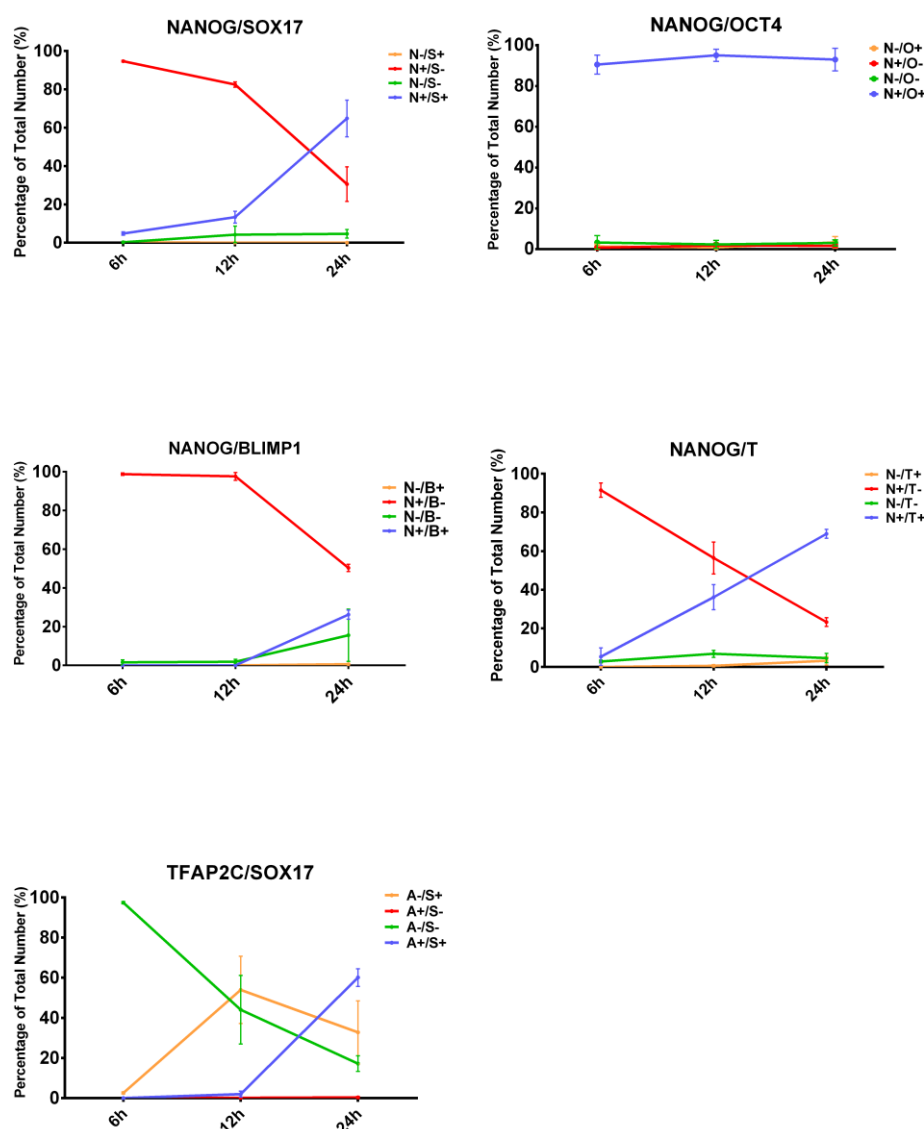


Figure 74. Quantification of PGC related genes at 6h, 12h and 24h.

From D1 to D4, the size of EB expands as shown in Figure 75, however the number of NANOG⁺ and SOX17⁺ cells is maintained. The intensity of NANOG increases drastically from D1 to D2. NANOG⁺ only cell number reduces until restricted to SOX17⁺ cells. As shown in Figure 75. TFAP2C initiates from D1, while OCT4⁺ maintains high proportion in early PGC differentiation. From D1-D4, the number of TFAP2C⁺ increases, colocalized with maintained OCT4⁺ cells. Tet1 is also detected in specified PGCLC, which is consistent with observation of E13 porcine PGC cluster (Chapter4 Figure 32).

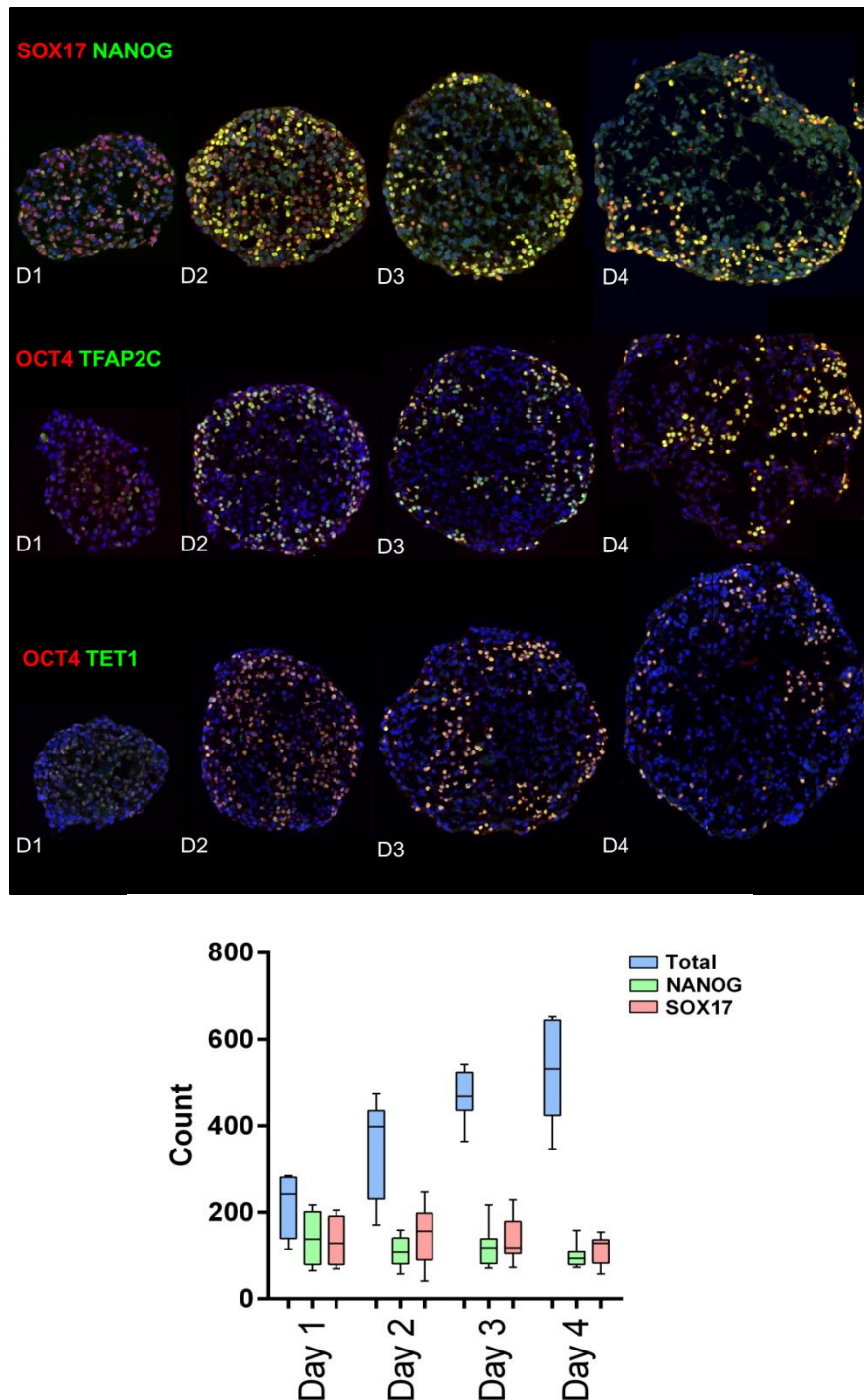


Figure 75. Quantification of colocalization of PGC markers.

Top, IF of D1-D4 EBs with SOX17/NANOG, OCT4/TFAP2G and OCT4 TET1. Images of same antibodies were taken under the same setting. Bottom, quantification of D1-D4 NANOG and SOX17 positive cells.

6.3.3 Determination of PGC specification pathways in vitro

To determine the key pathways participating in PGC induction, pharmacological inhibitors were used to block FGF, MAPK, PI3K, EGF and WNT pathways during PGC differentiation.

The results of the control (DMSO) group are consistent with previous PGC differentiation reported by Irie et al., (Irie, 2015). Nanog is prevalently positive from D1. Reduced T is still detectable in some cells. Bright Sox17 is detected in almost half cells. TFAP2C is only detected in a small sub population of cells (Figure 76).

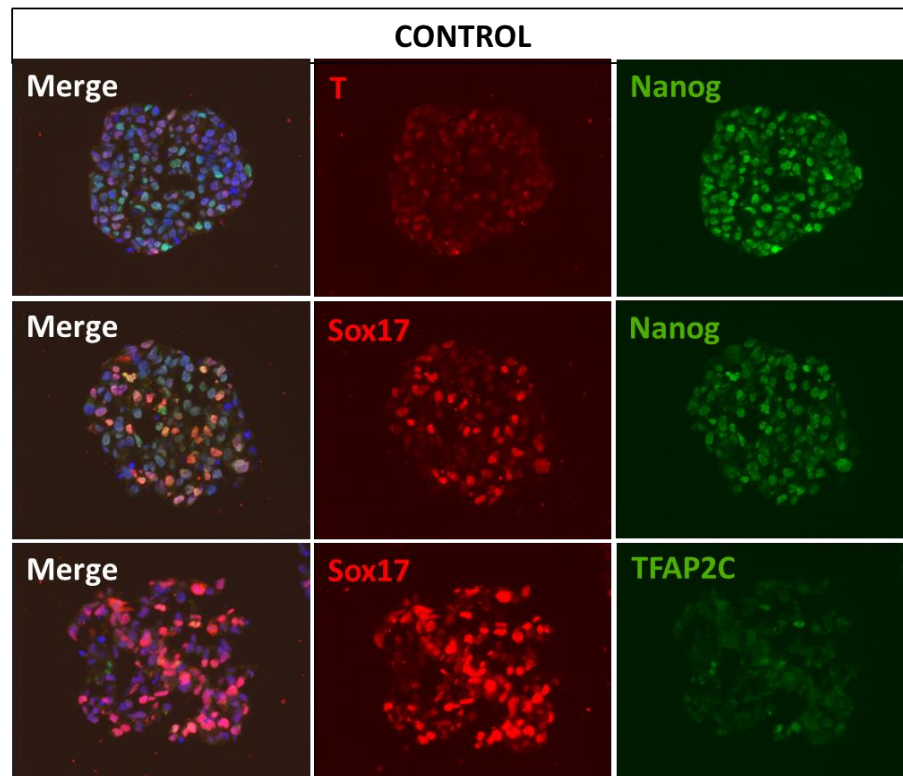


Figure 76. IF of D1 H9 PGC Differentiation in control group.

The size of WNTi treated EB is decreased at D1 (Figure 77), compared with control (Figure 76). Although T expression is significantly depressed, NANOG⁺/SOX17⁺ PGCs are still prevalent and TFAP2C is activated like control EB does. The absence of T together with SOX17 and Nanog colocalization suggests T is dispensable to PGC specification.

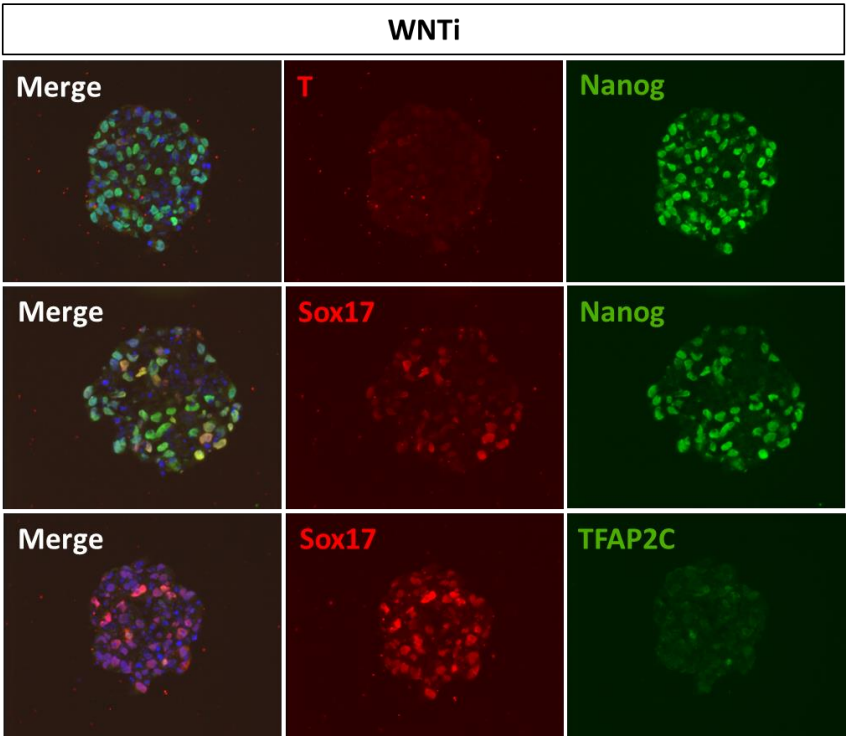


Figure 77. IF of WNTi treated EB at D1 during H9 PGC Differentiation.

As well as WNTi, FGFRi diminished the size of EB at Day1. Nanog is universally positive, and SOX17 is activated in a sub population of cells. TFAP2C is very rare (Figure 78), which is consistent with the effect of FGFRi cultured epiblast (Figure 67).

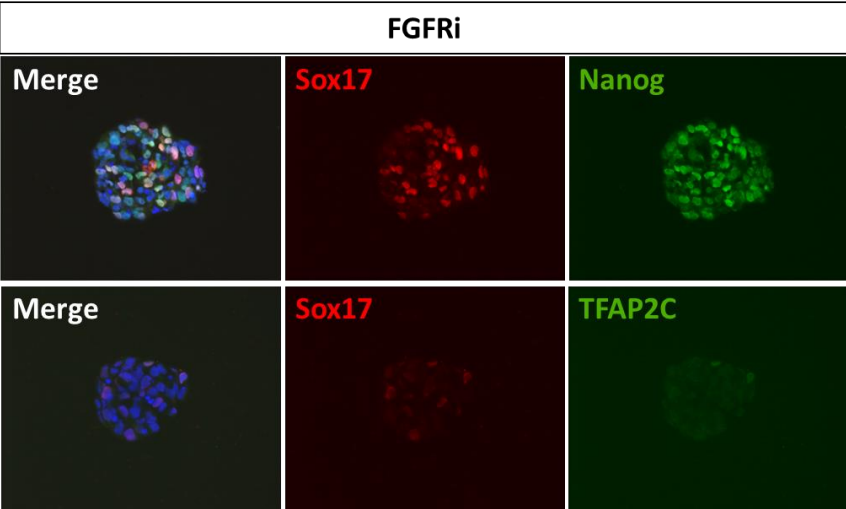


Figure 78. IF of FGFRi treatment EB at D1 during H9 PGC differentiation.

MAPK and PI3K are two downstream effectors of FGF. Both have been reported relevant for mouse PGC specification (De Miguel, Cheng et al. 2002; Kimura, Kaga et al. 2014). To dissect their function in PGC specification, we inhibited them respectively during PGC differentiation.

MAPKi treated EBs are smaller while gene expression profile is not altered significantly by D1 (Figure 79). This raises the PGC percentage, indicating that it impacts on PGC specification efficiency.

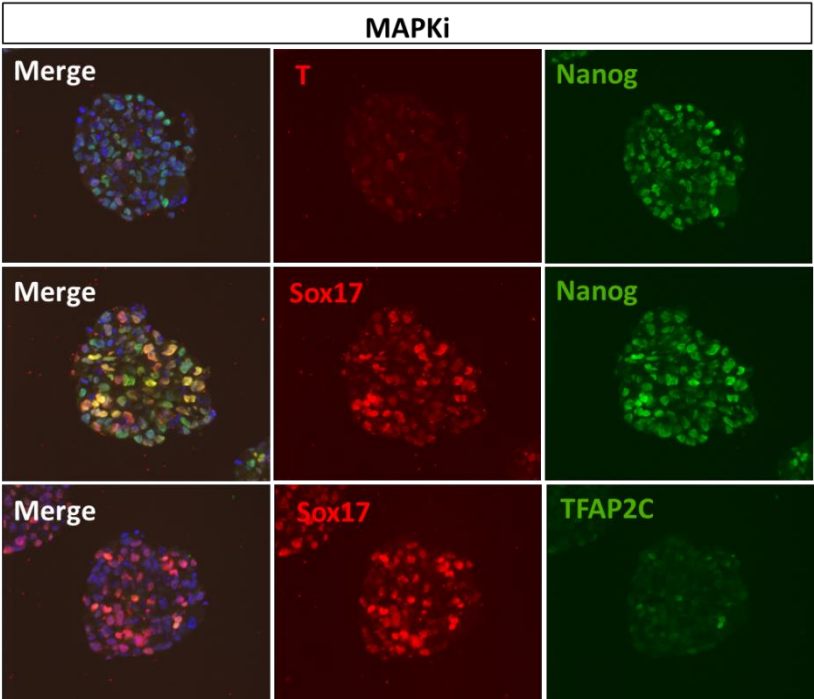


Figure 79. IF of MAPKi treated EB at D1 during H9 PGC Differentiation.

On contrary to MAPKi, when PI3Ki is supplemented into PGC differentiation system, more prevalent T was detected at D1, but PGC gene expression pattern is maintained (Figure 80).

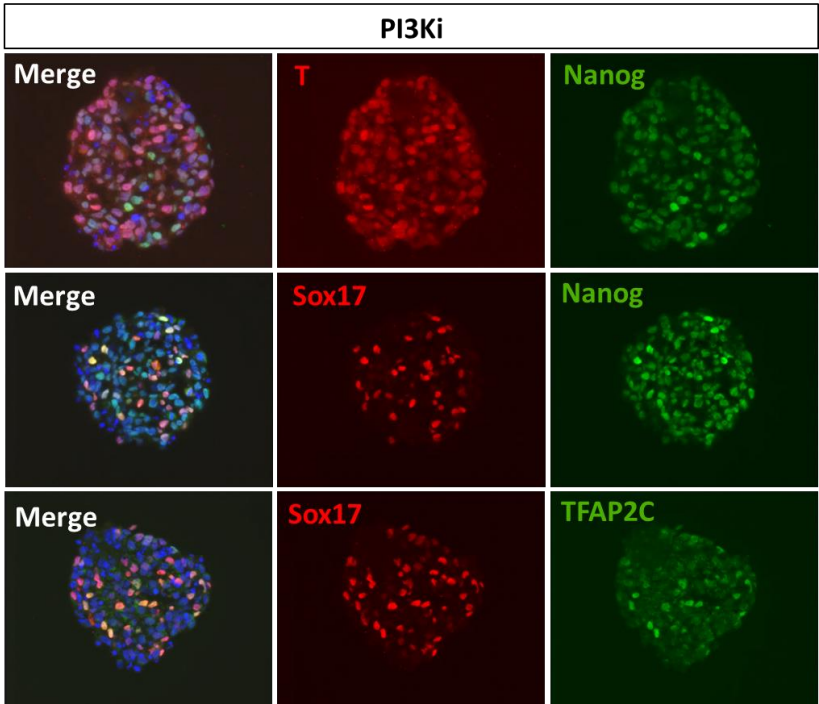


Figure 80. IF of PI3Ki treated EB at D1 during PGC Differentiation.

In EGFRi treatment, no obvious alteration in the shape of EB size, phenotype, nor NANOG/T/SOX17 expression patterns at D1 were detected (Figure 81).

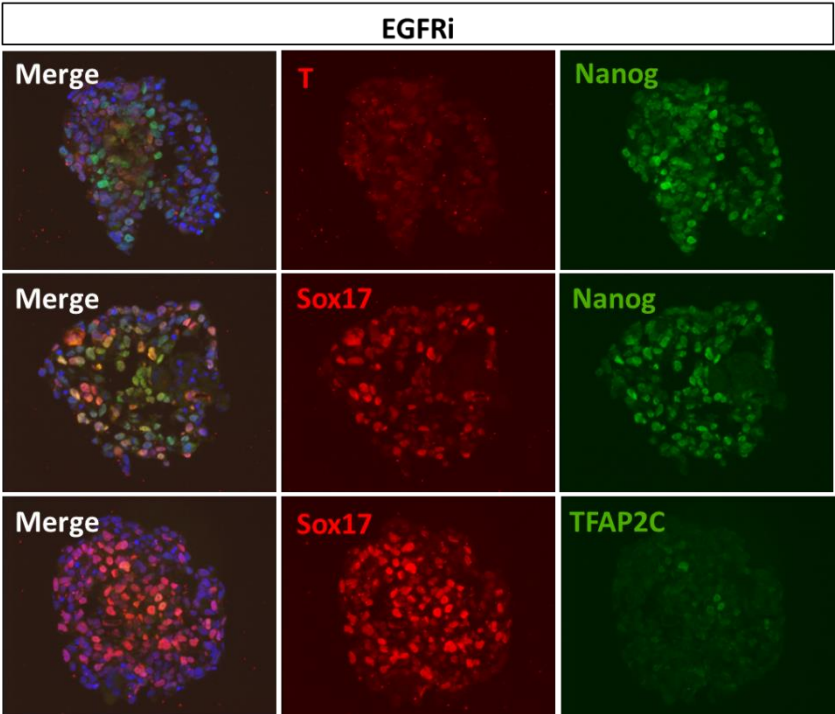


Figure 81. IF of EGFRi treated EB at D1 during H9 PGC Differentiation.

By D4, EBs in EGFRi and WNTi significantly reduce size, compared with control. MAPKi condition generates medium size EBs, and PI3Ki, EGFRi groups EBs

maintain similar size as control. Although the size is drastically different, the proportion of SOX17+/NANOG+ cells in FGFRi and WNTi remains as control. MAPKi and PI3Ki decrease PGC proportion significantly. EGFRi has no effect on PGC proportion (Figure 82).

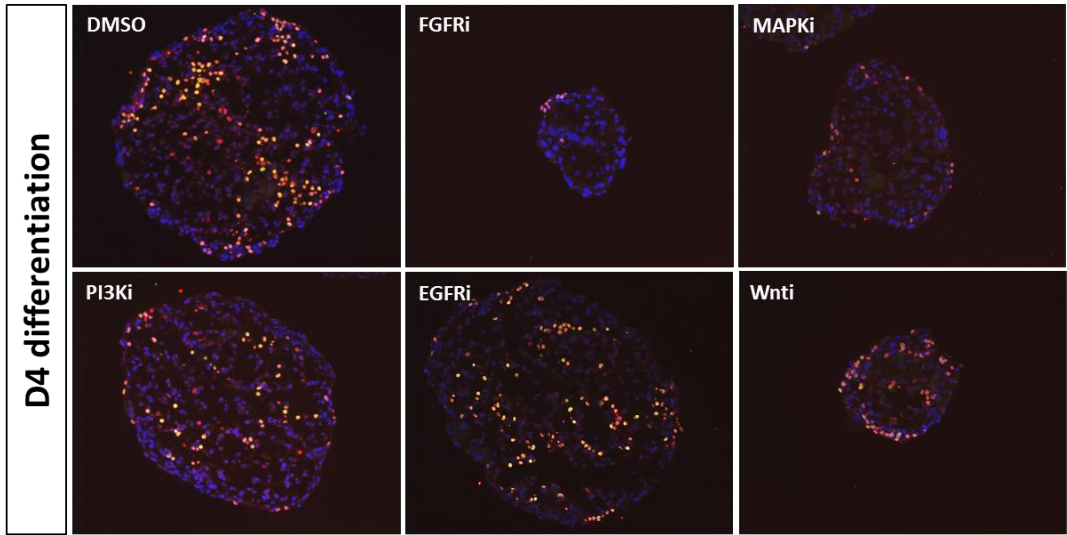


Figure 82. SOX17+/NANOG+ cell proportion in D4 EBs treated with inhibitors.

Refers to last experiment, candidate signalling pathways WNT, FGF and MAPK were selected accordingly to dissect their effects on PGC differentiation. Treated EBs were stained at D1 and D4, to distinguish stage of inhibitor effects.

In control group, besides the consistent NANOG/SOX17/T/TFAP2C expression pattern at D1, BLIMP1 is expressed in a small sub population of NANOG+ cells. As OCT4 is always colocalized with NANOG+ cells, we can conclude that NANOG+/SOX17+/OCT4+ cells are PGCs (precursors), within which, Blimp1 and TFAP2C are incipient at D1. Some NANOG+ cells are T+, while some are T-, no regime is aware. At D4, BLIMP1/NANOG/SOX17/OCT4/TFAP2C co-localizes and restricts to PGCs, while T is detected in some PGCs as well as other cells. PGCs are formed in clusters or individually throughout the sections without particular niche (Figure 83).

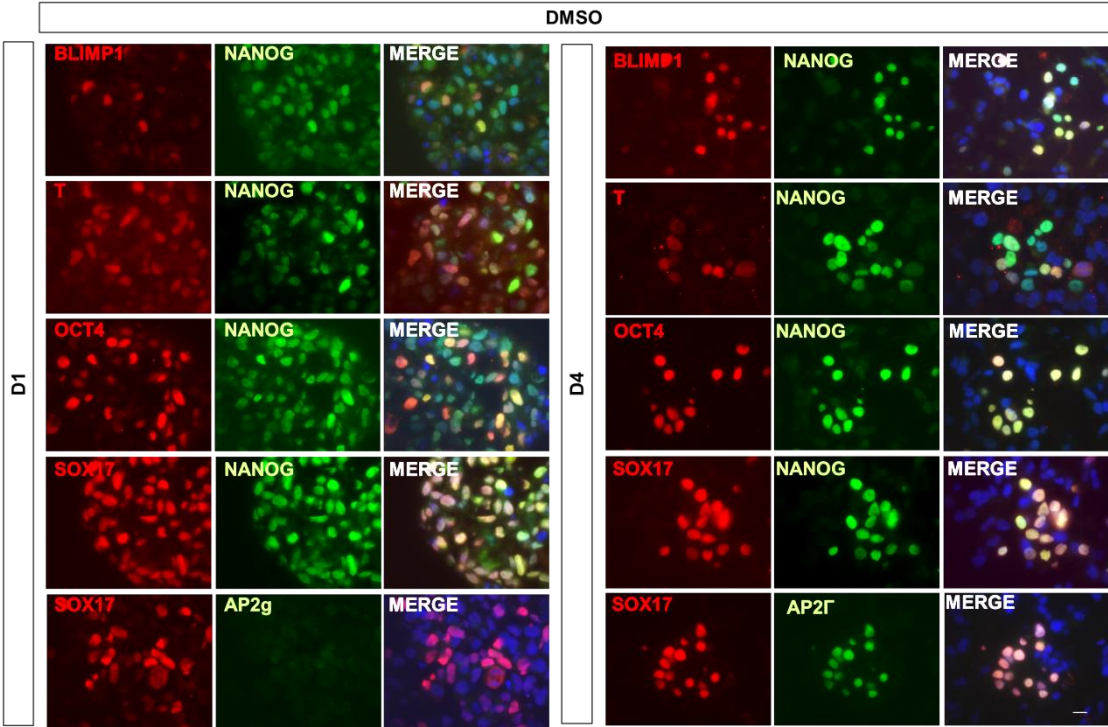


Figure 83. IF of DMSO treated EBs at D1 and D4 during PGC differentiation.

EBs subject to WNTi treatment presents smaller size, and the PGCs tend to form at the periphery of EB sections, particularly at D4. At D1, T is blocked with only a few exceptions. In this situation, BLIMP1/NANOG/SOX17/OCT4/TFAP2C colocalization is similar to DMSO control from D1 to D4. Some TFAP2C+ only cells are observed at D4 (Figure 84). These observations indicate that T is dispensable to human/pig PGC differentiation.

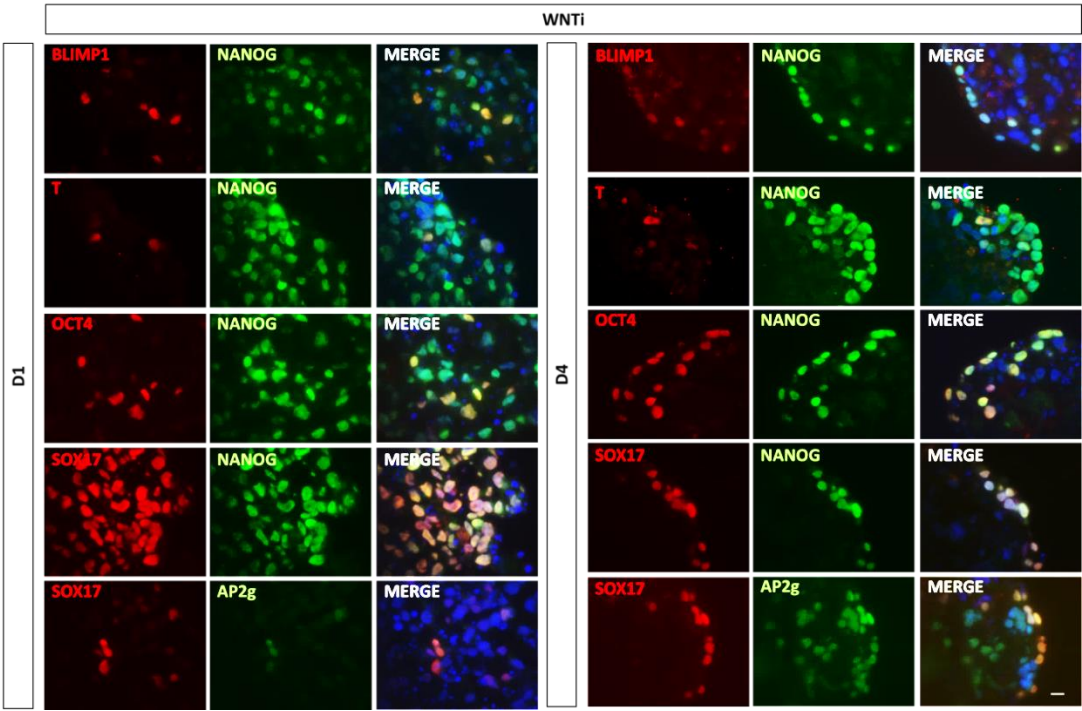


Figure 84. IF of WNTi treated EBs at D1 and D4 during PGC differentiation.

MAPKi treatment generates diminished EB size from D1. At D1, the gene expression pattern appears the same as control. However the number of D4 PGC drastically drops. A good number of TFAP2C+ only cells are recognizable by their morphology (Figure 85). These observations indicate that MAPK may not be required for PGC specification, but plays critical role in PGC maintenance, at least for nascent specified PGCs.

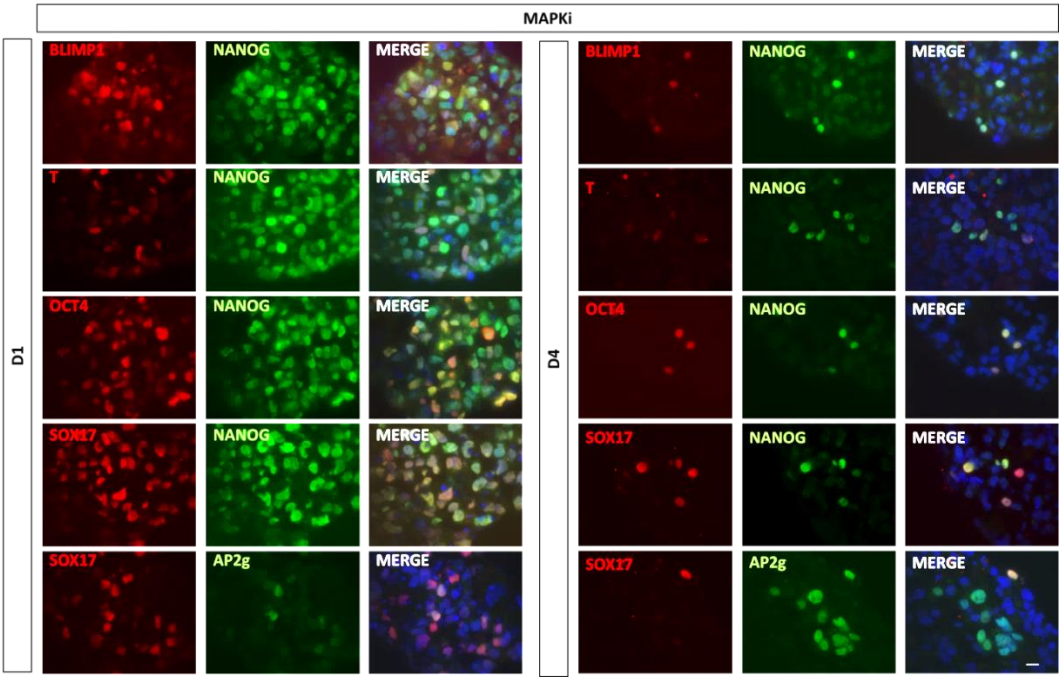


Figure 85. IF of MAPKi treated EBs at D1 and D4 during PGC differentiation.

EBs cultured in FGFRi are smaller with many apoptotic cells from D1. Although T is wiped out, there are still BLIMP1/NANOG/SOX17/OCT4/TFAP2C colocalized PGC forms from D1. Very few PGCs are maintained until D4. Some PGCs are detected with weak T (Figure 86).

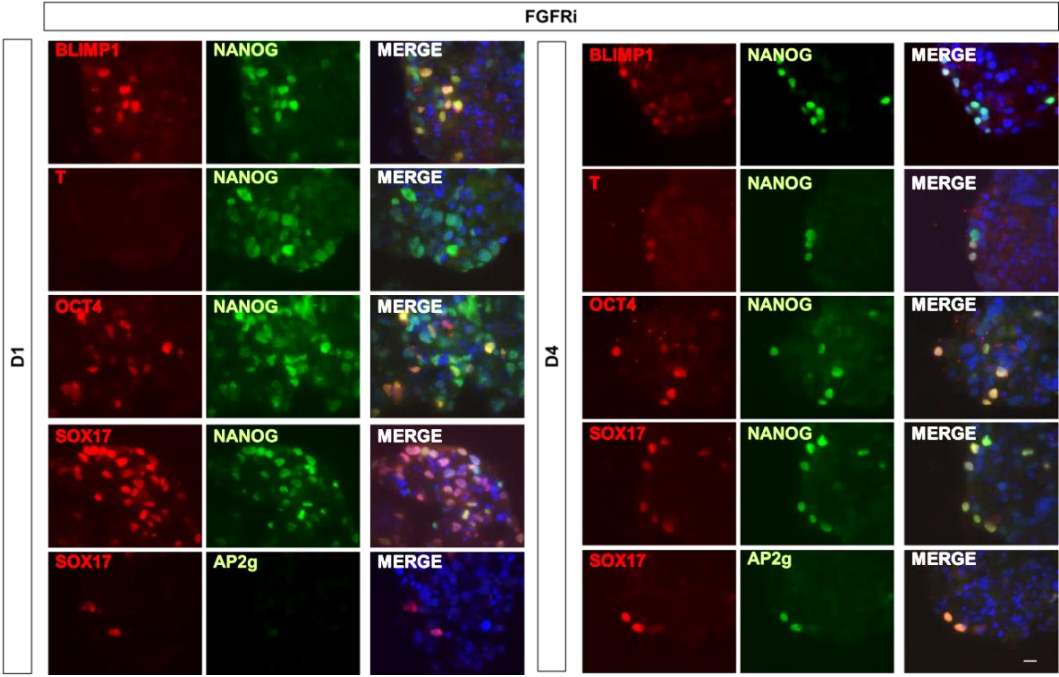


Figure 86. IF of FGFRi treated EBs at D1 and D4 during PGC differentiation.

Quantification of D1 and D4 differentiated PGCs in different inhibitors is shown in Figure 87. D1 EBs consists of similar proportion of NANOG⁺/OCT4⁺ cells in control (40.97%) and WNTi (36.72%), and less in FGFRi (20.38%) group. The reduced cells make contribution to higher NANOG⁻/OCT⁻ population, 23.20% in control, 21.74% in WNTi and 44.64% in FGFRi. Percentage of another population NANOG⁺/OCT4⁻ are control 31.29%, WNTi 40.32% and FGFRi 33.91%. At D4, only 0.35% of NANOG⁺/OCT4⁻ cells are maintained. The two major populations are NANOG⁺/OCT4⁺ and NANOG⁻/OCT4⁻ in three groups. NANOG⁺/OCT4⁺ proportions are still similar in control (19.67%) and WNTi (22.41%), and less in FGFRi (5.99%) treatment.

The trend of NANOG/SOX17 numbers is similar to NANOG/OCT4, except for different starting contribution. NANOG⁺/SOX17⁺ is the major population of D1 EBs in three inhibitors, 78.68% in control, 77.40% in WNTi and 58.07% in FGFRi treatment. Proportion of NANOG⁻/SOX17⁻ is less, 11.78% in control, 17.38% in WNTi and 32.52% in FGFRi.

As observed in previous experiment, BLIMP1 is initiated from D1. So D1 EB consists of three major populations: NANOG⁺/BLIMP1⁻, NANOG⁺/BLIMP1⁺ and NANOG⁻/BLIMP1⁻. WNTi and FGFRi significantly decreases proportion of NANOG⁺/BLIMP⁺, WNTi 5.36% and FGFRi 6.84%, compared with 26.23% in control group. Two inhibitors results in more NANOG⁻/BLIMP1⁻ cells, and FGFRi treatment generates less NANOG⁺/BLIMP⁻ cells. At D4, both WNTi and FGFRi treatments make less NANOG⁺/BLIMP⁺ cells, 8.82% in FGFRi and 16.90% in WNTi, compared with 23.90% in control. This discrepancy is possibly caused by the increased number of NANOG⁺/BLIMP1⁻ in two treatments, 7.28% in WNTi and 11.50 % in FGFRi, while control is 0.2%. NANOG⁻/BLIMP1⁻ cells are over 70% in all three groups.

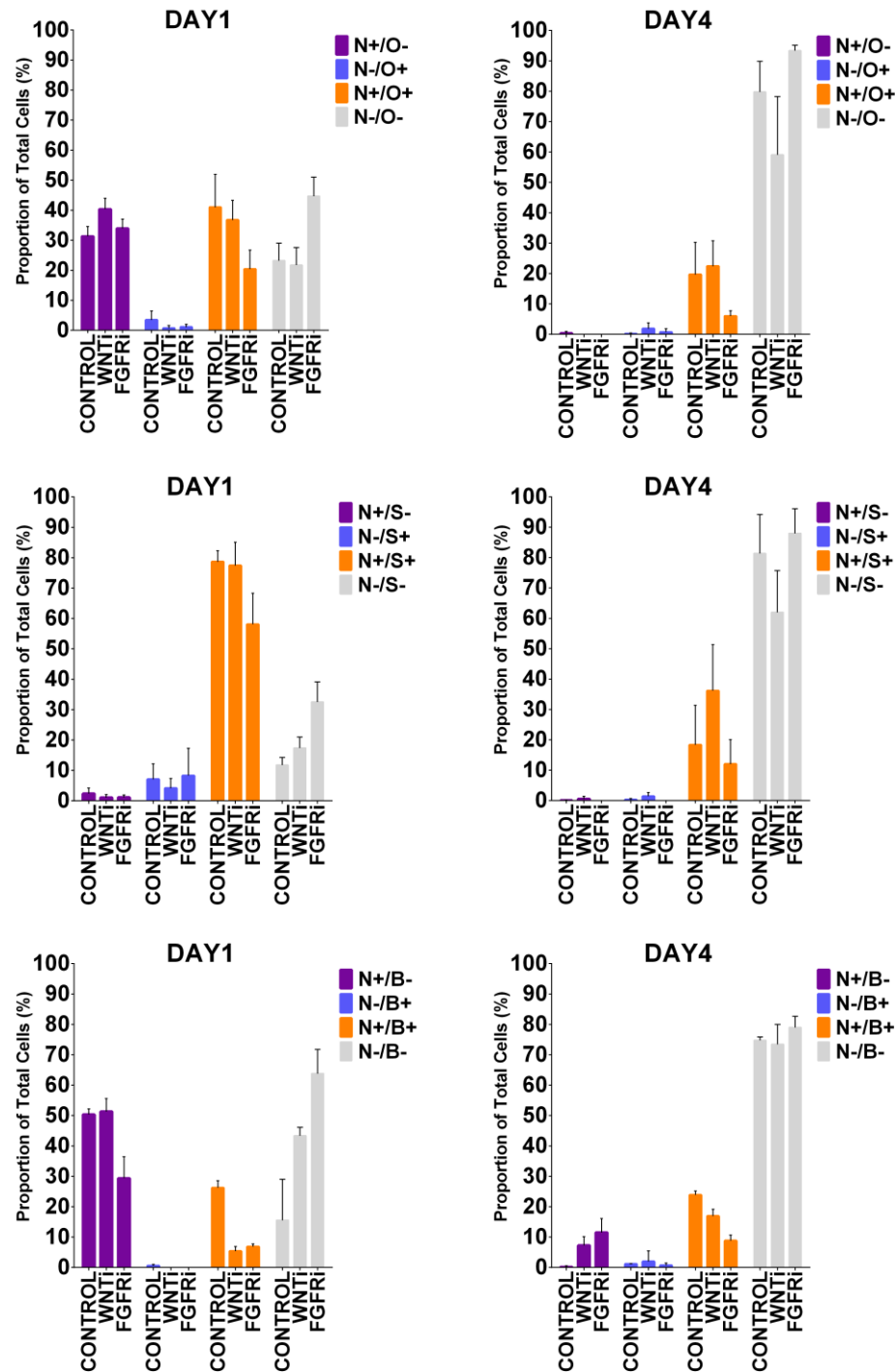
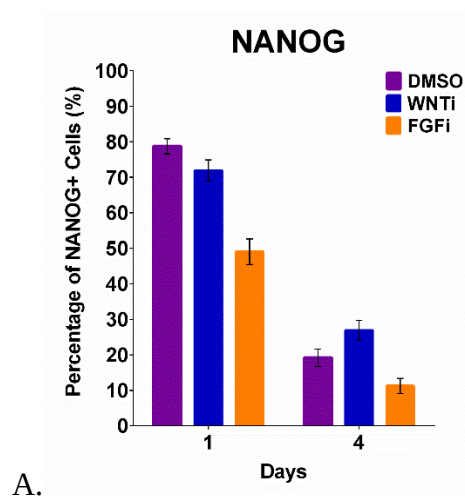


Figure 87. Quantification of positive cells of PGC related genes at D1 and D4 in inhibitors.

Proportion of indicated cell numbers in total EB cell number. Error bars are SD from three independent quantifications. N, NANOG. O, OCT4. S, SOX17, B, BLIMP1.

As NANOG is maintained in EB (Figure 70 and Figure 71), another way of quantification was applied to understand the PGC specification efficiency in maintained pluripotent cells (Figure 88). First of all, percentage of all NANOG+ cells,

including NANOG⁺ only and colocalized NANOG⁺ cells, were calculated (A). 78.75% control, 71.92% WNTi and 49.08% FGFRi of D1 are NANOG⁺ cells, and 19.17% control, 21.92% WNTi and 11.25% FGFRi are NANOG⁺ at D4. Among these NANOG⁺ cells, OCT4⁺ cell increases from 56.33% in control, 47.67% in WNTi and 37.33% in FGFRi at D1, to over 98% at D4 in all groups. Although the proportion of NANOG⁺/OCT4⁺ cell is slightly impaired in FGFRi at D1, but not affected at D4. Percentage of NANOG⁺/SOX17⁺ cell maintains high from D1 (over 86%) to D4 (over 83%). Proportion of NANOG⁺/BLIMP1⁺ cells increases drastically, although the starting number is different between treatments at D1. Overall, both WNTi and FGFRi impair the efficiency of BLIMP1⁺ cells differentiation from NANOG⁺ cells. Proportion of NANOG⁺/T⁺ is significantly higher in control group than the other two inhibitor treatments.



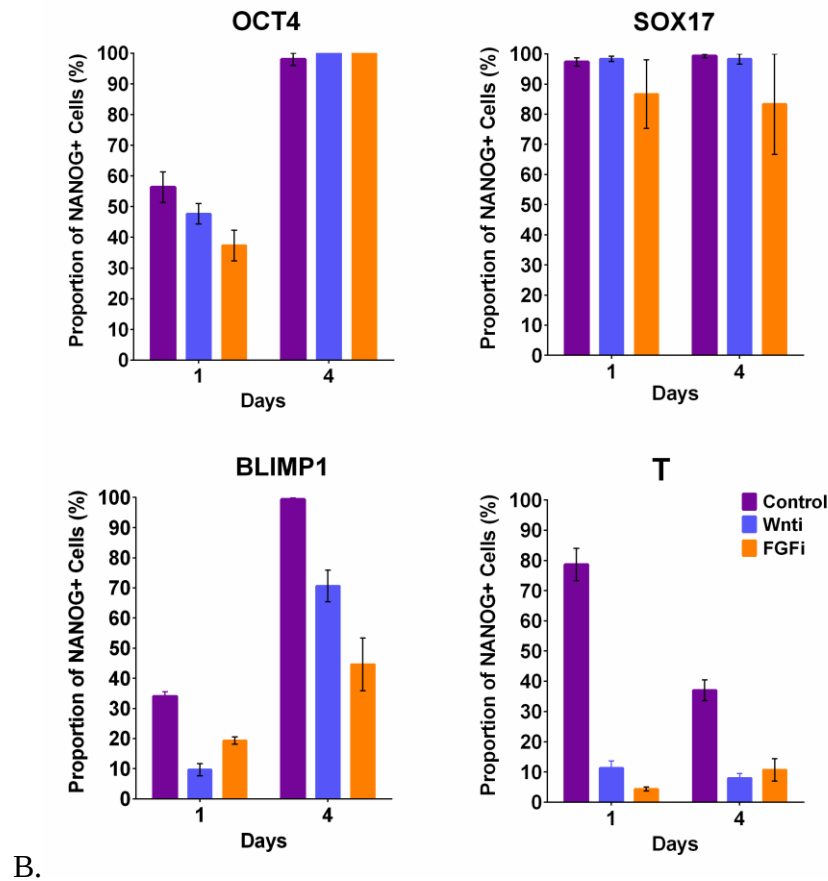


Figure 88. Quantification of colocalized cells at D1 and D4 of PGC differentiation with inhibitors.

A. Proportion of NANOG+ cells in total EB cells. B, Proportion of indicated positive cell numbers colocalized with NANOG+ cells. Error bars are SD from three independent quantifications. N, NANOG. O, OCT4. S, SOX17, B, BLIMP1.

As shown in chapter 5, transcription of WNT signalling related genes are downregulated in nascent PGCs (Figure 55). To further confirm the effect of WNT inhibition in PGC, PGC related genes i.e. NANOG, OCT4, SOX17, BLIMP1, and NANOS3, and WNT target genes LEF1 and TCF7L1 are detected in sorted hPGCLC (Figure 89). Treated in WNTi or control condition, D5 hPGCLC were double sorted by FACS with surface glycoprotein CD38 (Malavasi, Deaglio et al. 2008), and TNAP, according to Irie's method (Irie, Weinberger et al. 2015). CD38 and TNAP double positive cells are hPGCLCs. Although the proportion of WNTi treated hPGCLC is different to control group, the expression level of NANOG, OCT4, BLIMP1 and NANOS3 are similar. SOX17 is upregulated in WNTi group. Double negative cells have very low expression level of all the genes. This result suggests that WNTi is dispensable for specified hPGCLCs.

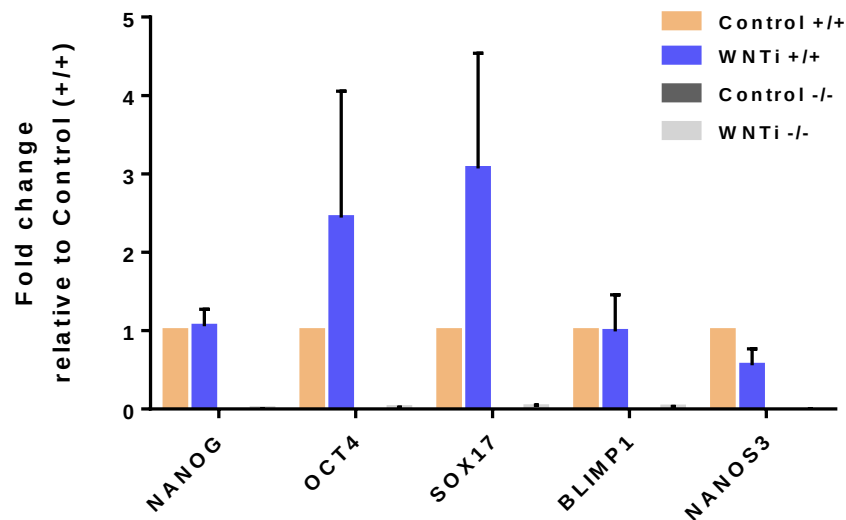


Figure 89. Expression levels of NANOG, OCT4, SOX17, BLIMP1 and NANOS3 in FACS sorted hPGCLC. +/+ : AP and CD38+ cells, -/- : AP and CD38 negative cells.

Considering downstream canonical WNT/ β -catenin targets, LEF1 is the cotranscription factor of active β -catenin. TCF7L1 is a transcriptional repressor in the presence of β -catenin (Wu, Hoffman et al. 2012). In double sorted PGCLC cells, LEF1 and TCF7L1 are significantly downregulated in both WNTi treated and control group, indicating that WNT signalling is inhibited in hPGCL cells (Figure 90).

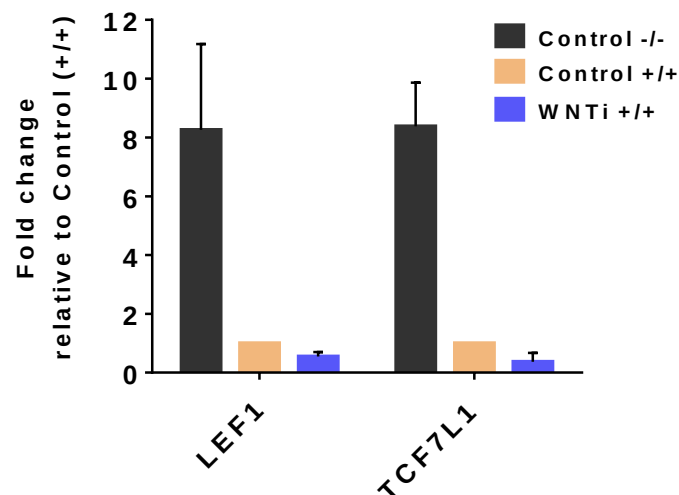


Figure 90. Expression levels of WNT targets LEF1 and TCF7L1 in FACS sorted hPGCLC. +/+ : AP and CD38+ cells, -/- : AP and CD38 negative cells

7 Discussion

In this chapter, the time window of *in vivo* porcine PGC differentiation, chronology of key genes, and role of key signalling pathways was investigated.

The higher efficiency of PGC differentiation in E (equals to PS) stage compared to VE (equals to pre-streak) and L (Late PS) stages (Figure 64) suggests that PGC specification occurs over a period of development including early PS epiblasts to PS stage embryos. Firstly, since VE embryos fail to differentiate efficiently, this suggests that the presence of mesoderm is required for the response to inducing signal. Second, early mesoendoderm (ME) appears to be the precursor of PGCs, rather than more differentiated mesoderm (M), indicated by the significantly lower PGC induction efficiency in late PS stage. Canonical WNT/ β -catenin signalling pathway plays a critical role in mesoderm lineage specification from pluripotent hESC (Davidson, Adams et al. 2012) or mouse epiblast (Hoffman, Wu et al. 2013) during gastrulation. When WNTi is supplemented into pre-streak epiblasts, the competence of mesoderm differentiation is repressed to some extent, therefore the PGC specification is hampered (Figure 65). Considering the off-target effects of WNTi treatment, different dosages of WNTi treatment might be worth trying to verify the effect. While in the other two stages (Early and late epiblasts) nascent mesoendoderm has already formed, inhibition of WNT signalling does not show as strong respond to induction. Late epiblasts are induced with less efficient, this suggests that long exposure to WNT signal doesn't benefit PGC induction. It is perhaps because that WNT promotes mesoendoderm conversion to mesoderm fate, unless that the cells initiated SOX17 expression. As described in the introduction, it is possible that SOX17 represses canonical WNT signalling, as well as indirectly activate BLIMP1 (Irie, Weinberger et al. 2015). In this occasion, canonical WNT signalling determines the PGC fate or mesoderm fate from a common mesendoderm precursor. Besides, as described in the introduction, it is possible that by inhibition of WNT signalling, SOX17 reduces the chances of tumorigenesis.

This model is different to the mouse PGC specification, which requires canonical WNT-T pathway to directly activate Blimp and Prdm14 (Aramaki, Hayashi et al. 2013). However, this WNT-T regulation can be bypassed by Nanog expression for mouse PGC specification (Murakami, Gunesdogan et al. 2016). In porcine PGC

specification, WNT needs to be inhibited. T expression dynamic seems to follow the kinetic of WNT in PGCs, as its levels drop as soon as BLIMP1 is expressed. This also explains why no correlation is observed between NANOG⁺ and T⁺ cells (Figure 70 and Figure 83).

Consistent with these observation, hPGCLC differentiation from H9 hESC shows that although mesoderm is successfully blocked by the WNTi, the differentiation of NANOG⁺/SOX17⁺/TFAP2C⁺ cells are not significantly affected, only with less efficiency (Figure 84). This is because the pre-culture of hESC in 4i medium, particularly the GSK3i inhibitor, activates WNT induced mesoderm differentiation (Patsch, Challet-Meylan et al. 2015). It is also possible that WNTi hampered survive of somatic cells, therefrom less PGC were induced. Taken together, WNT helps improving the mesoendoderm differentiation, therefore indirectly benefits PGC specification. Once the PGCs are induced, WNT is not required and it better be suppressed to prevent inappropriate signalling gradients for PGC. LEF-1/TCF transcription factors mediate the nuclear localization of canonical WNT signalling (Eastman and Grosschedl 1999). The significantly downregulated LEF-1 indicates inactivated WNT signalling. As Tcf7l1 prepares pluripotent epiblast for mesodermal differentiation during mouse gastrulation (Hoffman, Wu et al. 2013), its suppression may contributes to prevent mesoderm differentiation in PGCs.

The chronology of selected transcription factors in pig epiblast culture and *in vitro* hPGCLC system is similar to what is observed in early pig embryos. Meanwhile, it suggests that SOX17 is the first gene that initiated in the germ line, therefore could play lineage restrict role in pig and human PGC specification. Functional experiment is required in the future.

Here I propose the model of PGC determination by canonical WNT/ β -catenin signalling inhibition in non-rodent mammals (Figure 91). At the onset of gastrulation, pluripotent epiblast is induced by canonical WNT signalling, together with BMP and Activin, to become mesoendoderm (ME). When exposed to high concentration of WNT and BMP signalling, ME transits to mesoderm (M). While in the mesodermal location, SOX17 is activated, probably in response to BMP4. SOX17 binds and supresses WNT signalling, meanwhile indirectly upregulating PRDM1. PRDM1

inhibits the transcription of mesodermal or endodermal genes therefore prevent PGC precursors from further somatic differentiation. PRDM1 directly or indirectly activates TFAP2C, NANOS3, DND and KIT, which marks the specified PGCs. WNT signalling is downregulated in PGCs until postmitotic stages. In GCT, SOX17 is upregulated in undifferentiated seminoma, compared with differentiated embryonic carcinoma (EC). The canonical WNT signalling is significantly inhibited by SOX17 in seminomas.

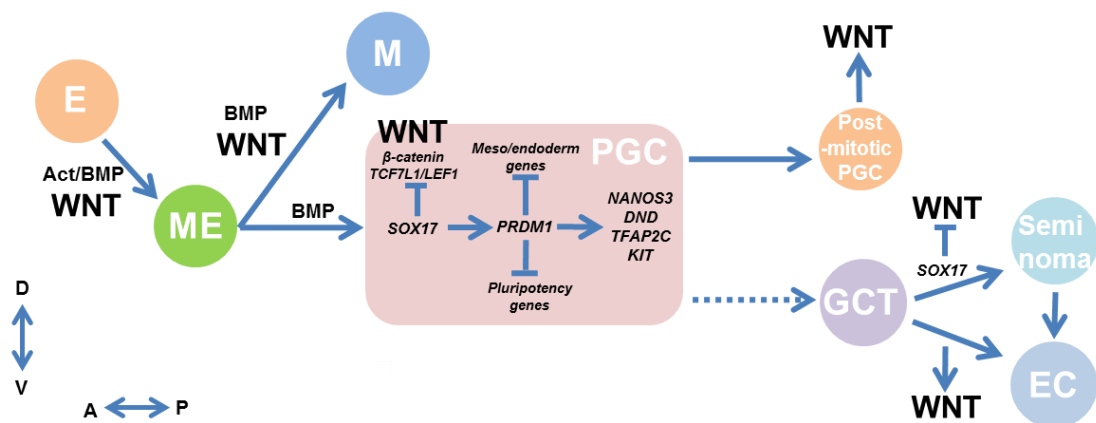


Figure 91. Schematic representation of canonical WNT signalling pathway in different cell lineage induction.

BMP4 is key mouse PGC inducer in mouse from extra embryonic ectoderm (Lawson, Dunn et al. 1999). It is also detected in mesoderm from posterior end of embryo at ovoid PSII-late stage (Valdez Magana, Rodriguez et al. 2014) and added to PGCLC differentiation medium (Irie, Weinberger et al. 2015). Activated BMP transducer, p-SMAD1, is also detected at posterior mesoderm around PGC domain at ovoid PSII-L stage. BMP2 is detected from spherical to ovoid stages (Valdez Magana, Rodriguez et al. 2014). It is possible that porcine PGCs are also induced by BMP-SMAD1/5/8 pathway. However, there is no reports directly detect the effect of BMP4 on PGC specification in non-rodent mammals in vivo. In this study, I first report the fundamental role of BMP in PGC inducing with porcine epiblast. No PGC were induced in BMPi treated epiblasts.

FGF4 is homogenously faint in epiblast and prominent in TE of ovoid PSII-L embryos (Valdez Magana, Rodriguez et al. 2014). FGF4 bound to FGFR2 is consistently faint in ovoid epiblast and hypoblast, and intensive in TE. Activated by

FGF4, nuclear p-MAPK was observed in TE cells surrounding epiblast at PSII-L stage (Valdez Magana, Rodriguez et al. 2014). This increased FGF signalling is essential for PGC specification, as FGFRi eliminates PGC competence from porcine PGC progenitors (Figure 67). hPGCLC specification system mimics this hampered PGC specification competence from D1 (Figure 78 and Figure 86).

MAPKi doesn't significantly prevent PGC specification at D1 but impact PGC maintenance as well as EB size at D4, although not as much as FGFRi and WNTi (Figure 79 and Figure 85). No obvious effects of PI3Ki or EGFRi were observed through the PGC specification from D1 to D4 (Figure 80), in terms of the expression patterns of NANOG/SOX17/TFAP2C.

Although *in vivo* PGC specification occurs in varying signalling gradients, while hPGCLC is cultured in constant environment with simpler components, the *in vitro* differentiation largely recapitulates *in vivo* PGC differentiation. PGC progenitors are induced to PGC in a mesodermal location; where T is highly activated in response to mesoderm signalling. This status is recapitulated by H9 cultured in 4i medium prior to differentiation. Specified PGCs are located at the posterior end of primitive streak in a mesodermal location. The key signalling gradient in PGC niche, such as BMP4, is provided in the PGCLC differentiation medium. The expression profile of T, NANOG, SOX17, BLIMP, OCT4 and TFAP2C during PGC differentiation shows that NANOG and OCT4 are maintained in PGC progenitors. SOX17 is activated in a subgroup of low NANOG⁺ cells, followed by BLIMP1. Activation of T is not a prerequisite to PGC differentiation, but more likely to be a response to the signalling gradients. The proof is that within WNTi treatment, the percentage of PGC is significantly increased considering that T expression is blocked (Figure 77 and Figure 84). The chronology of NANOG, OCT4, SOX17, BLIMP1 and TFAP2C is consistent with that in porcine PGC specification (chapter 3). Except for T, which is homogeneously low in the PGC cluster (Figure 83) but heterogeneous in hPGCLC (Figure 70). This is probably because of some unknown regulation during induction or specification.

Chapter 7

7. General discussion and conclusions

In this study I set out to investigate the mechanism of pig PGC specification and compare the findings with the observations reported in humans and mice.

I hypothesized that PGC specification in pigs and human would be more similar than it would be to the mechanisms operating in mice. To test the hypothesis I investigated three aspects of germ cells development: 1- gene regulatory network, 2- epigenetic reprogramming and 3- signalling pathways. Together, the results of this study support the hypothesis presented in this thesis.

In the next section I will integrate the results of this study and present the evidence that demonstrates that PGC specification mechanisms are conserved between humans and pigs.

7.1 The gene regulatory network of pig PGCs

I used a combination of IF and RNA seq. to determine the gene expression profile of early pig PGCs. One of the first aims was to establish whether *SOX17* played a role in pig PGC. Since the divergence in the role *SOX17* between humans and mice was clearly demonstrated (Irie, Weinberger et al. 2015), it was important to determine whether this marker also participates in establishing the pig germline. Analysis of embryos at multiple stages showed that *SOX17* is expressed in PGC precursors before *BLIMP1*, *TFAP2C* and the surface marker *Sda/GM2* is activated. *SOX17* is first detected in nascent mesoderm cells of pre-streak embryos, not in earlier bilaminar stage embryos. This is an important point, since in mice, 3-6 cells from the posterior epiblast activate *Blimp1/Prdm1* expression in response to *BMP4* from the ExE. These cells represent lineage restricted PGC precursors and are the founders of the germline (Ohinata, Payer et al. 2005). My observations, however, suggest that pig PGCs are induced as a large group of about ~60 cells from early mesendoderm precursors. The location of these precursors coincides with the area of *BMP4* expression in these embryos (Valdez Magana, Rodriguez et al. 2014), suggesting that these cells are responding to this inducer. Results from my in vitro experiments using epiblasts confirm that *BMP4* is a key inducer of pig PGCs. IF against p-SMAD1/T on sagittal

section of pre-streak stage embryo shows that nuclear p-SMAD1 is negative in T positive cells (data not shown). And nuclear p-SMAD1 is only detected faintly in both ends of ectoderm and strong in anterior endoderm. This suggests that BMP inducing progress doesn't beyond streak stage. Thus, a first conclusion from my work is that SOX17 is the first marker that identifies these PGC progenitors from a pool of early mesendodermal cells.

After the activation of SOX17 upregulation of NANOG is also observed from these PGC precursors. At the same time, T levels are reduced compared to neighbouring somatic cells, indicating a segregation of the germ cells from the rest of the mesoderm by this early stage. Another striking observation was the absence of *PRDM14* and *SOX2* expression in pig PGCs, which highlights a key difference with the mouse PGC program, where these two genes are expressed (Yabuta, Kurimoto et al. 2006; Yamaji, Seki et al. 2008). One weakness of IF based characterization is the impossibility of lineage tracing. Interesting questions are that which gene lineage restricts pig and human PGCs, and when does that happen.

Established early PGCs (~D13) analysed by RNA seq. express *bona fide* PGC markers *NANOS3* and *KIT*, as well as other important genes such as *OCT-4* (*POU5F1*), *GDF3* and *KLF4*. Based on these observations I propose a model of pig PGC specification whereby cells delaminating from the epiblast (which express *T*), initiate *BMP4* expression. These cells activate *SOX17*, and subsequently activate *BLIMP1* and other PGC markers.

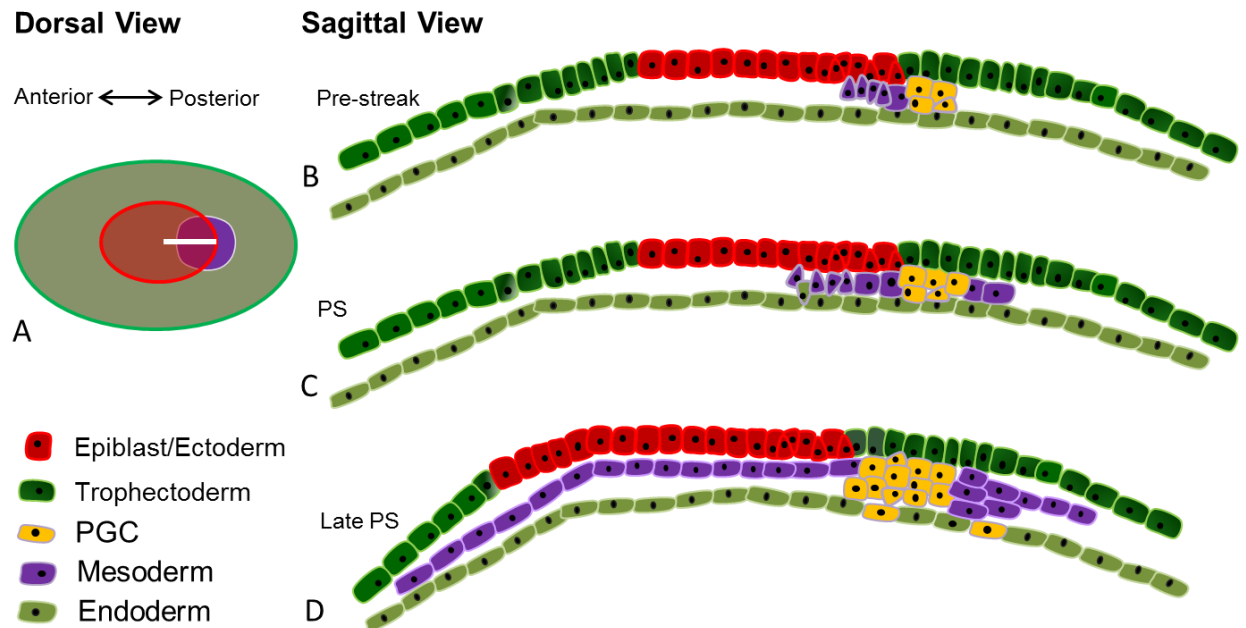


Figure 92. Model depicting the origin of PGC in the pig embryo.

A) Dorsal view of an early gastrulation embryo with a primitive streak (white line) emerging from the posterior end. **B)** Sagittal section through the midline of a pre-streak embryo shows early delaminating mesoderm from the posterior end of a pig embryo. Cells in yellow represent PGC precursors expressing SOX17/BLIMP1. **C)** Primitive streak (PS) embryo showing an expanded mesoderm and more PGCs, **D)** Late PS embryo shows an expanded PGC cluster and some cells starting their migratory path. Double arrows indicate anterior (A) and posterior (P) end of embryo. Different types of cells are indicated in legend.

Singe cell genome wide analysis of PGCs was carried out to gain further understanding of the key features of these cells early in their development. An interesting observation, that would not have become apparent if we had carried out bulk cell genome wide profiling, is the expression of SOX17 in each of the 6 PGCs. An unexpected profile of SOX17 was found, where 2/6 cells did not express this gene at all. These two cells however expressed BLIMP1 at the highest level. In contrast, another two cells, which expressed the highest levels of SOX17, had no BLIMP1. These dynamic differences in SOX17 expression suggest that the levels of this gene may be carefully regulated, perhaps to prevent these cells in becoming endoderm. It will be interesting to determine whether BLIMP1 plays a role in regulating SOX17 to ensure that a careful balance of expression is maintained during PGC specification. One of the important outcomes of the genome wide analysis was the identification of 568 genes that are uniquely expressed in pig PGCs. The role of these genes will need to be studied in future projects. GO analysis also highlighted features of PGCs that are

conserved with that of other species. Some common terms that represent downregulated genes include DNA methylation, positive regulation of ERK1/2 cascade, post embryonic development, and WNT signalling. These GO terms are all involved in the regulation of early embryo development, thus it seems that during the segregation of the germ cells from the soma it is critical that these pathways are downregulated to prevent diversion into a somatic fate. On the other side, GO terms that are enriched in PGCs include oogenesis, KIT and Jak/Stat signalling, DNA repair, and mesoderm commitment. These are all consistent with the commitment of early mesodermal cells to the germ cells fate, supporting the proposed hypothesis for this work.

7.2 Epigenetic profile of pig PGCs

One distinguishing feature of the germline development program is the extensive epigenetic reprogramming that these cells undergo prior to their differentiation into gonocytes. The comparison of epigenetic dynamics between pig, human and mouse is shown in DNA demethylation in mouse PGCs is sought to occur in phases (Seki, Yamaji et al. 2007; Yamaguchi, Hong et al. 2013). In pre-gonadal PGCs DNA is demethylated passively by the lack of Dnmt1 activity, determined by the low levels of UHRF1. Then when PGCs reach the gonad at E9.5-10.5, active demethylation is catalysed by Tet1/2 enzymes converting 5mC into 5hmC. In humans, there are only a few reports indicating that gonadal PGCs do not stain for 5mC/5hmC, however some Tet1 expression is detected in week 7 PGCs. However, migratory PGCs from 4 week old embryos have high 5hmC and low 5mC (Tang, Dietmann et al. 2015). My results extend these observations showing that in pig pre-migratory PGCs the levels of 5hmC are already higher than the surrounding somatic cells. Interestingly the levels of UHRF1 and Dnmt3B are also reduced in these cells, and the expression of Tet1/2 is high compared to somatic cells. These observations indicate that in pigs a combination of active and passive DNA demethylation mechanisms are operating in early PGCs. The unexpected high levels of 5hmC detected in pre-migratory PGCs raises the question whether these cells have inherited this epigenetic profile from their ME precursors or whether they upregulate Tets after specification. Because the levels of 5hmC are high, as those detected in the bilaminar epiblast, it is tempting to speculate that PGCs may be retaining the epigenotype of epiblast cells, and that the

surrounding mesoderm/endoderm initiates de novo methylation, and therefore shows reduced 5hmC levels. Future experiments will need to be performed to answer this question.

I next investigated the profile of H3K9me2, which in mice is downregulated in early migratory PGCs (Seki, Hayashi et al. 2005). In human this mark is low in gonadal cells (Tang, Dietmann et al. 2015). A previous study from our lab showed that D15 pig PGCs have no H3K9me2 (Hyldig, Croxall et al. 2011). Here, I extend these observations to the early PGCs cluster and show that a significantly lower staining is detected in pre-migratory cells. This is also consistent with the lack of EHMT2, the histone H3K9 specific methylase, in D13 PGCs. H3K27me3 is another very well studied chromatin mark that in mice is increased in gonadal PGCs. In humans (Guo, Yan et al. 2015) it follows an opposite trend, with reduced expression in gonadal stages. My findings in the pig shows a similar kinetic to the reported in the human gonad.

Together these results show that pig PGC reprogramming is fundamentally different to the mouse, and that extensive changes take place from the moment that the cells are specified.

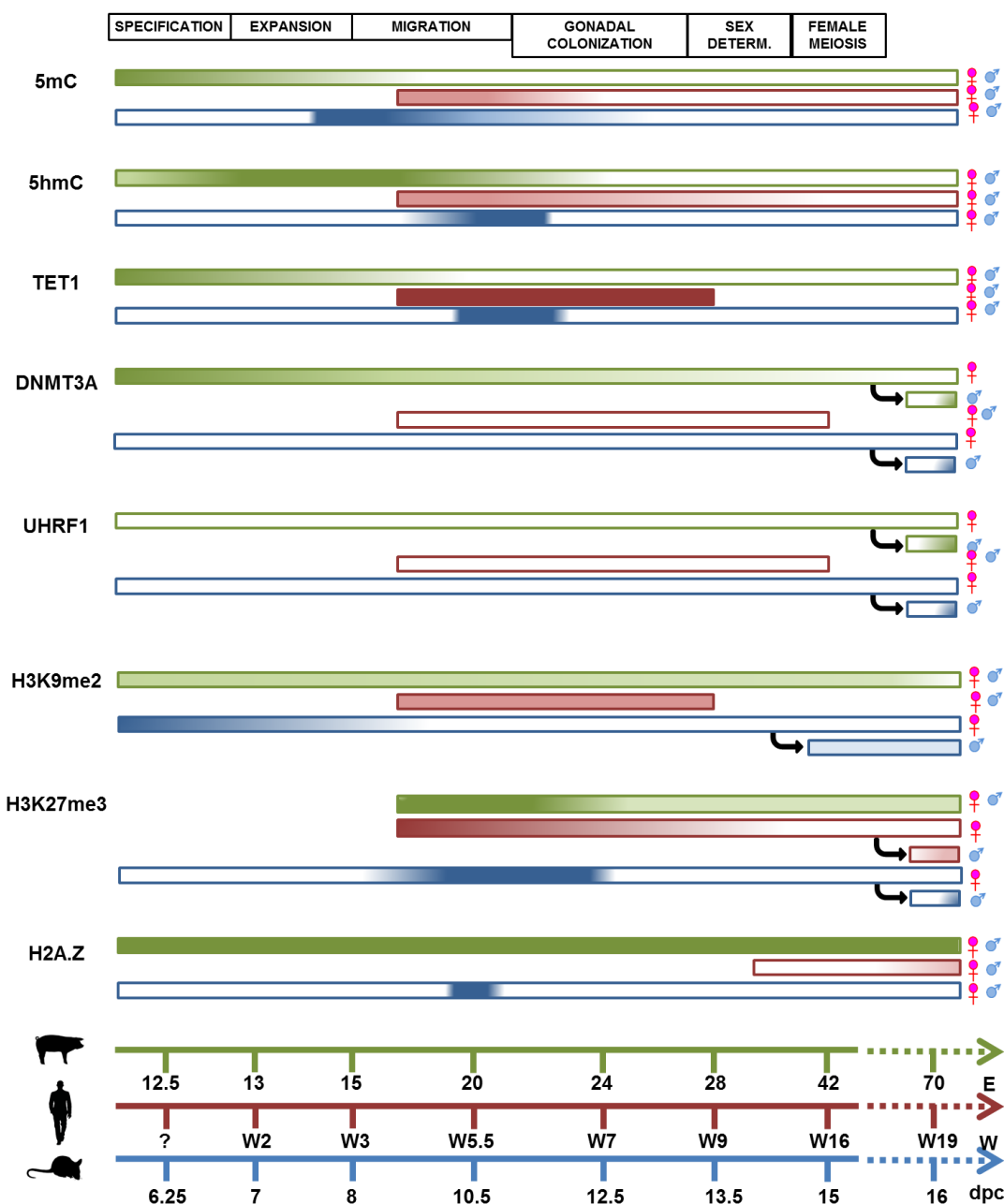


Figure 93. Comparison of selected epigenetic reprogramming events in pig, human, and mouse germ lines.

pPGC, hPGC, and mPGC epigenetic dynamics are compared based on the expression profile of 5-mC, 5-hmC, TET1, DNMT3A, UHRF1, H3K9me2, H3K27me3, and H2A.Z. Germ line development is divided into PGC specification, expansion, migration, gonadal colonization, sex determination, and female meiosis stages on top of the graph. Corresponding time window is labelled at the bottom of graph. pPGC is shown in green (unit in days (E)). hPGC is shown in red (unit is week (W)). mPGC is shown in blue (unit in days (dpc)). Female and male PGCs are distinguished in different symbols. Time course comparison refers to (Hyldig, Ostrup et al. 2011) and (Tang, Dietmann et al. 2015). The epigenetic dynamics refers to (Hackett, Sengupta et al. 2013), (Hyldig, Croxall et al. 2011), (Seki, Hayashi et al. 2005), (Seki, Yamaji et al. 2007), (Tang, Dietmann et al. 2015), (Kurimoto, Yabuta et al. 2008), (Gkoutela, Li et al. 2013), (Gkoutela,

Zhang et al. 2015), (Guo, Yan et al. 2015), (Vincent, Huang et al. 2013), (Hajkova, Ancelin et al. 2008)

7.3 Signalling pathways involved in pig PGC specification

In vitro PGC induction from isolated epiblasts showed that BMP4 is a critical cytokine required for PGC induction in the pig. The single cell transcriptome analysis identified downregulation of WNT signalling in early PGCs. This finding was unexpected, since in the mouse WNT signalling is required for PGC induction (Ohinata, Ohta et al. 2009; Murakami, Gunesdogan et al. 2016). Reduction in WNT/ β -catenin was shown in gonadal stages (Kimura, Nakamura et al. 2006), but not in early mouse PGCs (Yabuta, Kurimoto et al. 2006). This contrasting difference in WNT requirement for PGC development was extended to hPGCs, using an in vitro differentiation system, and by analysing the transcriptomic dataset from Irie et al., (2015). In this dataset I found that several members of the WNT pathway that were downregulated in pig PGCs were also low in human germ cells. These observations are consistent with my findings on the time window for PGC specification from epiblasts. Early bilaminar epiblasts failed to differentiate efficiently into PGCs in the presence of WNTi, whereas more advanced epiblasts were moderately affected by this intervention. This suggests that an initial requirement of WNT is important to generate the mesendoderm (ME) cells from where PGCs seem to originate (see model below). After the ME has formed, PGCs presents inhibited WNT pathway, and WNTi benefits PGC specification. It might be because that inhibition of WNT prevents mesoderm differentiation. However, the key role of WNT signalling, and whether WNT needs to be inhibited during PGC induction requires functional experiment to show direct evidence. Interestingly, the inhibition of WNT signalling seems associated with the potential role of SOX17 during PGC specification. SOX17 inhibits WNT/ β -catenin during endoderm differentiation (Zorn, Barish et al. 1999). Further evidence shows that SOX17 can directly inhibit LEF1 (Liu, Luo et al. 2010) and TCF7L1 (Sinner, Kordich et al. 2007) transcription. SOX17 is also recognised as an antagonist of WNT signalling in several cancers types (Fu, Wang et al. 2010; Jia, Yang et al. 2010) , and a recent report shows that during the conversion of seminoma into embryonic carcinomas SOX17 is downregulated at the expense of an upregulation of WNT/ β -catenin signalling (Nettersheim, Jostes et al. 2015). It will be

interesting to find out whether there is a direct relationship between SOX17 and WNT signalling inhibition.

Based on the results of my work I propose a model of PGC specification for the pig that may also be relevant to humans (Figure 94).

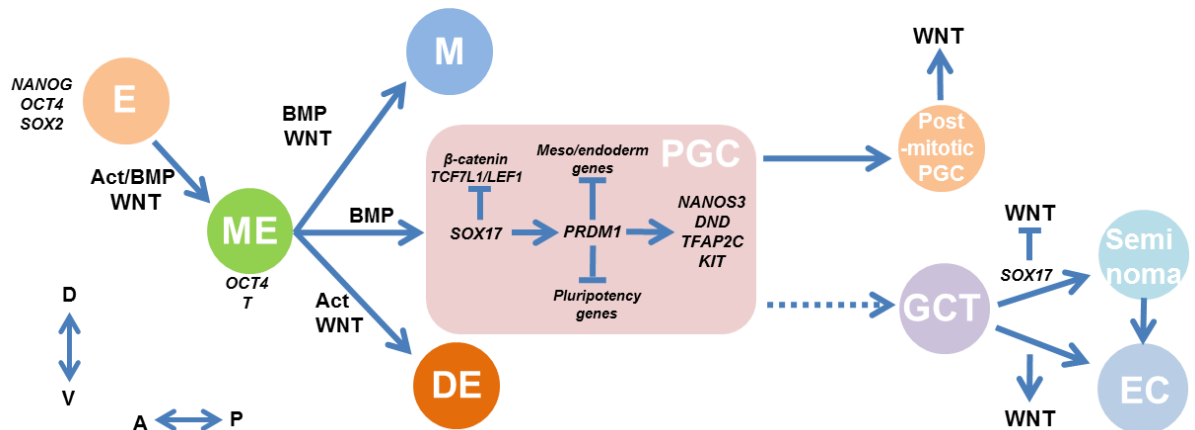


Figure 94. Proposed model of PGC specification (see below for full description of the model). E: epiblast, ME: mesendoderm, M: mesoderm, DE: definitive endoderm, GCT: germ cell tumour, EC: embryonal carcinoma.

Epiblast (E) expresses pluripotency genes, NANOG, OCT4, and SOX2. Induced by Activin (Act), BMP, and WNT signalling, a subpopulation of pluripotent epiblast differentiates to Mesoendoderm (ME), where T is activated. Mesoendoderm can differentiate into PGC, mesoderm (M), or definitive endoderm (DE), depending on the inducing signals. Given BMP and canonical WNT, ME differentiates into mesoderm. DE is derived in response to Activin and WNT. BMP induces ME into potential PGCs, where SOX17 is activated. SOX17 suppresses WNT signalling, and activates BLIMP1 (PRDM1), which prevents somatic differentiation and pluripotent gene expression. BLIMP1 also activates downstream germ cell related genes NANOS3, DND, TFAP2C, and KIT. Canonical WNT is suppressed throughout the mitotic stages. In germ cell tumours (GCT), undifferentiated seminoma maintain SOX17 expression, which downregulates WNT signalling. On the contrary, differentiated embryonic carcinomas (EC) maintain active WNT and have low levels of SOX17. Dorsal (D), ventral (V), anterior (A), and posterior (P) orientations are indicated at the bottom left. E, epiblast. ME, Mesoendoderm. M, mesoderm. DE, definitive endoderm. GCT, germ cell tumour. EC, embryonal carcinoma.

In pre-streak embryos nascent mesoderm cells expressing T start to delaminate in response to low levels of ActivinA, BMPs and WNT from the posterior epiblast to form mesendoderm (ME) cells. These mesendoderm cells expand and are still expressing high levels of *OCT-4* but reduced *NANOG* and no *SOX2*. ME cells will respond to gradients of BMP/WNT, which drive mesoderm differentiation, and ActA, which promote definitive endoderm. A subgroup of cells located in the midline of the posterior epiblast/PS exposed to BMP will activate *SOX17*. *SOX17* cells then inhibit

WNT signalling to prevent mesoderm differentiation of these PGC progenitors, and will subsequently activate BLIMP1, which also contributes to preventing somatic differentiation. These lineage restricted cells activate PGC markers, such as *TFAP2C*, *NANOS3* and *DND*. I also propose that SOX17 plays a key role during the transition of seminomas into embryonal carcinomas, whereby inactivation of SOX17 leads to the reactivation of WNT signalling, which contributes to the malignant conversion of these cells.

7.4 Conclusions

These experiments carried out in this thesis have led to several important conclusions about the mechanism of *in vivo* pig and *in vitro* human PGC specification.

- 1- A conserved gene regulatory network between pigs and humans was confirmed as shown by the expression of SOX17 in early PGCs, which is different to that reported for mice.
- 2- Epigenetic reprogramming initiates in pre-migratory PGCs as demonstrated by the reduced levels of 5mC, H3K9me2, UHRF1 and Dnmt3B, and the high levels of 5hmC, and Tet1. In migratory and gonadal stages the epigenetic profile of pig and humans are very similar, but different to mice.
- 3- Canonical WNT signalling is required for competence of PGC precursors, however it is sharply reduced once PGCs are specified. The potential mechanism is that WNT is initially required to induce ME (T expressing cells) from which PGCs will then differentiate in response to BMP. To counteract the somatic differentiation potential of BMP, WNT inhibition is needed in PGCs. WNT inhibition could be regulated by SOX17, the earliest marker of PGC progenitors. This mechanism is not present in the mouse germline, however hPGCL cells seem to follow similar mechanisms to the one described in pigs.

This study has highlighted critical differences between mice and humans/pigs in the process of germ cell specification. I provide evidence that the pig embryo is a useful model for the study of human development, and future studies will need to be directed to re-evaluate concepts of cell differentiation and early lineage commitment established in mice that may not apply to humans.

7.5 Future work

In chapter 3 and chapter 6, I have reported the chronology and expression profiles of transcription factors during porcine PGC specification and development. SOX17 is the earliest and critical gene. How does SOX17 activated? In the same PGC niche, when SOX17 is only activated in potential PGC precursors, rather than the mesodermal cells? With the help of transcription factors, we identified PGCs at different stages. The size of PGC cluster expands in pre-migratory stages. How does the PGC number enlarge in PGC niche? Either by recruiting like ‘Blimping’ in mouse PGC, or induced from mesoendodermal precursors?

In chapter 5, we discovered novel PGC related genes by single-cell RNA sequencing analysis. More exploratory analysis will be needed to explain the discrepancy of PGCs and somatic cells.

In chapter 4, I reported the high level of 5-hmC in nascent PGCs. Are the 5-hmC level inherited from epiblast or mesoendoderm? Or the PGC progenitors demethylate, increasing 5-hmC level upon specification? Mouse Prdm14 majorly regulates epigenetic reprogramming related genes in PGCs shortly after Blimp1 is initiated. Since Prdm14 is not active in human and pig germ line, what gene or mechanism triggers the onset of epigenetic reprogramming in human/pig PGCs? Could it be SOX17, as high levels of 5-hmC are already detected in nascent PGCs?

In chapter 6, I proved the critical role of WNT inhibition in PGC specification. WNT is downregulated by SOX17 in nascent PGCs. What is the resource of WNT signalling? Is there a negative feedback between SOX17 and canonical WNT signalling?

Mouse EG (embryonic germ) cells have been successfully derived from E8.5 migratory PGCs (Leitch, Tang et al. 2013). Human EG cells were induced from wk5-9 migratory and gonadal PGCs (Shamblott, Axelman et al. 1998). While in pig, no successful example has been reported. This suggests the different nature of mouse and human/pig PGCs. Besides different transcription factor expression profile, divergent

epigenetic reprogramming events could be one aspect that requires further understanding to optimize EG derivation in this species. For example, H2A.Z is constantly expressed in pig/human PGCs (chapter 4), but transiently in mouse E11.5 gonadal PGCs (Hajkova, Ancelin et al. 2008). H2A.Z facilitates the access of transcription factors to the chromatin (Sevilla and Binda 2014). In this sense, porcine PGCs are consistently under regulation from transcription factors. In addition, RNA-seq. and my functional experiments suggests that WNT, BMP4, ERK1/2, TGF β (NODAL) signalling should be down regulated and JAK-STAT and KIT signalling are upregulated in PGCs (chapter 5). In this sense, simply 2i+LIF condition from mouse EG derivation only blocked ERK and GSK3 β , meanwhile supplemented JAK-STAT pathway. In comparison, 4i medium adopted in this thesis added P38i and JNKi to block downstream of MAPK and PI3K in addition to GSK3 β i and ERK1/2i, as well as LIF, TGF β and bFGF. Inspired by the GO enrichment, optimize 4i medium might help deriving EG cells from porcine migratory PGCs. Such as add WNT inhibitor (for example, DKK-1) and remove TGF β to 4i medium.

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APPENDIX

- Appendix I. List and expression levels of uniquely and commonly expressed genes among PGC, group1 and group2 cells.
- Appendix II. List of DE genes in PGC, group1 and group2 cells.
- Appendix III. List of DE GO terms in PGCs compared with group1 or group2 cells.
- Appendix IV. List of DE genes among pig PGCs, human gonadal PGCs, hPGCLC and TCam2.