

NEW RESEARCH HORIZON

Modelling germ cell development *in vitro*

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Germ cells have a critical role in mediating the generation of genetic diversity and transmitting this information across generations. Furthermore, gametogenesis is unique as a developmental process in that it generates highly-specialized haploid gametes from diploid precursor stem cells through meiosis. Despite the importance of this process, progress in elucidating the molecular mechanisms underpinning mammalian germ cell development has been retarded by the lack of an efficient and reproducible system of *in vitro* culture for the expansion and *trans*-meiotic differentiation of germline cells. The dearth of such a culture system has rendered the study of germ cell biology refractory to the application of new high-throughput technologies such as RNA interference, leaving *in vivo* gene-targeting approaches as the only option to determine the function of genes believed to be involved in gametogenesis. Recent reports detailing the derivation of gametes *in vitro* from stem cells may provide the first steps in developing new tools to solve this problem. This review considers the developments made in modelling germ cell development using stem cells, and some of the challenges that need to be overcome to make this a useful tool for studying gametogenesis and to realize any future clinical application.

Keywords: germ cell; stem cell; spermatogenesis; oogenesis; infertility

Introduction

The germ cells of sexually reproducing organisms have three crucial functions, namely the preservation of genetic integrity, the generation of genetic diversity and the transmission of this information to the next generation through the production of haploid gametes from diploid precursors. Perturbations at any stage of the gametogenic process can result in subfertility, which within human populations is a major public health issue affecting ~0–15% of couples (De Kretser and Baker, 1999). A better understanding of gametogenesis could aid the development of new therapeutic approaches for subfertility and provide targets for novel contraceptives. Despite these potential benefits, our understanding of the molecular mechanisms that regulate the development of the mammalian germ lineage is poor when compared with that of most somatic cells.

A major reason for this is the lack of a robust *in vitro* culture system allowing the expansion of germline progenitor cells and their differentiation into mature gametes. This has rendered the study of gametogenesis, and in particular the role of germ cell endogenous factors in the regulation of this process, broadly resistant to recent technological developments in high-throughput analysis of gene function, such as the use of RNA interference to knockdown gene expression.

Previous efforts to establish *in vitro* models of germ cell development have focused primarily on attempting to immortalize post-natal

spermatogenic cells. By introducing the SV40 large T antigen and a temperature-sensitive mutant form of p53 into mouse testicular germ cells, Hofmann *et al.* (1994) reported the establishment of an immortalized germ cell line capable of undergoing meiosis *in vitro* when grown at a permissive temperature. However, further characterization of this line failed to detect the presence of transcripts expressed by post-meiotic cells, nor the presence of cells with a haploid DNA content, leading to the conclusion that this cell line was incapable of undergoing *trans*-meiotic differentiation *in vitro* (Wolkowicz *et al.*, 1996). Feng *et al.* (2002) used the *TERT* subunit of telomerase to immortalize mouse spermatogonia capable of undergoing meiosis and forming acrosome-like structures characteristic of haploid round spermatids at high efficiency when treated with stem cell factor (SCF, the ligand for the c-Kit receptor). Again this result has yet to be replicated by other groups, and despite the potential utility of this cell line to germ cell biologists, its use has not been widely reported. Although methods to maintain and expand mouse spermatogonial stem cells (SSCs) in culture have recently been developed, none appear to support full *trans*-meiotic differentiation of these cells *in vitro* (Kanatsu-Shinohara *et al.*, 2003).

Indeed, the range of tools available to reproductive biologists to determine the function of genes with a suspected role in mammalian germ cell development is essentially limited to the study of

homologous genes in lower model organisms and the production of transgenic mice with targeted disruptions of genes of interest (Cooke and Saunders, 2002; Matzuk and Lamb, 2002; Lee *et al.*, 2007). While the success of this approach is undeniable, it is laborious, time consuming and costly in comparison to *in vitro* cell-based assays of gene function, compounded by the difficulty of maintaining mouse lines with reproductive defects through breeding. Furthermore, targeted gene disruptions in transgenic mice classically result in a complete loss-of-function phenotype, and this may not accurately reflect some instances of currently unexplained subfertility seen in human populations that may stem from more subtle hypomorphic alleles.

Alternative animal-based strategies to investigate germ cell development have included the use of grafting gonadal fragments onto immunocompromised mice (Paris and Schlatt, 2007), a technique used elegantly by Naughton *et al.* (2006) to allow the study of the effects of disrupting the glial cell line-derived neurotrophic factor (GDNF) system on early spermatogenesis, overcoming the perinatal mortality seen in GDNF-null mice. Isolation and genetic modification of SSCs, followed by transplantation back into a host testis to restore spermatogenesis is another approach (Nagano *et al.*, 2001, 2002; Kanatsu-Shinohara *et al.*, 2006), however, both of these techniques require sufficient time for grafts or transplants to become established.

The ability to generate 'artificial' (i.e. *in vitro*-generated) gametes would also have potential therapeutic use where couples currently require gamete donation through absence of their own (Nagy and Chang, 2005). These include individuals suffering from a diverse range of conditions including genetic causes both known (e.g. Y chromosome deletions) and unknown, chromosomal abnormalities (such as Turner's and Klinefelter's syndromes), and iatrogenically following chemotherapy for cancer or inflammatory conditions. Additionally, there is a need for oocytes for the refinement of somatic cell nuclear transfer for stem cell derivation, progress in which is difficult with the restricted numbers of oocytes available through current donation programmes (Hall and Stojkovic, 2006).

What then are the alternative approaches available to study germ cell development? Recent advances in the derivation of germ cells from stem cells may hold some promise. The fields of research on pluripotent stem cells (i.e. those capable of indefinite self-renewal and differentiating into all three germ layers *in vitro* or *in vivo* when introduced into a blastocyst to form a chimera) and early germ cells are historically deeply interlinked (Zwaka and Thomson, 2005). Embryonal carcinoma cells (ECCs), the first mammalian pluripotent cells to be isolated and cultured, were derived from rare testicular germ cell tumours known as teratocarcinomas (Solter, 2006). Work establishing the optimal culture conditions for maintaining ECCs in the undifferentiated state laid the foundations for the isolation and propagation of embryonic stem cells (ESCs) from the pluripotent inner cell mass of the mouse, and much later primate and human blastocysts (Chambers and Smith, 2004; Solter, 2006).

Although themselves unipotent (capable only of giving rise to the cell types of one lineage), germ cells share many transcriptional and phenotypic similarities with ESCs (Zwaka and Thomson, 2005). Primordial germ cells (PGCs) are the last cells of the mammalian fetus to retain expression of transcription factors Oct-3/4 (encoded by the gene *Pou5F1*) and Nanog, involved in the maintenance of the undifferentiated state (Pesce *et al.*, 1998; Chambers *et al.*, 2003; Chambers *et al.*, 2007), and they express similar cell surface markers to pluripotent stem cells such as alkaline phosphatase (Chiquoine, 1954). The concept that a close relationship exists between germ cells and stem cells is reinforced by the finding that under appropriate conditions of *in vitro* culture, unipotent PGCs, and even post-natal and adult spermatogonia, are capable of undergoing reprogramming into

a pluripotent state (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Kanatsu-Shinohara *et al.*, 2004a; Guan *et al.*, 2006).

This review focuses on recent developments in the *in vitro* modelling of germ cell development using stem cells with particular reference to the implications of these for the understanding of human gametogenesis and defects therein. We address the major approaches taken by stem cell biologists to study germ cell development, and consider the challenges that need to be overcome to make these technological developments useful research tools. Finally, we consider how new work on the derivation of pluripotent stem cells from terminally differentiated adult cells through the introduction of reprogramming factors has the potential to impact on the *in vitro* modelling of germ cell development.

Germ cell development in rodents and humans

Producing accurate *in vitro* models of germ cell development is dependent on understanding the normal process of establishing the germ cell lineage *in vivo*. In lower organisms, such as *Drosophila* and *Caenorhabditis elegans* the germ cells are segregated from the soma at the earliest stage of development through the asymmetric distribution of proteins and mRNAs encoding factors required for germ cell development (Lehmann and Ephrussi, 1994; Seydoux and Schedl, 2001). In contrast, the formation of the first germ cells in mammals occurs comparatively late in development, through an inductive process known as epigenesis (Saitou *et al.*, 2002; McLaren, 2003). This process has been most extensively studied in the mouse, where PGCs arise in the proximal epiblast of the embryo through the inductive action of extraembryonic ectoderm-derived bone morphogenetic proteins (BMPs) (Lawson and Hage, 1994; Lawson *et al.*, 1999; Saitou *et al.*, 2002). In mice, the earliest detectable precursors of the germ cell lineage are a group of six to eight cells expressing the transcriptional repressor Blimp1 in the epiblast cell layer adjacent to the extraembryonic ectoderm (McLaren and Lawson, 2005; Ohinata *et al.*, 2005). Although this small group of Blimp1-positive cells is likely to constitute the first lineage-restricted germ cells in the embryo, surrounding cells may also be recruited to form the population of 40 or so alkaline phosphatase-positive 'founder' PGCs detectable at the base of the allantois at e7.5 (McLaren and Lawson, 2005). Blimp1 appears to be essential for the appropriate repression of the somatic *Hox* gene programme, which would otherwise direct them into same fate as the cells that surround them (Ohinata *et al.*, 2005). Repression of the somatic programme in PGC precursors occurs concomitantly with the up-regulation of *Stella* (*Dppa3*), the earliest definitive germ cell marker (Saitou *et al.*, 2002).

From e8.5 in mice, the founder PGCs migrate through the embryo, passing through the hindgut and the dorsal mesentery and arriving at the genital ridges around e10.5, wherein they become known as gonocytes (McLaren, 2003). Germ cell proliferation occurs throughout the migratory period, and continues for two to three days after colonizing the gonads, forming a population of around 25 000 gonocytes at e13.5 when proliferation ceases (Tam and Snow, 1981). Subsequently, these germ cells either cease mitosis and enter meiosis in the female, or progressively arrest in G0/G1 over several days in the male (Adams and McLaren, 2002; McLaren, 2003; Western *et al.*, 2008).

The precise events and timings surrounding the earliest events in the establishment of the germ cell lineage in human embryos remain unknown. Migratory human PGCs have been reported in the endoderm of the human embryo as early as the fourth week of development, following a similar migratory path to that seen in mice through the hindgut and dorsal mesentery arriving at the gonads around the fifth week of development (Witschi, 1948). Colonization

of the gonadal primordium is thought to be complete around 10 days later (Wartenburg, 1981).

The first overt signs of sexual dimorphism between the male and female gonads are detectable at around 6 weeks of development. Sex determination and the onset of expression of the testis-determining factor *SRY* in human fetal gonads occurs between 41 and 44 days post-ovulation in the gonads of male human fetuses, and the subsequent organization of the somatic and germ cells of the developing testis into the testicular cords is first visible at 44 days post-ovulation (Wartenburg, 1981; Hanley *et al.*, 2000).

Interestingly, at this stage of development the human fetal ovary is reported to contain around 30 000 germ cells; ten times as many as are present in the testis of a fetus of the same age (Bendsen *et al.*, 2003, 2006). The gonocytes of both sexes continue to expand by mitosis for a further 3 weeks, increasing 10-fold in number in both the testis and the ovary (resulting in 30 000 and 250 000–300 000 germ cells in the testis and ovary by 9 weeks post-fertilization, respectively) (Bendsen *et al.*, 2003, 2006). However, despite these radical differences in germ cell number, we have been unable to detect significant differences in the levels of expression of key germ cell-associated genes such as deleted in azoospermia-like (*DAZL*) and the Dead-box RNA helicase (*VASA*) between human fetal testis and ovaries, although we do observe a significant sexual dimorphism in the expression of *OCT-3/4* [A.J.C. and R.A.A. unpublished observations, Anderson *et al.* (2007)]. Interestingly, no such sex-specific differences in germ cell number have been reported in the mouse, in which both male and female fetal gonads contain roughly equal numbers of germ cells (Tam and Snow, 1981).

Key mouse/human differences in germ cell biology

Due to the regulatory, ethical and logistical difficulties in obtaining sufficient human fetal gonadal tissue for research, most studies into mammalian germ cell development have focused on the mouse, in the hope that these findings can be extrapolated to humans. However, work from our laboratory and others has begun to demonstrate that the differences between the two species in the sequence of events surrounding germ cell development extend beyond merely differences in timing.

Mouse PGCs express *Oct-3/4* throughout their migratory period, and in addition up-regulate expression of the cytoplasmic RNA-binding proteins *Dazl* and *Mvh* (mouse vasa homologue, also known as *Ddx4*) as they colonize the gonadal ridges and become gonocytes (Fujiwara *et al.*, 1994; Pesce *et al.*, 1998; Seligman and Page, 1998; Toyooka *et al.*, 2000; Kehler *et al.*, 2004). Expression of these markers continues in male germ cells throughout the period of mitotic arrest and beyond the resumption of spermatogenesis after birth. Human gonocytes, in contrast, express only *OCT-3/4* and *DAZL* proteins. Consistent with previous reports studying older specimens, *DAZL* protein localizes to the nuclei of gonocytes at this stage, the functional significance of which remains unknown (Rajpert-De Meyts *et al.*, 2004; Anderson *et al.*, 2007). *VASA* protein is undetectable in proliferating human gonocytes, although they do produce readily detectable amounts of *VASA* mRNA (Anderson *et al.*, 2007), indicating that the transcripts of this gene may be subjected to similar translational regulation as occurs in the mouse (Reynolds *et al.*, 2005).

In the human fetal ovary, the entry of gonocytes into meiosis is coincident with the loss of *OCT-3/4* expression, the concomitant translocation of *DAZL* protein to the cytoplasm and the onset of detectable *VASA* protein expression (Stoop *et al.*, 2005; Anderson *et al.*, 2007). Whether these events are linked is unknown, but it is tempting to speculate that the translocation of *DAZL* protein to the cytoplasm might be necessary to allow the onset of translation of *VASA*

mRNA. In the testis, germ cells undergo a similar maturational process, up-regulating *DAZL* and *VASA*, and down-regulating *OCT-3/4*, although this appears not to be linked to meiotic entry (Rajpert-De Meyts *et al.*, 2004; Anderson *et al.*, 2007).

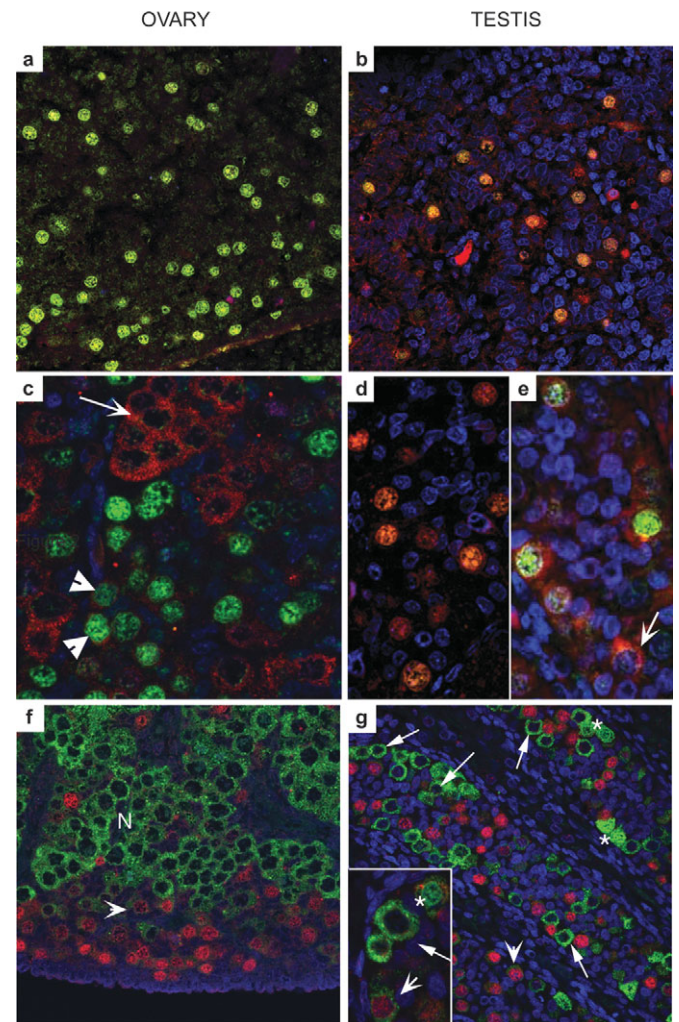


Figure 1: Distinct subcellular localization of *OCT-3/4*, *DAZL* and *VASA* in human fetal gonads.

In contrast to mouse gonocytes, which express *Oct-3/4*, *Dazl* and *Mvh* proteins, first trimester ovaries (a, 61 day), and testes (b, 64 day) express only *OCT-3/4* (green) and *DAZL* (red) both of which co-localize to germ cell nuclei. In ovaries from the second trimester (c, 14 week) *DAZL* protein is almost exclusively cytoplasmic and largely localizes to *OCT-3/4*-negative groups of cells (arrow); a few *OCT-3/4*-positive cells display low levels of *DAZL* expression in their cytoplasm (arrowheads). In second trimester testes (d, 16 week; e, 19 week), *DAZL* is still expressed in the nuclei of some *OCT-3/4*-positive germ cells but this pattern of expression is variable, with *DAZL* protein present in the cytoplasm of *OCT-3/4*-positive and *OCT-3/4*-negative (arrow, e) cells. (f) Mutually exclusive expression of *OCT-3/4* and *VASA* in the human fetal ovary; cells with intense immuno-positive staining for *OCT-3/4* are found at the periphery of the organ (red nuclei), while cytoplasmic *VASA* protein is most intense in cells located in nests (N) closer to the centre of the ovary. An intermediate population of cells with low intensity nuclear staining for *OCT-3/4* and low intensity staining for *VASA* (arrowheads) was also present. (g) In second trimester human fetal testis, *OCT-3/4*-positive and *VASA*-positive germ cells are found within the same seminiferous cords; germ cells with intense nuclear *OCT-3/4* expression (red nuclei) are *VASA*-negative and those with intense cytoplasmic expression of *VASA* (e.g. arrowed in inset g) were *OCT-3/4*-negative. Two other populations of male germ cells can be identified; a population with low intensity expression of both *OCT-3/4* and *VASA* (arrowheads) and cells with nuclear *VASA* expression (asterisks) which were typically found in pairs (adapted from Anderson *et al.* (2007)).

Spatial variations also exist in the expression of key germ cell markers between mouse and human during fetal germ cell development. In the human fetal ovary, proliferating OCT-3/4-positive germ cells are found at the periphery of the organ, with progressively more mature meiotic germ cells found in a gradient towards the centre (Fig. 1a–g) (Stoop *et al.*, 2005; Anderson *et al.*, 2007). This is in contrast with the mouse ovary, in which germ cells enter meiosis in a rostro-caudal wave along the long axis of the gonad (Menke *et al.*, 2003; Bullejos and Koopman, 2004). In mice, the switch from mitotic proliferation to meiotic differentiation has been reported to be initiated by the diffusion of retinoic acid (RA) from the mesonephric duct into the most rostral part of the ovary, and its subsequent diffusion along the gonad (Bowles *et al.*, 2006; Koubova *et al.*, 2006), although this claim remains controversial and other mechanisms for regulating meiotic entry may exist (Best *et al.*, 2008). Given the arrangement of germ cells in the human fetal ovary, it is difficult to envisage how such an analogous system could work, and the mechanisms regulating meiotic entry in human germ cells remain to be determined.

Another recently identified species-specific difference relates to the expression of the transcription factor SOX2, which is expressed in mouse, but not human germ cells (Perrett *et al.*, 2008). SOX2 is a component of the core transcriptional machinery involved in the maintenance of pluripotency in stem cells, and works through binding the promoters of many pluripotency-associated genes in concert with OCT-3/4 (Niwa, 2007). The functional significance of the lack of SOX2 expression in human germ cells is presently unclear, as they continue to express many of the genes considered dual OCT-3/4–SOX2 targets (Perrett *et al.*, 2008). A screen to identify other germ cell-expressed SoxB-class transcription factors (the subfamily to which SOX2 belongs) that might substitute for SOX2 found none, suggesting that no functional redundancy exists within the SoxB subfamily in germ cells (Perrett *et al.*, 2008). Looijenga and colleagues have suggested that SOX17 may fulfil this role instead (de Jong *et al.*, 2008). These data are interesting given the suggestion that OCT-3/4 may fulfil different functions in germ cells and stem cells through modulating different sets of target genes (Hubbard and Pera, 2003; Kehler *et al.*, 2004).

The differences in localization and onset of expression of these marker genes relative to the stage of germ cell maturation suggest significant differences may exist in the control of germ cell development between mouse and human. Differences between the two species are not limited to spatiotemporal variations in marker gene expression however. Rodents also lack some of the candidate fertility factors known to be clinically relevant in humans. For example, the human Y-linked *DAZ* genes, which are candidate azoospermia factors in humans, have only autosomal homologues in mice (Reijo *et al.*, 1995; Ruggiu *et al.*, 1997). Therefore, while the study of such homologues can provide critical insight into the biology of the *DAZ* family proteins (Ruggiu *et al.*, 1997), the clinically relevant condition of Y chromosome microdeletions encompassing the *DAZ* gene cluster cannot be replicated directly in rodents. There are, therefore, substantial limitations in using rodent models to understand human fertility and the aetiology of human infertility.

Strategies for *in vitro* culture of PGCs

A significant research effort by numerous labs worldwide has established roles for kit ligand/SCF, leukaemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), BMPs, forskolin and RA in the maintenance or expansion of isolated mouse PGCs and proliferating gonocytes *in vitro* (reviewed in De Felici and Pesce, 1994; Donovan, 1994; De Felici *et al.*, 2004). While the study of the effects of some

of these factors (especially SCF and RA) on germ cell development *in vitro* has provided significant insight into their role *in vivo*, the relevance of data acquired *in vitro* on others is unclear. For example, mice carrying a germ cell-specific deletion of gp130, the common receptor for interleukin-6 family ligands (which includes LIF), develop a normal complement of PGCs (Molyneaux *et al.*, 2003), whereas blocking the gp130 receptor with an antibody results in the death of isolated PGCs cultured *in vitro* (Koshimizu *et al.*, 1996), or suppression of meiotic entry (Chuma and Nakatsuji, 2001).

Despite the inclusion of these factors in culture media, PGCs or gonocytes have a limited proliferative lifespan *in vitro*, before undergoing either death or differentiation (Donovan, 1994). Furthermore, in the absence of appropriate programming from the somatic cells of the gonad, isolated male PGCs will enter meiosis *in vitro* at the same time as those isolated from female gonads, thus making the investigation of sex-specific differences in germ cell biology difficult to study *ex vivo* (Nakatsuji and Chuma, 2001).

Little is known about the behaviour of isolated human PGCs and gonocytes in culture. Shambloot *et al.* (1998) reported two populations of human gonocytes in culture, namely ‘stationary’ (round, non-migratory) and ‘migratory’ (motile cells with long processes) germ cells. Turnpenny *et al.* (2003) described populations of poorly-proliferating and vigorously-proliferating human germ cells, though whether these populations are linked to the populations reported by Shambloot *et al.* is unclear. More recently, Tu *et al.* (2007) have also identified that human gonocytes divide into two distinct morphological populations (‘round’ and ‘spindly’), and that treatment with SCF increased the numbers of ‘round’ germ cells in culture compared with those with the ‘spindly’ morphology’. We have identified similar phenomena while trying to culture human gonocytes isolated from fetuses of ~60 days gestational age on feeder layers of mitotically inactivated STO cells (Fig. 2a–d). How these populations relate to differences in the germ cells *in vivo* is unknown, but warrants further investigation.

EGCs: stem cells from PGCs

Matsui *et al.* (1992) and Resnick *et al.* (1992) reported the establishment of long term exponentially expanding cultures of mouse PGCs.

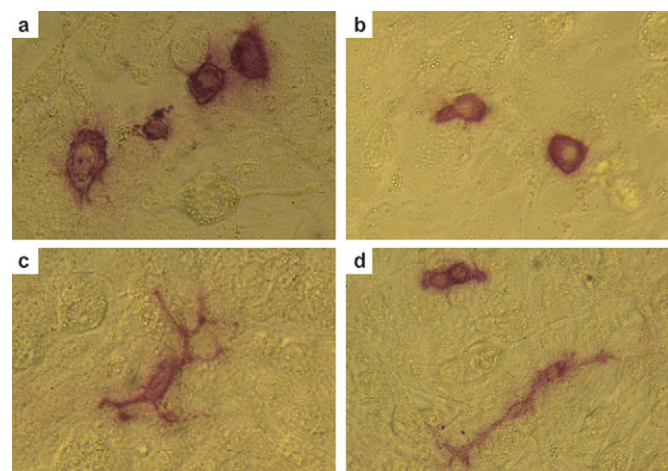


Figure 2: Morphology of human fetal gonocytes *in vitro*.

When cultured *in vitro* on feeder layers of mitotically inactivated STO cells, gonocytes isolated from first trimester testes or ovaries (~60–65 days gestational age) adopt one of two morphologies, namely round (a, b and d) or migratory (c and d). Both populations can be found in close proximity (d). Germ cells, but not feeders, display strong alkaline phosphatase activity (red).

When cultured on a mitotically inactivated mouse fibroblast feeder layer in the presence of LIF, SCF and bFGF, small numbers of PGCs escaped the *in vitro* block on proliferation and acquired the ability to divide indefinitely (Matsui *et al.*, 1992; Resnick *et al.*, 1992). These proliferating germ cells could also contribute to a chimera when introduced to a blastocyst (Labosky *et al.*, 1994). Germ cells that have undergone this 'reprogramming' event in culture cells are known as embryonic germ cells (EGCs).

Derivation of EGCs from human PGCs/gonocytes was first reported shortly after the derivation of human ESCs (hESCs), using culture conditions similar to those used to derive mouse EG cells (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2003; Liu *et al.*, 2004). Unlike hESCs however, hEGCs do not appear to be fully pluripotent (Aflatoonian and Moore, 2005; Turnpenny *et al.*, 2006). Although capable of giving rise to representative tissues of all three germ layers *in vitro*, it has not yet been demonstrated that hEGCs are capable of forming teratomas when introduced into immunocompromised mice (the standard test of pluripotency for hESCs in the absence of a chimera-forming assay) (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2003; Turnpenny *et al.*, 2005).

The exact status of hEGCs remains unclear as the repertoire of cell surface antigens they express differs from that expressed by hESCs. Undifferentiated hESCs express the stage-specific embryonic antigens SSEA3 and SSEA4, and up-regulate the SSEA1 antigen on differentiation (Thomson and Odorico, 2000). hEGCs appear to express all three simultaneously, although this too differs from the *in vivo* situation where human gonocytes express SSEA1, and the expression of SSEA4 is not restricted to the germ cells in the gonad (Shamblott *et al.*, 1998; Kerr *et al.*, 2008). The recent finding that both human gonocytes and hEGCs do not express SOX2 indicates that the latter are at least in part representative *in vitro* derivatives of the former, and the lack of SOX2 expression in hEGCs may in part explain their lack of total pluripotency (Perrett *et al.*, 2008).

Surprisingly, given their germ cell origin, the possibility of using EGCs as an *in vitro* model for studying germ cell behaviour has not been extensively explored. Toyooka *et al.* (2000) reported that culturing mEGCs in aggregates with somatic gonadal cells resulted in the up-regulation of Mvh expression (and by extension germ cell formation *in vitro*), a response not seen when mESCs were used in similar aggregate cultures. Although this overlooked result perhaps represents the first demonstration of germ cell derivation from a pluripotent stem cell, it more importantly indicates that unlike mESCs, mEGCs may inherit the ability to respond to signals from the gonadal microenvironment from the germ cells from which they were derived (Toyooka *et al.*, 2000). EGCs may therefore represent a potential source of reproducible *in vitro*-derived germ cells for the study of germ-soma gonadal interactions.

There are difficulties however in the use of hEGCs. They are difficult to maintain in culture compared with hESCs (Aflatoonian and Moore, 2005; Turnpenny *et al.*, 2005), they appear not to retain their pluripotency for longer than 15–20 passages and cryopreservation has proved difficult (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2005). Despite this, EGCs may still provide a valuable model for some studies of germ cell development.

Within our own laboratory we have recently investigated the potential for hEGCs to act as a useful model system for investigating early human germ cell development. As with previous reports, we have found these cells difficult to identify in culture by light microscopy without fixing and staining for alkaline phosphatase activity, and the efficiency of derivation in our hands is significantly lower than that previously reported (Shamblott *et al.*, 1998). We have however, succeeded in establishing proliferative cultures of putative hEGCs from fetal gonads lasting several weeks (Fig. 3a–d) and efforts to determine

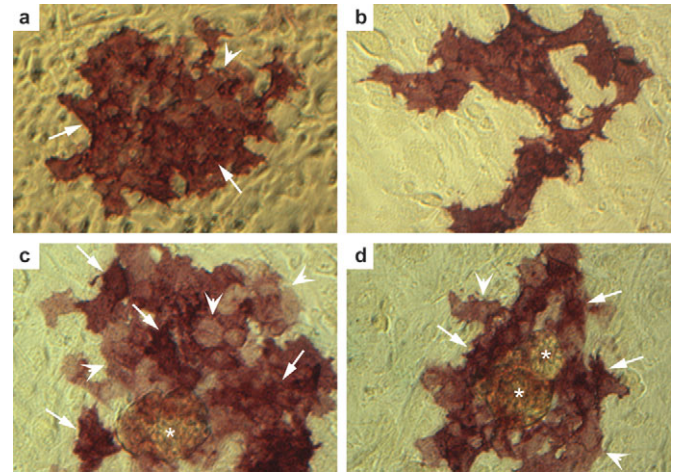


Figure 3: Colonies of putative human EGCs derived from cultured human gonocytes.

Proliferative cultures of putative human EGCs at late passage 2 (3 weeks in culture), stained for alkaline phosphatase activity (red). Note the variable staining intensity within colonies from regions of strong (arrows, a, c and d) to weak (arrowheads, a, c and d) staining. Cultures were often found in association with aggregations of feeders carried over during subculture (asterixes).

which, if any, characteristics of PGCs are retained following their conversion to EGCs are underway.

Pluripotent stem cell-derived gametes: making germ cells from stem cells

Although pluripotent ESCs are capable of contributing to the germline of chimaeric embryos and forming fully functional gametes *in vivo* (Bradley *et al.*, 1984), Toyooka *et al.* (2000) were unable to detect the induction of Mvh expression in co-cultures of mESCs and gonadal somatic cells, suggesting that mESCs were either unresponsive to the gonadal signals inducing germ cell development, or that the signals produced by somatic cells at this stage were insufficient to induce germ cell development. Subsequent successful derivation of germ cells from ESCs *in vitro* in 2003 was a significant breakthrough, and one likely to have significant impact on the study of germ cell development (Hubner *et al.*, 2003). Table I gives the major findings of the publications in this field to date.

Hubner *et al.* (2003) first reported the derivation of germ cells from mESCs. They harnessed a green fluorescent protein (GFP) reporter construct fused to part of the mouse Oct-3/4 promoter gene that restricted expression of the reporter gene to early germ cells and were thus able to detect increasing numbers of GFP-positive cells when mESCs were bulk differentiated in a monolayer. Analysis of the GFP-positive cells after sorting revealed that they had differentiated into early germ cells on the basis of the expression of germ cell markers *c-Kit* and *Mvh*. Extended culture of these cells resulted in the formation of aggregates that detached from the culture surface and expanded in suspension, forming structures resembling ovarian follicles, comprising an oocyte with associated somatic cells. Consistent with these structures being follicle-like, they were found to release estradiol into the culture medium. Eventually, the follicle-like structures extruded the putative oocytes, and in some instances the formation of blastocyst-like structures was detected, apparently through a parthenogenetic mechanism (Hubner *et al.*, 2003).

Shortly after the report of mESC-derived 'oocytes', two other groups reported the derivation of male germ cells from mESCs. Geijsen *et al.* (2004) differentiated mESCs into three-dimensional cellular aggregates known as embryoid bodies (EBs), a system that

Table I. Major developments in pluripotent stem cell-derived gametes.

Source material	Method	Endpoint	Reference
Mouse ES cells	Monolayer differentiation, followed by sorting on cell surface markers	Formation of follicle-like structures, progressing to blastocyst-like structures by possible parthenogenesis	Hubner <i>et al.</i> (2003)
Mouse ES cells	Formation of EBs, isolation of SSEA1-positive cells	EG-like cells isolated when rare residual SSEA1-positive cells isolated from EBs and cultured in presence of RA Isolation of cells with haploid DNA content expressing post-meiotic male germ cell markers after extended EB differentiation Fertilization of oocytes with sperm-like cells, progression to morula stage embryo	Geijsen <i>et al.</i> (2004)
Mouse ES cells	<i>Mvh-LacZ</i> ES cells cultured in aggregates with BMP4-expressing somatic cells LacZ-positive ES cells isolated and aggregated with fetal gonadal somatic cells, transplanted into testis capsule	Aggregates formed new tubule structures <i>de novo</i> under testis capsule. All stages of spermatogenic cells detectable Functionality of sperm not tested	Toyooka <i>et al.</i> (2003)
Human ES cells	ES cells differentiated into EBs	Transcripts for PGC, pre-meiotic and post-meiotic germ cells expressed in time-dependent fashion during course of differentiation. Detection of VASA protein in cells at periphery of EBs Failure of meiosis, simultaneous detection of male and female germ cell transcriptional programme irrespective of ES cell karyotype	Clark <i>et al.</i> (2004)
Porcine skin-derived stem cells	Differentiation into sphere-like structures, oocyte-like cells cultured in presence of porcine follicular fluid	Follicle-like structures containing oocytes with zona pellucida-like casings Progression to blastocyst stage when fertilized	Dyce <i>et al.</i> (2005)
Mouse ES cells	Differentiation into EBs in the presence of testicular cell-conditioned medium	Ovary-like structures containing oocyte-like cells produced at high efficiency	Lacham-Kaplan <i>et al.</i> (2006)
Mouse ES cells	Stimulation with RA, isolation and culture of <i>Stra8</i> -GFP-positive cells, subsequent differentiation into 'haploid' cells expressing post-meiotic <i>Prm1</i> -DsRed reporter	Haploid sperm like-cells isolated, used in ICSI producing live mice, although these died shortly after birth due to suspected imprinting defects	Nayernia <i>et al.</i> (2006b)

EB, embryoid bodies; EG, embryonic germ; BMP, bone morphogenic protein; ES, embryonic stem; RA, retinoic acid, PGC, primordial germ cell; SSEA, stage-specific embryonic antigen.

promotes differentiation in the absence of LIF. Due to the lack of surface markers that can be used to sort PGCs from ESCs, Geijsen *et al.* sorted rare SSEA1-positive cells from EBs that may represent early germ cells or remaining undifferentiated mESCs, and cultured them in the presence of RA, which promotes PGC proliferation but mESC differentiation. Under these conditions, colonies of alkaline phosphatase-positive cells formed that could be cultured indefinitely under mESC culture conditions. These cells progressively underwent imprint erasure, a hallmark of germ cell development *in vivo*. This, coupled with the unlimited proliferative potential of these cells led the authors to suggest that these cells were probably equivalent to mEGCs—pluripotent derivatives of the EB-derived PGCs (Geijsen *et al.*, 2004). Extending the culture period of the EBs for a further 3–4 weeks, followed by sorting of cells based on the expression of a marker of the acrosome, a structure found only in post-meiotic spermatids—revealed the existence of a haploid cell population. Importantly, isolation of these haploid sperm-like cells and their use in intracytoplasmic sperm injection (ICSI) resulted in oocyte fertilization and embryonic progression to a morula-like stage but no further, probably due to imprinting defects in the mESC-derived sperm-like cells (Geijsen *et al.*, 2004). Transcripts for anti-Müllerian hormone (*Amh*) and the luteinizing hormone receptor (*Lhr*) were found in EBs, suggesting that gonadal somatic-like cells were arising in tandem with germ cells (Geijsen *et al.*, 2004). If indeed germ cells and their supporting gonadal somatic cells are closely associated within EBs, this may lead to new insights into the molecular architecture of the germ cell niche.

Toyooka *et al.* (2003) found that when co-cultured with somatic cells expressing *Bmp4*, small numbers of mESCs up-regulated *GFP*- or *LacZ*-reporter genes under the control of the *Mvh* promoter, reflecting commitment to the germ cell lineage. Mixing the *GFP*- or *LacZ*-positive cells with fetal gonadal somatic cells and implantation of the aggregates beneath the testis capsule resulted in the formation of seminiferous tubule-like structures distinct from those of the host testis. These tubules appeared to contain normal spermatogenesis, and morphologically normal spermatozoa with condensed nuclei and tails. Sperm isolated from the transplant-derived tubules were found to contain the *LacZ* transgene, demonstrating that they were indeed derived from mESCs, although the fertilizing ability of the sperm was not reported (Toyooka *et al.*, 2003).

Subsequently, Lacham-Kaplan *et al.* (2006) reported that EBs cultured in media conditioned by cultures of neonatal testicular cells gave rise to ovary-like structures containing cells expressing oocyte-specific markers with remarkably high frequency (83%), although replication of this result has yet to be reported. Novak *et al.* (2006) derived follicle-like structures similar to those seen by Hubner *et al.* (2003) but found that they were unable to correctly progress through meiosis, indicating that although *in vitro* haploid germ cell formation has been reported, synapsis, recombination and appropriate segregation of chromosomes may not occur.

Building on earlier work in teratocarcinoma and bone-marrow-derived stem cells, Nayernia *et al.* (2006b) reported the production of live offspring from sperm produced *in vitro*. Using two fluorescent reporter genes (*GFP* and *DsRed*) under the control of spermatogonia-

(*Stra8*) and post-meiotic spermatid-specific (*Prml1*) promoters respectively, they were able to track the progression of mESC-derived germ cells through the spermatogenic process and isolate cells with a sperm-like morphology. Use of these cells for ISCI resulted in a number of pregnancies, although the majority died *in utero* and the remainder died within a few months of being born, probably as a result of imprinting defects (Nayernia *et al.*, 2006b). This development, if able to be replicated, would represent a significant step forward, and suggest that differentiation of mESCs into germ cells has the potential to produce fully functional gametes.

Progress in producing germ cells from human ESCs has been less successful. To date, Clark *et al.* (2004) are the only group to have reported the successful formation of germ cells from hESCs differentiated into EBs. Differentiation of hESCs over a period of 2 weeks resulted in a steady decline in the levels of pluripotency markers *NANOG* and *OCT-3/4*, and an up-regulation of germ cell-specific transcripts including *VASA*, *SYCP1* and *PUMILIO-2*. Of particular interest is the finding that differentiating hESCs expressed both the oocyte-specific gene *GDF9* and the spermatid-specific gene *TEKTI* irrespective of whether the cells were karyotypically female (XX) or male (XY) (Clark *et al.*, 2004). Consistent with the report that mESC-derived oocytes were unable to correctly undergo meiosis (Novak *et al.*, 2006), Clark *et al.* (2004) were unable to detect hESC-derived germ cells undergoing normal meiosis as judged by the formation of synaptonemal complexes. The simultaneous activation of both the male and female germ cell transcriptional programmes in hESC-derived germ cells may explain the meiotic failure seen in hESC-derived germ cells.

Uses and limitations of stem cell-derived gametes

Despite initial promise there has been little progress using this system as a model for gametogenesis. Germ cell derivation remains very inefficient with less than one of one million starting cells becoming a germ cell (Geijsen *et al.*, 2004). Why the efficiency of this process is so low is unclear, though it may be related to the failure of meiosis seen by Novak *et al.* and/or the simultaneous activation of both male and female germ cell programmes in ESC-derived gametes resulting in meiotic catastrophe (Clark *et al.*, 2004; Novak *et al.*, 2006). Methodologically, culturing cells in high glucose media appears to be beneficial for deriving germ cells from mESCs (Mizuno *et al.*, 2006), and supplementing culture media with a cocktail of recombinant BMP4, 7 and 8b has been reported to enhance the efficiency of germ cell derivation from hESCs (Kee *et al.*, 2006).

The recent finding that some hESC lines display greater propensities to differentiate along certain lineages than others is of particular interest (Osafune *et al.*, 2008) and suggests that some ESC lines could produce gametes more efficiently than others. Although Clark *et al.* (2004) were unable to find significant differences in the expression levels of multiple germ cell-associated genes in three independent undifferentiated hESC lines, the quantitative RT-PCR method used in their experiment could only provide a global overview of the transcriptional landscape of the entire hESC population analysed. It is likely that stochastic differences in gene expression between individual ESCs within a differentiating population will have some influence on cell fate decisions. Indeed, immunostaining of undifferentiated mESCs and hESCs reveals a non-uniform pattern of markers including *Nanog*, *Stella*, and *Dazl* within individual colonies (Clark *et al.*, 2004; Payer *et al.*, 2006; Chambers *et al.*, 2007).

A major potential benefit of using stem cells to model germ cell development is the ability to carry out genetic modifications in the starting cell population. Although the genetic modification of PGCs has been demonstrated using retroviruses (De Miguel *et al.*, 2002),

low survival rates and poor differentiation of isolated PGCs *in vitro* makes this a laborious system. Transgenes on the other hand can be readily introduced to ESCs, and clonal lines in which the construct has integrated can be isolated using drug selection. Furthermore, rather than having to use homologous recombination to target a gene for disruption, it should be possible to introduce siRNA constructs that target-specific genes of interest into the undifferentiated ESC population prior to differentiating them into germ cells. Knocking down specific genes in ESCs then assaying their ability to form germ cells when differentiated *in vitro* could provide a more rapid and efficient method for identifying new candidate fertility genes. The apparent failure of stem cell-derived gametes to undergo meiosis efficiently could also provide opportunities to study the molecular mechanisms regulating meiotic progression and provide insight into the causes of meiotic failure.

Stage-specific approaches to modelling germ cell development

Rather than attempting to recapitulate the entire germ cell differentiation pathway from ESCs *in vitro*, which appears to happen only with very low efficiency, recent developments in stem cell research may offer the opportunity to study specific stages of the process *in vitro* using cell lines derived from different stages.

In vivo the germ cell population arises from the proximal epiblast of the post-implantation embryo (McLaren, 2003). Whether the cells within an EB that give rise to germ cells *in vitro* go through a similar epiblast-like stage early in their differentiation is yet to be established, although the finding that treatment with BMPs increases the efficiency of ESC-derived germ cell formation might suggest that this is the case (Kee *et al.*, 2006). Two groups have recently reported the isolation and culture of pluripotent stem cells from the mouse epiblast (Brons *et al.*, 2007; Tesar *et al.*, 2007). These cells can be expanded and maintained in the undifferentiated state under the same culture conditions as hESCs (in the presence of activin/nodal and FGF, rather than LIF) and display a morphology and transcriptome more akin to that of hESCs than mESCs. Treatment of epiblast stem cells with BMP4 resulted in the up-regulation of *Blimp1* within 24 h, followed by later *Stella*, thus recapitulating the sequence of changes in gene expression seen during the earliest stages of germ cell formation (Tesar *et al.*, 2007). The efficiency of germ cell induction from epiblast stem cells has not yet been reported, but as undifferentiated epiblast stem cells do not express germ cell markers such as *Dazl* and *Stella* (Tesar *et al.*, 2007), they may provide a cleaner model for the study of early germ cell development than ESCs, which express many supposedly 'germ cell-specific' transcripts.

New developments in the study of germline stem cells may also present opportunities to study the later stages of gametogenesis *in vitro*. Several groups have reported the isolation of SSCs and their expansion in culture (Kanatsu-Shinohara *et al.*, 2003; Kubota *et al.*, 2004). Although no reports of *in vitro* differentiation of SSCs to haploid sperm have been forthcoming, these cells can be transplanted back into recipient testes and re-establish spermatogenesis (Kanatsu-Shinohara *et al.*, 2003). The genetic modification of SSCs *in vitro* has been demonstrated (Nagano *et al.*, 2001, 2002; Kanatsu-Shinohara *et al.*, 2004b, 2006). Combining the introduction of overexpression or knockdown constructs into SSCs with their subsequent transplantation back into recipient testes may offer a viable alternative approach for investigating the effect of disrupting candidate fertility genes on spermatogenesis.

Comparable developments may not be possible in the female as there is believed to be no analogous germ stem cell in the ovary. Work by Johnson *et al.* (2004, 2005) has challenged this with a

report claiming the identification of germline stem cells in the post-natal mouse ovary, possibly deriving from bone marrow, which can replenish the primordial follicle pool. Although this work remains highly controversial (Telfer *et al.*, 2005), it does reopen the prospect that a rare germline stem cell population exists within the post-natal ovary. The finding that bone marrow-derived stem cells from both mouse and human can form cells with spermatogenic characteristics may support this (Nayernia *et al.*, 2006a).

Other strategies for manipulating cell differentiation

A major breakthrough likely to impact on the field of germ cell research is the recent discovery that differentiated adult cells can be reprogrammed into pluripotent stem cells. By introducing a cocktail of transgenes (*Oct-3/4*, *Sox2*, *Klf4* and *c-Myc*), mouse fibroblasts could be reprogrammed to ESC-like 'induced pluripotent cells' (iPS cells) capable of differentiating into all three germ layers *in vitro* and *in vivo* (Takahashi and Yamanaka, 2006). The same group and others have since demonstrated that the same effect is achievable in terminally differentiated human dermal fibroblasts (Takahashi *et al.*, 2007). It has since been reported that the use of *c-Myc*, the inclusion of which resulted in tumours in chimaera mice produced using iPS cells, is dispensable for the derivation of both human and mouse iPS cells, although the use of viral vectors to introduce transgenes still poses a risk of insertional mutagenesis (Yu *et al.*, 2007; Nakagawa *et al.*, 2008). Whether human iPS cells are capable of generating germ cells *in vitro* has yet to be demonstrated but this approach has the potential to greatly increase access to pluripotent stem cells, especially for researchers in countries where hESC derivation or research is limited or banned by law.

Therapeutic potential

The use of ESC-derived gametes in assisted reproduction treatments remains a distant prospect. At present it would require the cloning of an individual, derivation of stem cells from the resultant blastocyst and the subsequent derivation of gametes from them. The recent discovery of iPS technology may offer a shortcut by allowing the creation of pluripotent stem cells directly from a patient's tissues that could then be used as a source of cells from which gametes could be derived *in vitro*. Even if these challenges could be overcome, major obstacles to the use of *in vitro*-derived gametes will still exist. The greatest of these is safety. Although cells with a haploid DNA content have been created *in vitro* whether they consistently form with the correct chromosome complement has yet to be demonstrated. hESCs have been shown to show chromosome gain during extended periods of culture (Draper *et al.*, 2004), so any future use in therapy would require tight quality control procedures and frequent assaying for chromosomal aberrations.

Perhaps most importantly however, the appropriate erasure and re-establishment of sex-specific patterns of genomic imprints will need to be demonstrated. The mEGC-like cells generated by culturing mESC-derived PGCs were found to erase imprints, but no data were presented as to whether the correct androgenetic imprinting pattern was installed when EBs were allowed to differentiate for longer and produce haploid sperm-like cells (Geijsen *et al.*, 2004). The inability of mESC-derived sperm to support development beyond the morula stage would suggest that, at least in part, it was not (Geijsen *et al.*, 2004). Inconsistent erasure and re-establishment of imprints in mESC-derived sperm has been reported and is likely to underpin the premature death and phenotypic abnormalities seen in the offspring produced using *in vitro*-produced sperm (Nayernia *et al.*, 2006b).

However, quite what level of proof would be required before *in vitro*-derived gametes could be used therapeutically remains unclear. Indeed, it could be argued that existing reproductive technologies (most notably ICSI) have been adopted without extensive safety analysis, and there remains a concern that assisted reproductive technologies may result in a higher frequency of imprinting defect-associated conditions such as Beckwith–Wiedeman syndrome (Cox *et al.*, 2002; Maher *et al.*, 2003; Allen and Reardon, 2005). Certainly, robust molecular and biochemical documentation of the appropriate resetting of imprints in hESC-derived gametes would be a prerequisite, as too would large-scale karyotyping analyses demonstrating that *in vitro*-derived gametes were repeatedly produced with the appropriate chromosome number. Determining whether meiosis and recombination occur appropriately during the process of deriving germ cells from pluripotent stem cells is also of some importance, given the suggestion that ESC-derived gametes are unable to complete meiosis (Clark *et al.*, 2004; Novak *et al.*, 2006). Should the meiotic process proceed *in vitro* without recombination, it would produce gametes genetically identical to the donor cells from which they were derived, albeit with half the number of chromosomes, which arguably has ethical implications for the identity of the offspring.

In the shorter term, ESC-derived artificial gametes have the potential to make a significant clinical impact in two ways. First, a major practical application for all stem cell-derived tissues is in toxicity screening for drugs in development (Rubin, 2008). The effects of candidate drugs on stem cell-derived gametes could provide useful pre-clinical insight into the potential effects of any drug on reproductive function both in screening for toxicity and for application in contraception, and could be extended to investigate the impact of environmental chemicals on germ cell development. Secondly, the short supply of good-quality human oocytes for use in somatic cell nuclear transfer experiments is a major factor limiting research into the generation of so-called 'patient-specific' hESC lines with great potential therapeutic benefit (Hall and Stojkovic, 2006). Should the derivation of oocytes from mESCs be repeated using hESCs, it may be that this route could provide a potentially limitless source of *in vitro*-derived oocytes that can be used as recipients for somatic cell nuclear transfer (Hubner *et al.*, 2003; Nagy and Chang, 2007).

Conclusions

The use of stem cells to model germ cell development *in vitro* remains an avenue of research which is still very much in its infancy. Confirmation of the reports of Hubner *et al.* (2003), Toyooka *et al.* (2003) and Geijsen *et al.* (2004) at least in part by other groups (Novak *et al.*, 2006; Kerkis *et al.*, 2007), demonstrates that this approach will be of scientific use, and it is likely that issues of efficiency and robustness will be resolved. Information obtained from *in vitro* germ cell models will reciprocally inform the study of human germ cell development, complimenting data derived from fetal material. As the progression of *in vitro*-produced human germ cells has yet to pass the premeiotic stage (Clark *et al.*, 2004), the prospect of this technology being used for therapeutic benefit remains a distant but exciting possibility. The rapid advancement of stem cell research, particularly with respect to iPS technology, suggests that useful *in vitro* approaches to modelling germ cell development are unlikely to be a long way off.

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