Focus on Stem Cells

Germ cells from mouse and human embryonic stem cells

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Abstract

Mammalian gametes are derived from a founder population of primordial germ cells (PGCs) that are determined early in embryogenesis and set aside for unique development. Understanding the mechanisms of PGC determination and differentiation is important for elucidating causes of infertility and how endocrine disrupting chemicals may potentially increase susceptibility to congenital reproductive abnormalities and conditions such as testicular cancer in adulthood (testicular dysgenesis syndrome). Primordial germ cells are closely related to embryonic stem cells (ESCs) and embryonic germ (EG) cells and comparisons between these cell types are providing new information about pluripotency and epigenetic processes. Murine ESCs can differentiate to PGCs, gametes and even blastocysts – recently live mouse pups were born from sperm generated from mESCs. Although investigations are still preliminary, human embryonic stem cells (hESCs) apparently display a similar developmental capacity to generate PGCs and immature gametes. Exactly how such gamete-like cells are generated during stem cell culture remains unclear especially as *in vitro* conditions are ill-defined. The findings are discussed in relation to the mechanisms of human PGC and gamete development and the biotechnology of hESCs and hEG cells.

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Introduction

Detailed investigations of the earliest stages of germ cell development in the human are curbed by the practical and ethical difficulties associated with obtaining human tissue samples. An *in vitro* model system that can recapitulate the development of human germ cells and gametes would be an extremely valuable research tool, which might lead in due course to novel reproductive toxicological assays and eventually new clinical applications to overcome infertility. Embryonic stem cells (ESCs) are defined as pluripotent stem-cell lines derived from early embryos before the formation of the tissue germ layers (Smith 2006). ESCs are derived usually from the pre-implantation blastocyst and exhibit indefinite proliferative capacity under appropriate conditions *in vitro* (Evans & Kaufman 1981, Martin 1981, Thomson *et al.* 1998, Draper *et al.* 2004). In the last few years, landmark investigations have demonstrated that murine embryonic stem cells (mESCs) can differentiate to primordial germ cell (PGCs) and subsequently early gametes and blastocysts (Hubner *et al.* 2003, Geijsen *et al.* 2004). Recently, immature sperm cells derived from mESCs in culture have generated live offspring (Nayernia *et al.* 2006a, 2006b). Preliminary data

indicate that human embryonic stem cells (hESCs) most likely display a similar developmental capacity (Clark *et al.* 2004, Aflatoonian *et al.* 2005). This ESC technology offers great potential for new types of reproductive investigations including a readily accessible system to investigate early stages of human gametogenesis including epigenetic modifications of the germline (Allegrucci *et al.* 2005). Indeed, a range of pluripotent stem cell lines have now been derived from different stages of germ cell development in the mouse and human (Fig. 1). Here, we review the derivation of germ cells and their formation from embryonic stem cells *in vitro* and discuss how these investigations may provide fresh insight into some of the mechanisms of human germ cell development.

Development of primordial germ cells

Germ cells hold a unique place in the life cycle of an animal as they carry the genome onto the next generation (Donovan & de Miguel 2003, McLaren 2003). In mammals, gametes are derived from a small founder pluripotent population, which segregated early in embryogenesis in the developing yolk sac, become PGCs. Elucidating the mechanisms controlling human PGC and gamete development is crucial for

Figure 1 The germ cell cycle. Primordial germ cells (PGCs) are first specified in the proximal epiblast (1) and migrate into the genital ridge of the developing gonad (2). Embryonic germ cell lines have been derived by transformation of PGCs *in vitro*. In the female, germ cells enter meiosis and remain in an arrested state until follicular development occurs after puberty (3). In the male, germ cells retain mitotic capacity for proliferation (3) and pluripotent murine spermatogonial and multipotent adult germ stem cell lines have been reported. From the inner cell mass of the pre-implantation blastocyst, pluripotent embryonic stem cells have been derived (4).

understanding the aetiology of various aspects of infertility. For example, it has been hypothesised that endocrine disrupting chemicals either in the environment or the workplace may interfere with early germ cell development thereby increasing susceptibility to testicular cancer and infertility in adulthood – the so-called ‘testicular dysgenesis syndrome’ (Skakkebaek *et al.* 2001). Indeed, there is growing evidence for effects of environmental chemicals on the various early stages of germ cell and gamete development (Baillie *et al.* 2003). Recently, organochlorines were implicated in affecting the ratio of X and Y sperm in human semen (Tiido *et al.* 2005) and transient exposure of pregnant rats in early gestation to the fungicides and endocrine disrupting chemicals vinclozolin (antiandrogenic compound) or methoxychlor (an oestrogenic compound) increased the incidence of male infertility in offspring (Anway *et al.* 2005). Significantly, this infertility was transferred through the male germ line to subsequent generations possibly indicating modification to epigenetic pathways.

Specification of PGCs, their proliferation, maintenance, then differentiation to primary oocytes and prospermatogonia (precursor spermatogonia stem cells) all have a profound bearing on the number and function of germ cells that are available subsequently for

gametogenesis. Over many years, the activity of tissue non-specific alkaline phosphatase (TNAP) has been used to mark PGCs and monitor their transition from the base of the allantois, through the hindgut to the dorsal body wall where they enter into the genital ridges of the gonadal anlagen (McLaren 2003). The high expression of this enzyme in PGCs is a feature also shared with ESCs, embryonic germ (EG) cells and embryonal carcinoma (EC) cells, all of which have pluripotent capabilities. PGC migration (whether active or passive) occurs at about 7–10 days post-conception (dpc) in the mouse and between weeks 5 and 8 of human gestation (Freeman 2003, Molyneaux & Wylie 2005). Because of the difficulties of undertaking detailed investigations of early human fetal development, relatively little is known of the specification of human PGCs, although it is probable that common signalling pathways occur in mammals and possibly all vertebrates (Donovan & de Miguel 2003). In the mouse, PGCs arise from the proximal epiblast, a region of the early mammalian embryo that also contributes to the first blood lineages of the embryonic yolk sac (Ginsburg *et al.* 1990). Recent studies indicate that as early as 6.25 d.p.c, germline competence can be identified in a founder population of perhaps as few as six epiblast cells that express the

protein Blimp1 (B-lymphocyte-induced maturation protein 1, McLaren & Lawson 2005, Ohinata *et al.* 2005, Vincent *et al.* 2005). Blimp1 was first identified as a transcriptional repressor that enables the further differentiation of immunoglobulin-secreting plasma cells by inhibiting the expression of genes involved in alternative B-cell development. Mutant null-allele mice lacking *Blimp1* generate very few PGCs and those that develop lack normal migratory behaviour, unlike the cells of wild-type individuals where PGCs will multiply rapidly as they migrate to the genital ridges to eventually become non-migratory gonocytes (Vincent *et al.* 2005). In females, the gonocyte surrounded by a cortical interstitial layer initiates meiosis and becomes a primary oocyte and follicle, thereby ending precursor proliferative potential. In males, the gonocyte surrounded by the fetal sex cord of the gonadal ridge (pre-seminiferous tubules) arrests in G0/G1 of mitosis as a prospermatogonium, but retains a proliferative precursor potential. Following birth, prospermatogonia migrate to the basement membrane of the seminiferous tubule and differentiate into spermatogonial stem cells (SSCs). Like adult stem cells, SSCs can both self-renew and provide daughter cells, which differentiate into one or more terminal cell types (Brinster 2002).

Germ cell competence is induced in the murine proximal epiblast in response to signals emanating from the extra embryonic ectoderm including the synergistic action of the growth factors, particularly bone morphogenic proteins (BMP) 4 and 8b; both members of the transforming growth factor- β (TGF- β) superfamily of secreted proteins (Shimasaki *et al.* 2004). Mature BMP4 is a dimer that binds to and signals through heteromeric receptor complexes and downstream SMAD proteins. However, BMP4 or BMP8b alone are unable to induce PGCs from cultured epiblast, while they can when combined, which suggests that signalling for various BMPs may occur through separate receptor complexes. BMP2, a close relative of BMP4, is expressed in visceral endoderm at the time of PGC specification, and inactivation of BMP2 results in a reduction in PGC number, revealing a function of visceral endoderm in PGC generation in the mouse at least (Lawson *et al.* 1999, Ying *et al.* 2001, de Sousa Lopes *et al.* 2004, Shimasaki *et al.* 2004).

The genes *fragilis* and *stella* also have key roles in germ cell competency and development. *Fragilis* is a transmembrane protein and part of a larger interferon-inducible family of genes that is evolutionarily conserved and has human homologues. Interferon-inducible proteins such as *fragilis* have an anti-proliferative function and may serve to increase the length of the cell cycle in PGCs. As germ cell fate is induced, there is only transient expression of *fragilis*, but this gene is also expressed in ESCs and embryonic germ (EG) cells, suggesting a potential role in pluripotency status (Saitou *et al.* 2002). Similarly, *stella* may have a function during the

development of pluripotency and is associated with chromatin remodelling or RNA processing. It is expressed in the oocyte, through pre-implantation embryo development and in germ cell tumours (Payer *et al.* 2003). *Stella*-positive nascent germ cells exhibit repression of homeobox genes, which may explain their escape from a somatic cell fate and the retention of pluripotency (Saitou *et al.* 2002). Transgenic mice have been generated that express a green fluorescent protein (GFP) – *stella* reporter transgene, which appears to accurately mark PGC development (Payer *et al.* 2006).

A number of other factors have been implicated in PGC derivation and maintenance. Immunohistochemical analyses demonstrate that mouse vasa homologue (mvh) protein was exclusively expressed in PGCs just after their colonisation of embryonic gonads and in germ cells undergoing gametogenic processes until the post-meiotic stage in both males and females (Toyooka *et al.* 2000). The tyrosine-kinase receptor c-kit and its ligand, stem cell factor (SCF), are also 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 follicle-stimulating hormone stimulation (Rossi *et al.* 2000).

Another set of genes involved in germ cell development is *DAZ* (deleted in azoospermia) genes. Men with deletions encompassing the Y-chromosome *DAZ* 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 required 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* through the same functional domain required for RNA binding, protein–protein interactions (Moore *et al.* 2003) suggesting mechanisms of germ cell development are highly conserved.

The POU domain transcription factor Oct4 has been shown to have a role in PGC survival. It has been known for some time that this transcription factor is crucial for maintaining pluripotency in the inner cell mass of the blastocyst and in ESCs. By use of conditional gene targeting with the Cre/LoxP system, Kehler *et al.* (2004) showed that loss of Oct4 leads to apoptosis (Kehler *et al.* 2004). In human fetal tissue, Oct4 is strongly expressed in migrating PGCs as well as in human germ cell tumours and EG cells (Looijenga *et al.* 2003, Rajpert-De Meyts *et al.* 2004). Oct4 expression is down-regulated rapidly in the human female gonad and silenced as oocytes enter the first meiotic prophase. In contrast, the same process occurs much more gradually in the male with Oct4 expression often persisting in some gonocytes and infantile spermatogonia. This 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.* 2003).

Thus, some of the genes that play a crucial role in germ cell differentiation are *Blimp1*, *Stella*, *fragilis*, *c-Kit*, *vasa* and *DAZL*. Their expression is stage-specific, therefore, allowing solid identification of germ cells at different developmental phases. In addition to the expression of these genes, other markers associated with germ cell development are non-specific alkaline phosphatase activity, the stage-specific embryonic antigen 1, the transcription factor Oct4 and beta1- and alpha6-integrins (Lacham-Kaplan 2004; Fig. 2).

Embryonic stem cells to germ cells

ESCs have been defined as pluripotent (Smith 2006) as they differentiate along somatic cell lineages but were not believed to form either trophoblast or germline cells. However, it may be more appropriate to use the term totipotent for hESCs as they can spontaneously generate

trophoblast and trophoblast-stem cells, while mESCs form trophoblast if Oct4 expression is conditionally down-regulated (Xu *et al.* 2002, Hay *et al.* 2004, Harun *et al.* 2006). Moreover, ESCs can apparently form germ cells which have a totipotent potential. In the last few years it has become clear that murine ESCs can develop into PGCs *in vitro* (Hubner *et al.* 2003, Geijsen *et al.* 2004, Payer *et al.* 2006) and after further culture may occasionally form early spermatids or oogonia, which form follicle or ovarian-like structures and oocytes (i.e. having morphological similarities to follicles and gametes from gonads) that subsequently develop to blastocyst-like structures, presumably due to parthenogenetic activation (Hubner *et al.* 2003, Geijsen *et al.* 2004, Lacham-Kaplan *et al.* 2006). Clearly, as these germ-cell derivatives have not arisen from true gonadal structures it is crucial that biological function is properly characterised. Some biological functions of mESC-derived spermatids were tested *in vitro* using ESCs initially carrying an Oct 4 promoter-driven 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-ESCs were isolated by flow cytometry and injected intracytoplasmically into oocytes, the diploid chromosome complement was restored and green fluorescent blastocysts generated. Noce and co-workers (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 mESCs could also develop to some extent *in vivo*. To achieve this, cells were aggregated with day 12.5–13.5 (d.p.c) gonadal tissue, which formed seminiferous tubules (but separate from host tubules) within 6–8 weeks when co-transplanted under the testis capsule. These tubules participated in spermatogenesis to produce elongated β -gal-stained spermatids. This demonstrated that such *mvh-lacZ* ESCs formed PGCs with developmental potential to integrate into a somatic epithelium and undergo meiosis to form early sperm cells. There had remained considerable uncertainty as to the biological fidelity of these various culture systems, but Nayernia *et al.* (2006a, 2006b) recently produced viable transgenic offspring from sperm produced from mESCs using a novel two-stage culture system. First, mESCs 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. Following ESC culture, green cells expressing the gene and therefore presumably marking pre-meiotic germ cells were isolated by fluorescent-activated flow cytometry and cultured initially in the presence of RA as a proliferative cell line. Subsequently, the cells were transfected with a second fusion reporter gene *Prm1-dsred* (protamine1 gene fused to red fluorescent protein gene construct) to identity post-meiotic sperm cells. After further RA induction for a few days, red post-meiotic sperm cells were isolated and microinjected

Figure 2 Schematic diagram 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 ESCs in culture.

into mouse eggs to generate viable offspring although these exhibited abnormalities and died prematurely (5 days to 5 months).

While investigations with hESCs are more preliminary, 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. In our lab, PGCs (and on rare occasions, early spermatid cells but so far not oocytes) have been identified after the appropriate culture conditions (Aflatoonian *et al.* 2005).

Gamete determination

During murine and human ESC culture, markers of female germ cells are expressed in both XX and XY cell lines (Toyooka *et al.* 2003, Geijsen *et al.* 2004). The prevailing hypothesis to date has been that whatever their 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). In this case, it would be unsurprising for both male and female ESC 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 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. By contrast, in the embryonic mouse testis, RA is metabolised and inactivated by the P450 enzyme CYP26 (B1) thereby preventing early germ cell entry into meiosis with down-regulation of genes such as SCP3 (synaptonemal complex protein; associated with meiotic events) and the induction into the alternative pathway of mitotic arrest as G0/G1 prospermatogonia. Therefore, the induction of presumptive PGCs into meiosis in culture medium containing RA might be expected although local concentrations within cell aggregates may differ significantly and affect the timing. When RA has not been added as a supplement to basal medium, the spontaneous induction of PGCs from ESCs might potentially still arise if RA is endogenously generated by ESC derivatives. Concentrations of RA in plasma or serum (or serum replacement) that supplement ESC culture medium are normally in the 2–8 nM range (Eckhoff & Nau 1990), much lower than the 1–5 μ M concentrations often used with induction protocols. However, the sensitivity of cells to RA can vary considerably depending on composition of medium.

When hESCs are cultured the process of sex determination seems even more dysregulated as 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 implication 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 hESCs. For example, for male germ cell development *in vivo*, seminiferous epithelium with Sertoli cells is required. This is normally dependent on the expression of the transcription factor SRY, the gene of which is normally located on the Y chromosome. Therefore, for XX hESCs to generate presumptive male germ cells either an artefactual differentiation of Sertoli cells occurs, for example, by female supporting cells differentiating to a Sertoli-like phenotype (Adams & McLaren 2002) or meiosis inhibitor factors are produced (or metabolised in the case of RA) from a different origin. Interestingly, in vitamin A-deficient adult mice, where only spermatogonia and Sertoli cells populate the seminiferous epithelium, the addition of retinoids such as RA and retinol may induce the resumption of spermatogenesis (Baleato *et al.* 2005).

The germ cell niche and ESCs

An intriguing aspect of the generation of ESC-derived germ cells *in vitro* is how the normal checkpoints of the developmental process, which would naturally span a timeline from early fetal development to puberty and lead to arrest of cells at different stages, seem to be overcome and then apparently compressed into a relatively much short culture period. It has been suggested that in the absence of environmental cues, germ cells may develop according to an intrinsic clock. Using *stella*-GFP murine ESCs, Payer *et al.* (2006) showed somewhat surprisingly that ESC colonies displayed a well-defined subpopulation of *Stella*-positive cells and unlike the uniform distribution of cells expressing Oct4 or TNAP. Significantly, there was overlap of expression of Oct4 and *stella* in some cells and, since this combination of markers identifies germ cells it may indicate a population of germ cell-like ESCs (Payer *et al.* 2006). Whether *stella*-positive ESCs are destined to form PGCs in the mouse and whether their specification requires subsequent exposure to a set of environmental cues, awaits further investigation but these results suggest that there is perhaps an inherent propensity of ESC culture systems to form PGCs. Thereafter, these cells may create a stem cell niche continuum for induction of germ cell development.

Exactly how ESC cultures may mimic the somatic environment that encapsulates either developing oogonia (the follicle) or the sperm stem cell (seminiferous epithelium) is unclear. The appropriate growth factor and hormonal microenvironment required to support and sustain these complex niches probably depends to some extent on the type of culture system adopted for the

differentiation process. ESCs are cultured usually in two basic ways. Monolayer adherent cultures of ESCs can be allowed to differentiate directly to form an appropriate niche or ESCs can be induced to aggregate to form embryoid bodies that form a more three-dimensional micro-environment. In conjunction with these two culture methods, ESCs can be initially co-cultured with feeder layers (i.e. mouse embryonic fibroblasts and 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 these conditions are far from being specific. Apart from the absence of a defined culture medium, the various procedures for passaging ESCs (i.e. enzymatically with trypsin to single cells or as clumps with collagenase or manually), the influence of feeder cells and extracellular matrix (e.g. Matrigel) and the cell line itself can all contribute to whether germ cells are specified. If a progenitor cell population is not selected (i.e. using fluorescent reporter systems), then only a small proportion of cells (usually less than 5%) will normally display PGC or gonocyte/spermatogonia markers, but the fact that some cells progress further to a post-meiotic stage seems remarkable given the complex hormonal requirements of gamete development. Interestingly, oestradiol production (50–100 pg/ml) was detected after 12 days in mESC cultures that generated follicle-like structures (Hubner *et al.* 2003), while appreciable levels (60 pg/ml) of dihydrotestosterone can be detected in hESCs cultures that generate PGCs and spermatids (Aflatoonian & Moore, unpublished). Presently, the source of steroidogenesis is unclear. It may originate from additives to the culture medium (i.e. serum or serum replacement supplement) or gonadal cell types (granulosa, Leydig cells) that co-differentiate with germ cells. This appeared to be the case for oocytes generated from mESCs where increased oestradiol levels were correlated with increased proportion of apparent follicular cells (Hubner *et al.* 2003). Alternatively, steroids may be produced from non-reproductive cell phenotypes (e.g. liver or adrenal cells) that could conceivably differentiate from ESCs in culture at the same time as germ cells.

The proliferative capacity of germ cells in culture will depend to some extent on their fate. Since, at least *in vivo* germ cells are programmed by default to enter meiosis, the efficiency at which ESC cultures will generate germ cells will depend on the initial rate of differentiation to PGCs and subsequently whether factors are present to divert development down spermatogenesis when mitotic proliferation of prospermatogonia can potentially occur. Recent studies (Hamra *et al.* 2005, Ryu *et al.* 2005) indicate that the self-renewal of these spermatogonial stem cells (SSCs) in rodents (and possibly all mammals) is dependent on glial cell line-derived neurotrophin factor (GDNF), the GDNF-family receptor α -1 and basic

fibroblast growth factor, providing candidate factors to be tested in ESC systems.

In the mouse, pluripotency of ESCs is mirrored also by EG cells (Matsui *et al.* 1992, Resnick *et al.* 1992) but additionally extends to a population of SSCs in the neonatal mouse testis (Kanatsu-Shinohara *et al.* 2004) and perhaps more remarkably in 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*. When injected into mouse pre-implantation blastocysts they contribute to the development of all the major organs and display germline transmission (Guan *et al.* 2006) and therefore are termed multipotent adult germline stem cells. 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 the translation from the murine to the human system may not be straightforward. While human EG cell lines have been reported (Shamblott *et al.* 1998), their proliferative and pluripotent capacity presently as true EG cells has been questioned (Turnpenny *et al.* 2003, Aflatoonian & Moore 2005).

Since the evidence is becoming more compelling that in culture ESCs can generate PGCs and germ cells, an important question (and the subject of considerable debate) is whether germ cells can be derived (or transdifferentiated) from adult stem cells residing outside the gonad? In the mouse, it has been claimed 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 were criticised (Telfer *et al.* 2005) and subsequently have not been substantiated (Eggan *et al.* 2006). Germ-cell derivation from pig fetal skin cells *in vitro* has also been reported (Dyce *et al.* 2006) with the 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. Clearly, if any these recent reports can be fully corroborated, they may indicate that PGC and germ cell formation *in vitro* and possibly *in vivo* is much more plastic than previously believed (Fig. 3). At present, 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 not due to true germ cell phenotype. The use of a wider battery of specific protein and functional phenotypic assays are required to resolve this issue.

Figure 3 Schematic diagram showing the relationship between *in vivo* development of germ cells and the *in vitro* pluripotent stem cell lines that have been reported to be derived from mouse and human cells (ESC and EG) and their developmental potential. The relationship between ESC, EG and AGS cells still remains unclear although they express similar markers of pluripotency.

Conclusion

ESCs differentiation remains hard to control and accurately monitor *in vitro* but with the advent of highly specific transgene reporter systems to monitor germ-cell differentiation, the evidence that primordial germ cells can be derived readily from mouse ESCs *in vitro* appears strong. Moreover, there are growing data indicating that very similar processes are likely to occur with hESCs. It seems that occasionally gametes, albeit immature, can be generated under appropriate culture conditions although their complete developmental competence remains uncertain. The promiscuous nature of spontaneous ESC differentiation in culture makes it especially difficult to determine at present whether PGC and germ cell development follow similar programmes to those occurring *in vivo* or whether the conditions merely provide suitable conducive conditions that permit an intrinsic developmental process but not necessarily the same as the gonadal environment. For example, the findings that the development of both haemopoietic plasma cells and PGCs depend on expression of the same protein (blimp1) indicate conservation in these differentiation systems and therefore there is potential for interference between pathways during *in vitro* development. However, with careful selection of cells with the appropriate markers, it should be feasible to devise stage-specific protocols (with specific growth factors) for differentiation of ESCs to PGCs and subsequently PGCs

to gonocytes and male or female germ cell fate. Given the continuing advances in the proliferation and maturation of germ cells *in vitro*, the production of *in vitro* gametes may be a practical proposition in the not too distant future. However, there remains major uncertainty about the genetic/epigenetic processing of germ cells *in vitro* and very careful consideration would need to be given to safety aspects of such cells if ever they are to be used for clinical applications.

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