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GERM CELL SPECIFICATION IN MAMMALS

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Duration : Oct2010-Dec2010

Department: Cancer Genomics and Developmental Biology

Supervisors of Project

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PGCs, primordial germ cells, are germ cell precursors which arise early in development in mammals and lower organisms. In lower organisms, the ‘germ plasm’, the accumulation of granular cytoplasmic components containing ribonucleoproteins and RNAs, is inherited maternally, hence the PGCs form by ‘preformation’. Mammals, on the other hand, do not inherit material but induce germ cells in epiblast (~E6.0) by BMP4 signaling and various epigenetic modifications, by ‘epigenesis’. The germ cells go through more changes and migrate to gonadal ridges, where they serve as gametogonia. In order to underdtand the mechanism of PGC specification and their scientific/clinical value, *in vitro* techniques have been developed as well as *in vivo.* In this paper, we will explore how gametes are formed, how germ cells are specified and how they migrate. Finally we will go through the advancements in embryonic stem cell and embryonic germ cell cultures.

Keywords: primordial germ cells, germ plasm, BMP4, epiblast, preformation, epigenesis, embryonic stem cells, embryonic germ cells

1. INTRODUCTION

"Germ plasm" is a term used to describe the region in egg cytoplasms containing molecular determinants for germ cell differentiation and/or specification. Described by August Weismann in 1885, it is theorized and evidenced to inherit information to the daughter cells early in embryonic development for the formation of initial germ cell lineage, also known as "primordial germ cells" (PGCs), in lower model organisms such as *Drosophila melanogaster, Xenopus laevis, Gallus gallus, Danio rerio* and *Caenorhabditis elegans* [Saitou et. al., 2002]. There are several determinants found to be effective in germ cell specification in these organisms, including mRNAs and proteins that specifically localize or form gradients to initiate the signals specifying PGCs [Johnson et. al., 2003].

In these lower organisms, identification of the germ plasm resulted from germ layer transplantation experiments. After transplantation, PGCs formed in the region to which the cells were transplanted and further research led to the thought of preformation; the inheritence of maternal determinants from the eggs to the early embryos. However, in most mammals, these transplantation experiments resulted in PGCs forming only in a specific region in the embryo, and the transplanted to-be-germ-cells "blended in" the region of transplantation, hence did not differentiate into PGCs. In contrast with non-mammalian model organisms that already have PGC determinants localized in cells, this led to the thought of external signals being implemented around the native PGC locality, cueing epigenetic changes to specify germ cells. Despite this initial understanding and the existing knowledge about how germ cells develop after specification, there has not yet been groundbreaking discoveries to elaborate the germ cell specification mechanisms in mammals as clearly as in lower organisms [Extravour and Akam, 2003].

Epigenesis (mammals) and preformation (lower organisms) in germ cell specification seems to be distinct and some researchers state that the epigenetic transformation of the mammalian PGCs is merely a novel evolutionary trait. Yet, some others rely on the recent evidence that may lead to an evolutionary relation between the two ways of germ cell specification. In this thesis, firstly we will attempt to address the key aspects of germ cell development, then we will compare and comment on the germ plasm view in lower organisms, and finally we will lay out the overall findings about mammalian germ cell specification and elaborate the *in vitro* and *in vivo* methods used to perform the related research.

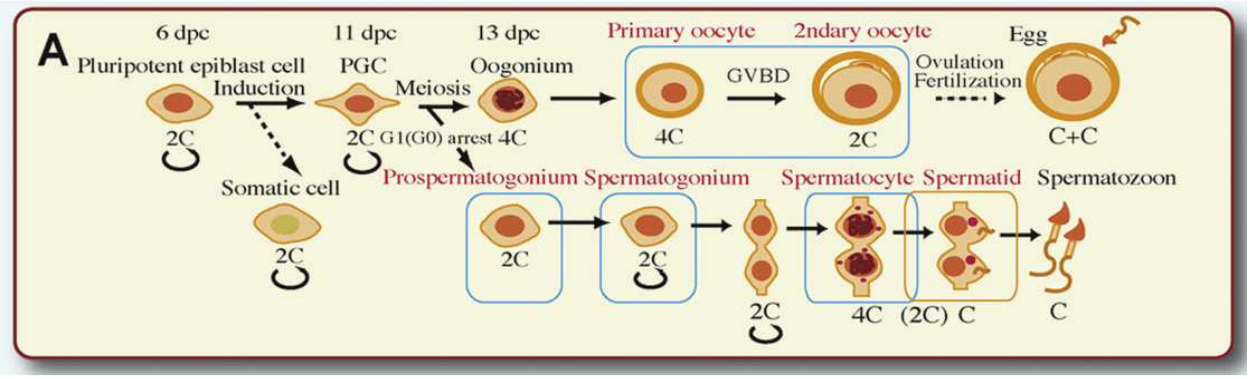

Figure 1. ***Germ cell development (gametogenesis) in mice.*** After their specification between E6.0-E8.0, primordial germ cells migrate towards the genital ridges where they start to be called gametogonia. After proliferation steps, both male and female gamets undergo meiosis and get ready for fertilization, although this process spans E12.5-postnatal stages in mice. Image from Chuma et. al., 2009.

2. GAMETOGENESIS IN MAMMALS AND LOWER ORGANISMS

In order for species to continue their presence, they have to reproduce. It is important for them to have healthy reproductive organs and this lies in a successful embryonic development, most importantly a successful germ cell development and regeneration. Gametogenesis is the process in which germ cells, gametogonia, proliferate and meiotically divide to form spermatids or ootids, which will eventually morph into spermatozoa and eggs (Figure 1).

2.1. Spermatogenesis in mammals

The aim of spermatogenesis is to produce haploid male gametes in sexually mature males, which will fertilize with and recombine with female gametes at fertilization. The process occurs in testes, in seminiferous tubules. Leydig cells, which produce testosterone, and Sertoli cells, which aid the spermatogenesis with the environmental conditions, division and regulatory cues such as the hormones inhibin and estrogen and proteins, are crucial for this process.

Spermatogonia, also called male gametogonia, are germline stem cells that descend from PGCs. After PGC specification in the embryo, they migrate to the gonads and further get specified to be called gonocytes. They localize adjacent to the mesonephric mesenchyme-derived supporting cells and together form the seminiferous tubules. After birth, they start rapidly proliferating by forming Type A spermatogonia but do not differentiate until sexual maturation. These spermatogonia are localized in the basal area created by Sertoli cells, which is separated from the luminous area via junctional complexes between Sertoli cells (blood-testis barrier).

Of the three spermatogonial subtypes, Type A(d), which has dark nuclei, maintains the spermatogonial pool and re-proliferates before the end of spermatogenesis to ensure the

preservation of the stem cell count. Type A(p), which has pale nuclei, mitotically divides and differentiates into Type B spermatogonia to create meiotic division potential. From a spermatogonium to a spermatozoon, there are three distinct phases. The first phase, spermatocytogenesis, is the proliferative phase. In this phase, Type A(p) nuclei go through 1 to 5 divisions with incomplete cytokinesis to create semi-connected cell chains. After this, the fate of the daughter cells are destined to be same as each other. These daughter cells go through a differentiation step and 5 successive divisions to form Type B spermatogonia, which are called primary spermatocytes after additional division and differentiation [Zhou and Griswold, 2008]**.** The second phase is the meiotic phase. Meiosis I produces the secondary spermatocytes and Meiosis II produces haploid spermatids. The third phase, spermiogenesis includes the metamorphosis of the spermatids into spermatozoa to get them ready for fertilization. This phase includes condensation of chromatin, acrosome, neck and tail formation, mitochondrial enhancement and reduction of excess cytoplasm. After the spermatozoa are formed the Sertoli adherence/protection of the cells are removed, and the readily available sperms migrate towards the lumen and leave the seminiferous tubules until ejaculation, a process called spermiation.

Throughout spermatogenesis, various hormones are important. Testosterone from Leydig cells is cued to be produced by Luteinizing Hormone (LH) to drive spermatogenesis, the necessary activation of Follicle Stimulating Hormone (FSH) from pituitary gland induces Sertoli cells to produce androgen binding protein and estradiol for testosterone reception, inhibin and activin from Sertoli cells account for FSH regulation. [Parrish JJ, n.d.].

2.2. Spermatogenesis in lower organisms

Alhough similar to mammals in many levels, there are differences of organism systems in spermatogenesis. We will go through the mechanisms in some model organisms shortly.

C. elegans is a hermaphrodite, which produces sperm from L4 larval stage to adulthood and then switches to oocyte production. Similar to mammals which from spermatocytes that are semi-connected, primary spermatocytes in *C. elegans* from a syncytium (a circular formation of spermatocytes with connective bridges) with the cytoplasmic core “rachis”. When meiosis initiates, these primary spermatocytes leave the rachis and start acting individually. In meiosis II, spermatids form by budding off the secondary spermatocytes taking many mitochondria and organelles that carry sperm proteins with them to initiate spermiation. Cytoplasmic reduction and mitochondrial enhancement occurs in this budding step. The membraneous organelles leave their cargo into the cytoplasm which also cause pseudopod formation without flagella. The resulting spermatozoa are stored until oocyte production. After first ovulation, sperms are pushed out of the oviduct to the spermatheca. They become motile and fertilize an oocyte. They go in and out the uterus because of the embryo movement, and then wait in the spermatheca until the next oocyte is produced [L'Hernault, 2006].

Drosophila has many similarities to mammals in spermatogenesis. As in mammals, the testes are composed of tubular structures, which have germline cells and supporting cells. Post-mitotic "hub" cells maintain, signal and supply the surrounding stem cell pool, while cyst progenitor cells (CPCs) have an analogous function to Sertoli cells, two of which support one germline stem cell (GSC), a.k.a. spermatogonium. The spermatogonium goes through four divisions with semi-cytokinesis as in mammals to form spermatocytes and after meiosis I and II with semi-cytokinesis, it results in 64 spermatids with connective cytoplasmic bridges. These spermatids and the surrounding cysts are very asymmetric, which eventually distinguishes the sperm head and tail structure development. After this, spermatids lose their bridges while reducing their excess cytoplasm and finalize their differentiation until fertilization [White-Cooper, 2008].

Xenopus laevis, is similar to *Rana sylvatica* and *Rana cantabrigensis* in spermatogenesis. The testes, as some other organisms, have seminiferous tubes and the PGC-descendant spermatogonia are surrounded by helper somatic cyst/follicle cells in a circular form in the basal lamina, first detected at stage 57-59. Dark and pale nuclei spermatogonia are also present in *Xenopus* as in mammals. After metamorphosis, primary spermatocytes form. [Kerr and Dixon, 1974]. After meiosis, spermatids form in close proximity with follicle cells and in spermiogenesis acrosome and flagellum of the sperm are present and the sperm has a clear anteroposterior polarity [Reed and Stanley, 1972].

Chicken and turkey also form syncytial structures of spermatogonia/spermatocytes. The resulting spermatozoon from meiosis is highly polar and has an acrosome and a tail [Thurston and Korn, 2000].

Evidently, there is strict conservation of spermatogenesis between lower organisms and between lower organisms and mammals. The seminiferous tubules and the cytoplasmic bridges between spermatocytes are a given in all the model organisms. In the next segment we will explain oogenesis in various model organisms.

2.3. Oogenesis in mammals

Oogenesis is a process that is, in principal, similar to spermatogenesis but largely different. After the differentiation of oogonia from PGCs and further mitosis to result in approximately 7 million oogonia, oogenesis starts. It has three phases. Folliculogenesis, which is parallel to oogenesis itself, forms the follicles that support and help the oocytes to transform into ootids. These follicles are paralogous to Sertoli cells in spermatogenesis. Oocytogenesis, which occurs before or shortly after birth, produces primary oocytes from oogonia by mitotic division and localization into surrounding follicle cells, paralogous to spermatocytes. The fundamental difference between spermatogenesis and oogenesis occurs in this step; no additional primary oocytes develop after the fetal stage unlike the lifelong spermatocyte supply in mammalian males. The next step is ootidogenesis, in which primary oocytes meiotically divide to produce

ootids and polar bodies. However, this stage is prenatally suspended in prophase I and does not continue until sexual maturity. After puberty, meiosis I continues to produce one secondary oocyte and one polar body to discard the excessive set of chromosomes with little cytoplasm. The immediate start of meiosis II leads to another halt in metaphase II until fertilization. However, in case of fertilization, meiosis II continues to produce three more polar bodies and an ootid which is then released from the follicles to mature into an ovum [Gilbert, 2000a].

Folliculogenesis occurs parallel to oogenesis, both assisting and helping the auto- and paracrine effects. From primordial follicle to mature follicle, gametogenic follicles surround oocytes and produce steroids. The primordial follicle is the first developmental stage, in which follicular cells surround the oocyte. When the primordial follicle differentiates into the primary follicle, the flat cells around the oocyte become cubical. The secondary follicle has two layers of cells, namely granulosa cells surrounding the oocyte that is now growing in volume, and epitheloid cells connecting the surrounding capillaries and stroma with the follicle. The 2 types of cells are separated by a membrane. The tertiary follicle has increasing number of follicular cells and an increasing cavity in between granulosa and epitheloid cells. This cavity supplies hormones and cellular signals for the oocyte, produced by Leydig-paralog cells. When the follicle matures to ~50 million cells, it is called mature/preovulatory follicle and after the maturation of the ootid to an ovum, it releases its contents by rapturing. These events are controlled first by FSH and estrogen, and then LH and progesterone [Guerin, 1993].

In almost all eutherian mammals, oogenesis occurs during fetal stages in the gonads and no additional production ensues during adulthood but the maturation and the release of the present oogonia from the follicles [Bukovsky et. al., 2009]. Although there is novel disputing evidence that mouse bone marrow and peripheral blood can supply and sustain oocytes in adulthood from putative germ cells extragonadally [Johnson et. al., 2005], further accomplishments need to be made in this hypothesis.

2.4. Oogenesis in lower organisms

In teleost fish, after oogonia turn into oocytes by mitotically dividing, oocytes start growing and accumulating vitellogenin with the help of estrogen inside two layers of follicle cells (granulosa and epitheloid/theca) separated by a membrane. After growth, oocytes enter meiosis I and arrest in prophase I stage. In order for meiosis to continue, germinal vesicle (oocyte nucleus) migrates to the animal pole. After this, the oocytes finish meiosis I and enter meiosis II, in which they arrest again in metaphase II until fertilization [Nagahama et. al. 1995].

In *C. elegans*, oogenesis which starts in adulthood is very similar to spermatogensis. In oogenesis, an oogonial syncytium with incomplete cytokinesis forms with a rachis inside. They share fates until the halt in prophase I [Matova and Cooley, 2001]. The continuation of meiosis is

triggered by the sperm itself, and oocyte maturation leads to fertilization [Yamamoto et. al., 2006].

Drosophila has egg chambers which have aligned units called ovarioles. One oogonium in an ovariole divides asymmetrically to produce one oogonium and one cystoblast, which will mitotically divide to produce a cyst of 16 cells with semi-cytokinesis, surrounded by somatic stem cells. One of these cells become an oocyte and the other 15 differentiate into nurse cells to supply the oocyte. This cyst is then surrounded by 16 follicle cells descending from the somatic cells and the oocyte starts growing, accumulating material (yolk material, maternal mRNA, proteins) and maturing. By this time, the nurse cells apoptose to leave the mature oocyte alone for meiosis [Gigliotti et. al., 2004; Matova and Cooley, 2001]. Follicle cells, other than supplying vitellogenin to form the yolk, also create the eggshell for the egg, which then moves in the uterus. After insemination, the eggs are released [Society for Developmental Biology, 2009].

Xenopus is one of the most studied organism in oogenesis. Oocytes are protected by an outer epithelial layer, a thecal layer and an inner epithelial cell layer. Each oocyte and its follicle cell is also surrounded by a cell layer [Rasar and Hammes, 2006]. After the oocyte is ready to mature it enters meiosis and arrests in prophase I to accumulate cytoplasmic material and grow. When the maturation is complete, progesterone signals the eggs to proceed with meiosis until the second arrest in metaphase II until fertilization [Marteil et. al., 2009].

Chick oogonia, as the above organisms, also form cysts with incomplete cytokinesis. They enter meiosis I but halt in prophase while breaking down the cyst. In order for the meiosis-arrested oocytes to grow and enrich their cytoplasm, folliculogenesis ensues and after fertilization, the egg is laid [Liu et. al., 2007].

2.5. Summary of gametogenesis

Evolutionarily, the system for gametogenesis has the same basis in all organisms. What we did not focus on above are the genes that are the main players and how/why egg/sperm polarity is created, e.g. sperm anteroposterior polarity and egg animal-vegetal pole polarity [FINDREF]. Another thing we did not mention is that in both spermatogenesis and oogenesis, apoptosis plays a great role and that not all the designated progenitors turn into sperm or eggs [Matova and Cooley, 2001]. Lastly, and most importantly, we tried to explain that gametogonia descend from PGCs, but we did not enter the subject of how PGCs arise. The specification of PGCs, in fact, answers all that is unmentioned above.

3. GERM CELL SPECIFICATION AND MIGRATION IN LOWER ORGANISMS

As mentioned above, we have wide range of knowledge about gametogenesis, both in mammals and lower organisms. But what about how germ cells come to be? In lower organisms, by scientists who carefully examined the electron micrographs of spermatogenic and oogenic samples of different organisms, this phenomenon has been successfully explained by a concept called "germ plasm". Therefore, the connection between germ cell determinants, primordial germ cells, germinal granules and germ plasm dates as early as the 1960s and 1970s although the first discoveries were made decades before that. Before the direct evidence was not clear, August Weismann already had discovered the possibility of the notion of germ plasm in 1885 [Weismann, 1885]. Germ plasm in *Rana temporaria* was examined by Bounoure in 1929 [Bounoure, 1929]. Afterwards, in zebrafish, chick, *C. elegans, Drosophila* and *Xenopus*, scientists found maternal inheritence of cytoplasmic material with granular structures that persist throughout life, first in specific localizations in early embryogenesis and later in germ cells. They called the cytoplasm "germ plasm", and the mitochondria-associated electron-dense polar granular structures that are rich in RNAs and ribonucleoproteins (RNPs) "germinal granules". In this caption, we will attempt to summarize the explanations made about the germ plasm and germinal granules of lower organisms, and the related genes.

3.1. Germ plasm and PGC formation in lower organisms

In early embryos, only the cells that inherit the cytoplasm containing germ plasm can differentiate into PGCs. The accumulation takes place in oocyte maturation, hence the cytoplasm and its components including germinal granules and germ plasm are provided and inherited maternally, mostly as vegetal pole mRNAs migrating to the yolk in early cleavages. Since its discovery as a germ cell marker in Drosophila, vasa and vasa homologous genes (DEAD-box family proteins of ATP-dependent RNA helicase activity [Noce et. al., 2001]) have been detected in almost every animal species localizing in either germline granules, or in animals seemingly lacking germ plasm, like mammals.

The cell lineage in *C. elegans*, which only has specifically 959 cells in adulthood, is completely defined. Its initial divisions create P1, and then P2, P3 and P4 germline blastomeres and the latter three inherit the germinal granules from P1 (called P-granules in *C. elegans*) by the movement of the granules to the posterior side of the embryo either by

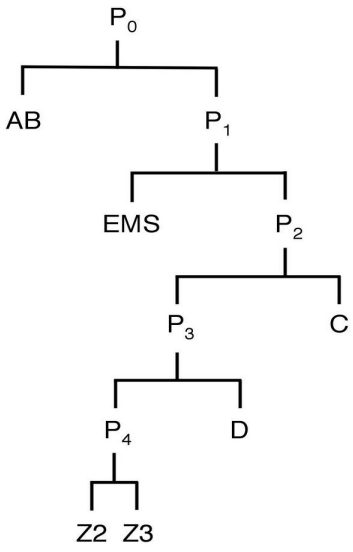

Figure 2. ***The first germ cell lineages in C. elegans.*** The first cell divisions give rise to future germ cell lineages in the posterior side of the embryo.

cytoplasmic current or by harnessing to the nucleus. They also eliminate the granules in the cells which have somatic fate. P4 granules, in this manner, collects almost all granules to itself and subsequently divides in ~100 cell stage into the first two PGCs: Z2 and Z3 cells (Figure 2).As mentioned before, these granules are rich in RNAs, RNPs and several proteins and most of them have RNA-binding sequences to putatively regulate translation. Moreover, most of the defined components are associated with both embryonic and adult P-granules. MES, MEP and PIE proteins are in inhibitory and activating interactions to decide on the expression pattern of germline or somatic cells to maintain the fate [Wormbook: Specification of the germ line - Susan Strome html pdf]. The Z3 and Z4 cells start dividing only in the larval stage, after hatching and spermatogenesis starts. Granular proteins and mRNAs are present temporally. GLH1 and GLH2 proteins are also a part of P-granules and they are homologous to Drosophila vasa protein. Their RNAi results in sterile worms [Ikenishi, 1998]. Nanos 1 and 2, the RNAs of which also localize on P-granules, are interchangably required for germline development [Subramaniam and Seydoux, 1999].

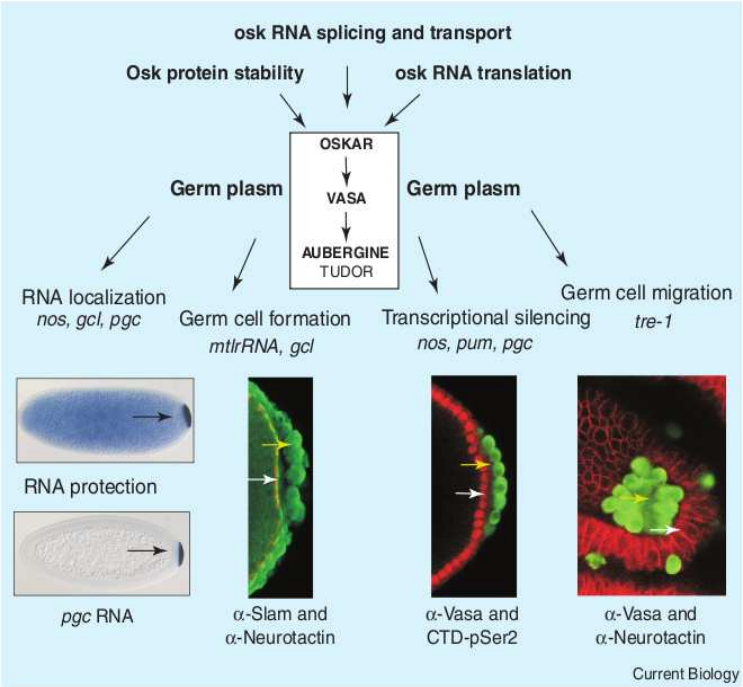

Figure 3. Germ cell formation in *Drosophila.* Gene and protein interactions lead to various effects, eventually to the correct localization, formation, differentiation and migration of germinal granules, hence PGCs.

Drosophila is, as mentioned above, the first organism that the vasa gene was found [Hay et. al., 1988]. Oskar, vasa, tudor and aubergine are the essential RNA binding proteins in germ plasm and granule formation (Figure 3). Nanos, also a very important gene, is important in and after PGC formation. Germ plasm is also important for the maintenance of maternal cellular components and the activation of germ-specific genes or inactivation of transcription. Maternal RNAs and proteins in germ plasm and granules are crucially important; their elimination cancels germ cell formation while zygotic gene elimination only causes somatic cell defects. *Drosophila* embryos form a syncytium of nuclei initially, and germ plasm is located at the posterior pole. The nuclei that encounter the germ plasm immediately form plasma membranes, stop

transcription and divison and become germ cells with the help of nanos, germ cell-less, polar granule component [Santos and Lehmann, 2004].

Germ plasm and polar granules are explained in zebrafish as well as other organisms. In 4-cell stage, vasa mRNA-containing polar electron dense materials are present in the 4 cleavage furrows, led by the 3'-UTR of the RNA. From that point on, first the granules move into the cells and until approximately the end of 4 hours of development, these germinal polar granule containing cells do not change count [Yoon et. al., 1997]. Similar to *Drosophila*, they stop dividing and transcription and they eliminate the other somatic cell granules. They get the help of mainly vasa and nanos, and other granular proteins like dead end, dazl and cxcr4b/sdf1a help the later stages. After the 4^{th} hour of development, at ~4000 cell stage, they divide to produce 25-50 granule-containing cells now called "PGCs" until the end of their first 24 hours [Raz, 2003; Yoon et. al., 1997].

Xenopus is very similar to zebrafish in germ plasm inheritence to daughter cells. In 4-cell stage germ plasm is present in 4 animal pole cells, but after the vegetal pole divisions the granule-containing cells do not increase number until gastrula stage. Thus, 2-6 cells are the presumptive PGCs [Ressom and Dixon, 1988]. The granules are first seen in maturing oocytes as a mitochondrial cloud or "Balbiani body". This cloud slowly transforms into granules until tadpole stage [Ikenishi, 1998]. The vasa-homologue VLG1 and nanos homologue cat2 are localized in granules. Dazl, dead end and cxcr1/sdf1a systems also take action after PGC specification [Raz, 2003].

Chick-Quail chimera experiments show that avian PGCs originate from epiblast (area pellucida) [Eyal-Giladi et. al., 1981], and upon PGC depletion gonads develop normally [McCarrey and Abbott, 1982]. Chicken vasa homologue (Cvh) is present in germ cells throughout development and adulthood, including the first cleavage furrows basally. Cvh, as other vasa homologues, is localized in granules (mitochondrial cloud in oocyte maturation). Before gastrulation, Cvh expressing cells are found in the epiblast (~33 cells at stage X), and PGCs in the 1 day embryo are localized in the germinal crescent, anterior to the headfold. The movement is passive due to the formation of primitive streak [Tsunekawa et. al., 2000].

3.2. PGC migration in lower organisms

PGC migration studies started early. When posterior pole plasm of *Drosophila* was transferred to anterior pole plasm, the PGCs still formed and migrated to the gonads and only contributed to germ cells. However, in anurans *Rana* and *Xenopus,* the transplated cells contributed to endoderm, mesoderm and ectoderm which states that the possible PGC fate is reversible, or external cues in posterior endoderm has equal importance [Wylie et. al., 1985;

Houston and King, 2000]. Before migration, PGCs are clustered together, but while entering the active migratory route they lose their attachments putatively by downregulating their cadherins [Richardson and Lehmann, 2010]. Therefore, in most organisms, PGCs travel independent of each other cued by the ECM and extracellular signals.

In *C. elegans,* not many things are known about the migration of PGCs. However, it has been foung that nanos 2 has function in the correct migration of Z2 and Z3 cells around the Z1 and Z4 somatic gonadal cells [Subramaniam and Seydoux, 1999].

Drosophila PGCs first migrate passively into posterior midgut pocket with tissue invagination, and then they start their active migration transepithellially through the gaps formed in primordial posterior midgut epithelium by the expression of Trapped in endoderm 1 (TRE1). However, the cells do not leave the posterior midgut but move along it by the help of the repellents wunen 1 and 2 expressed by the anterior midgut. From this point, PGCs get attracted by the gonadal mesoderm by HMG-CoA-Reductase and connect with somatic gonadal cells [Santos and Lehmann, 2004; Jaglarz and Howard, 1995; Richardson and Lehmann, 2010].

Zebrafish PGCs, as mentioned above, are formed localizing at four different locations. They first downregulate their cadherins using the protein dead end to dissociate their junctions and start migrating freely. Zebrafish use the Cxcr4/Sdf1 (G-protein coupled chemokine receptor and its ligand Stromal derived factor, respectively) and HMG-CoA-Reductase system to I. migrate dorsally in shield stage, II. locate laterally close to dorsal midline, III,IV,V. align laterally and anteroposteriorly on either side of the first to the third somite, VI. finalize migration at ~24 hpf by locating around the 8^{th} somite between the spherical yolk and its extention [Raz, 2003; Richardson and Lehmann, 2010; Weidinger et. al., 1999] .

In frogs and toads, after the first cleavages, germ cell containing cells localize in the four vegetal-most cells in 32-cell stage, then the endodermal lining of blastocoel, which eventually forms the posterior gut (hindgut). The cells then migrate to the dorsal gut, dorsal mesentery between the gut and mesoderm, and finally along the abdominal wall before reaching the gonads. Until *Xenopus* PGCs migrate to the gonads, they go through three cleavages which result in approximately 30 cells [Wylie and Heasman, 1993; Gilbert, 2000b].

After chick presumptive PGCs form in the area pellucida and migrate to the germinal crescent with vasa homologue-positive cells, blood vessels develop and PGCs enter the bloodstream, unlike other organisms. They go out of the bloodstram in the hindgut and follow a path similar to other organisms; they reach first the dorsal gut, then the dorsal mesentary, and finally the genital ridges to form gonads together with somatic cells [Wylie and Heasman, 1993; Gilbert, 2000b, Fujimoto Ukeshima Kiyofuji 1976].

3.3. Summary of PGC specification and migration in lower organisms

Although PGC specification and migration amongst different organisms seem different, they all use the germ plasm, germinal granules, or mitochondrial cloud to initiate the germinal lineages. We mentioned the transport of the germ plasm, however we did not go into detail. Mitochondria is central to the application of germ plasm; controlling apoptotic pathways for excess germ cells, maintaining the homeostasis, controlling polarity with non-uniform density distribution in the egg, even helping fertilization. Male gametes do not retain the germ plasm and lose it usually during spermiogenesis and still use the mitochondria very efficiently. This efficient central use of mitochondria is also substantial in mammals [Dumollard et. al., 2007]. In the next chapter, we will explain PGC specification and migration in mammals.

4. GERM CELL SPECIFICATION AND MIGRATION IN MAMMALS

Although there have been many proteins like vasa, dead end, SDF-1 found in mammals that are homologous to lower organisms, the general view is that there is no germ plasm inherited from the eggs in mammals and there are other prominent cellular interactions that specify and epigenetically modify PGCs. Unlike lower organisms, all the cells resulting from first cleavages are totipotent and there is no prelocalized determinants in the oocytes [Yeom et. al., 1996]. This provides an evolutionary gap between non-mammals and mammals, because although intercellular signals take role in PGC specification in lower organisms as well as mammals, mammals, using a presently unknown evolutionary reason, developed a different way of germ cell specification, predominated by epigenetic control. However, PGC specification is very similar especially to chick, given that the origin is epiblast and the migration occurs [Eyal-Giladi et. al., 1981], and the migratory route resembles that of anuran amphibians [Gilbert, 2000b] and *Drosophila*. Most of the studies about mammalian PGC specification was and is made in mice, and *in vitro* systems have been tried to be developed.

Primordial germ cells were identified in mouse cells at the stage of 7-7.25 days post coitum (dpc) after the alkaline phosphatase [Chiquoine, 1954] marked golgi-associated spots were found posterior to the primitive streak (E6-6.5) [McLaren, 1999]. Subsequently, lineage tracing experiments brought the realization that PGCs, in fact, originate from the epiblast [Lawson and Hage, 1994]. It is now known that mouse embryos express Bmp4 in extraembryonic ectoderm and the null mutants lack PGCs completely [Lawson et. al., 1999]. New marker and antibody screens open new options in the elaboration of PGC specification, as alkaline phosphatase staining is not solely limited to PGCs due to subtypes. Moreover, because of embryonic staining, it is very hard to visualize PGCs before 8 dpc [Yeom et. al., 1996]. Besides elaborating PGC specification, marker studies also may highlight the now-unknown evolutionary conservation of the specification systems between non-mammals and mammals; for instance, a rabbit

monoclonal antibody has been found to be associating with mitochondria from when germ cells leave the hindgut until at least right before birth, in gametogonia [Viebahn et. al., 1998]. In lower organisms, germinal granules show themselves as a "mitochondrial cloud" and that is where the initial markers localize (see 3.1. Germ plasm and PGC formation in lower organisms). A connection of the initial mammalian PGC markers with mitochondria may be an indication of conversion in some level, although germ plasm material may not be as in lower organisms. Here, we will try to explain what has been found about mammals before getting thoroughly into the *in vitro* techniques and new developments.

4.1. PGC migration in mammals

PGC migration in mammals has more similarities with lower organisms than PGC specification. After their state of presence posterior to the primitive streak, they passively invaginate into the forming hindgut, attaching to the inner surface (E7-7.5). At E8.5, there are approximately 100 detectable PGCs. At E9.5 stage, PGCs emigrate from the hindgut but as dorsal mesentery has not formed yet, they directly move to the genital ridges, where the gonads also are not present yet. By the stage of E11.5, all PGCs (~3000 cells) have moved to the genital ridges; some late-comers indeed transepithelially migrate through the dorsal mesentery. Aggregation occurs in gonads until they do their job and are named as gametogonia [Bendel-Stenzel et. al., 1998].

4.2. PGC-specific gene identification involved in specification and migration

Genes defined by mutant phenotype, genes defined by their lower organism homologues, growth factors, antibodies and molecules defined by marker screens, transcriptional repressors/activators,, surface proteins and cell-cell or cell-ECM contact proteins have been found to be specifically expressed in PGCs. Although some of them have known function, some are not yet functionally described. We will list the related proteins and markers that have been found to be important in PGC specification and/or migration in mammals.

Most Bmp4-null mutants die at E6.5 while some survive until somite stages, giving advantage in observing what the lack of the protein does. As expected, Bmp4-null embryos do not have PGCs and heterozygous embryos have less PGCs. The initiation of the germ line (and allantois) might be dependent on Bmp4 expression in a concentration-dependent manner in extraembryonic endodermal cells that are very close to proximal-most epiblast cells. Another theory would be that allantois and PGC differentiation occurs in a stepwise manner from epiblast; that is, allantois and PGCs may have a "common ancestor" cell directly differentiated by Bmp4 cues, and a second signal might cue for each cell type. The latter theory is more likely because some mutations affect only the allantois differentiation while PGCs are normal and vice

versa. [Lawson et. al., 1999]. Bmp4 expression affects the PGCs from its expression in extraembryonic mesoderm close to PGCs, and PGCs show responsiveness from E5.25-E5.5 until they form at E6.0 [Okamura et. al., 2004]. Bmp8b is also found coexpressing with Bmp4 in extraembryonic ectoderm [Saitou et. al., 2002], and its mutants, independently from Bmp4 mutants, cause either reduction or complete depletion of PGCs and aberrant allantois, although embryos are more sensitive to Bmp4 mutations than Bmp8b mutations [Ying et. al., 2000].

Fragilis1-2-3, an interferon-induced transmembrane protein, controls attachment of the same kind of cells together, and in leukocytes and endothelial cells, it transfers signals to stop proliferation. Fragilis1, 2 and 3 expressions are temporal and spatial. Expression of Fragilis3 is restricted to PGC precursors in proximal epiblast before gastrulation as the first marker of specification [Saitou et. al., 2002] and they are thought to help the clustering of cells. During gastrulation, mid-streak stage and late-streak stage marks the initiation of Fragilis1 and 2 expression in the epiblast, respectively. Fragilis1 is downregulated in PGCs during passive invagination, and during migration from the posterior gut, the Fragilis1-negative PGCs run away from Fragilis1 expressing cells in a repelled fashion. This action may be aided also by Lhx1 (Lim1) [Tanaka et.al., 2010]. Fragilis3, however, contrasts Fragilis1 effects and are localized specifically close to PGCs, possibly helping the PGCs define the localization pattern. The effect of Fragilis 1 and 3 have an analogous function to wunen1-2 in *Drosophila* [Tanaka et. al., 2005; Tanaka and Matsui, 2002]. They are also thought to repress homeobox proteins in germ cell precursors [Saitou et. al., 2002].

Blimp1, (B-lymphocyte-induced maturation protein-1) is a transcriptional repressor specifically expressed in PGCs during PGC specification and migration. Its major role is leading the terminal differentiation of B-cells by inducing them into plasma cells secreting immunoglobulins. In E6.25 stage, it marks 6 PGCs in proximal epiblast, together with the future primitive streak (20-28 PGC cells) in a very restricted expression pattern, narrower than that of Fragilis. As the PGCs proliferate, Blimp1 marks more and more cells until the cluster of 40 cells in E7.5. Blimp1 deletions do not cause defects until E8.5, but the PGC count gets reduced dramatically. [Ohinata et. al., 2005].

Lim1 (Lhx1), is also a gene thought to promote the specification of PGCs. During gastrulation, it is expressed at proximal epiblast, posterior primitive streak and developing gonads in organogenesis. Lim1 null mutation causes a spectrum of defects, which includes allantois and genital primordium defects, but the most important is PGC loss or lack of survival. In Lim1 mutants, Bmp4 expression is not restricted to extraembryonic ectoderm, it is also present in proximal germ layer, therefore the induction of germ layer may be rendered impossible. Other mutations that may disrupt the body plan and cause germ cell phenotypes are Hnf3β (Foxa2) and Otx2 but not brachyury (T). Further research is needed [Tanaka et. al., 2010; Tsang et. al., 2001; Kinder et. al., 2001]. Despite the negative effect of the Lim1 mutation on the

PGCs, it is asserted that Lim1 needs to be repressed as well as Hox genes in order for the PGCs to be specified [Saitou et. al., 2002].

Stella (Dppa3, PGC7) starts expression in nascent PGCs at E7.0-E7.25 and is thought to be involved in the maintenance of pluripotency in ESCs. It is considered to be the first marker of lineage restricted PGCs. Its expression persists in PGCs through migration and is downregulated in gametogonia stage (E13.5 in females, E15.5 in males). It is also maternally inherited while the oocytes are maturing and pre-implantation embryos express maternal Stella from 2-cell stage to blastocyst stage (E1.5-E4.5) [Payer et. al., 2006]. Stella is not homologous to any known proteins and may be involved in nucleic-cytoplasmic shuttling and binding DNA [Saitou et. al., 2002; Sato et. al., 2002]. However, a key study reveals that Stella deficient embryos do not suffer from PGC defects; the defects rather occur from the deficiency of the maternally inherited protein [Bortvin et. al., 2004].

Oct4, a maternal POU transcription factor, marks the undifferentiated embryonic stem cells, and is used in almost all *in vitro* cultures for marking pluripotency, not only in PGCs. Two enhancers, germline and epiblast enhancers, regulate the expression in the named tissues. The expression in the embryo first occurs through the maternally inherited from the pronuclei of eggs, and in 8-cell stage zygotic expression starts [Palmieri et. al., 1994]. Preimplantation embryos are controlled by the epiblast enhancer of the Oct4 gene, and when the embryo is implanted (4.5 dpc) the switch to germline enhancer occurs. [Yeom et. al., 1996]. GCNF (Germ Cell Nuclear Factor) represses Oct4 expression together with its corepressors Nco-R and SMRT, while in the mutants Oct4 expression is not restricted to the PGCs [Fuhrmann et. al., 2001]. Retinoic Acid is also a factor thought to control the downregulation the Oct4 protein in the epiblast [Yeom et. al., 1996]. Oct4 is expressed in gonadal PGCs decrease in females entering meiosis at E14.5, but in males it continues until adult spermatogonia [Sabour et. al., 2010; Payer et. al., 2006].

Steel factor (kit ligand, stem cell factor SCF) is a cytokine crucial for PGC survival. It has a soluble and membrane bound splice variant, possibly for signaling and direct cell-cell contact, respectively. Its receptor is c-kit which is expressed during PGC migration. The deprivation of embryos from Steel factor or c-kit results in less PGCs, flawed migration and results in sterile mice. The mutations strongly correlate with testicular germ cell tumors [TesticularCancer-PGCRelationReview2010]. It expresses in the surrounding somatic cells in the migratory route, which decides the fate of PGCs; the cells that are left in the midline without Steel factor expression after its downregulation there at E10.5 soon die, and the PGCs that are in the genital ridges survive where Steel factor is still expressed. From their specification, PGCs that do not fail to migrate to the gonadal ridges are surrounded by a moving wave of Steel factor expression [Gu et. al., 2009].

c-kit Receptor Tyrosine Kinase is a product of *White* spotting (W) gene expressed in PGCs and its effect on PGCs was identified by the mutation phenotype. As mentioned above, it is the receptor for Steel factor or SCF. Similar to *Steel* mutants, *W* mutants decrease PGC count dramatically. The monoclonal antibody Ack2 binds the extracellular region of c-kit. There is evidence that the SCF/c-kit interaction results in cytoskeletal rearrangements for the formation of circumferential actin to mediate ECM binding and/or migration, together with Integrins [Anderson et. al., 1999; De Felici and Pesce, 1994].

E-cadherin, the Ca^{++} mediated monotypic transmembrane interaction molecule, helps the clustering of PGC precursors in the extra-embryonic mesoderm and this is crucial for PGC commitment before migration. E-cadherin expression slowly starts being downregulated during migration and is undetectable in the mesoderm [Okamura et. al., 2003]. It may also take a role in migration according to *in vitro* data [Di Carlo and De Felici, 2000].

Murine Vasa homologue (Mvh), unlike in lower organisms, does not persist its expression in PGC specification and migration. It is expressed in a low level starting at E9.5-E10.5, and in an increased level gonadal ridges. This expression is analogous to zygotic vasa expression in *Drosophila.* [Noce et. al., 2001]. Null mutants cause spermatogonial proliferation and differentiation defects in males, while reduction in Oct4 is visible [Tanaka et. al., 2000]. Its translation is initiated by a conserved protein Dazl, the mutations of which commonly lead to infertility by oligozoospermia or azoospermia [Reynolds et. al., 2005].

LIF (Leukemia Inhibitory Factor) influences the growth and differentiation of PGCs and ESCs activating different pathways. Its receptor is expressed on PGC plasma membrane. LIF works with a family of cytokines; interleukin-6 (IL-6), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) also can promote growth of PGCs. LIF also promotes the proliferation and totipotency of ESCs in culture [De Felici and Pesce, 1994; Cheng et. al., 1994]. Its involvement in the Jak/Stat3 and PI3K/Akt pathways regulate self renewal while the MEK/ERK pathway induces ESC differentiation in mice [Buecker and Geijsen, 2010]. Its *in vivo* effects for PGCs are not certain; LIF receptor (LIFR) mutants die short after birth because of organ system failures with a normal number of PGCs. LIF mutant females have implantation failure but viable and normal oocytes, eggs and embryos of these eggs. The mutants of the low affinity LIF receptor GP130 working in the RAS/MAPK pathway die at E15.5; comparing to STAT3 mediated high affinity receptor (LIFR, OSMR, IL6R) mutants which die at E7.5. GP130 mutants have been found to have fewer numbers of PGC but later it was clarified that the importance of GP130 lies in oogenesis although it is expressed from E10.5 in PGCs. The problems also extend to folliculogenesis [Molyneaux et. al., 2003a].

TGFβ1 (Transforming Growth Factor) secreted growth factor inhibits proliferation of PGCs *in vitro* and it was thought to have the same function *in vivo* [Godin and Wylie, 1991], but it is

not so. When PGCs are isolated from E8.5 embryos, TGFβ1 acts similar to the effect of gonadal ridges of E10.5 embryos. However, *in vivo* data shows that TGFβ1 receptor Alk5-null embryos have not only normal migration until E10, but also incorporate into gonadal ridges without fault and even more efficiently than wild type by reducing the concentration of ECM and increasing the Integrin concentration [Chuva de Sousa Lopes et. al., 2005].

FGF (Fibroblast Growth Factor) receptors I and II are expressed in the developing gonad and can directly interact with PGCs, and PGCs can directly bind to basic FGF (bFGF) *in vitro*, while the binding decreases in oogonia. FGF expression, together with LIF and Steel factor, promotes growth and proliferation of PGC in culture [Resnick et. al., 1998]. basicFGF (bFGF) mutations viable and fertile without gametogenesis problems [Molyneaux et. al., 2003a]. FGF proteins work both paracrine and autocrine on PGCs, but FGF2 induces proliferation in culture but not in the embryo. FGF4, 8 and 17 are expressed in neighboring somatic tissues along the path of PGC migration and FGF4 and 8 promote proliferation *in vivo*. After gonadal ridge localization, PGCs start expressing FGF1, 2 and 4 receptors themselves [Kawase et. al., 2004]. In Embryonic Stem Cells, Oct4 is thought to be able to form a complex with Sox2 to start FGF4 transcription, therefore may also take an effect in this process, although there is not enough data collected [Pesce and Schöler, 2001].

RA (Retinoic Acid), stimulated by Stra8 in females and degraded by CYP26B1 in male cells in gonads, regulates entrance into meiosis positively and negatively, respectively, while FGF9 negatively regulates meiosis entry in concert with RA regulation by promoting male germ cell fate at the same time [Bowles et. al., 2010]. In culture, RA also cues the differentiation of ESCs and helps the proliferation of PGCs [NielsMaleGameteDerivation].

Nanog is a homeodomain containin transcription factor which is explained as “the new center of pluripotency”. Its role is being cleared out using mostly *in vitro* studies, as well as *in vivo* studies. Its importance was understood when it was shown that LIF was not enough for pluripotency and that with Nanog the feeder cell layers or LIF was not necessary. In Nanog-null ESC cultures, a bias towards extraembryonic endoderm differentiation occurs and the other cell-type markers are decreased; including pluripotency marker Oct4. In Nanog-null mouse blastocysts, the inner cell mass differentiates into endoderm-like cells and stops proliferating.. *In vitro,* it is controlled through LIF, TGFβ, FGF, RA, BMP4 and GPCRs (PKA signaling) by STAT3, PI3K, Smad2/3, Oct4/Sox2 complex (activation) and MEK, RA, p53 (inhibition) pathways. It expresses in ESCs, epiSCs, EGCs, ECCs, and in embryos from morula stage to ICM until differentiation, and it is present in the PGCs of E11.5 embryos in the gonadal ridges [Buecker and Geijsen, 2010; Mitsui et. al., 2003; Pan and Thomson, 2007; Chambers et. al., 2003].

As explained in lower organisms, SDF1 and its receptor CXCR4 are required also for mammals, but only for the incorporation of PGCs in gonadal ridges. They do not pave the way for PGC migration as they do in zebrafish before gonadal localization; although CXCR4 is expressed in migrating PGCs, SDF1 is only present in genital ridges [Molyneaux et. al., 2003b; Ara et. al., 2003]. However, when SDF1-coated beads are implanted in the embryo or exogenous SDF1 is injected to culture, PGC migration has failures, probably because of the disruption in directional cues [Raz, 2004].

The 4C9 antigen is found to be a very prominent marker for mouse PGCs. Starting at inner cell mass of late blastocysts, it is expressed while the PGC form, migrate and enter the gonads. After gonadal localization, it expresses in polarized granular structures in spermatogenesis and in oogenesis it is uniformly distributed in the cytoplasm and then the cell surface [Yoshinaga et. al., 1991].

Other monoclonal antibody markers are EMA1, and SSEA1 (Stage-Specific Embryonic Antigen), which recognize different chains of the same carbohydrate chain. They start marking undifferentiated embryonic stem cells from 8-cell blastomeres until some tissues in adulthood, and additionally they mark embryonic carcinoma and embryonic stem cells, most probably in embryonic germ cells as well. While EMA1 and SSEA1 interact with 8-13 dpc PGCs, EMA6 interacts with 8-10 dpc PGCs [Hahnel and Eddy, 1986; Sasado et. al., 1999].

4.3. Model for PGC specification control with known gene expression patterns

Lawson and Hage's epiblast lineage tracing experiments to detect PGCs [Lawson and Hage, 1994], or transplantation experiments of Tsang et al. from proximal to distal epiblast and vice versa [Tsang et. al., 2001] are only two examples of many experiments that led to the conclusion that PGC form by induction and the cells themselves do not inherently have properties to produce PGCs. The gene functions, however, are harder to define. Because of the hardship of *in vivo* studies, the scientists felt the need to replicate the embryonic processes *in vitro.* The drawback of cultures is that the components may not behave the same as in the embryonic environment; e.g. TGFβ1 promotes proliferation of PGCs *in vitro* but it is not crucial for PGC proliferation *in vivo* [Godin and Wylie, 1991]. In this segment, we will attempt to summarize the possible interactions and mechanisms of protein action that leads to PGC specification and gonadal localization.

The present data about PGC specification shows that Bmp4 that is expressed in extraembryonic ectoderm is responsible for induction of PGCs. Bmp4 expression is regulated by its antagonists Bmp Receptor I and II and its cleavers like Bmp1 [Lawson et. al., 1999]. Fragilis is the first PGC-related protein that starts to get expressed, and as soon as its expression is detectable it localizes to the epiblast cells adjacent to the extraembryonic ectoderm. It is thought to be induced by Bmp4, because when the distal epiblast is excised next to extraembryonic

ectoderm by discarding proximal epiblast in culture, the fragilis positive cells occur at distal epiblast close to Bmp4 expressing cells. Moreover, in Bmp4-/- embryos fragilis is not detected. Blimp1 is expressed in a narrower region in the proximal epiblast than fragilis and very close to the extraembryonic ectoderm in probably a single cell layer, while fragilis spans a region of 3-5 cell layers. Blimp1 expression, given a single cell cDNA analysis, occurs before Stella expression. Moreover, intriguing preliminary data shows that in Bmp4 mutants no Blimp1 positive cells are detected, which might mean an induction relationship similar to fragilis [Ohinata et. al., 2005]. Stella, on the other hand, is expressed in the center of fragilis and alkaline phosphatase stained cells, where the fragilis and Hoxb1 concentration is the highest [Saitou et. al., 2002]. But although it is thought to maintain pluripotency, it is not crucial for PGC specification, and it does not induce Oct4 transcription [Bortvin et. al., 2004]. It is thought that Nanog may be functional in conserving the pluripotency of germ cells as well [Chambers et. al., 2003]. More data is needed for the function of Stella in PGCs, however, it is thought that Blimp1 expressing cells are almost always positive for Stella. Homeobox (Hox) gene repression is an important phase of PGC specification, and when Stella is knocked out, Blimp1 and alkaline phosphatase positive cells fail to repress Hox genes and have proliferative deficiencies [Ohinata et. al., 2005].

Single cell QPCR gene expression profiling studies reveal temporal expression and gene repression/upregulation in PGC specification and migration. PGC-related genes sequentially get activated after Blimp1 positive cells differentiate into Stella positive cells. Yabuta et. al. (2003) divided PGC genes in 5 groups. (1) Housekeeping genes are highly present in both somatic and germ cells, (2) some genes have higher expression in germ cells, (3) some genes in somatic cells, (4) some genes are constantly present in germ and somatic cells, and finally (5) some genes have no detectable expression. Fragilis1, Blimp1, Stella, Sox2, Nanos3, Nanog and Kit are some genes that are expressed higher in germ cells than the surrounding somatic cells between E6.75–E8.25, Sox2 possibly in a complex with Oct3/4. Conversely, between E7.25-E7.75, Hoxa1 and Hoxb1, together with Dnmt3b, Snail homologue 1 (Snai1) and DNA methyltransferase 3b (Dnmt3b), get highly repressed in germ cells. The sensitivity to BMP signals decrease upon PGC specification. These results overlap with the qualitative experimental data on separate genes. [Yabuta et. al., 2006].

4.4. Epigenesis in PGC specification

Embryos emerge with paternal genetic imprints, and during embryogenesis [Li, 2002] and especially PGC specification to be complete, these imprints has to be erased and the cells have to be reprogrammed in order to render and maintain their totipotency. X chromosome activation/deactivation also needs to be modulated. Various DNA and histone methylation/demethylation types has been found to take place during PGC specification and migration.

H3K9 dimethylation, which is involved in the repression of arms of euchromatin, starts to occur in PGCs around E8.0 and this repression goes on until at least E12.5 with low expression status, when PGCs have migrated to the gonadal region. Connected to this process, PGC DNA gets demethylated twice; once with the whole genome at around E8.0, and in gonadal region at E12.5. At E8.0, also the loss of DNA methyl-transferase activity occurs and lasts for ~16 hours. At E8.5-E9.0, H3K27 trimethylation in germ cells spans wide euchromatic genomic regions, while in somatic cells it is restricted to the X chromosome marking the start of X-inactivation. By replacing H3K9 dimethylation, H3K27 trimethylation creates more flexible control over transcription. H3K4 methylation and H3K9 acetylation, which means transcriptionally active chromatin, increases upon PGC migration to the gonadal ridges [Seki et. al., 2005].

X-chromosome-linked HMG-lacZ reporter gene activity studies show that X-inactivation and reactivation shows chronological relevance to PGC migration status. Before migration, PGCs identified by alkaline phosphatase activity mostly (85%) do not show X-inactivation to reduce the gene dosage effect. This status remains constant in migration through hindgut until the PGCs reach dorsal mesentery, where a dramatic X-inactivation occurs. From their reach to genital ridges until meiosis starts, 80%, and during meiosis, 90% of the cells reactivate the X chromosome. The regulation, although it has chronological tracibility, may be induced by the cellular behavior and interactions through and after the migration route. Because the PGC migration is in waves of cells and those who are temporarily left behind the gonadal ridges do not yet re-activate their X-chromosomes, and some cells which have mesonepric fate never do [Tam et. al., 1994; Tsang et. al., 2001].

4.5. Similarities and differences of mammals with lower organisms

Although we emphasized the preformation method as the only method for specifying PGCs in lower orgnisms, mammals are not the first or only organisms to efficiently use epigenesis for germ cell specification. Urodele amphibians (axolotls, salamanders, newts) present evidence of epigenetic PGC specification rather than preformation. Any cell from the animal half of the urodele blastula can give rise to PGCs as long as there is induction (possibly BMP) from ventral endoderm. Axolotls do not have germ plasm or the mitochondrial cloud and this leads to the thought of induction playing a role in PGC specification [Johnson et. al., 2003; Extavour and Akam, 2003]. Likewise, from the non-model organisms, *Mnemiopsis leidyi,* a comb jelly, *Blatta germanica*, a cockroach, the turtles *Trachemys scripta* and *Sternotherus odoratus* and other reptiles do not show evidence for preformation and are also thought to epigenetically induce germ cells, although the specification is in different stages between species [Extavour and Akam, 2003]. Rotifers, despite the fact that they express vasa and nanos homologoues, are also thought to specify germ cells by epigenesis, although preformation cannot be ruled out completely [Smith et. al., 2010].

From the primitive organisms, germinal vesicles are observed in Hydra, the ancestor of metazoans, although Tannreuther (1909) has not seen a continuous germ plasm. Still, vasa homologous proteins are expressed in Hydra [Mochizuki et. al., 2001; Tannreuther, 1909]. Starfish also presents evidence for germinal vesicles [Zhang et. al., 1990]. This proves the idea that in an evolutionary perspective, epigenetic germ line specification was not "invented" for mammals only. It was, perhaps, first evolved aside of germ plasm in preformation organisms, and turned out to be as efficient. For instance, as mentioned above, in rabbits, a monoclonal antibody was found in mitochondria, specific to the PGCs from hindgut migration to at least birth [Viebahn et. al., 1998], which may lead to the thought that germinal vesicles and mitochondrial clouds are not absent from mammals. After all, the germ plasm is only there to induce the necessary cellular changes for germ cells to arise and it is also an induction mechanism. Convergent evolution; the evolution of similar solutions to similar problems without the spatial or evolutionary relation of the species, may also be an explanation. Dixon (1994) has proposed the theory that germ cells are produced where they get influenced the least by body plan formation determinants, and that the inductive signals or the germ plasm is merely a way to prevent germ cells to become somatic cells [Dixon, 1994].

Although the knowledge about mice are gathering up, detailed information is dependent on studies on culture, *in vitro*. Next, we will try to elaborate the contemporary techniques used to culture and mimic the migration of PGCs.

5. PGC and GAMETOGENESIS STUDIES *in vitro*

ESs (or EBs), ECs, and EGs are three culturable cell types derived from early mammalian embryos. ESs are embryonic stem cells from blastocysts/inner cell mass, which either stay in monolayer culture (humans) or differentiate in 3D culture (mice) into artificial "embryoid bodies", EBs, cystic structures that contain early and preliminary embryonic lineages. ECs are embryonic carcinoma cells which have been found to arise from PGCs that abberantly localize/proliferate to form teratocarcinomas. EGs are embryonic germ cells directly derived from isolated PGCs. Although their pluripotency characteristics are similar to each other, they differ in behavior and their external needs in culture. We will give examples of key studies and try to elaborate how far the *in vitro* studies go in understanding the behavior of cells.

When trophectoderm is removed from human donors or mice, the inhibition on the ESC formation is also removed, and they can grow on a feeder cell layer (usually MEF, mouse embryonic fibroblasts) because they cannot attach to the plates efficiently, or in suspension. ESCs have been used in many mammals up till now (buffaloes, pigs, sheep, mice, rats, humans etc.), and they have been differentiated into many cell types *in vitro* (nerve, cardiac muscle, germ

cells etc.). [Pera et. al., 2000; Zhou et. al., 2010]. As our focus is germ cells, we will maintain the subject there.

Many scientists have attempted to obtain functional, that is, able to produce progeny, germ cells from ESCs, although the choice for oocyte or sperm cell formation depends on conditions. The first successful attempt for male germ cell differentiation came from Toyooka et. al. in 2003, where a mouse vasa homologue (Mvh1) knock-in mouse that expressed GFP or lacZ was used as a marker for the germ cell differentiation from ESCs. Regular PGC pluripotency markers are usually present also in ESCs which show totipotency, so Mvh1, which is solely expressed in germ cells in the mouse, is an appropriate marker. Interestingly, the presence of LIF hindered GFP production. Without LIF, the EB formation yielded GFP-positive cells, but monolayer culture did not. When BMP4 and 8b induction was introduced to the culture by helper cells, culture PGCs increased. After this production, the GFP expressing germ cells were FACS sorted and co-cultured with germ cells derived directly from male gonadal ridges at E12.5-E13.5 and transplanted into a host testis capsule, where they formed testicular tumors separately from the host and went through meiosis to form mature sperms [Toyooka et. al., 2003].

The second successful experiment of male germ cell differentiation came from Geijsen et. al. in 2004, but their approach to the marker problem was different; their basis was that the expression of many markers decreases upon EB differentiation while still expressed in PGCs. So SSEA1 and Oct4 double positive cells would mark either true PGCs or undifferentiated ESCs. RA promotes ESC differentiation and PGC proliferation in culture, and in EB cultures SSEA1-positive cells were still present after a week of culturing with MEF feeder cell layer. Similar to the experiments of Toyooka et. al. (2003), the PGC count peaked at 5 days. Interestingly, a part of the population resembled migratory PGCs. The PGC colonies were then seeded on gelatin coated plates with LIF, SCF and bFGF as an EGC culture, and it was found out that in time, all colonies lost somatic methylation and gained the characteristic pattern of methylation loss of IGF2R in PGCs. Similar to *in vivo* differentiation, hematopoietic cell precursors were located adjacent to the PGC population in EB cysts, indicating that the inductions coming from the microenvironment was still conserved in cystic EB culture conditions. In the EGCs, the male germ cell marker Sry was found. In time, other markers such as Acrosin started being expressed, meaning the specification was more defined between day 7-11 with the presence of GCNF. Moreover, the cells were positive for a male meiosis marker although meiosis seemed to be rather inefficient. Still, the cells positive for Acrosome were already polarized, indicating some level of maturation until round spermatid stage. The next step was the microinjection of these isolated male germ cells into donor oocytes, which were successful in fertilization until either 2-cell stage (50%) or blastocyst stage (20%) [Geijsen et. al., 2004].

The third male gamete derivation experiment came from Nayernia et. al. in 2006, in which the male germ cell markers were Stra8 and and protamine1 (Prm1) which mark premiotic and

haploid male germ cells. The male mice ESC cell line R1 was cultured for stable expression of the two constructs and transgenesis in the animal was not involved. The EGC culture plates were coated with gelatine and cultured without LIF and with RA for successful differentiation and meiotic activity. After successive FACS sorting steps of the cells for both markers it was confirmed that markers such as Mvh1, stella, fragilis, Dazl, c-Kit, Oct4 was also expressed. Moreover, similar to the results of Geijsen et. al. (2004), the Prm1 expressing cells looked motile and presented tail-like structures. Meiotic activity and sperm maturation (acrosin presence and tail-like structures, nuclear condensation) was confirmed. Parental germ cell methylation markers like IGF2R were reduced, although not all that was expected from the data in *in vivo* studies. The male germ cells were then moved to a sperm-depleted testes and successfully completed spermatogenesis. The final experiment was the injection of the Prm1 positive gametes into oocytes. After successful fertilization, two-cell embryos were placed into host oviducts and 12/65 birth rate was observed, although the babies had abnormal size and died after 5 days to 5 months; presumably because of methylation, hence, germ cell reprogramming defects [Nayernia et. al., 2006a]. The same group managed to derive male gametes from bone marrow stem cells as well, but the cells failed to express post-meiotic markers when transplanted into the testes of live mice [Nayernia et. al., 2006b].

Hübner et. al. (2003) was the first group to derive oocytes from ESCs. They used a germ cell-specific reporter Oct4-GFP as a marker for initial germ cell detection. They generated stable ESCs expressing the reporter and they cultured these ESCs without a feeder cell layer and without the usual factors (LIF, SCF etc.). They detected an increasing number of germ cells between day 4 and 8, the peak being 25-40% in the end. Separating these germ cells according to reporter Oct4, endogenous c-kit and endogenous Mvh1 expression, they found evidence that (1) premigratory/early migratory (Oct4+, c-kit+, Mvh1-), (2) early postmigratory (Oct4+, c-kit-, Mvh1+), (3) postmigratory premeiotic (Oct4-, c-kit-, Mvh1+) germ cells were present in this ESC-to-EGC transitionary culture. The same pattern was observed in EGC culture, moreover, some Oct4- c-kit- Mvh1+ cells were found to be aggregated in the supernatant, usually together with follicule-like cells. When these aggregates were cultured, 20% of them succeeded to mature oocytes and secrete estradiol in the culture medium and express GDF9 (required for folliculogenesis *in vivo*). Later on, the presumably "matured" oocytes were let off into the culture supernatant with zona pellucida-like structures surrounding them with the expression of zona pellucida proteins ZP2 and ZP3, but not ZP1. Some oocytes were proven of their capability of going through meiosis I. After longer culturing, morula- and blastocyst-like structures were formed by parthenogenesis [Hübner et. al., 2003].

Lacham-Kaplan et. al. (2006) attempted a new way of deriving oocytes from EBs; they fed the culture medium with testicular cells from newborn mice, which naturally contain hormones very similar to females. EBs were checked for germ cell markers Oct 3/4, Mvh, c-kit, Stella, and DAZL. The most efficient ovarian structures were formed without LIF and with testicular cell

conditioning. Follicular structures helped the formation of early oocytes surrounded by two layers of flattened cells but without zona pellucida. ZP3, Sry and Stra8 was also expressed [Lacham-Kaplan et. al., 2006].

Qing et. al. (2007) connected the methods of Geijsen et. al. (2004) and Lacham-Kaplan et. al. (2006) for deriving oocytes in a different way. EGCs were isolated from day 4 EBs which were grown without LIF and with RA. But instead of the testicular cell boost, the EGC culture was made using high density (50%<) ovarian granulosa cells from newborn mice. In the resulting differentiated cells (PGC and granulosa culture), amongst the markers Nobox (important in folliculogenesis and oocyte-specific gene expression), Figα (important germ cell marker in zona pellucida gene expression), ZP1-2-3 and GDF9, only Nobox was not expressed. Later on, Mvh1 and the meiosis marker SCP3 were confirmed to express together with GDF9 inside the colonies in a relatively big (25 μm) cell, although this seemingly early-oocyte expressed neither ZP2 nor any male germ cell specific marker [Qing et. al., 2007].

The handling of PGCs *in vitro* is not restricted to EB-to-EG differentiation; the differentiation process can also be in reverse. In 1992, Matsui et. al. showed that when PGCs are left to proliferate and differentiate on a feeder cell layer which only expresses membrane associated SCF in the presence of LIF, soluble SCF and bFGF in the culture medium, the resulting cells show pluripotency markers such as SSEA1 and global AP expression, and they form EB-like cystic colonies which do not need outside factors after passaging. When these seemingly de-differentiated colonies were passaged onto STO feeder cell layer, in the classic ESC culturing method, the cells kept their undifferentiated state for a long time (20< passages), although some ECCs were also present with trisomy. When the PGC-derived EBs were injected subcutaneously into mice, teratocarcinomas occurred. And when cells from these ESCs were injected into blastocysts, they contributed to chimerism. Although a 50% chimeric mouse died in 11 days, the 90% chimeric mouse lived at least 12 weeks [Matsui et. al., 1992]. A similar study has been successfully done in testes as well [Conrad et. al., 2008].

PGCs were also attempted to be differentiated into functional gametes after direct derivation. In one study by Shen et. al. (2006), E12.5 mouse fetal ovaries were transplanted under the kidney capsule of a live mouse and “incubated” for oocyte formation and maturation from the premiotic stage germ cells. The full maturation was allowed to be completed *in vitro* by culturing, and the resulting follicular structures and the oocytes were highly similar comparing to the *in vivo* state. After complete maturation with EGF and hCG, the oocytes were fertilized *in vitro* and more than half succeeded to go through 2-cell stage and blastocyst stage, while some were allowed to continue with the pregnancy and biologically normal live offspring was acquired [Shen et. al., 2006]. In another study, sterilized female mice ovaries were delivered with a 6-month GSC culture and ovaries were replenished by oocytes and follicles with normalized fertility and successful offspring production [Zou et. al., 2009]. A similar study of Qing et. al. (2008) reveals

that the synchronization of the somatic (follicle) cell stage and the oocyte stage is crucial for successful oocyte maturation *in vitro* [Qing et. al., 2008].

There have been many studies about the *in vitro* derivation of oocytes and spermatids in mice, which cannot all be spanned by this literature study. Some of them give rise to progeny, some of them do not, **d**epending on the culture conditions. Some research enlightens the view of *in vivo* studies, such as the view on aged ovaries; the premitotic germ cells can be transplanted to young mice and successfully produce oocytes [Niikura et. al., 2009]. Primate studies have also been promising in the last years; ESC-derived germ cells can produce offspring [Fukunaga et. al., 2010].

Nevertheless, in none of the organisms the conditions have been optimized into one successful "gamete generating **m**edium" despite the recent understandings. One reason is the difference between organisms in developmental time and potential; for instance, oogenesis in mice occurs between E12.5 and early postnatal stages, while in humans the developmental span is 12-13 years. ESCs in mice form tightly packaged three-dimensional structures, while human ESCs form two-dimensional colonies with bFGF & ActivinA/TGFβ signaling in effect rather than BMP4 & LIF that is effective in mice [Buecker and Geijsen, 2010].

Another reason for the hardship is that there are many affectors in the process which are not yet well understood. Growth factor interactions, tissue and signaling interactions in embryoid bodies and whether they are close to the *in vivo* state, the exact need for the methylation status specific to PGCs, the related X-inactivation conditions, the involvement of small RNAs such as piRNAs and miRNAs, the exact reasons for the need for helper cells (Sertoli and follicle cells) and how to mimic them in culture... These are only a few comparing to all the unknowns of germ cell differentiation. For the enhancement of experimental success *in vitro*, the marker visualization techniques should be optimized, the drawback that gametes are not able to finish meiosis in culture should be circumvented, the fertility potential of the derived gametes should be improved, and the problematic zona pellucida or the cell layer around the oocytes should be corresponded [Zhou et. al., 2010].

Even so, the tableau does not seem to be dark and the pieces of puzzle are gathering up. The difference in culturing conditions and behavior between humans and mice are also a subject being pressed on. The addition of various growth factors and compounds overrides the need for a feeder cell layer and other somatic cells [Farini et. al., 2005]. Without natural growth factors in the serum, the undifferentiated maintenance of PGCs into an EGC culture occurs [Horii et. al., 2003]. It is also known that SCF and LIF presence in the culture reverses an otherwise apoptotic fate for cultured PGCs and contributes to their survival [Pesce et. al., 1993]. The inhibition of FGF RTK, GSK3β and MEK/ERK signaling helps the ESCs maintain their undifferentiated state, which might mean that the self-renewal of ESCs has a self-sufficiency that is disrupted by

the culture medium and is perceived "cancerous" in case of subcutaneous injection/transplantation in mice [Ying et. al., 2008]. The ectopic expression of Dazl bypasses the need for EB formation as a mediator step for germ cell derivation; it reduces pluripotency by decreasing Nanog and maintains germ cell markers [Yu et. al., 2009]. Akt activation in mouse ESCs leads to a metastatic/migratory ESC-PGC intermediate phenotype and is reversible [Yamano et. al., 2010]. As seen by this small fraction of the growing piles of information, the control over PGC, ESC-to-PGC and PGC-to-ESC cultures is increasing.

6. CONCLUSION

While there is progress on the studies of PGC specification and migration *in vivo* and *in vitro*, some problems remain unsolved. Firstly, the theory that preformation and induction are distinct processes needs to be re-evaluated. There is not enough evidence that a kind of germ plasm does not exist in humans. Maternal factors, as accumulated scientific experience shows, have not been completely understood in mammals because of the lack of techniques present to examine very early embryos. Mitochondrial and granular RNA and protein accumulations are also present in mammals although the exact compositions are not known. The start of PGC specification can only be observed by the morphological changes or the known markers, and the exact start of the differentiation process is not known. Also in culture EGCs highly resemble ECS and various studies need to be made. The related methylation patterns specifying PGCs have not been explored and comparative studies have not been made for lower organisms. A systems biology perspective would give more data about markers and DNA-protein or protein-protein interactions leading to PGC specification and migration.

On the bright side, culturable PGCs and ESCs have been promising for fertility treatments and even cancer. When EC cultures are understood deeper, the related cures might be experimented clinically as well. From the aging studies, it is already proven that the remaining premiotic germ cells can be "revived" with the appropriate somatic cell microenvironment, that is, by transplantation to the young. If meiosis can be re-started with the learned necessary factors, aged or prematurely aged ovaries can be functional again by gene therapy.

The ethics side of the issue, however, is controversary. Human pluripotent stem cell sources are bound to be limited. As donated germ or embryonic stem cells, even tissues can be differentiated and de-differentiated in culture, virtually endless progeny can be produced from one donor material. This may lead to the choice of babies, which everybody likes to debate in the last years. The cultures have the "potential" to be alive, but will they be accepted so in the future? Or will the governmental and/or social restrictions cause science to achieve its goal and slow down? Still, research has more probability to cure patients than create a clone army in the

next following years, and the new developments are leading rapidly towards clinical applications.

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