Differentiation of germ cells and gametes from stem cells

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Background: Advances in stem cell research have opened new perspectives for regenerative and reproductive medicine. Stem cells (SC) can differentiate under appropriate in vitro and in vivo conditions into different cell types. Several groups have reported their ability to differentiate SCs into germline cells, and some of them have been successful in obtaining male and female gamete-like cells by using different methodologies.

Methods: This review summarizes the current knowledge in this field and emphasizes significant embryological, genetic and epigenetic aspects of germ cells and gametes in vitro differentiation in humans and other species, highlighting major obstacles that need to be overcome for successful gametogenesis in culture: studies reporting development of germ cell-like cells from murine and human embryonic (ESC) and somatic SCs are critically reviewed.

Results: Published studies indicate that germ cells can be consistently differentiated from mouse and human ESC. However, further differentiation of germ cells through gametogenesis still has important genetic and epigenetic obstacles to be efficient.

Conclusions: Differentiation of germ cells from SCs has the potential of becoming a future source of gametes for research use, although further investigation is needed to understand and develop the appropriate niches and culture conditions. Additionally, if genetic and epigenetic methodological limitations could be solved, therapeutic opportunities could be also considered.

Key words: differentiation / gametes / germ cells / embryonic stem cells / reproductive medicine

Introduction

Stem cells (SC) are undifferentiated cells that have the potential to self-replicate and give rise to specialised cells. These include cells lost from normal turnover or from a disease in specific organs and tissues, reflecting on their possible use in future therapies.

SCs can be obtained from the embryo at cleavage or blastocyst stages (embryonic stem cells, ESC), but also from extra-embryonic tissues such as the umbilical cord blood obtained at birth (McCuckin et al., 2005), the placenta (Miki et al., 2005) and the amniotic fluid (De Coppi et al., 2007). SC can also be obtained in adult mammals from specific niches. These somatic SCs can be found in a wide range of tissues including bone marrow (Pittenger et al., 1999), blood (Goodman and Hodgson, 1962; Barnes and Loutit, 1967), fat (Zuk et al., 2002), skin (Toma et al., 2001; Alonso y Fuchs, 2003) and also the testis (Guan et al., 2006). ESCs exposed to appropriate and specific conditions differentiate into cell types of all three germ layers (endoderm, ectoderm and...
mesoderm) and also into germline cells. The latter had raised specula-
tions that ESCs may have a potential role in reproductive medicine.

According to the World Health Organisation (WHO), infertility is
considered a disease affecting millions of people in Europe, where
prevalence is 14% of couples in reproductive age. Based on the
2005 National Survey on Family Growth American report, there
was a 20% increase in American couples experiencing impaired
fecundity between 1995 and 2002. This may be related to a tendency
to delay motherhood to the third decade of life due to professional
and social reasons. As a consequence, oocyte quality in females is
reduced (Howe, 1985; Spira, 1988; Maroulis, 1997; Ziebe et al.,
2001).

The already short reproductive period in women can be even shor-
tened if oocytes or granulosa cells are damaged following cancer treat-
ments; consequently, assisted fertilization is required.

In males, sperm are produced continuously during the adult life,
and hence, spermatogenesis may be re-established through progenitor
germ SC within the testis. In the case of radiation, the damage is
dose dependent, leading from transient to permanent infertility in
men, and consequently, to the necessity of assisted fertilization treat-
ments (Kinsella, 1989; Lampe et al., 1997). The option of storing
mature sperm prior to treatment is a common practice, but this possi-
bility does not exist for pre-pubertal cancer patients. For these
patients, transplantation of spermatogonial stem cells (SSCs) obtained
before treatment is the only possible strategy to restore fertility,
although with the risk of reseeding cancer cells back to them.

Failure of patients to obtain viable embryos and pregnancies
through assisted reproductive techniques (ART) due to the reduced
quality of their gametes leaves them with the option of receiving
donated oocytes and sperm. From ART cycles performed in 2000
in 49 countries worldwide, 32.3% of the procedures involved
oocyte donation (Adamson et al., 2006). In the USA alone in 2004,
12.5% of ART cycles were carried out with donor oocytes (ART
Data published by the European Society of Human Reproduction
and Embryology (ESHRE), showed that the proportion of ART
cycles with oocyte donation increased by approximately 20% from
2003 to 2004 in Europe (Andersen et al., 2007, 2008). Although preg-
nancy rates with these procedures exceed 50%, oocyte donation
always raises ethical, legal and personal concerns. In addition, patients
value biological parenthood and desire their own biological
descendant.

**Methods**

The development and improvement of the research on ESC-derived
gametes has gained attention from the reproductive biology field in
recent years. Several studies reported that murine ESCs can develop
into germ cell precursor cells in vitro (Hubner et al., 2003; Toyooka
et al., 2003; Geijsem et al., 2004; Lacham-Kaplan et al., 2006; Neymaria
et al., 2006b; Novak et al., 2006; Qing et al., 2007). Also human ESCs
(hESCs) show spontaneous and induced differentiation into cells with
markers of germ cells (Clark et al., 2004; Kee et al., 2006; Chen et al.,
2007).

The present critical review will outline progress in research into ESC-
derived gametes in mice and humans in recent years, focusing on the
potential benefits that this development may hold for reproductive thera-
pies. In addition, this paper will outline possibilities to improve the
outcome of these procedures to result in viable gametes, which will be
accepted for clinical use. For this purpose, the more knowledge about
the processes of gametogenesis in vivo the better to replicate them prop-
erly to obtain viable gametes in vitro.

**Specification of germ cells in mammals**

Germ cells are the biological route for genetic transmission from one
generation to the next. These cells constitute a very different cell
population from somatic cells, with unique characteristics, and
display a haploid chromosome number after a delicate process of
meiosis. But, how do these cells acquire a different fate than the
other cells of the individual? Can we induce this same behaviour in
undifferentiated SC in vitro?

In humans, and mammals in general, germ cells are derived from
a specific pluripotent population known as primordial germ cells
(PGCs), which segregate early in embryogenesis and become progeni-
tors of adult gametes. The first PGCs in humans are located in the yolk
sac outside the embryo at 2–3 weeks of development (Witschi,
1948). In mice, they are originated from a founder population of
cells located at the base of the allantois on Day E7.25 (Ginsburg
et al., 1990) in response to inductive signals emanating from the adja-
cent extra-embryonic ectoderm, including bone morphogenetic pro-
teins (BMPs), particularly BMP-4 and -8 (Lawson et al., 1999; Ying
et al., 2000, 2001; Fujiwara et al., 2001; Saitou et al., 2002). In addition
to PGCs, the pluripotent epiblast cells also give rise to the three
embryonic lineages: the mesoderm, ectoderm and endoderm. While
these three lineages continue their differentiation into other tissues
in the body, PGCs retain pluripotency as identified by the expression
of specific markers such as Oct4 and Nanog (Scholer et al.,
1990; Pesce et al., 1998; Mitsui et al., 2003) and high alkaline phosphatase
activity (Chiquoine et al., 1954, Ginsburg et al., 1990) (Fig. 1).

Mouse and human PGCs migrate in the developing embryo towards
the gonadal ridge, proliferate and differentiate into gonocytes, the
primitive germ cells. In human, this migration takes place between
weeks 4–6 (Witschi, 1948; Fujimoto et al., 1977; Goto et al., 1999)
and in mice between days E8.5–E12.5 (Wylie et al., 1986).

The differentiation of gonads into a primitive ovary or testis
coincides with time of arrival of the PGCs to the genital ridge, creating
the proper microenvironment or niche necessary for sex determi-
nation (Byskov, 1986).

In males, gonocytes arrest in G0 and stay mitotically quiescent until
birth (McLaren, 1984; Goto et al., 1999). At birth, quiescent cells are
triggered to enter mitotic cell cycles and differentiate into sperma-
togonia that undergo multiple cell divisions and differentiate into sperma-
tocytes. These cells continue their meiosis into haploid spermatids,
which proceed into mature sperm. A small population of SSCs
(capable of renewing themselves or producing spermatogonia for
further differentiation) remains in the testis through adulthood (Bellve
et al., 1977). These cells continuously enter the meiotic cell pool to
result in haploid cells. Similar germ stem cells (GSCs, multipotent
SCs derived from PGCs that generate sperm or oocytes) are not
thought to be present in the adult ovary although controversial
studies by Johnson and Bukovsky have challenged this dogma
(Johnson et al., 2004, 2005; Bukovsky et al., 2005). These researchers
have observed that adult mouse and human ovaries possess mitotically active GSCs, allowing continued renewal of the follicle pool in adult ovaries.

In females, PGCs initiate meiosis at the same time in development that male germ cells enter mitotic arrest. This occurs at the beginning of week 12 of human gestation (Gondos et al., 1986) and in mice on day E13.5 (Monk and McLaren, 1981). PGCs in the female embryo do not complete meiosis, and are arrested at prophase I of the first meiotic cycle. Progress in meiosis occurs post-natally just prior to ovulation followed by another arrest in meiosis II, which is completed just after fertilization (McLaren, 1995; Goto et al., 1999) (Fig. 1).

### Epigenetic modifications in the germline

Genomic imprinting is a DNA modification pattern which is common and unique to all cells of an individual. This imprinting can be found at both genomic areas without a specific function and areas where the expression of specific genes is controlled. Thereby imprinting can control the proper expression levels of genes required for normal embryonic development and cell function, without changing the genome itself. In an organism the imprinting pattern is partially inherited from both paternal and maternal genomes. After fertilization the imprinting marks and epi-mutations are erased by the epigenetic machinery of the zygote, and then re-established in a new and unique pattern depending on the sex of the new individual.

These epigenetic marks, which define the imprinting, include modifications in DNA and histones, especially methylation, acetylation, phosphorylation and ubiquitination, DNA methylation being the best characterized of these mechanisms, which has been shown to have essential functions in the germline and the embryo development. These chromatin modifications provide a mechanism for the adequate expression and repression of genes and hence for their temporal or permanent inactivation (Razin and Cedar, 1991; Yeivin and Razin, 1993; Wolfe et al., 1998).

Epigenetic modifications are sequentially established and erased in the germinal lineage. PGCs undergo DNA demethylation of the imprinted loci as soon as they reach the gonadal ridge and the parental patterns are erased (Monk, 1987; Brandeis et al., 1993; Lee et al., 2002; Yamazaki et al., 2003). Re-establishment of different imprints in germ cells of both sexes with new patterns according to the gender of the new individual is initiated during male and female gamete differentiation. During spermatogenesis, de novo methylation occurs before the onset of meiosis, while in oogenesis it occurs after the onset of meiosis (Kafri et al., 1992; Brandeis et al., 1993; Kono et al., 1996; Davis et al., 2000; Obata and Kono, 2002) (Fig. 2). For the zygote to acquire totipotency extensive epigenetic reprogramming occurs. Shortly after fertilization the paternal genome undergoes rapid demethylation, while the maternal genome is slowly demethylated during the first cleavages (Reik and Walter, 2001; Santos et al., 2002). Then, around the time of implantation of the blastocyst there is a wave of de novo methylation to establish an individual specific pattern (Reik et al., 2001; Hajkova et al., 2002).

Parthenogenesis, a mechanism by which an oocyte is activated without fertilization by a spermatozoid, has been described in some studies after differentiation of oocyte-like cells from SC in culture (Hübner et al., 2003; Dyce et al., 2006). However, the formed embryos arrested in early stages of the process with no further development. It is likely that the levels of imprinted genes are unbalanced in these embryos, suggesting that some imprints are not correctly reprogrammed in the differentiated gametes.

Factors that mediate epigenetic resetting are not known, but they must be present in early embryos and pluripotent SC. The pluripotent cells of the inner cell mass (ICM) and the PGCs share a common feature: expression of the homeobox gene Nanog (Mitsui et al., 2003; Yamaguchi et al., 2005). Nanog is responsible for the maintenance of pluripotency in SC, and loss of its expression leads to cell differentiation (Mitsui et al., 2003; Wang et al., 2003). During mice
embryonic development Nanog is expressed in preimplantation embryos (morula stage and ICM of blastocysts) and in migrating and gonadal PGCs from Day E7.75–E12.25 (Mitsui et al., 2003; Yamaguchi et al., 2005). Nanog expression is down-regulated in female germ cells with the onset of meiosis at E13.5–E14.5, and in male germ cells after mitotic arrest in E14.5 embryos (Yamaguchi et al., 2005).

A recent study by Chambers et al. (2007) shows that gonadal ridges of chimaeras resulting from aggregation of Nanog null ESCs with wild-type morulae lack PGCs beyond E11.5, suggesting that Nanog is required for germline development and maturation, although, the defect is rescued by repair of the mutant allele. The authors propose that the primary function of Nanog may be to establish ICM and PGC states in vivo and to promote nuclear reprogramming into a state with minimal epigenetic modifications and consequently with a broad developmental potential. However, further work needs to be performed to show that this is indeed the case.

In vitro differentiation of gametes from SC

In vitro development of germ cells from SC to obtain mature, haploid male and female gametes with the capacity to participate in normal embryo and fetal development has been attempted for the last 5 years. To date, some studies have been published involving differentiation of mouse and human SC, either embryonic or somatic, into germ cells and both male and female presumptive gametes. Nevertheless, the accurate functionality of these structures still needs to be demonstrated (Table I).

GSCs appear spontaneously in ESC cultures indicating that, similar to their ability to give rise to many other lineages following the removal of pluripotency maintaining factors, such as feeder cells and leukaemia-inhibiting factor (LIF) (in the mouse), ESCs are also reprogrammed to go through the germline lineage (Hübner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Lacham-Kaplan et al., 2006; Chen et al., 2007; Qing et al., 2007). Exogenous addition of factors such as BMP4 and retinoic acid (RA) (Toyooka et al., 2003; Geijsen et al., 2004, Nayernia et al., 2006b) improves recruitment of GSCs and enhances their maintenance in vitro, but does not support their progress through meiosis.

One of the major difficulties in the in vitro differentiation of germ cells is the lack of appropriate molecular markers for the characterization of the differentiated germ cells to distinguish them from the somatic cells. Most of the markers used for PGCs are present in ESCs as well: Fragilis, Stella, DAZL, c-Kit (Clark et al., 2004). Therefore, it is very difficult to distinguish early germ cells from undifferentiated ESCs. Meiotic and post-meiotic markers are more reliable markers, but it has been demonstrated that the progression through the meiotic process is still a challenge in the in vitro differentiation of gametes (Table II). The transfection of ESC lines with marked or fluorescent proteins linked to specific gene promoters (genes implicated in pluripotency or germ cell line development) enables the visualization of the cells in which the specific gene of interest is expressed during the differentiation process. However, the use of transfected lines disqualifies the putative gametes obtained for their application in clinical procedures.

In vitro germ cells differentiation from ESCs

Basically, two different methods, with some variants, have been used for germ cells differentiation from human and murine ESCs. One of
them consists in spontaneous differentiation in adherent culture after removing factors that promote pluripotency as feeders and basic fibroblast growth factor (bFGF) or LIF (Hübner et al., 2003; Novak et al., 2006; Chen et al., 2007), whereas the second one implicates the formation of three-dimensional structures known as embryoid bodies (EBs, Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Lacham-Kaplan et al., 2006; Novak et al., 2006; Nayernia et al., 2006a; Kee et al., 2006; Qing et al., 2007; Chen et al., 2007). Using the first method, Hübner et al. (2003) differentiated a mESC transgenic line with specific green fluorescent protein (GFP)-tagged Oct4 expression in germ cells and reported the obtaining of floating structures mimicking ovarian follicles, which extruded a central cell after gonadotrophin stimulation. PCR analysis showed the expression of oocyte-specific markers as ZP-2, ZP-3 and FlGa but not ZP-1, maybe this is the reason why the zona pellucida was very fragile. Although the presence of the meiotic protein SCP3 indicated entry of the putative oocytes in the meiotic process, Novak et al. (2006) did not detect other meiotic proteins as SCP1 and SCP2 or Rec8 when they replicated the same experiment. Furthermore, no evidence of chromosomal synopsis formation was detected. Then, although the meiotic process is initiated in some cells, the meiotic programme fails to progress correctly in vitro. Some of these structures were spontaneously activated leading to the formation of parthenogenetic embryos which arrested and degenerated in early stages of development.

Toyooka et al. (2003) and Geijsen et al., (2004) obtained male germ cells from mouse ESCs through EBs formation combined with the use of knock-in cell lines with markers associated with pluripotency or germline characteristic genes. The EBs are three-dimensional structures formed by the aggregation of undifferentiated ESCs, in which different cell types from the three embryonic germ layers can be formed, but also cells of the germ lineage.

**Table I** Summary of the included studies on germ cell differentiation from stem cells

<table>
<thead>
<tr>
<th>Publication</th>
<th>Origin</th>
<th>Strategy for differentiation</th>
<th>Cell type obtained</th>
<th>Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germ cell differentiation from ESCs</td>
<td>mESC—XX, XY</td>
<td>SD in adherent cultures</td>
<td>Oocyte-like cells</td>
<td>No, PB</td>
</tr>
<tr>
<td>Hübner et al. (2003)</td>
<td>mESC—XX, XY</td>
<td>EBs formation+AC with growth factors</td>
<td>PGCs in vitro, sperm in vivo when transplanted</td>
<td>NT</td>
</tr>
<tr>
<td>Toynooka et al. (2003)</td>
<td>mESC—XX, XY</td>
<td>RA addition to EBs-derived cells</td>
<td>Spermatids</td>
<td>No, OF</td>
</tr>
<tr>
<td>Geijsen et al. (2004)</td>
<td>hESC—XX, XY</td>
<td>SD through EBs formation</td>
<td>Oocyte-like cells (although TEKT1 expression was found)</td>
<td>NT</td>
</tr>
<tr>
<td>Clark et al. (2004)</td>
<td>mESC—XX, XY</td>
<td>EBs with conditioned media from testicular cell culture</td>
<td>Immature oocyte-like cells</td>
<td>NT</td>
</tr>
<tr>
<td>Lacham-Kaplan et al. (2006)</td>
<td>mESC—XX, XY</td>
<td>RA addition to SSC lines-derived EBs</td>
<td>Oocyte-like cells</td>
<td>NT</td>
</tr>
<tr>
<td>Novak et al. (2006)</td>
<td>mESC—XX, XY</td>
<td>SD in adherent cultures and through EBs formation</td>
<td>Ovarian follicles</td>
<td>NT</td>
</tr>
<tr>
<td>Nayernia et al. (2006a)</td>
<td>mESC—XX, XY</td>
<td>RA addition to SSC lines-derived EBs</td>
<td>Sperm</td>
<td>Yes</td>
</tr>
<tr>
<td>Kee et al. (2006)</td>
<td>hESC—XX</td>
<td>SD through EBs+growth factors addition</td>
<td>Oocyte-like cells</td>
<td>NT</td>
</tr>
<tr>
<td>Qing et al. (2007)</td>
<td>mESC—XX</td>
<td>EBs co-cultured with ovarian granulosa cells</td>
<td>Oocyte-like cells</td>
<td>NT</td>
</tr>
<tr>
<td>Chen et al. (2007)</td>
<td>hESC—XX</td>
<td>SD in adherent cultures and through EBs formation</td>
<td>Oocyte-like cells</td>
<td>No, FD</td>
</tr>
<tr>
<td>Germ cell differentiation from somatic SCs</td>
<td>GSC in OSE</td>
<td>Histological analysis, Mvh and SCP3 IHC, BrdU-Mvh coexpression, ovarian fragments graft</td>
<td>Formation of ovarian follicles after graft</td>
<td>NT</td>
</tr>
<tr>
<td>Johnson et al. (2004)</td>
<td>GSC in female BM and blood</td>
<td>Analysis of germ cells markers, BM and blood transplantations</td>
<td>Formation of ovarian follicles after graft</td>
<td>NT</td>
</tr>
<tr>
<td>Johnson et al. (2005)</td>
<td>GSC in OSE</td>
<td>Oocyte-like cells and granulosa-like cells</td>
<td>Culture medium with estrogenic stimuli, Morphological and IHC analysis</td>
<td>NT</td>
</tr>
<tr>
<td>Bukovsky et al. (2005)</td>
<td>GSC in OSE</td>
<td>Oocyte-like cells and blastocyst-like structures</td>
<td>Medium with follicular fluid, addition of gonadotrophins, Oocytes markers and steroids production</td>
<td>PB</td>
</tr>
<tr>
<td>Dyce et al. (2006)</td>
<td>Female porcine skin</td>
<td>Spermatogonia-like cells</td>
<td>BM cells in adherent culture, RA addition, transplantation into testis</td>
<td>NT</td>
</tr>
<tr>
<td>Nayernia et al. (2006b)</td>
<td>SCs in mouse BM</td>
<td>Analysis after graft</td>
<td>Analysis of PGC, SSC and spermatogonia markers</td>
<td>NT</td>
</tr>
<tr>
<td>Drusenheimer et al. (2007)</td>
<td>SCs in human BM</td>
<td>Expression of early germ cell markers and male germ cells specific markers</td>
<td>Male germ cells</td>
<td>NT</td>
</tr>
</tbody>
</table>

Different strategies have been used to differentiate germ cells from embryonic stem cells, most of them based in spontaneous differentiation through EBs formation with or without combining addition of soluble factors to the culture media. Several studies have described the possibility of post-natal oocyte generation in females, not without controversy. The source of these cells (ovarian epithelium, bone marrow or fetal skin) still needs to be confirmed in most of them. AC, aggregation cultures with cells producing growth factors; BM, bone marrow; SD, spontaneous differentiation; SSC, spermatogonia-like cells. NT, not tested; OF, oocyte fertilization; OSE, ovarian surface epithelium; PB, parthenogenic blastocyst; PGCs, primordial germ cells; RA, retinoic acid; SD, spontaneous differentiation; SSC, spermatogonal stem cells.
Table II Molecular markers expressed during the different development and maturation stages of germ cells and gametes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein function</th>
<th>ESCs</th>
<th>PGCs</th>
<th>Migrating/premeiotic</th>
<th>Meiosis</th>
<th>Post-meiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct4</td>
<td>TF associated with pluripotency</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Nanog</td>
<td>TF associated with pluripotency</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Blimp1</td>
<td>First indicator of germ cell fate</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fragilis</td>
<td>Early indicator of germ cell fate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Stella</td>
<td>Early germline gene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c-Kit</td>
<td>Migration and survival of PGCs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dazl</td>
<td>Differentiation and maturation of PGCs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VASA/Mvh</td>
<td>Premeiotic germ cell-specific marker</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCP1</td>
<td>Meiotic-specific markers</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCP2</td>
<td>Elements of the synaptonemal complex</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCP3</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dmc1</td>
<td>Meiotic-specific marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FGa</td>
<td>Oocyte marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GDF9</td>
<td>Oocyte marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ZP1–ZP3</td>
<td>Zona pellucida markers</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tekt-I</td>
<td>Spermatocyte marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acrosin</td>
<td>Male gametes marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Haprin</td>
<td>Haploid marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TPI</td>
<td>Sperm marker</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ESCs, embryonic stem cells; ND, not determined; PGCs, primordial germ cells (in vivo); TF, transcription factor.

Toyooka et al. (2003) formed aggregates of selected cells positive for the germ cell marker VASA from the EBs with dissociated male embryonic gonads, and transplanted them into the testes of male mice to test the developmental potential of the differentiated cells. Although marked spermatozoaids were detected in the seminiferous tubules of these animals, no further analysis of the functionality of these sperm was performed.

Geijsen et al. (2004) analysed the imprinting status of the obtained cells and reported that these cells showed erasure of the methylation pattern in some imprinted loci, suggesting that PGCs obtained in vitro display some properties of PGCs developing in vivo. Although some haploid cells were found, the results suggested that meiosis was highly inefficient in the EBs environment. Finally, the authors investigated the biological function of the haploid cells obtained via their capacity to fertilize oocytes by intracytoplasmic injection. About 20% of the fertilized oocytes progressed to blastocyst stage, but it was not tested if the embryos were capable to develop normally with uterine transfer.

Although the number of imprinted genes represents a minority of the whole human genome (2–5%), most of them seem to have critical effects on fetal growth and development. A correct imprinting is mandatory in gametes and the fertilized zygote for proper development and cell function. Imprinting alterations are implicated in abnormal fetal development and several diseases such as Prader-Willi, Angelmann, Beckwith-Wiedemann, Turner and Russell-Silver (Cassidy et al., 2000; Tycko and Morison, 2002).

The imprinting regulation of the germline must be considered when differentiation of gametes from ESCs is attempted. It is possible that generation of gametes in vitro from ESCs results in suboptimal epigenetic programming inducing alterations in embryonic growth and development. The only group that reported on the birth of live offspring from ESC-derived gametes (Nayernia et al., 2006b) analysed the methylation status of several imprinted regions in the differentiated cells and found that methylation imprints were not correctly profiled in pups. This study is the first that demonstrates abnormalities in imprinting related to in vitro-derived germ cells and the progeny generated using them. The pups showed growth abnormalities (some of them were larger and others smaller), and most died during the first months of life, emphasizing the crucial concept that imprinting status of the derived gametes from stem cells must be carefully studied (Fig. 2).

Taking a step back, scientists need to reconsider their strategies when related to in vitro gametogenesis by looking at the in vivo processes. Considerations need to include stage-specific events of imprint erasure and re-establishment, global demethylation and re-methylation and meiosis, leading to completion of oogenesis and spermatogenesis in relation to the gonad environment as a whole.

Meiosis is a specific germ cell skill. During meiosis, a single DNA replication step is followed by two consecutive cell divisions, generating haploid gametes. RA is one of the participating factors in meiosis and also in controlling differentiation into male or female phenotypes. Low levels of RA generally prevent germline cells from entering meiosis and increased levels induce meiotic resumption (Bowles et al., 2006; Doyle et al., 2008). This is done through activation of Stra8, Scp3 and Dmc1. While male gametes exposed to lower RA concentrations stay quiescent until birth, female gametes exposed to...
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high RA concentrations enter the meiotic cycle with their arrival to the genital ridge (McLaren, 2003).

The RA induces differentiation of ESC while stimulating the proliferation of precursors of the germ cells when added to the culture medium. In vivo, the timing of germ cells entry into meiosis is dependent on the gonadal environment. Several lines of investigation suggest that the ovary and testis have the same signalling system to induce meiosis, but at different timing. RA signalling is the key regulator responsible for the induction of germ cell meiosis in the fetal ovary by inducing the expression of the germ cell-specific gene, stimulated by RA, Stra8 (Bowles et al., 2006; Koubova et al., 2006). In contrast, in the fetal testis this process is blocked by the metabolism of RA to an inactive form mediated by the protein product of the Cyp26b1 gene (Yashiro et al., 2004; Bowles et al., 2006; Koubova et al., 2006). This gene is expressed in the early gonad of both male and female embryos, preventing meiosis in the germ cells when they first arrive at the gonad and come into contact with RA. When the somatic sex is determined, the action of Sry leads to the up-regulation of Cyp26b1 expression and the reduced activity of RA in the male gonad, while it is down-regulated in the female gonad, where germ cells respond to RA (Bowles et al., 2006). The source of RA in the fetal urogenital region is the mesonephros (Bowles et al., 2006), suggesting that the effect of RA inducing meiosis is direct. In the post-natal testis, it has been suggested that RA could be produced and delivered by the Sertoli cells close to the spermatogonia, inducing entry into meiosis in the same way as in the developing ovary, via the up-regulation of Stra8 expression (Bowles and Koopman, 2007).

During murine and hESCs in vitro differentiation, markers of both male and female germ cells have been detected in culture regardless of the sex of the cell line (Hübner et al., 2003; Clark et al., 2004; Lacham-Kaplan et al., 2006). The cells may be responding to an external signal instead to an intrinsic programme to enter meiosis. It is possible that RA present in the culture conditions propitiate meiosis commencement and gamete determination. In fact, the bovine serum added to cultures probably provides enough RA to induce meiosis; media supplemented with 10% fetal bovine serum is estimated to contain 3.6 x 10-8 M RA (Fuchs and Green, 1981).

The most advanced progress in meiosis and formation of male haploid gametes was obtained following transplantation of in vitro-derived GSCs into the testis (Toyooka et al., 2003; Nayernia et al., 2006b). Only two studies to date have explored co-culture systems to establish oogenesis from ESCs in mice (Lacham-Kaplan et al., 2006; Qing et al., 2007) and in both, established EBs were introduced into biological systems. Lacham-Kaplan et al. (2006) explored the effects of conditioned medium obtained from testicular cell cultures of new born male mice on the appearance of germ cells within mouse ESC-derived EBs. They discovered that a higher number of EBs produce oocyte-like cells enclosed within follicular structures when EBs were cultured in the conditioned medium. Appearance of putative oocytes was also evident in EBs transferred to conditioned medium at 120 h from initiation. These EBs already contained germ cells, an indication that the formation of oocyte-like cells was dependent on the conditioned medium but not the appearance of germ cells. Similarly, Qing et al. (2007) transferred established EBs onto ovarian granulosa cell monolayer identifying oocyte-like cells within the EBs after 10 days of culture. In both studies, the oocyte-like cells did not contain the zona pellucida and appeared similar to resting stage of in vivo gonocytes. Interestingly, the putative oocytes obtained in both studies did not undergo spontaneous cleavage as described by Hübner et al. (2003). The latter study described the appearance of oocytes in differentiated mouse ESC cultures maintained in LIF-free medium with fetal calf serum.

Attempts to derive germ cells from human ES resulted in similar findings as described in mice. Cells within hESCs-derived EBs express markers for human germ cells including c-KIT, DAZL and VASA, as well as SSEA-1 and Tra-1-60 (Clark et al., 2004; Alfantoonian and Moore, 2005; Kee et al., 2006; Chen et al., 2007). This spontaneous differentiation is line-specific (Kim et al., 2007; Chen et al., 2007). The addition of exogenous factors such as BMP-4, BMP-7 and BMP-8 to hESCs cultures increases the number of germ cells expressing VASA, but does not necessarily induce their progress in meiosis (Kee et al., 2006). From the few studies exploring the ability of hESCs differentiation into germ cells, Chen et al. (2007) was the only one to describe follicular-like structures appearing within monolayer or EBs of differentiated hESCs. Disappointingly, the study did not explore the characteristics of cells enclosed within these follicular structures to identify if they do indeed resemble oocytes.

It has been demonstrated that germ cells and gametes, both male and female, can be differentiated from ESCs in culture. However, the efficiency of the different methods to obtain the precursors or early germ cells is still low. The latest studies recently published develop different strategies to increase the number of PGC-like cells to induce the formation of mature gametes with higher efficiency. The different protocols include strategies as prolonged cultures with low availability of nutrients (Bucay et al., 2008), co-culture with mouse embryonic fibroblasts in the presence of bFGF (West et al., 2008) and enrichment of the population of PGC-like cells by FACS (fluorescence activated cell sorting) using the marker SSEA1 (stage-specific embryonic antigen 1) (Tilgner et al., 2008). In the last study, the authors reported that obtained cells showed expression of markers such as Oct4, Stella, VASA and SCP3, but no further differentiation was carried out.

Recently, a new study using a mESC line transfected with a reporter gene associated with the GDF9 gene promoter (an oocyte-specific gene) showed that GFP-positive cells with an oocyte phenotype appeared surprisingly quickly in culture after differentiation in monolayer as well as through EBs formation (Salvador et al., 2008). The authors suggest that a subpopulation of cells within the initial undifferentiated mESC might be already pre-programmed to undergo female germline differentiation. These oocyte-like cells degenerated rapidly in culture although evidence suggests that meiosis and parthenogenesis may have occurred in some of them. Oocyte-specific structures such as polar bodies and zona pellucida were observed, as well as embryo-like structures with two or four cells. When LIF was added to the culture medium the number of oocyte-like cells (GFP-positive cells) significantly increased together with the expression of oocyte-specific genes such as ZP3.

In vitro germ cells differentiation from somatic SCs

Germline stem cells and mature gametes can be derived in vitro from multipotent stem cells other than ESCs. In mice, teratocarcinoma and mesenchymal stem cells (MSC) and induced iPSC are considered multipotent cells, which are able to differentiate into various cell types
representative of all three germ layers (Kassem, 2004; Solter, 2006; Takahashi and Yamanaka, 2006). With the exception of iPS cells, the other cell types have been shown to differentiate into either germ-line stem cells or early germ cells in vitro and to advanced male gametes in vivo following transplantation.

Embyronal carcinoma (EC) cells derived from teratocarcinomas, like ESCs, are pluripotent and able to differentiate through EBs to germline stem cells (Garcia-Sanz et al., 1996; Nayernia et al., 2004). When transplanted into sterile mice, EC-derived germline stem cells are able to complete meiosis and spermatogenesis (Nayernia et al., 2004). Regardless of these promising abilities, sperm numbers in the ejaculate are reduced and 43% are morphologically abnormal (compared with only 8% in normal males). Sperm are able to fertilize oocytes through intracytoplasmic injection but the resultant zygotes proceed to the 6–8-cell stage only.

Teratocarcinoma-derived EC cells have similar imprinting patterns as PGCs. As such, the chromatin is globally hypomethylated and the promoter of imprinted genes such as H19 is unmethylated (Okamoto and Kawakami, 2007). Hence, global methylation erosion is not required when teratocarcinoma cells are used to derive GSCs in vitro.

It appears, therefore, that re-establishment of imprinting in post-meiotic gametes obtained from teratocarcinoma-derived EC cells is also unsuccessful, resulting in preimplantation embryo developmental abnormalities.

MSCs obtained either from the bone marrow (BM) or alternatively from fat tissue, were also shown to differentiate into several lineages (Kassem, 2004). It has also been proposed that MSCs are progenitors for oocytes in adult ovarian tissue (Johnson et al., 2005). That latter revolutionary proposal has been regarded as unreliable and has sparked controversy and several solid arguments have been raised against it (Byskov et al., 2005; Eggan et al., 2006). A recent work published by Liu et al. (2007) showed that meiosis, neo-oogenesis and GSCs are unlikely to occur in normal adult human ovaries. If post-natal oogenesis is finally confirmed in mice, then this species would represent an exception to the rule. Stronger evidence is needed to confirm this new theory indicating that these somatic SC-derived oocytes enter meiosis or support the development of offspring in cases of patients with allogenic BM transplant.

However, the authors of these controversial studies have come into discussion refuting the arguments against post-natal oogenesis in adult human ovary arguing, among others, that this reasoning derives from the inability of the authors to detect markers of germ cell mitosis and meiosis, and that an absence of evidence does not mean an absence of the possibility (Tilly and Johnson, 2007). Curiously, a recent work published by the same group presents a mouse model in which BM transplant helps to preserve or recover ovarian function of recipient females, but all offspring generated derived from the host germline and not from the transplanted BM cells (Lee et al., 2007).

Nayernia et al. (2006a) demonstrated that mouse MSCs are able to give rise to germline stem cells in vitro. As for germline stem cells derived from teratocarcinoma and ESCs, MSCs-derived germline stem cells arrest at premeiotic stages upon transplantation into the testes of adult sterile mice. More recently, this group announced the differentiation of sperm from human BM-derived stem cells (Drusenheimer et al., 2007). However, they have no ability to examine the functional competence of these cells, the successful establishment of which will remain unexplored due to ethical constrains.

Attempts to develop potential germ cells from somatic cells were demonstrated by Dyce et al. (2006) who have been able to differentiate oocyte-like cells from fetal porcine skin. Skin SCs in this study were isolated and cultured in follicular fluid with the addition of exogenous gonadotrophin. This resulted in the formation of follicular structures containing putative oocytes. The oocyte-like cells underwent spontaneous cleavage in culture similar to that described by Hübner et al. (2003). While it remains unclear if the skin SCs de-differentiated into ES-like cells before differentiation into the germ-line the authors postulated precociously that these events are similar to those described by Johnson et al. (2005) in mice. In humans, Bukovsky et al. (2005) reported that new oocytes can be derived from ovarian cortical mesenchymal cells. While as previously discussed, mice BM MSCs can give rise to germ cells in vitro, it has not been proven that human MSCs from the BM or any specific tissue are able to do so. On the contrary, Liu et al. (2007) clearly demonstrated that early meiotic-specific or oogenesis-associated mRNAs for SPO11 (involved in the early steps of meiotic prophase, Shannon et al., 1999), PRDM9 (essential for progression through early meiotic prophase, Hayashi et al., 2005), SCP1 (necessary for establishment of synaptonemal complex, Yuan et al., 2000), TERT (role in stem cells proliferation, Martin-Rivera et al., 1998) and NOBOX (required for follicle development, Rajkovic et al., 2004) were undetectable in adult human ovaries using RT–PCR, compared with fetal ovary and adult testis controls. These findings are further corroborated by the absence of early meiocytes and proliferating germ cells in adult human ovarian cortex probed with markers for meiosis (SCP3), oogonia (Oct4, c-Kit) and cell cycle progression (Ki-67, PCNA), in contrast to fetal ovary controls.

In summary, when male gametes differentiation has been attempted, the best results have been obtained with transplantation of premeiotic germ cells selected from differentiated EBs into in vivo systems (Toyooka et al., 2003; Nayernia et al., 2006b). For female germ cells differentiation, both culture in monolayer and through EBs formation succeeded although the oocyte-like cells obtained seemed to be in an immature stage, in most cases with a fragile structure. Their use for the generation of live progeny has not been reported.

The addition of exogenous factors to the culture media such as BMPs (Toyooka et al., 2003; Kee et al., 2006) or RA (Geijsen et al., 2004; Nayernia et al., 2006b), which play basic roles in germ cells and gametes development in vivo, seem to help to expand the germ cell population and push them to the meiotic process in vitro, but it is not enough to direct them through that process properly. At this point, a gonadal-like three-dimensional structure or specific cell-to-cell contact might be required to progress through meiosis and to acquire a correct epigenetic status. An environment similar to the in vivo niche might be a necessary requisite.

**Demanding biological descent: how to obtain patient-specific gametes**

The growing demand for biological offspring for patients with impaired fertility has put its hope in scientific research and the obtaining of patient-specific differentiated gametes. Theoretically, it can be
accomplished through two different techniques: somatic cell nuclear transfer (SCNT) and reprogramming. In both cases, the newly derived gametes will be genetically identical to the individual whose gametes are trying to be replaced.

Nuclear transfer can be defined as the creation of somatic embryos (no gametes are involved) using the oocyte cytoplasm as the reprogramming conduit following transfer of somatic nuclei into enucleated mature oocytes. In this case, hESCs can be derived from these somatic embryos and differentiated gametes will match the adult individual.

Compared with the hundreds of published reports on animal SCNT, few studies to date have been published on human SCNT (Cibelli et al., 2001; Stojkovic et al., 2005; Lavoir et al., 2005). Results from these studies indicate that human SCNT is a highly inefficient technique that often results in poor embryonic development with high aneuploidy rates. No hESCs obtained from SCNT human embryos have been reported so far, although very recently successful derivation of primate ESCs from cloned embryos has been reported by Byrne et al. (2007), and the genotyping of these Rhesus SCNT pluripotent cell lines was verified by an independent group (Cram et al., 2007). These new reports encourage the continuation of the use of SCNT for therapeutic purposes.

To overcome ethical, social and legal problems, iPSCs may, in the future, replace ESCs for clinical therapies. iPSCs are fibroblast cells from mouse or human tissue that are reprogrammed to the pluripotent state by introducing factors into their genome known to induce pluripotency such as Oct4, Sox2, c-Myc and Kif4 through retroviral transfection (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). This simple and revolutionary approach might overcome the use of oocytes and the production of somatic embryos as a source for ESC lines. This new type of stem cell, iPSC, resemble ESCs by morphology and growth properties, expression of ESCs marker genes and teratoma formation (Takahashi and Yamanaka 2006; Maherali et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). However, their global gene expression and DNA methylation patterns are similar but not identical to that of ESCs. Three studies presented a second generation of iPSC adding a new factor (Nanog) to the cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). The selection of Nanog resulted in germ line competent iPSC cells leading to the formation of chimeric mice. A high proportion (20%) of chimeric mice developed tumours resulting from the c-Myc gene expression (Okita et al., 2007), which eliminates the possibility of their current use as prospective germline stem cell progenitors.

Applications and future prospects

Seemingly, the germ cell differentiation process is dependent on spatial distribution of differentiating cells. EBs represent a three-dimensional structure with a microenvironment that propitiates differentiation, although other cell types besides germ cells arise from them (Itskovitz-Eldor et al., 2000).

Currently, one of the most critical steps after differentiation is selection and isolation of differentiated germ cells. Since ESCs and PGCs share some common markers, detection of post-migratory and meiotic markers is a useful method, as well as the use of transgenes with fluorescent reporter genes under the control of specific promoters of male and female germ cells. These have been the most employed strategies in the studies of germ cell differentiation developed to date (Hübner et al., 2003; Toyooka et al., 2003; Nayernia et al., 2006b). The problem is that methods based on gene modification and the use of retroviral vectors limit the use of gamete-like cells in future clinical treatments.

Future translational application of these ESC-derived gametes in ART when functional gametes are not available from patients require still further investigation into gamete differentiation. Ultimately, the process needs to be reproducible and efficient. The focus of current and future studies should be on meiotic completion to avoid unwanted aneuploidies and the determination and establishment of accurate epigenetic modifications and imprinting status to provide reproductive hope to humans lacking gametes.

References


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