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► To cite this version:

Harry Moore, Ramya Udayashankar, Behrouz Aflatoonian. Stem Cells for Reproductive Medicine. Molecular and Cellular Endocrinology, 2008, 288 (1-2), pp.104. <10.1016/j.mce.2008.02.019>. <hal-00532007>

HAL Id: hal-00532007

<https://hal.science/hal-00532007v1>

Submitted on 4 Nov 2010

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HAL Authorization

Accepted Manuscript

Title: Stem Cells for Reproductive Medicine

Authors: Harry Moore, Ramya Udayashankar, Behrouz Aflatoonian

PII: S0303-7207(08)00107-X
DOI: doi:10.1016/j.mce.2008.02.019
Reference: MCE 6832

To appear in: *Molecular and Cellular Endocrinology*

Received date: 28-11-2007
Revised date: 19-2-2008
Accepted date: 25-2-2008

Please cite this article as: Moore, H., Udayashankar, R., Aflatoonian, B., Stem Cells for Reproductive Medicine, *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2008.02.019

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Stem Cells for Reproductive Medicine

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Keywords: Embryonic stem cells, primordial germ cells, gametes, trophoblast

Abbreviation of cell types

BM – bone marrow

CTBS – cytotrophoblast stem.

ES – embryonic stem; mES – mouse embryonic stem; hES – human embryonic stem.

EG – embryonic germ.

GSC – germline stem cell; maGSC – mouse adult germline stem cell

PGC – primordial germ cell.

SSC- spermatogonial stem cell.

Abstract

The generation of various pluripotent stem cell lines provides a new route to investigate developmental process of germ cell and embryo development, which until now was difficult to access in the human. In the future these cells may be used for new therapies in reproductive medicine. This brief review outlines the development of germ cells and their pluripotent capabilities, how embryonic and germline stem cells can mimic developmental processes *in vitro* and generate gamete and trophoblast phenotypes for research and potential treatments.

1. Introduction

The last 30 years has seen major advances in the treatment of infertility with the introduction of such treatments as hormonal induction of follicular development and ovulation; *in vitro* fertilisation and embryo transfer; intracytoplasmic sperm injection; and embryo and egg cryopreservation. For many patients, a combination of these assisted conception procedures can bypass critical physiological barriers such as spontaneous ovulation, fertilisation and embryo transport to alleviate infertility. However, when there is a primary failure of germ cell production as a result of developmental abnormalities, age, disease or toxic insult (e.g. chemotherapy), or embryo implantation failure, the clinician is often severely hampered in providing an effective treatment. In the last few years, many investigations have moved to

understanding the basis of early germ cell development with the hope that in time, treatments can be developed that stimulate *de novo* gamete development from the primary stem cells to rescue the gonad. Moreover, a new paradigm for studying the very earliest stages of germ cell commitment and differentiation, or early stages of trophoblast and placental differentiation has been created by the finding that embryonic stem (ES) cells can differentiate to a germ cell lineage and early gametes, and to trophoblast lineages in culture. It is anticipated that this research will provide a more accessible route to investigate some of the origins of human gonadal dysfunction (e.g. testicular cancer, early menopause), the effects of environmental chemicals on germ cells and embryos (Skakkebaek *et al.*, 2001; Anway *et al.*, 2005) and the developmental processes of early placentation. It also raises prospects of novel treatments for infertility sometime in the future.

The relationship between germ cells and embryos *in situ* and pluripotent stem cells *in vitro* has become increasingly complex as germ cell lines at different stages of development are demonstrated to have pluripotent/multipotent potential and show germ cell differentiation (figure 1).

2. The Primordial Germ Cell

The primordial germ cell (PGC) is the primary undifferentiated stem cell of spermatozoa and oocytes. Since PGCs provide the fundamental hereditary link from one generation to another, they are in this respect both immortal and totipotent. Early in evolution, unicellular organisms segregated immortal germplasm from the mortal soma probably as a way of protecting the fidelity of the genome when nutrients are scarce. This evolutionary legacy is still manifest in mammals today in the early foetal segregation of PGCs in extra embryonic tissue (Donovan & de Miguel, 2003;

McLaren, 2003), which subsequently migrate to the gonadal anlagen and differentiate to the gonocytes; progenitor cells committed to form oocytes or spermatozoa depending on the genetic sex of the individual. In the female, the gonocyte surrounded by a cortical interstitial layer initiates meiosis and becomes a primary oocyte and follicle, thereafter ending precursor proliferative potential. In the male, the gonocyte surrounded by the foetal sex cord of the gonadal ridge (pre-seminiferous tubules) arrests in G0/G1 of mitosis as a prospermatogonium but maintains a proliferative precursor potential. Following birth, prospematogonia migrate to the basement membrane of the seminiferous tubule and differentiate into spermatogonial stem cells (SSCs) with self-renewal capacity. Therefore, PGCs have a relatively short window of proliferative activity although they can be transformed *in vitro* to embryonic germ cells with prolonged proliferative and pluripotent capacity (figure 1) as is also the case for some of the later stages of germline stem cells.

While ethical constraints limit our knowledge of the specification of human PGCs, it is clear that common signalling pathways operate across mammals and possibly all vertebrates (Donovan & de Miguel, 2003). In the mouse, PGCs originate in the proximal epiblast and by 6.25 days post conception (d.p.c.) a small germline founder population can be identified that express the protein Blimp1 (B-lymphocyte-induced maturation protein 1, Oninata *et al.*, 2005; Vincent *et al.*, 2005; McLaren & Lawson, 2005). Blimp1 was initially identified as a transcriptional repressor that enables the further differentiation of immunoglobulin-secreting plasma cell by inhibiting the expression of genes involved in alternative B-cell development. Mutant null-allele mice lacking *Blimp1* generate very few PGCs, and any that do still develop lack the normal migratory behaviour towards the genital ridge (Vincent *et al.*, 2005). Germ

cell competence is induced in response to signals secreted by extra embryonic ectoderm including the synergistic action of the growth factors; particularly bone morphogenic proteins (BMP). In their mature dimeric form BMPs bind and then signal through heteromeric receptor complexes and downstream SMAD proteins (Shimasaki *et al.*, 2004). Induction of PGCs seems to require BMP4 or BMP8b in combination possibly indicating that signalling for various BMPs occur through separate receptor complexes.

The genes *fragilis* and *stella* also appear to have major roles in germ cell competency and development. *Fragilis* is a trans-membrane protein and a member of a large interferon- inducible family of genes that is evolutionarily conserved with human homologues. A characteristic of interferon –inducible proteins is their anti-proliferative function and *fragilis* may serve to increase the length of the cell cycle in PGCs. During induction of germ cell fate, there is only a transient expression of *fragilis* but this gene is also expressed in ES cells and embryonic germ (EG) cells indicative of a potential role in pluripotency status (Saitou *et al.*, 2002). Likewise, the gene *stella* may have a function during the development of pluripotency. It is expressed in the oocyte, through pre-implantation embryo development and in germ cell tumours (Payer *et al.*, 2003). *Stella*-positive germ cells exhibit a repression of homeobox genes, which is probably important in order to escape a somatic cell fate (Saitou *et al.*, 2002). Transgenic mice expressing a green fluorescent protein (GFP) – *stella* reporter transgene can be utilised to accurately follow PGC development (Payer *et al.*, 2006).

A number of other factors have been implicated in PGC derivation and maintenance (figure 2). The tyrosine-kinase receptor c-kit and its ligand, stem cell factor (SCF), are essential for the maintenance of PGCs in both sexes. In the adult testis, the c-kit receptor is re-expressed in differentiating spermatogonia, but not in spermatogonial stem cells, whereas SCF is expressed by Sertoli cells under FSH stimulation (Rossi *et al.*, 2000). Also the POU domain transcription factor Oct4 has been shown to have a role in PGC survival. This transcription factor is a 'gatekeeper' for maintaining pluripotency in the inner cell mass cells of the blastocyst and in ES cells. By use of conditional gene targeting with the Cre/LoxP system, Kehler and co-workers (2004) showed that loss of Oct4 leads to apoptosis (Kehler *et al.*, 2004). In keeping with their pluripotent capability, Oct4 is strongly expressed in migrating PGCs in human foetal tissue as well as in human germ cell tumours and EG cells (Looijenga *et al.*, 2003; Rajpert-De Meyts *et al.*, 2004). On the other hand, Oct4 expression is down regulated rapidly in the human female gonad and silenced as oocytes enter the first meiotic prophase. In contrast, the equivalent process occurs much more gradually in the male with Oct4 expression often persisting in some gonocytes and spermatogonia of neonates and infants. This intriguing observation has prompted the suggestion that differential germ cell expression of Oct4 between the sexes might contribute to the fact that germ-cell derived cancer is much higher in men than women (Rajpert-De Meyts *et al.*, 2004).

Other markers of germ cells with a wider expression include *vasa* and *DAZL*. Mouse vasa homologue (mvh) protein is exclusively expressed in PGCs just after their colonization of embryonic gonads and in germ cells undergoing gametogenic processes until the post-meiotic stage in both males and females (Toyooka *et al.*,

2000). Men with deletions encompassing the Y - chromosome *DAZ* (deleted in Azoospermia) genes have few or no germ cells, indicating they are defective in the formation or maintenance of germ cells. A *DAZ* homolog, *DAZL* (*DAZ-Like*), found in diverse organisms, including humans is necessary for germ cell development in males and females. Significantly, *PUM2*, a human homologue of pumilio, a protein required to maintain germ line stem cells in *Drosophila* and *Caenorhabditis elegans*, forms a stable complex with *DAZ* (Moore *et al.*, 2003), again suggesting mechanisms of germ cell development are highly conserved.

3. Germ cells from ES cells

While the pluripotency of ES cells has been well established for many years it came as a revelation that murine ES (mES) cells could develop into PGCs *in vitro* and with extended culture could occasionally form early spermatids or oogonia (Hubner *et al.*, 2003; Geijsen *et al.*, 2004). In retrospect, differentiation to PGCs might be expected as these cells develop so early in foetal development. However, given the complexity of gametogenesis it is surprising that formation of either early spermatids, or ovarian-like structures and oocytes (that subsequently developed to blastocyst-like structures) can occur (Hubner *et al.*, 2003; Geijsen *et al.*, 2004; Lacham-Kaplan *et al.*, 2006). Since these germ cell derivatives do not originate from true gonadal structures there is an imperative to properly characterise biological function. Mouse ES cell-derived spermatids were tested *in vitro* by using ES cells initially carrying an Oct 4 –promoter driven green fluorescent protein (GFP) reporter gene which enabled the tracking of these cells (Hubner *et al.*, 2003; Geijsen *et al.*, 2004). When haploid spermatids generated from these GFP-ES cells were isolated by flow-cytometry (fluorescent activated cell sorting, FACS) and injected intracytoplasmically into oocytes the

diploid chromosome complement was restored and green fluorescent blastocysts generated. In a different approach, Noce and coworkers (Toyooka *et al.*, 2003) showed that *Mvh-LacZ* murine germ cells (transgene with germ cell- specific mouse vasa homolog and *LacZ* reporter) generated *in vitro* from mES cells could also develop to some extent *in vivo*. These cells were aggregated with day 12.5 -13.5 (d.p.c) gonadal tissue at the time of initial testis development which formed seminiferous tubules within 6-8 weeks when co-transplanted under the testis capsule. Such tubules (which remained separate from host testis tubules) participated in spermatogenesis to produce elongated β -gal-stained spermatids. This demonstrated that such *mvh-lacZ* ES cells formed PGCs with developmental potential to integrate into a somatic epithelium and undergo meiosis to form early sperm cells. However the functional competence of these cells remained to be tested. Instead, Nayernia and coworkers (Nayernia *et al.*, 2006) recently produced viable transgenic offspring from sperm produced from mES cells using a two-stage culture system. First mES cells were transfected with the reporter gene *Stra8-EGFP*, a retinoic acid (RA) responsive gene expressed in pre-meiotic mouse germ cells and fused with enhanced green fluorescent protein gene construct. After an initial mES culture, green cells marking a pre-meiotic germ stage were isolated by FACS and cultured with RA as a proliferative cell line. These cells were then transfected with a second fusion reporter gene *Prm1-dsred* (protamine1 gene fused to red fluorescent protein gene construct) to subsequently identify post-meiotic sperm cells after further RA induction, which were isolated and microinjected into mouse eggs. Embryos generated in this way and transferred to recipient females produced viable offspring although they displayed abnormalities that resulted in premature death (5 days to 5 months). This may have

been due to inappropriate epigenetic control mechanisms during *in vitro* development of the germ cells.

Investigations with human ES (hES) cells are more preliminary but they also show that spontaneous or induced differentiation in culture can generate cells displaying mRNA expression profiles and cell surface markers consistent with PGCs (Clark *et al.*, 2004, Clark & Reijo Pera, 2006) and of subsequent meiosis with on rare occasions early spermatid identification after the appropriate culture conditions (Aflatoonian *et al.*, 2005). Interestingly, markers of female germ cells are expressed in both XX and XY cells from murine and human ES cell culture, (Toyooka *et al.*, 2003; Geijsen *et al.*, 2004). The prevailing hypothesis has been that pathways intrinsic to the cell rather than inductive signals regulate PGC development. Thus, whatever the complement of sex chromosomes, germ cells are cell – autonomous and intrinsically programmed to undergo meiosis, enter prophase and develop as oocytes, unless otherwise prevented from doing so by a meiosis inhibiting factor(s) (McLaren, 2003). Under these circumstances it is perhaps unsurprising for both male and female ES cell lines to display female germ cell markers since culture conditions may be sub-optimal and lack meiosis inhibition. However, recent studies (Bowles *et al.*, 2006; Koubova *et al.*, 2006) indicate that instead of an intrinsic programme to enter meiosis, germ cells can respond to the external signal of RA and its metabolism. Thus, in the embryonic mouse ovary, RA induces germ cells to express the pre-meiotic marker *Stra-8* (stimulated by retinoic acid 8) and initiate meiosis. On the other hand, in the embryonic mouse testis RA is metabolised and inactivated by the P450 enzyme CYP26 (B1) thereby preventing early germ cell entry into meiosis. Down-regulation of genes such as *Scp3* (synaptonemal complex protein; associated with meiotic

events) occurs with induction into the alternative pathway of mitotic arrest as G0/G1 prospermatogonia. In this case, the induction of presumptive PGCs into meiosis in culture medium containing RA might be expected although local concentrations within cell aggregates could differ significantly and affect the timing. Concentrations of RA in plasma or serum (or serum replacement) that supplement ES cell culture medium are normally in the 2 – 8 nM range (Eckhoff & Nau, 1990). This level is much lower than the 1 –5 μ M concentration often used with induction protocols but the sensitivity of cells to RA can vary considerably depending on composition of medium.

This process of sex-specific germ cell determination seems even more dysregulated with hES cells. Markers of both male and female germ cell development have been detected regardless of the sex of the cell line (Clark *et al.*, 2004; Aflatoonian *et al.*, 2005). The inference from these findings is that RA or possibly other factors affecting meiosis and gamete determination are present in the culture conditions possibly generated by male and female hES cells. For male germ cell development *in vivo*, functional seminiferous epithelium with Sertoli cells is necessary and the development of these cells is dependent normally on expression of the transcription factor SRY (Sex determining Region on the Y chromosome), which triggers a complex gene expression cascade leading to testicular differentiation. Hence, the generation of presumptive male germ cells with XX hES cells would require either an artifactual differentiation of Sertoli cells, for example, by female supporting cells differentiating to a Sertoli-like phenotype (Adams & McLaren, 2002) and/or production of meiosis inhibitor factors (or metabolised in the case of RA) from a different origin. Testicular tissue differentiation can be observed in 46,XX

individuals lacking SRY due to over expression of SOX9 (closely related to SRY) but other unknown genes may also trigger testis development (Vidal *et al.*, 2001; Maciel-Guerra). While during such development *in vivo* the presence of two X chromosomes will inhibit spermatogenesis this is not necessarily the case *in vitro* where germ cells may be exposed to a whole range of factors either from medium supplement (i.e RA) or from other cell phenotypes (steroidal hormone – see below) to promote spermatogenesis. Of note in this regard are vitamin A deficient mice, where only spermatogonia and Sertoli cells populate the seminiferous epithelium. The addition of retinoids such as RA and retinol can induce the resumption of spermatogenesis in these animals (Baleato *et al.*, 2005).

3.1 The Germ Cell niche and ES Cell culture

An intriguing aspect of the generation of ES cell-derived germ cells *in vitro* is how normal checkpoints of the developmental process which would naturally span a timeline from early foetal development to puberty and lead to arrest of cells at different stages is apparently overcome and compressed into a relatively much short culture period. How ES cell cultures can mimic the somatic environment that encapsulates either developing oogonia (the follicle) or the spermatogonial stem cell (seminiferous epithelium) is largely unknown. The appropriate growth factor and hormonal microenvironment required to support and sustain such complex niches probably depends on the type of culture system adopted for the differentiation process. ES cells are usually cultured in a combination of two basic methods. Monolayer adherent cultures of ES cells can be allowed to differentiate directly to form an appropriate niche or ES cells can be induced to aggregate to form embryoid bodies that form a more three-dimensional micro-environment. In conjunction with

these two protocols, ES cells can be initially co-cultured with feeder layers (i.e. mouse embryonic fibroblasts, neonatal gonadal cell culture) or conditioned-medium from feeder cells and various growth factor and serum supplementation. Generally, these culture conditions attempt to create an environment conducive for germ cell proliferation and differentiation but they are not specific. Aside from the absence of a defined culture medium, the various procedures for passaging ES cells (i.e. to single cells or as clumps), the influence of feeder cells and extracellular matrix (e.g. Matrigel) and the cell line itself can all contribute to proportion of germ cells specified, if at all. If a phenotype is not selected (i.e. using fluorescent reporter systems) then only a small proportion of cells (usually less than 5%) will normally display primordial germ cell or gonocyte /spermatogonia markers but the fact that some cells progress further to a post-meiotic stage at first sight does seem extraordinary given the complex hormonal requirements of gamete development. However, there can be considerable intrinsic capacity of cells to organise tissue. Foetal and neonatal rodent and pig gonadal tissue demonstrates great propensity for self-reorganisation when single cell suspensions are transplanted under the skin of immunodeficient mice (Honaramooz *et al.*, 2007; Kita *et al.*, 2007). Extensive rearrangement of cells can regenerate complete functional testis tissue with somatic cells and germ cells forming the endocrine and spermatogenic compartment of the testis. Such processes require hormones and it is of note that oestradiol production (50-100 pg/ml) was detected after 12 days in mES cell cultures that generated follicle-like structures (Hubner *et al.*, 2003) while appreciable levels (60 pg/ml) of dihydrotestosterone can be detected in hES cell cultures that generate PGCs and spermatids (Aflatoonian and Moore, unpublished). Presently, the source of steroidogenesis is unclear but some may arise from gonadal cell types (granulosa,

Leydig cells) that co-differentiate with germ cells. This appeared to be case for oocytes generated from mES cells since increased oestradiol levels correlated with increased proportion of cells resembling a follicular morphology (Hubner *et al.*, 2003). Alternatively, sex steroids may be produced extopically from non-reproductive cell phenotypes (e.g. liver or adrenal cells) that could conceivably differentiate from ES cells in culture at the same time as germ cells.

In the mouse, pluripotency of ES cells is mirrored also by EG cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992) and we can now include a population of germline stem cells from the culture of spermatogonial stem cells (SSCs) from the neonatal mouse testis (Kinatsu-Shinohara *et al.*, 2004); and perhaps more remarkably cells from the adult mouse testis (Guan *et al.*, 2006). These adult SSCs can be transformed in culture to ES-like cell lines displaying pluripotency both *in vitro* and significantly *in vivo*. SSCs are generated during early testis development from the gonocyte and they continue to self-renew and subsequently form spermatogonia committed to sperm production within a post-pubertal hormonal testis environment (additional issue cross-reference to be inserted). That SSCs represent the true stem cell of the testis has been shown by a number of transplantation experiments where SSCs are infused into testes of infertile recipients. Each colony of spermatogenic cells usually arises from a single SSC and when fertility is restored, offspring result from spermatozoa originating from the donor cells. Since SSCs represent a small proportion of cells in the normal adult testis (1 in 3,000 – 4,000 cells in the mouse testis) and a short development stage in the process of germ cell development from ES cells, it is clearly important to develop methods to isolate and proliferate SSCs *in vitro* as a distinct cell population or line. Recent studies suggest that mouse testis cells selected by flow cytometry on the basis

of MHC class 1⁻ / Thy⁻1⁺ are enriched for SSCs. When co-cultured in serum-free conditions using STO feeder cells and supplemented with glial cell line-derived neurotrophin factor (GDNF), the GDNF-family receptor [alpha]-1 and basic fibroblast growth factor (bFGF) (Hamra *et al.*, 2005; Ryu *et al.*, 2005) then colonies of SSCs proliferate which can be maintained for several months in culture and retain the ability to reconstitute normal spermatogenesis if transplanted to a suitable recipient.

When injected into mouse pre-implantation blastocysts, adult germline stem cells (maGSCs) contributed to the development of all the major organs and displayed germline transmission (Guan *et al.*, 2006). Moreover, if GFP-labelled maGSCs are mixed with a single cell suspension of testicular cells of rodents and transplanted under the skin of recipients, they integrate in newly formed reconstituted tubules and differentiate beyond meiosis into spermatids, which produce normal offspring by the microinjection into oocytes. The obvious conservation in many of the mechanisms for germ cell development in mammals suggests that it might be possible to develop similar cell lines from human adult spermatogonial populations although it may not be straightforward to translate from the murine to the human system. While human EG cell lines have been reported (Shamblott *et al.*, 1998), their proliferative and pluripotent capacity currently as true EG cells has been questioned (Turnpenny *et al.*, 2003; Aflatoonian & Moore, 2005).

Whereas progenitor SSCs generated from ES cells create the opportunity to separate the initial germ cell differentiation from subsequent proliferative and spermiogenesis steps, this is not possible with the germ cell committed to form an oocyte as it is destined to cease proliferation and enter meiotic arrest. Thus, to create large numbers

of oocytes from ES cells requires a process of either selecting primordial follicles directly or temporarily inhibiting the progress of meiotic arrest so that the pool of potential oocytes can be increased prior to the generation of primordial germ cells. However even if this process was attainable it remains a considerable challenge to mature primordial follicles in culture. There has been some success in culturing human primordial follicles up to secondary stages in organ cultures using tissue slices within extracellular matrix (Hovatta *et al.*, 1997). Secondary follicles with several layers of granulosa cells around the oocyte are frequently seen in these cultures, but early antral follicles only occasionally.

4. Reprogramming adult somatic cells to germ cells and stem cells

While the evidence is becoming more compelling that in culture ES cells can generate PGCs and germ cells, there remains the question as to whether germ cells can be derived (or transdifferentiated) from adult stem cells residing outside the gonad? There are reports that germ cells can be derived from bone marrow (BM) and peripheral blood cells (Johnson *et al.*, 2004; 2005; Nayernia *et al.*, 2006b) and BM-derived germ cells can repopulate the ovarian follicular reserve (Johnson *et al.*, 2004; 2005). However, the latter investigations have not been substantiated (Eggan *et al.*, 2006). Germ cell derivation from pig foetal skin cells *in vitro* has also been reported (Dyce *et al.*, 2006) with formation of morphological structures resembling follicles, that secrete oestradiol and progesterone; and oocyte-like cells displaying markers of germ cells (oct4, vasa, GDF9b) or exhibiting the meiosis marker, Scp3 and displaying apparent parthenogenetic embryo structures. Provided these report of transdifferentiation are fully corroborated, they indicate that PGC and germ cell formation *in vitro* and possibly *in vivo* is much more plastic than previously believed.

However, it is very difficult to reconcile some of these observations with known reproductive physiology in mammals and an alternative explanation is that detection of many germ cell markers is due to aberrant expression or detection in culture and does not correspond to true germ cell phenotype. A wide battery of specific protein and functional phenotypic assays will be necessary to fully resolve this issue.

Finally, perhaps the most significant finding since the derivation of hES cells has been the recent discovery that overexpression of a relatively small number of 'pluripotency' genes by retroviral transfection of adult or foetal fibroblasts can lead to efficient reprogramming of a small proportion cells to an ES cell equivalent state in both the mouse and human (Okita *et al.*, 2007; Takahashi *et al.*, 2007; Yu *et al.*, 2007). These induced pluripotency stem (iPS) cells can be subsequently isolated and cultured as a cell line providing a relatively direct route to producing person-specific stem cells with high pluripotency capabilities. If methods can be perfected that enable these iPS cells to be generated so the health risk is minimal (i.e without use of retrovirus or oncogene) then genetically matched cells may be produced by a simpler and much more effective means than using human eggs.

5. Trophoblast stem cells from ES cells

As well as using hES cells for investigating germ cell development, the findings that trophoblast cell phenotypes can also be generated *in vitro* means that early placentation events can also be investigated. An effective embryonic–maternal interaction is crucial for successful human pregnancy. Failure of this process is a major cause of infertility resulting in recurrent miscarriage, foetal retardation and pre-eclampsia but of course investigation of the earliest stage of human gestation is

severely constrained by ethical and practical considerations. HES cells differentiate spontaneously to trophoblast-like cells in culture (Thomson *et al.*, 1998; Draper *et al.*, 2002; Gerami-Naini *et al.*, 2004) but these cells are terminally differentiated and display a limited proliferative capacity. Hence the production of distinct cytotrophoblast stem cell (CTBS) lines isolated from hES cells represents an important advance (Harun *et al.*, 2006). These CTBS cells displayed typical cyto- and syncytio-trophoblast characteristics and exhibited further differentiation to invasive endovascular cell phenotype allowing them to be used to mimic the early invasive stages of implantation. Whether human CTBS cells originate from the immediate trophectoderm or following later extra embryonic differentiation remains to be resolved. However, trophectodermal stem cells have been isolated recently from rhesus monkey blastocyst (Vandevoort *et al.*, 2007) and if similar human trophectodermal stem cells could be generated, this raises the question whether human trophoblast/ES chimeras could be constructed to also mimic early embryo development.

6. Epigenetic Mechanisms

Notwithstanding that embryonic stem cells can undergo differentiation to PGCs and possibility early gametes there is the added complexity of a range of epigenetic processing required for germ cell development (Allegrucci *et al.*, 2005). Female PGCs reactivate their silenced X chromosome thereby allowing each oocyte to have an active X chromosome. However this characteristic is not exclusively confined to female PGCs as XXY male germ cells also may have this capability (Mroz *et al.*, 1999). Moreover, as PGCs enter the gonads they become hypomethylated resulting in a loss of imprinting and biallelic gene expression. Recent evidence suggests that cell

demethylation at this stage may control the expression of genes required for gametogenesis (Maatouk *et al.*, 2006) but exactly how such processes might operate in embryonic stem cell differentiation to germ cells in culture remains unclear.

7. Summary

HES cells are a powerful tool to investigate developmental processes in human reproduction and it seems fitting if research leads to new therapies to treat reproductive disorders given that hES cell lines are the result of altruistic donation of embryos for research from couples attending infertility clinics. While the potential creation of germ cells, gametes and trophoblast via stem cell routes raises important ethical questions, this should not preclude research in this area if suitably regulated. Rapid advances in the maintenance and proliferation of various germline stem cells produced from hES cells or from gonadal sources along with refined transplantation techniques provide new opportunities to treat primary reproductive conditions which to date have been refractory to treatment. Moreover, the manipulation of germline stem cells provides a new route eventually to gene therapy applications (Brinster, 2002; Kubota and Brinster, 2006).

References

- Adams, I.R., McLaren, A., 2002 Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* 129, 1155-1164.
- Aflatoonian, B., Moore, H., 2005. Human primordial germ cells and embryonic germ cells, and their use in cell therapy. *Curr. Opin. Biotech.* 16, 530-535.
- Aflatoonian, B., Fazeli, A., Ruban, L., Andrews, P., Moore, H., 2005 Human embryonic stem cells differentiate to primordial germ cells as determined by gene expression profiles and antibody markers. *Proc. 21st Annual Meeting of the European Society for Human Reproduction and Embryology, Copenhagen.* Hum. Reprod. 20, suppl 1 i6.
- Allegrucci, C., Thurston, A., Lucas, E. and Young, L., 2005 Epigenetics and the germline. *Reprod.* 129, 137-149.
- Anway, M.D., Cupp, A.S., Uzumcu, M. and Skinner, M.K. (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* 308: 1466-1469.
- Baleato, R.M., Aitken, R.J., Roman, S.D., 2005 Vitamin A regulation of BMP4 expression in the male germ line. *Develop. Biol.* 286, 78-90.
- Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M.J., Rossant, J., Hamada, H., Koopman, P., 2006. Retinoid Signaling Determines Germ Cell Fate in Mice. *Science* 312, 596-600.

Brinster, R.L., 2002 Germline stem cell transplantation and transgenesis. *Science* 296, 2174-2176.

Clark, A.T., Bodnar, M.S., Fox, M., Rodriguez, R.T., Abeyta, M.J., Firpo, M.T., Pera, R.A.. 2004. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum. Mol. Gen.* 13, 727-739.

Clark, A.T., Reijo Pera, R.A., 2006. Modeling human germ cell development with embryonic stem cells. *Regen. Med.* 1, 85-93.

Donovan, P.J., de Miguel, M.P., 2003. Turning germ cells into stem cells. *Curr. Opin. Genet. Develop.* 13, 463-471.

Draper, J.S., Moore, H.D., Ruban, L.N., Gokhale, P.J., Andrews, P.W., 2004. Culture and characterization of human embryonic stem cells. *Stem Cell Develop.* 13, 325-336.

Dyce, P.W., Wen, L., Li, J., 2006. In vitro germline potential of stem cells derived from foetal porcine skin. *Nature Cell Biol.* 8, 384-390.

Eckhoff, C., Nau, H., 1990. Identification and quantitation of all-*trans*- and 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in human plasma. *J. Lipid Res.* 31, 1445-1454.

Eggan, K., Jurga, S., Gosden, R., Min, I.M., Wagers A.J., 2006. Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* 441, 1109-1114.

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., Daley, G.Q., 2004. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427, 106-107.

- Gerami-Naini, B., Dovzhenko, O.V., Durning, M., Wegner, F.H., Thomson, J.A., Golos, T.G., 2004. Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. *Endocrinol.* 145, 1517-1524.
- Guan, K., Nayernia, K., Maier, M.S., Wagner, S., Dressel, R., Lee, J.H., Nolte, J., Wolf, F., Li, M., Engel, W., Hasenfuss, G., 2006. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 440, 1199-1203.
- Hamra, F.K., Chapman, K.M., Nguyen, D.M., Williams-Stephens, A.A., Hammer, R.E., Garbers, D.L., 2005. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. *Proc. Nat. Acad. Sci., U S A.* 102, 17430-17435.
- Harun, R., Ruban, L., Martin, M., Draper, J., Jenkins, N.M., Liew, G.C., Andrews, P.W., Li, T.C., Laird, S.M., Moore, H.D., 2006. Cytotrophoblast stem cell lines derived from human embryonic stem cells and their capacity to mimic invasive implantation events. *Hum. Reprod.* 21, 1349-1358.
- Honaramooz, A., Megee, S.O., Rathi, R., Dobrinski, I., 2007. Building a testis: formation of functional testis tissue after transplantation of isolated porcine (*Sus scrofa*) testis cells. *Biol Reprod.* 76, 43-47.
- Hovatta O., Silye, R., Abir, R., Krausz, T., Winston, R.M., 1997. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod.* 12, 1032-1036.

Hubner, K., Fuhrmann, G., Christenson, L.K., Kehler, J., Reinbold, R., De La Fuente, R., Wood, J., Strauss, J.F. 3rd, Boiani, M., Scholer, H.R., 2003. Derivation of oocytes from mouse embryonic stem cells. *Science* 300, 1251-1256.

Johnson, J., Canning, J., Kaneko, T., Pru, J.K., Tilly, J.L., 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* 428, 145-150.

Johnson, J., Bagley, J., Skaznik-Wikiel, M., Lee, H.J., Adams, G.B., Niikura, Y., Tschudy, K.S., Tilly, J.C., Cortes, M.L., Forkert, R., Spitzer, T., Iacomini, J., Scadden D.T., Tilly, J.L., 2005. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* 122, 303-315

Kita, K., Watanabe, T., Ohsaka, K., Hayashi, H., Kubota, Y., Nagashima, Y., Aoki, I., Taniguchi, H., Noce, T., Inoue, K., Miki, H., Ogonuki, N., Tanaka, H., Ogura, A., Ogawa, T., 2007. Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. *Biol Reprod.* 76, 211-217.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., Toyoshima, M., Niwa, O., Oshimura, M., Heike, T., Nakahata, T., Ishino, F., Ogura, A., Shinohara, T., 2004. Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 119, 1001-1012

Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomeli, H., Nagy, A., McLaughlin, K.J., Scholer, H.R., Tomilin, A., 2004. Oct4 is required for primordial germ cell survival. *EMBO*. 5, 1078-1083.

Kubota, H., Brinster, R.L., 2006. Technology Insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Endocrinol. Metab.* 2, 99 – 108.

Lacham-Kaplan, O., Chy, H., Trounson, A., 2006. Testicular cell conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes. *Stem Cells* 24, 266-273.

Koubova, J., Menk, D.B., Zhou, Q., Capel, B., Griswold, M.D., Page, D.C., 2006. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc. Nat. Acad. Sci., U S A.* 103, 2474-2479.

Looijenga, L.H., Stoop, H., de Leeuw, H.P., de Gouveia Brazao, C.A., Gillis, A.J., van Roozendaal, K.E., van Zoelen, E.J., Weber, R.F., Wolffenbuttel, K.P., van Dekken, H., Honecker, F., Bokemeyer, C., Perlman, E.J., Schneider, D.T., Kononen, J., Sauter, G., Oosterhuis, J.W., 2003. POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Res.* 63, 2244-2250.

Maatouk, D.M., Kellam, L.D., Mann, M.R., Lei, H., Li, E., Bartolomei, M.S., Resnick, J.L., 2006. DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages. *Develop.* 133, 3411-3418.

Maciel-Guerra, A.T., de Mello, M.P., Coeli, F.B., Ribeiro, M.L., Miranda, M.L., Marques-de-Faria, A.P., Baptista, M.T., Moraes, S.G., Guerra-Júnior, G., 2007. XX maleness and XX true hermaphroditism in SRY-negative monozygotic twins: additional evidence for a common origin. *J Clin Endocrinol Metab.* Dec 4; [Epub ahead of print]

Matsui, Y., Zsebo, K., Hogan, B.L., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 70, 841-847.

McLaren, A., Lawson, K.A., 2005. How is the mouse germ-cell lineage established? *Diff.* 73, 435-437.

McLaren, A., 2003. Primordial germ cells in the mouse. *Develop. Biol.* 262, 1-15.

Moore, F.L., Jaruzelska, J., Fox, M.S., Urano, J., Firpo, M.T., Turek, P.J., Dorfman, D.M., Pera, R.A., 2003. Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with DAZ (Deleted in AZoospermia) and DAZ-like proteins. *Proc. Nat. Acad. Sci., U S A.* 100, 538-543.

Mroz, K., Carrel, L., Hunt, P.A., 1999. Germ cell development in the XXY mouse: Evidence that X chromosome reactivation is independent of sexual differentiation. *Develop. Biol.* 207, 229-238.

Nayernia, K., Nolte, J., Michelmann, H.W., Lee, J.H., Rathack, K., Drusenheimer, N., Dev, A., Wulf, G., Ehrmann, I.E., Elliott, D.J., Okpanyi, V., Zechner, U., Haaf, T., Meinhardt, A., Engel, W., 2006. In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. *Develop. Cell* 11, 125-132.

Nayernia, K., Lee, J.H., Drusenheimer, N., Nolte, J., Wulf, G., Dressel, R., Gromoll, J., Engel, W., 2006b. Derivation of male germ cells from bone marrow stem cells. *Lab. Invest.* 86, 654-663.

Ohinata, Y., Payer, B., O'Carroll D., Ancelin, K., Ono, Y., Sano, M., Barton, S.C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., Surani, M.A., 2005.

Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 436, 207-213.

Okita, K., Ichisaka, T., Yamanaka, S. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature* 448, 313-317.

Payer, B., Saitou, M., Barton, S.C., Thresher, R., Dixon, J.P., Zahn, D., Colledge, W.H., Carlton, M.B., Nakano, T., Surani, M.A., 2003. Stella is a maternal effect gene required for normal early development in mice. *Curr. Biol.* 13, 2110-2117.

Payer, B., Chuva de Sousa Lopes, S.M., Barton, S.C., Lee, C., Saitou, M., Surani M.A., 2006. Generation of stella-GFP transgenic mice: A novel tool to study germ cell development. *Genesis* 44, 75-83.

Rajpert-De Meyts, E., Hanstein, R., Jorgensen, N., Graem, N., Vogt, P.H., Skakkebaek, N.E., 2004. Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. *Hum. Reprod.* 19, 1338-1344.

Resnick, J.L., Bixler, L.S., Cheng, L., Donovan, P.J., 1992. Long-term proliferation of mouse primordial germ cells in culture. *Nature* 359, 550-551.

Rossi, P., Sette, C., Dolci, S., Geremia, R., 2000. Role of c-kit in mammalian spermatogenesis. *J. Endocrinol. Invest.* 23, 609-615.

Ryu, B.Y., Kubota, H., Avarbock, M.R., Brinster, R.L., 2005. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. *Proc. Nat. Acad. Sci., U S A.* 102, 14302-14307.

Saitou, M., Barton, S.C., Surani, M.A., 2002. A molecular programme for the specification of germ cell fate in mice. *Nature* 418, 293-300.

Shamblott, M.J., Axelman, J., Wang, S., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R., Gearhart, J.D., 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Nat. Acad. Sci., U S A.* 95, 13726-13731.

Shimasaki S., Moore, R.K., Otsuka, F., Erickson, G. F., 2004. The Bone Morphogenetic Protein System In Mammalian Reproduction. *Endocr. Rev.* 25, 72 - 101

Skakkebaek, N.E., Rajpert-De Meyts, E., Main, K.M., 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum. Reprod.* 16, 972-978.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131, 861-872.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147.

Toyooka, Y., Tsunekawa, N., Takahashi, Y., Matsui, Y., Satoh, M., Noce, T. 2000. Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. *Mech. Develop.* 93, 139-149.

Toyooka, Y., Tsunekawa, N., Akasu, R., Noce, T., 2003. Embryonic stem cells can form germ cells in vitro. *Proc. Nat. Acad. Sci., U S A.* 100, 11457-11462.

Turnpenny, L., Brickwood, S., Spalluto, C.M., Piper, K., Cameron, I.T., Wilson, D.I., Hanley, N.A., 2003. Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. *Stem Cells* 21, 598-609.

Vandevoort, C.A., Thirkill, T.L., Douglas, G.C., 2007. Blastocyst-derived trophoblast stem cells from the rhesus monkey. *Stem Cells Dev.* 16, 779-788.

Vidal, V.P., Chaboissier, M.C., de Rooij, D.G., Schedl, A., 2001. *Sox9* induces testis development in XX transgenic mice. *Nat. Genet.* 28, 216–217

Vincent, S.D., Dunn, N.R., Sciammas, R., Shapiro-Shalef, M., Davis, M.M., Calame, K., Bikoff, E.K., Robertson, E.J., 2005. The zinc finger transcriptional repressor *Blimp1/Prdm1* is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development* 132, 1315-1325.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., Thomson, J.A., 2007. Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science* 318, 1917-1920.

Figure 1. The complex inter-relationship *in vitro* between different pluripotent stem cells and embryo and germ cell development. (dotted arrows indicates potential differentiation) which has evolved over the last few years. Bold – from human and mouse; italics – from mouse.

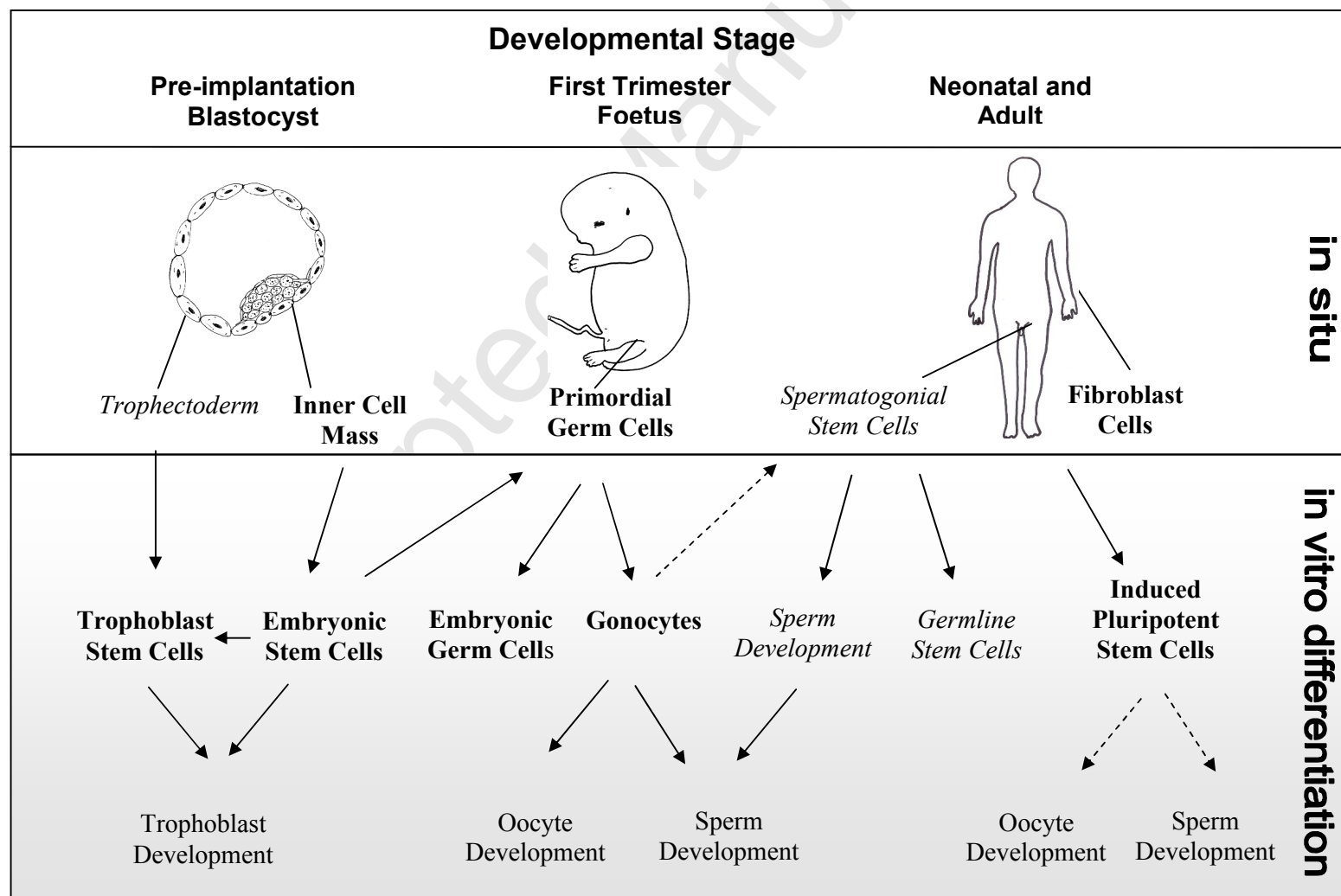


Figure 2. A table of the developmental stages of mammalian germ cell development and some of the factors and genes (*italics*) involved. The genes have been used as markers of PGC and gamete development from ES cells in culture.

Specification of PGCs		
BMPs	<i>Blimp1</i>	
	<i>Stella</i>	
	<i>Fragilis</i>	
SCF	<i>Oct4</i>	
	<i>Nanog</i>	
	<i>TNAP</i>	
	<i>C-Kit</i>	
Migration to Genital Ridge		
Gamete determination		
RA	<i>VASA</i>	
	<i>DAZL</i>	
	<i>Stra8</i>	
	<i>SCP3</i>	
Gonadal Development		
Gametogenesis		
RA	<i>H1t</i>	<i>ZP1</i>
	<i>TNP1</i>	<i>ZP3</i>
Steroid	<i>TNP2</i>	<i>GDF9</i>
Hormones	<i>PRM1</i>	
	<i>PRM2</i>	
	<i>1.97</i>	
Sperm		Egg