Generation of male differentiated germ cells from various types of stem cells

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*(J Hou and S Yang contributed equally to this work)

Abstract

Infertility is a major and largely incurable disease caused by disruption and loss of germ cells. It affects 10–15% of couples, and male factor accounts for half of the cases. To obtain human male germ cells ‘especially functional spermatids’ is essential for treating male infertility. Currently, much progress has been made on generating male germ cells, including spermatogonia, spermatocytes, and spermatids, from various types of stem cells. These germ cells can also be used in investigation of the pathology of male infertility. In this review, we focused on advances on obtaining male differentiated germ cells from different kinds of stem cells, with an emphasis on the embryonic stem (ES) cells, the induced pluripotent stem (iPS) cells, and spermatogonial stem cells (SSCs). We illustrated the generation of male differentiated germ cells from ES cells, iPS cells and SSCs, and we summarized the phenotype for these stem cells, spermatocytes and spermatids. Moreover, we address the differentiation potentials of ES cells, iPS cells and SSCs. We also highlight the advantages, disadvantages and concerns on derivation of the differentiated male germ cells from several types of stem cells. The ability of generating mature and functional male gametes from stem cells could enable us to understand the precise etiology of male infertility and offer an invaluable source of autologous male gametes for treating male infertility of azoospermia patients.

Reproduction (2014) 147 R179–R188

Introduction

It has been estimated that human infertility affects 10–15% of the couples, and male factors account for about 50% of cases, which has become a severely social issue (De Kretser & Baker 1999). Azoospermia has been observed in 10–15% of male infertility and 1% of general population, and non-obstructive azoospermia has been diagnosed in 60% of azoospermic men (Matsumiya et al. 1994). Low quantity and poor quality of haploid spermatids are the main causes of male infertility in non-obstructive azoospermic men (Kee et al. 2009). Currently, semen cryopreservation is the only way to preserve male fertility by the assisted reproductive technologies (ART), such as ICSI or round spermatid injection (ROSI); however, its success rate is as low as 25% (Blackhall et al. 2002). Studies on mouse models as well as human gene mutation have suggested a correlation between genetic causes (e.g. chromosomal aberrations and single gene mutation) and male infertility (Cooke & Saunders 2002, Ferlin et al. 2007, Walsh et al. 2009, Hwang et al. 2010, Shelling 2010). For male infertility with a normal genetic background, stem cell therapy to generate male gametes may represent a promising treatment strategy.

Stem cells, by definition, have the potentials of both self-renewal and differentiation. Currently there are three major stem cell sources for generating male differentiated germ cells: the embryonic stem (ES) cells, the induced pluripotent stem (iPS) cells, and spermatogonial stem cells (SSCs). ES cells are derived from the inner cell mass (ICM) of developing blastocysts, and the first human ES cell line has been established in 1998 (Thomson et al. 1998). Notably, much progress has been made in the derivation of male differentiated germ cells from mouse or human ES cells (Clark et al. 2004, Kee et al. 2006, Mikkola et al. 2006, Nayernia et al. 2006, Chen et al. 2007, Tilgner et al.
In 2006, transcription factors have been used to reprogram somatic cells to the iPS cells (Takahashi & Yamanaka 2006). We and peers have recently demonstrated that the iPS cells could generate haploid spermatids (Park et al. 2009, Imamura et al. 2010, Hayashi et al. 2011, Easley et al. 2012, Yang et al. 2012). SSCs are able to self-renew and differentiate into male gametes called mature spermatozoa in the testis throughout the life in the male (de Rooij 1998, Brinster 2002). Recent studies have shown that spermatogonia including SSCs can be induced to differentiate into male differentiated germ cells and eventually result in haploid spermatids.

In this review, we discuss the advancements in the derivation of male differentiated germ cells from several types of stem cells, including ES cells, iPS cells, SSCs, as illustrated in Fig. 1. There are certain differences with regards to the origins, potentials and phenotype among these stem cells: i) ES cells are derived from the ICM of blastocysts, and they are totipotent since they are capable of differentiating into all cell lineages of three germ layers; ii) iPS cells are originated from the reprogramming of somatic cells, and these cells have pluripotency to generate numerous types of cells; iii) SSCs are a subpopulation of type A spermatogonia, and previously they are regarded as unipotential in that they can produce sperm only within the testis. Nevertheless, it has recently been shown that SSCs can acquire pluripotency in vitro to become ES-like cells. The phenotype for ES cells, iPS cells, SSCs, spermatocytes and spermatids was summarized and listed in Table 1. The iPS cells share pluripotent markers with ES cells, including OCT4, NANOG, SOX2, SSEA3, SSEA4, TRA-1–81 and TRA-1–60. In contrast, SSCs express the hallmarks for adult stem cells, such as CD90, GPR125 and GFRA1, although they are also positive for OCT4 and PLZF, transcription factors for ES cells and iPS cells. Furthermore, we compare the methodologies applied to generate various stages of male germ cells, including spermatogonia, spermatocytes, and spermatids, from different kinds of stem cells. Significantly, adult non-obstructive azoospermia patients with SSCs could have own biological children via the differentiation of SSCs into haploid spermatids, while boy cancer patients can cryopreserve their SSCs before chemotherapy and/or irradiation therapy and these SSCs with expansion in culture could be transplanted back to the patients after treatment or induced to differentiate into functional spermatids (Jahnukainen & Stukenborg 2012, Struijk et al. 2013), since a number of parents of these boy cancer patients desire to preserve their son’s fertility (Sadri-Ardekani et al. 2013). The Sertoli cell-only syndrome patients without male germ cells might father own biological children using their iPS cells-derived male gametes.

**Male germ cells derived from ES cells**

ES cells are totipotent cells derived from the ICM of blastocysts, and they have the ability of self-renewal and differentiating into all cell types of three germ layers in the body, including male germ cells (Martin 1981, West et al. 2006). When introduced into preimplantation embryos, ES cells can develop into germline in chimeric embryos and produce normal sperm (Bradley et al. 1984). Much progress has been made in the derivation of male germ cells from mouse and human ES cells, as shown in Tables 2 and 3, respectively.

In mice, several groups utilized the embryoid body (EB) differentiation strategy to generate male germ cells from ES cells. Toyooka et al. (2003) first demonstrated that mouse ES cells could differentiate into male germ cells using EB formation combined with bone morphogenetic proteins (BMP4) induction. Mouse ES cells with the endogenous vasa homolog (Mvh) as a reporter gene were cultured in medium without LIF (Leukemia inhibitory factor) and they formed EBs. MVH-positive cells were purified and further stimulated with BMP4 by co-culture with BMP4 producing cells and subsequently transplanted into mouse testes. Interestingly, ES-derived MVH-positive cells could participate in spermatogenesis and gave rise to sperm. However, the fertilization capacity of the sperm was not assessed in this study. Similarly, Geijsen et al. obtained male germ cells from mouse ES cells via EB formation and retinoic acid (RA) treatment (Geijsen et al. 2004, West et al. 2006). The SSEA1<sup>+</sup> cells, namely primordial germ cells (PGC), were enriched from EBs and treated with RA. EB microenvironment supported male germ cell development and meiotic maturation. It is worth noting that EB-derived haploid cells were demonstrated to have the capacity to fertilize oocytes. The same differentiation protocol was also used to obtain mouse spermatogonia, spermatocytes,
spermatids and sperm-like cells positive for FE-J1, Dazl, Fragilis, Mvh, Acrosin and acetylated α-tubulin (Kerkis et al. 2007). Significantly, Nayernia et al. (2006) first reported that haploid spermatids derived from mouse ES cells have actual function, as evidenced by the live birth of offspring from ES cell-derived male gametes. Mouse ES cells were transfected with Stra8-EGFP reporter genes and cultured with RA induction. Then Stra8+ cells were sorted and transfected with Prm1-DsRed reporter genes. Subsequently, Prm1+ cells were selected and injected into mouse testes, and they could form seminiferous tubule-like structures and differentiate into sperm. Moreover, Prm1+ cells could fertilize the eggs and generate offsprings. A 2-step adherent cell differentiation protocol was used to generate male germ cells from mouse ES cells (Hayashi et al. 2011). Mouse ES cells were first stimulated with Active A, bFGF and 1% KSR (knockout serum replacement) to generate epiblast-like cells and subsequently treated with numerous cytokines, including BMP4, BMP8b, stem cell factor (SCF), LIF (Leukemia inhibitory factor) and EGF, to induce PGC-like cells (PGCLCs) that were transplanted into seminiferous tubules to allow the complete spermatogenesis. Those PGCLCs-derived spermatooza could fertilize the eggs and give birth to normal offspring, which is the gold standard for gamete function.

In humans, male germ cells can be derived by spontaneously differentiating human ES cells into EBs (Clark et al. 2004). Human ES-derived EBs could express markers for germ cells and haploid cells, including

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**Table 1** Phenotype of ES cells, iPSCs, spermatocytes and spermatids.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Phenotypic markers (proteins)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES cells</td>
<td>CD133, SSEA1, OCT3/4, SOX2, NANOG, SSEA4, TRA-1–60, REX-1</td>
<td>Clark et al. (2004), Geijsen et al. (2004), Conrad et al. (2008), Tilgner et al. (2008), Bucay et al. (2009), Hayashi et al. (2011) and Easley et al. (2012)</td>
</tr>
<tr>
<td>SSCs</td>
<td>CDH1, GFRA1, CD49f, GPR125, SOGGY, CD90, CD9, CD24, PLZ2, UTF1</td>
<td>Shinohara et al. (1999), Kubota et al. (2003), West et al. (2006), Seandel et al. (2007), Conrad et al. (2008), He et al. (2010) and Riboldi et al. (2012)</td>
</tr>
<tr>
<td>Spermatocytes</td>
<td>SCP3, SCP1, PIWIL2, LDH-C4, HILI, HIWI</td>
<td>Feng et al. (2002), Nayernia et al. (2006), Beyret &amp; Lin (2011), Easley et al. (2012), Zhu et al. (2012) and Li et al. (2013)</td>
</tr>
<tr>
<td>Spermatids</td>
<td>ACROSIN, PRM1, PRM2, TP1, TP2, SP-10, SP-56, FE-J1, TEKT1, Fragilis</td>
<td>Feng et al. (2002), Clark et al. (2004), Geijsen et al. (2004), Lee et al. (2006, 2007), Kerkis et al. (2007), Kerkis et al. (2011), Sato et al. (2011b) and Easley et al. (2012)</td>
</tr>
</tbody>
</table>

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**Table 2** The in vitro differentiation potential of mouse ES cells into male germ cells.

<table>
<thead>
<tr>
<th>Methods</th>
<th>RNA markers</th>
<th>Protein markers</th>
<th>Functional assays</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB formation combined with BMP4 induction</td>
<td>Oct4, Mvh, Fragilis, Stella, Bmp8b</td>
<td>GCN1A, SYCP3</td>
<td>Not determined</td>
<td>Tanaka et al. (2003) and Toyooka et al. (2003)</td>
</tr>
<tr>
<td>EB formation combined with RA induction</td>
<td>Oct4, Stella, Fgl5, Dazl, Piwil2, Rnf17, Rnf2, Tdrd1, Tex14, Gcnf, Acrosin, Haqpri, LHR, Stra8, Soggy, Gfra1, Tp1, H1t, H1ls1, Prm1, Prm2</td>
<td>Not determined</td>
<td>Not determined</td>
<td>West et al. (2006)</td>
</tr>
<tr>
<td>EB formation</td>
<td>Oct4, stell, fragilis, Dazl, Piwil2, Rnf17, Rnf2, Tdrd1, Tex14, Sry, Gcnf, Acrosin, Haqpri, LHR, MIS, Zp1, Zp2, Zp3, Hprt</td>
<td>FE-J1</td>
<td>Not determined</td>
<td>Geijsen et al. (2004)</td>
</tr>
<tr>
<td>Transfected with Stra8-EGFP reporter genes and cultured with RA</td>
<td>Oct4, Fragilis, Stella, Mvh, Ddx4, Stra8, Rbm, Rnf17, c-Kit, Ddx4, Scp3, Arc, Dmc1, Tp2</td>
<td>STRA8, PRM1, HSP-90α, DAZL, RBMY, PIWIL2, hnRNPΓ, OAM, ACROSIN, TP2</td>
<td>ICSI</td>
<td>Nayernia et al. (2006)</td>
</tr>
<tr>
<td>Two-step adherent cell differentiation protocol</td>
<td>Oct3/4, Sox2, Nanog, Prdm14, Zip42, Tbx3, Tcf1, Esrrb, Klf2, Klf4, Klf5, Wnt3, Fgf5, Dnmt3b, Sox17, Blimp1, Prdm14, Tcap2c, Nanos3, Stella, Tdrd5, Dnd1, Hoxa1, Hoxb1, Snai1, Dnmt3a/3b, Npp95, Mvh, Dazl</td>
<td>OCT3/4, SOX2, NANO</td>
<td>ICSI</td>
<td>Hayashi et al. (2011)</td>
</tr>
<tr>
<td>Standardized mouse SSC culture conditions</td>
<td>Dazl, Vasa, Cxcr4, Piwil1, Nanog</td>
<td>DAZL, VASA, NANO, PLZ2, UTF1, CDH1, HILI, HIWI, ACROSIN, PROT1, TP1, RET, GFRα1</td>
<td>Healthy offspring</td>
<td>Easley et al. (2012)</td>
</tr>
<tr>
<td>EB formation</td>
<td>Oct4, Gdf3, Stellar, Nanog, Nanos, Vasa, Bol, Kit, Pum1, Pum2, Gdf9, Tektn1, Dazl, Scp3, Ncam1, Scp1</td>
<td>BOULE, SCP3, DAZL, PUM2, OCT4, NANO51, DAZL, VASA, SSEA3, TRA-1–61, MLH1</td>
<td>Healthy offspring</td>
<td>Clark et al. (2004)</td>
</tr>
</tbody>
</table>
BMPs induction and co-culture with human fetal reducing ES cells colony size

A 3-step differentiation approach to generate male germ cells from human ES cells. Tilgner (2014) compared monolayer differentiation protocol and EB differentiation for optimal PGC production from human ES cells. They found both methods had no obvious difference in the ability to get PGCs, although greater cell numbers could be obtained by monolayer differentiation. Other approaches, including co-culturing with human fetal gonadal cells, were used to generate PGCs from human ES cells (Bucay et al. 2008). However, neither of these studies showed that mouse iPS cells could differentiate into SSCs and late-stage male germ cells (Zhu et al. 2012) with reprogramming of fetal and adult somatic cells. Rodent iPS cells are used for appraising the generation of male germ cells derived from iPS cells. It has recently been shown that mouse iPS cells could differentiate into SSCs and late-stage male germ cells (Zhu et al. 2012) with an approach of EB formation and RA induction or through RA or testosterone induction of EB formation from ES cell lines would be genetically unrelated to the patient (Eguizabal et al. 2011). Furthermore, there are ethical problems concerned with the use of human ES cells and sources of human ES cells are limited. Therefore, other pluripotent stem cells, such as the iPS cells, were considered to be another cell resource for derivation of male germ cells.

Table 3 The in vitro differentiation potential of human ES cells into male germ cells.

<table>
<thead>
<tr>
<th>Methods</th>
<th>RNA markers</th>
<th>Protein markers</th>
<th>Functional assays</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer differentiation and EB formation</td>
<td>Vasa, Stella, Sep3, Sep1, Oct4, Pax6, Nanog, scl, Brachyury, Gata4, Hoxa3, Hoxa4, Hoxa5</td>
<td>SSEA4, TRA-1–60, c-KIT, VASA, SCP3, TRA-1–60</td>
<td>Not determined</td>
<td>Tilgner et al. (2008)</td>
</tr>
<tr>
<td>Reducing ES cells colony size and manipulating the number of feeding cycles</td>
<td>Cxcr4, Nanog, Rex-1, Prdm1, c-KIT, Dppa3, Dazl, Vasa, Acrosin, MIS, FSHR, Sox9, LHR</td>
<td>CXC4, SSEA4, TRA-1–60, AP, VASA, FSHR, SOX9</td>
<td>Not determined</td>
<td>Bucay et al. (2009)</td>
</tr>
<tr>
<td>Co-culture with human fetal gonadal stromal cells</td>
<td>Vasa, c-KIT, PLAP, SSEA1, AMH</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Park et al. (2009)</td>
</tr>
<tr>
<td>BMPs induction and overexpression of DAZL, DAZ, BOULE</td>
<td>VASA, DAZL, γH2AX, SCP3, TEKT1, ACROSIN</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Kee et al. (2009)</td>
</tr>
<tr>
<td>A 3-step differentiation protocol</td>
<td>VASA, Stra8, Lin28</td>
<td>VIMENTIN, VASA, NESTIN, 3β-HSD, SCP3, γH2AX</td>
<td>Not determined</td>
<td>Eguizabal et al. (2011)</td>
</tr>
</tbody>
</table>

VASA, BOL, SCP1 and SCP3. The adherent cell differentiation protocol was often used in obtaining male germ cells from human ES cells. Tilgner et al. (2008) compared monolayer differentiation protocol and EB differentiation for optimal PGC production from human ES cells. They found both methods had no obvious difference in the ability to get PGCs, although greater cell numbers could be obtained by monolayer differentiation. Other approaches, including co-culturing with human fetal gonadal cells, were used to generate PGCs from human ES cells (Bucay et al. 2008). However, neither of these studies showed that mouse iPS cells could differentiate into SSCs and late-stage male germ cells (Zhu et al. 2012) with reprogramming of fetal and adult somatic cells. Rodent iPS cells are used for appraising the generation of male germ cells derived from iPS cells. It has recently been shown that mouse iPS cells could differentiate into SSCs and late-stage male germ cells (Zhu et al. 2012) with an approach of EB formation and RA induction or through RA or testosterone induction of EB formation from ES cell lines would be genetically unrelated to the patient (Eguizabal et al. 2011). Furthermore, there are ethical problems concerned with the use of human ES cells and sources of human ES cells are limited. Therefore, other pluripotent stem cells, such as the iPS cells, were considered to be another cell resource for derivation of male germ cells.

Male germ cell generation from the iPS cells

One of the exciting breakthroughs in stem cell research is the establishment of the iPS cells from somatic cells via overexpressing one or more transcription factors, including Oct4, Sox2, Klf4 and c-Myc, or another combination of Oct3/4, Sox2, Lin28 and Nanog (Okita et al. 2007, Yu et al. 2007, Liu et al. 2008, Nakagawa et al. 2008, Park et al. 2009, Wolfen et al. 2009, Zou et al. 2009). The iPS cells are very similar to ES cells in many aspects, including morphology, gene expression patterns, especially the pluripotent ability to differentiate into all cell lineages of three germ layers (Yamanaka 2007, 2008, Kang et al. 2009). Compared with human ES cells, iPS cells have some advantages: i) there is no ethical issue for using human iPS cells; ii) the source for obtaining human iPS cells is more abundant; and iii) male gametes derived from patients’ own iPS cells have genetic information. Due to human iPS cells can be generated from patients’ somatic cells, a large number of patient-specific human iPS cell lines have been established and might be used for reproductive medicine (Yamanaka 2007, Dimos et al. 2008, Park et al. 2008, Saha & Jaenisch 2009). The in vitro differentiation potential of the iPS cells into male germ cells was summarized in Table 4.

Recently, there are many studies demonstrating the feasibility of obtaining PGCs from the iPS cells via reprogramming of fetal and adult somatic cells. Rodent iPS cells are used for appraising the generation of male germ cells derived from iPS cells. It has recently been shown that mouse iPS cells could differentiate into SSCs and late-stage male germ cells (Zhu et al. 2012) with an approach of EB formation and RA induction or through RA or testosterone induction of EB formation from ES cell lines would be genetically unrelated to the patient (Eguizabal et al. 2011). Furthermore, there are ethical problems concerned with the use of human ES cells and sources of human ES cells are limited. Therefore, other pluripotent stem cells, such as the iPS cells, were considered to be another cell resource for derivation of male germ cells.

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Table 4 The in vitro differentiation potential of the iPS cells into male germ cells.

<table>
<thead>
<tr>
<th>Sources of stem cells</th>
<th>Methods</th>
<th>RNA markers</th>
<th>Protein markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse iPS cells</td>
<td>EB formation and RA induction</td>
<td>Oct4, Dppa3, Pitx1, Tex1, Stra8, Dazl, Scp1, Scp3, Msy2, Otf2, ACT, Akap3, Prm1</td>
<td>OCT4, c-KIT, MVH</td>
<td>Cai et al. (2013)</td>
</tr>
<tr>
<td>Mouse iPS cells</td>
<td>EB formation and RA or testosterone stimulation</td>
<td>Oct4, Stra8, Dazl, Vasa</td>
<td>MVH, CDH1, SCP3</td>
<td>Lee et al. (2006)</td>
</tr>
<tr>
<td>Mouse iPS cells</td>
<td>EB formation and RA induction</td>
<td>Oct4, Stra8, Dazl, Vasa</td>
<td>VASA, SCP3, CDH1, GFRA1</td>
<td>Zhu et al. (2012)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>BMP and overexpression of DAZL, BOULE and DAZ</td>
<td>Oct3/4, Nanog, Ifitm1, Pelota, Prdm1a</td>
<td>VASA, DAZL, SCP3, CENPA, ACROSIN, DAZL, VASA, NANOG, PLZF, UTF1, CDH1, HILLI, HIWI, ACROSIN, PROT1, TP1, RET, GFRA1</td>
<td>Panula et al. (2011), Easley et al. (2012)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>Standardized mouse SSC culture condition</td>
<td>Dazl, Vasa, Cxcr4, Pww1, Nanog</td>
<td>VIMENTIN, VASA, NESTIN, 3β-HSD, SCP3, γH2AX</td>
<td>Eguizabal et al. (2011)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>A 3-step differentiation protocol</td>
<td>Oct4, Nanog, Sox2, Klf4, c-Myc, Dppa4, Dnmt3b, Rex1, Sall2, Lin28, SSEA3, Tra-1-81, Vasa, Stra8, Lin28, Phlda2, Cdkn1c, Mst, Igl2, Nnat</td>
<td>VASA, CENPA, SCP3, ACROSIN</td>
<td>Medrano et al. (2012)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>Overexpression of VASA and/or DAZL</td>
<td>Vasa, Frailis, Dazl, Blimp1, Gcnf, c-Kit, Pelota, Scp3, Gdf3, Gdf9, Mlh1, Dnc1</td>
<td>VASA, CENPA, SCP3, ACROSIN</td>
<td>Medrano et al. (2012)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>Co-culture with human fetal gonadal stromal cells</td>
<td>Sry, Cyp19, Anh, Vasa, Prmde1, Dpp3a, Dazl, Hoxa2, Hoxc5</td>
<td>VASA, c-KIT, PLAP, SSEA1, AMH</td>
<td>Park et al. (2009)</td>
</tr>
</tbody>
</table>

(Li et al. 2013). However, there is no significant increase in the expression of SCP3, a hallmark for spermatocytes, in mouse iPS cells-derived cells with RA treatment, suggesting that these approaches did not differentiate into spermatocytes in vitro. Park et al. (2009) first showed that human iPS cells could differentiate to PGCs by co-culturing with human fetal gonadal stromal cells. They developed a triple biomarker assay of SSEA1/c-KIT/PLAP for identifying and isolating PGCs by evaluating the formation of PGCs during the first trimester in vivo. BMPs were demonstrated to be effective for inducing the differentiation of human iPS cells into PGCs and meiotic cells (Panula et al. 2011). Furthermore, male germ cell differentiation may occur more spontaneously in human iPS cells than in human ES cells. However, neither of these studies mentioned above could differentiate to post-meiosis stage from iPS cells. Another significant method for male germ cells differentiation is manipulation of gene expression which can regulate the lineage decision of germ cells in iPS cells differentiation. It has been demonstrated that RNA-binding proteins like VASA and/or DAZL enhanced meiotic progression of human iPS cells-derived germ cells in vitro (Medrano et al. 2012). Eguizabal et al. (2011) first achieved differentiation of human iPS cells into haploid cells using human ES cell medium without bFGF but with RA for 3 weeks. Furthermore, they sorted the whole cells utilizing a combination of markers, including CD9<sup>+</sup>, CD49f<sup>+</sup>, CD90<sup>-</sup> and SSEA4<sup>-</sup>, and cultured the positive cells with LIF, bFGF, FRSK and CYP26 inhibitor for 4 more weeks to get male germ-like cells. Notably, the conditioned-medium containing Forskolin, human recombinant LIF, bFGF and the CYP26 inhibitor R115866 was demonstrated to be useful to coax human iPS cells to generate more differentiated male germ cell lineages, as shown by the expression of the markers for spermatogonia, premeiotic spermatocytes, post-meiotic spermatocytes, and round spermatids (Eguizabal et al. 2011). However, it needs to be defined whether round spermatids derived from human iPS cells have the capacity of fertilizing oocytes to form embryos.

Similar to ES cells in potential, the iPS cells can proliferate extensively and differentiate into a variety of cell types, including male germ cells. Nevertheless, one major issue remains to be resolved with regard to clinical application of iPS cells: the use of oncogenic factors or vectors in inducing the pluripotency of iPS cells may cause tumorigenesis, since the reprogramming of somatic cells to iPS cells requires an oncogenic factor to achieve a higher transfection efficiency (Aasen et al. 2008, Mali et al. 2008, Yu & Thomson 2008). Currently, male germ cells derived from human iPS cells may not be used for treating male infertility due to tumor-forming risks, which might result from cancer-formation risks and their genetic instability. Therefore, more attention has been paid to generate male gametes from human SSCs.

**Male differentiated germ cells derivation from SSCs**

Numerous evidence confirmed the existence of SSCs in human adult testes (Conrad et al. 2008, Golestaneh et al. 2009, Kossack et al. 2009, He et al. 2010, Mizrak et al. 2010, Izadyar et al. 2011). SSCs are the foundation of spermatogenesis and male fertility (de Rooij 2006). Unlike other stem cells, SSCs are unique in that they are the stem cells that transmit genetic information to subsequent generations. Spermatogenesis is a complex differentiation process in which SSCs self-renew and
differentiate into spermatozoa with the support of Sertoli cells, peritubular myoid cells and extracellular matrix components (Clermont 1972). Therefore, researchers have a long way to recapitulate the process in vitro, including the mechanism and the conditions for spermatogenesis (Kierszenbaum 1994, Staub 2001). The differentiation potential of SSCs and testicular cells into male differentiated germ cells were addressed in Table 5.

Several studies have reported the completion of meiosis from spermatocytes to spermatids in vitro. In rodents, rat testicular cell mixture was cultured for 4 weeks and they could differentiate into spermatocytes and eventually spermatids in vitro, as shown by morphologic and biochemical analyses (Staub et al. 2000). Mouse spermatogonia were isolated and immortalized using mouse TERT, and they were cultured with SCF to differentiate into spermatocytes and round spermatids (Feng et al. 2002). However, the functionality of round spermatids derived from spermatogonia remains unknown. Several mouse cell lines, including the SSC line C18-4 cells (Hofmann et al. 2005) and spermatogonal cell line (e.g. GC-1 spg germ cell line), have been established via overexpressing the SV-40; however, these cell lines are unable to be induced to differentiate into spermatocytes and spermatids. Although mouse spermatogonia cell line created by overexpressing TERT has the potential to generate haploid spermatids, it seems rather hard to maintain the stability in vitro due to the missing the niche for SSCs.

A 3D cell culture system was used to mimic the microenvironment or niche within seminiferous tubes for inducing haploid production in vitro from rat testes issues (Lee et al. 2006). Likewise, there were other studies using 3D cell culture system to gain more insights into the interactions among germ cells, somatic cells and various extracellular matrices for the in vitro spermatogenesis (Hue et al. 1998, Tanaka et al. 2003, Stukenborg et al. 2009, Abu Elhija et al. 2012). The 3D blood–testis barrier model was also used for rat germ cell differentiation with production of haploid cells (Legendre et al. 2010). A novel organ culture condition has been shown to support the whole spermatogenesis of neonatal mouse testicular issues and cultured with serum-free medium (Sato et al. 2011a). The fertility of round spermatids and sperm produced in vitro was assessed by ROSI and ICSI to obtain offspring. The same group also showed another new culture system that can induce spermatogenesis in vitro from SSCs or SSC lines (Sato et al. 2011b). Colonies were formed in the seminiferous tubules after SSC injection, and meiosis, including the generation of spermatocytes and spermatids, occurred in recipient mouse testis issues. Moreover, the fertility of resultant haploid cells and rise of healthy offspring were examined by micro-insemination. Mouse SSCs were co-cultured with Sertoli cells in the presence of hormones and vitamins to differentiate into spermatid-like cells in vitro (Minnaee Zanganeh et al. 2013). Thus far, it remains to be a difficult issue to induce the differentiation of SSCs into functional gametes with high efficiency and reproducibility.

Apart from the in vitro and 3D cell culture, transplantation of SSCs or testicular cells provides an efficient functional approach for identifying SSCs and inducing the differentiation of these cells in vivo. In rodents, male germ cell transplantation was first developed in 1994 (Brinster & Zimmermann 1994) and followed by numerous studies demonstrating that meiosis and post-meiosis occur in the

![Table 5 The differentiation potential of SSCs and testicular cells into male differentiated germ cells.](attached_image)

<table>
<thead>
<tr>
<th>Sources of cells</th>
<th>Methods</th>
<th>RNA markers</th>
<th>Protein markers</th>
<th>Functional assays</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat testicular cell mixture</td>
<td>Direct culture</td>
<td>Tp1, Tp2</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Staub et al. (2000)</td>
</tr>
<tr>
<td>Rat testis issues</td>
<td>Direct culture</td>
<td>Tp2</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Lee et al. (2006)</td>
</tr>
<tr>
<td>Mouse type A spermatogonial cells</td>
<td>Co-cultured with supportive cells</td>
<td>Oc14, Protamine-2</td>
<td>TP2, PRM2, 3BHSD, c-KIT, DAZL, SP-10, SCP3, LDH-C4</td>
<td>Not determined</td>
<td>Feng et al. (2002)</td>
</tr>
<tr>
<td>Mouse testis issue</td>
<td>Cultured with serum-free medium and induced by KSR</td>
<td>Not determined</td>
<td>SYCP3, SYCP1</td>
<td>Round spermatid injection (ROSI) and ICSI</td>
<td>Sato et al. (2011a,b)</td>
</tr>
<tr>
<td>Mouse SSCs</td>
<td>Co-cultured with Sertoli cells in presence of hormones and vitamins</td>
<td>Dazl, Stra8, H2A, Th2b, Scp3, Ube1y, Tp1, Tp2, Prm1</td>
<td>66-integrin, β1-integrin</td>
<td>Not determined</td>
<td>Minnaee Zanganeh et al. (2013)</td>
</tr>
<tr>
<td>Mouse SSCs</td>
<td>Testes fragments cultured with injection of Acgerninal stem cells</td>
<td>Not determined</td>
<td>SYCP3, SYCP1</td>
<td>Round spermatid injection (ROSI) and ICSI</td>
<td>Sato et al. (2011b)</td>
</tr>
<tr>
<td>Human CD49f+ cells</td>
<td>Co-culture with Sertoli cells</td>
<td>Thy-1, c-Kit, Dazl, Vasa, Stella, Scp3, Piwll2, Boule, Dcm1, Prm1, Tnp2, Blimp1</td>
<td>CD49f, THY-1, GPR125, SCP3, CREST, MLH1</td>
<td>Not determined</td>
<td>Riboldi et al. (2012)</td>
</tr>
<tr>
<td>Human testicular cell mixture</td>
<td>3D cell culture</td>
<td>Not determined</td>
<td>PRM2</td>
<td>Not determined</td>
<td>Lee et al. (2007)</td>
</tr>
</tbody>
</table>
recipient animals after SSC transplantation in other species including goat (Honaramooz et al. 2003). In primates, autologous transplantation of SSCs into the testes of monkey could lead to producing sperm with fertility and developmental potentials (Hermann et al. 2012), reflecting a functional spermatogenesis by SSC transplantation.

In human, late stage of spermatids could be obtained from mixture cells isolated from testicular biopsies of non-obstructive azoospermic patients when they were co-cultured with the Vero cells-conditioned medium or the medium containing FSH or FSH plus testosterone (Sousa et al. 2002). Although meiosis induction could be achieved by FSH, significant increases only appeared under the condition of medium with FSH and testosterone. Moreover, developmental potential of induced spermatids in vitro was tested by microinjecting into oocytes. The 3D cell culture system was also applied to obtain haploid cells from non-obstructive azoospermic patients (Lee et al. 2007). The CD49f-positive cells from testicular tissues of azoospermic patients were co-cultured with Sertoli cells to mimic the niche and resulted in generation of haploid cells (Riboldi et al. 2012). Nevertheless, CD49f is not a specific maker for SSCs, since it is also expressed in human Sertoli cells (He et al. 2010). As noted, the generation of functional haploid spermatids from SSCs in vitro has not yet been achieved in human.

As discussed above, great efforts have been made to achieve spermatogenesis in vitro. On the one hand, it is essential to enrich and expand SSCs due to the limited number of SSCs in testes issues and no human SSC line is currently available. In the adult mouse testes, SSCs population is only about 0.03% of total male germ cells (Tegelenbosch & de Rooij 1993). On the other hand, it is required to mimic meiotic and post-meiotic niche as in vivo the appropriate microenvironment. To date, xenotransplantation of human SSCs to rodent recipients only leads to the colonization of these cells but without differentiation into spermatocytes or spermatids.

Concerns on generation of differentiated male germ cells from stem cells

Since the 1960s, much progress have been achieved in generation of male differentiated germ cells from a variety of stem cells, which offers a promising therapeutic prospective for infertility in male. However, several issues remain to be solved before the clinical application of stem cell-derived male gametes. First, it is prerequisite to establish an appropriate culture system for differentiation of mature and functional male spermatids from different types of stem cells. As shown in Tables 2, 3, 4 and 5, we summarized numerous culture systems used to induce various kinds of stem cells into male germ cells. Co-culture with human fetal gonadal stromal cells (Park et al. 2009), Sertoli cells (Takahashi & Yamanaka 2006, Minae Zanganeh et al. 2013), and Vero cells (Sousa et al. 2002) can increase the efficiency of male differentiated germ cells derived from stem cells. Although these studies highlight the importance of culturing with feeder cells to mimic a microenvironment as in vivo to induce stem cells differentiate into male differentiated germ cells, it remains to be defined which factors play an important role in differentiation and their action mechanisms. Cytokines and signaling molecules, including BMP4, RA, and SCF have been used to enhance male differentiated germ cells generation from stem cells. However, it is unknown about the precise effect of these molecules and how to achieve an efficient differentiation of male gametes from stem cells. In addition, some researchers overexpressed key regulators to regulate the cell lineage decisions to promotes meiosis and the formation of haploid cells in differentiating stem cells (Panula et al. 2011, Medrano et al. 2012). Nevertheless, the low frequency of haploid cell production suggests that approaches have not yet achieved the best meiotic progression. Second, it is crucial to use appropriate markers to identify various stem cells and germ cell lineages, as illustrated in Table 1. In mouse SSCs, α6-Integrin (CD49f) was the first identified surface marker (Shinohara et al. 1999); however, it is expressed in human both SSCs and Sertoli cells (He et al. 2010). Other surface molecules for SSCs were identified, including CD90, CD9, CD24 and GPR125 (Kubota et al. 2003, Kanatsu-Shinohara et al. 2004, Seandel et al. 2007). Recently, we found that human SSCs share some but not all phenotypes with rodent SSCs, since human SSCs express GPR125, GFRA1, UCHL1, PLZF, CD90 and MAGEA4 but are negative for OCT4 (He et al. 2010). Thus, a uniform standard using a combination of markers should be established to obtain a pure population of SSCs. Finally, complete spermatogenesis in vitro to obtain functional male gametes has not yet been achieved in human, although progress has been made in the derivation of male differentiated germ cells from ES and iPS cells. The most important thing for patients with infertility diseases is to seek an ideal stem cell type and efficient differentiation system for cell-based therapy of male infertility.

Summary

In short, we have addressed the advancements on differentiation of male differentiated germ cells from various kinds of stem cells. Nevertheless, the use of stem cells to generate male gametes in vitro remains an avenue of research which is still in its infancy. Although several questions remain to be answered before the stem cell-derived spermatooza can be used successfully in clinical therapies, exciting progress has been made. More and more researchers are dedicated to the study of kinds of stem cells for generating mature and
functional spermatozoa. The rapid development of stem cell research, particularly with respect to iPSCs and SSCs, lays a solid basis for offering an invaluable source of functional male gametes for treating male infertility with their own genetics.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding
This study was supported by key grants from Chinese Ministry of Science and Technology (2013CB947901, 2014CB943101) and National Nature Science Foundation of China (312030048), grants from National Science Foundation of China (31171422, 31201109), The Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning, a key grant from the Science and Technology Commission of Shanghai Municipality (12JC1405900). Key Discipline and Specialty Foundation of Shanghai Municipal Commission of Health and Family Planning, and Shanghai Pujiang Program (11PJ1406400).

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Received 16 December 2013
First decision 27 January 2014
Revised manuscript received 10 February 2014
Accepted 17 February 2014