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

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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.

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Introduction

It has been estimated that human infertility affects 10–15% of the couples, and male factors account for about 50% of causes, 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.*

2008, West *et al.* 2008, Aflatoonian *et al.* 2009). 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 unipotent 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⁺ 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,

Figure 1 Schematic diagram shows the derivation of male differentiated germ cells from various types of stem cells. ES cells, embryonic stem cell; FISH, fluorescence in situ hybridization; ICM, inner cell mass; ICSI, Intracytoplasmic sperm injection; iPS cells, induced pluripotent stem cells; ROSI, round spermatid injection; SSCs, spermatogonial stem cells.

Table 1 Phenotype of ES cells, iPS cells, SSCs, spermatocytes and spermatids.

Cell types	Phenotypic markers (proteins)	References
ES cells	CD133, SSEA1, OCT3/4, SOX2, NANOG, SSEA4, TRA-1-60, REX-1	Clark <i>et al.</i> (2004), Geijsen <i>et al.</i> (2004), Conrad <i>et al.</i> (2008), Tilgner <i>et al.</i> (2008), Bucay <i>et al.</i> (2009), Hayashi <i>et al.</i> (2011) and Easley <i>et al.</i> (2012)
iPS cells	OCT4, NANOG, SOX2, KLF4, C-MYC, DPPA4, DNMT3B, REX-1, SALL2, LIN28, SSEA3, TRA-1-81, TRA-1-60, GDF3, Integrin-β3, STELLA	West <i>et al.</i> (2006), Eguizabal <i>et al.</i> (2011), Cai <i>et al.</i> (2013) and Li <i>et al.</i> (2013)
SSCs	CDH1, GFRA1, CD49f, GPR125, SOGGY, CD90, CD9, CD24, PLZF, UTF1	Shinohara <i>et al.</i> (1999), Kubota <i>et al.</i> (2003), West <i>et al.</i> (2006), Seandel <i>et al.</i> (2007), Conrad <i>et al.</i> (2008), He <i>et al.</i> (2010) and Riboldi <i>et al.</i> (2012)
Spermatocytes	SCP3, SCP1, PIWIL2, LDH-C4, HILI, HIWI	Feng <i>et al.</i> (2002), Nayernia <i>et al.</i> (2006), Beyret & Lin (2011), Easley <i>et al.</i> (2012), Zhu <i>et al.</i> (2012) and Li <i>et al.</i> (2013)
Spermatids	ACROSIN, PRM1, PRM2, TP1, TP2, SP-10, SP-56, FE-J1, TEK1, Fragilis	Feng <i>et al.</i> (2002), Clark <i>et al.</i> (2004), Geijsen <i>et al.</i> (2004), Lee <i>et al.</i> (2006, 2007), Kerkis <i>et al.</i> (2007), Kee <i>et al.</i> (2009), Eguizabal <i>et al.</i> (2011), Sato <i>et al.</i> (2011b) and Easley <i>et al.</i> (2012)

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 spermatozoa could fertilize the eggs and gave 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

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

Methods	RNA markers	Protein markers	Functional assays	References
EB formation combined with BMP4 induction	Oct4, Mvh, Fragilis, Stella, Bmp8b	GCNA1, SYCP3	Not determined	Tanaka <i>et al.</i> (2003) and Toyooka <i>et al.</i> (2003)
EB formation combined with RA induction	Oct4, Stella, Fgls, Dazl, Piwil2, Rnf17, Rnh2, Tdrd1, Tex14, Gcnf, Acrosin, Haprin, LHR, Stra8, Soggy, Gfra1, Tp1, H1t, H1LS1, Prm1, Prm2	Not determined	Not determined	West <i>et al.</i> (2006)
EB formation	Oct4, stell, fragilis, Dazl, Piwil2, Rnf17, Rnh2, Tdrd1, Tex14, Sry, Gcnf, Acrosin, Haprin, LHR, MIS, Zp1, Zp2, Zp3, Hprt	FE-J1	Not determined	Geijsen <i>et al.</i> (2004)
Transfected with Stra8-EGFP reporter genes and cultured with RA	Oct4, Fragilis, Stella, Mvh, Ddx4, Stra8, Rbm, Rnf17, c-Kit, Ddx4, Scp3, Arc, Dmc1, Tp2	STRA8, PRM1, HSP-90a, DAZL, RBMY, PIWIL2, hnRNPGT, OAM, ACROSIN, TP2	ICSI	Nayernia <i>et al.</i> (2006)
Two-step adherent cell differentiation protocol	Oct3/4, Sox2, Nanog, Prdm14, Zfp42, Tbx3, Tcf1, Esrrb, Klf2, Klf4, Klf5, Wnt3, Fgf5, Dnmt3b, Sox17, Blimp1, Prdm14, Tcfap2c, Nanos3, Stella, Tdrd5, Dnd1, Hoxa1, Hoxb1, Snai1, Dnmt3a/3b, Np95, Mvh, Dazl	OCT3/4, SOX2, NANOG	ICSI	Hayashi <i>et al.</i> (2011)
Standardized mouse SSC culture conditions	Dazl, Vasa, Cxcr4, Piwil1, Nanog	DAZL, VASA, NANOG, PLZF, UTF1, CDH1, HILI, HIWI, ACROSIN, PROT1, TP1, RET, GFRA1	Healthy offspring	Easley <i>et al.</i> (2012)
EB formation	Oct4, Gdf3, Stellar, Nanog, Nanos, Vasa, Bol, Kit, Pum1, Pum2, Gdf9, Tektin1, Dazl, Scp3, Ncam1, Scp1	BOULE, SCP3, DAZL, PUM2, OCT4, NANOS1, DAZL, VASA, SSEA3, TRA-1-81, MLH1	Healthy offspring	Clark <i>et al.</i> (2004)

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

Methods	RNA markers	Protein markers	Functional assays	References
Monolayer differentiation and EB formation	Vasa, Stella, Scp3, Scp1, Oct4, Pax6, Nanog, Scl, Brachyury, Gata4, Hoxa3, Hoxa4, Hoxa5	SSEA4, TRA-1-60, c-KIT, VASA, SCP3, TRA-1-60	Not determined	Tilgner <i>et al.</i> (2008)
Reducing ES cells colony size and manipulating the number of feeding cycles	Cxcr4, Nanog, Rex-1, Prdm1, c-Kit, Dppa3, Dazl, Vasa, Acrosin, MIS, FSHR, Sox9, LHR	CXCR4, SSEA4, TRA-1-60, AP, VASA, FSHR, SOX9	Not determined	Bucay <i>et al.</i> (2009)
Co-culture with human fetal gonadal stromal cells	Sry, Cyp19, Amh, Vasa, Prmd1, Dppa3, Dazl, Hoxa2, Hoxc5	VASA, c-KIT, PLAP, SSEA1, AMH	Not determined	Park <i>et al.</i> (2009)
BMPs induction and overexpression of DAZL, DAZ, BOULE	Dazl, Prdm1, Dppa3, Vasa, Lin28, Nanog, Oct4, Tert, Dazl, Daz, Boule	VASA, DAZL, γ H2AX, SCP3, TEKT1, ACROSIN	Not determined	Kee <i>et al.</i> (2009)
A 3-step differentiation protocol	Vasa, Stra8, Lin28	VIMENTIN, VASA, NESTIN, 3 β -HSD, SCP3, γ H2AX	Not determined	Eguizabal <i>et al.</i> (2011)

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.* 2009, Park *et al.* 2009). However, neither of these studies reported that they could differentiate human ES cells beyond meiosis stage. Importantly, Kee *et al.* (2009) revealed that overexpression of DAZ gene family could promote PGC formation from human ES cells and further induced the meiotic progression and haploid formation. Without the overexpression of any developmentally related genes, Eguizabal *et al.* (2011) developed a 3-step differentiation approach to generate male germ cells from human ES cells. However, the process using this differentiation protocol took 10 weeks. Although postmeiotic cells were observed in human ES-derived cells, human ES cells failed to complete meiosis without yielding haploid cells. Remarkably, Easley *et al.* (2012) first demonstrated that human ES cells could directly differentiate into haploid cells without gene manipulation. After 10 days' adherent culture with mouse SSC medium containing minimum essential medium (MEM) α , BSA, insulin, transferrin, putrescine, L-glutamine, β -mercaptoethanol, bFGF (basic fibroblast growth factor), GDNF (Glial cell-derived neurotrophic factor), sodium selenite, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and HEPES, human ES cells could differentiate directly into male germ cell lineages, including post-meiotic and spermatid-like cells. Nevertheless, the functionality of these human spermatid-like cells remains to be determined.

Collectively, derivation of male germ cells from ES cells has promising applications for male infertility treatment and provides an ideal platform for elucidating molecular mechanisms of male germ cell development. However, in regard of fertility treatment, gametes derived

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, Woltjen *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

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

Sources of stem cells	Methods	RNA markers	Protein markers	References
Mouse iPS cells	EB formation and RA induction	Not determined	OCT4, c-KIT, MVH	Cai <i>et al.</i> (2013)
Mouse iPS cells	EB formation and RA or testosterone stimulation	Oct4, Dppa3, Piwil, Tex14, Stra8, Dazl, Scp1, Scp3, Msy2, Odf2, ACT, Akap3, Prm1	MVH, CDH1, SCP3	Lee <i>et al.</i> (2006)
Mouse iPS cells	EB formation and RA induction	Oct4, Stra8, Dazl, Vasa	VASA, SCP3, CDH1, GFRA1	Zhu <i>et al.</i> (2012)
Human iPS cells	BMP and overexpression of DAZL, BOULE and DAZ	Oct3/4, Nanog, Ifitm1, Pelota, Prdm1a	VASA, DAZL, SCP3, CENPA, ACROSIN	Panula <i>et al.</i> (2011)
Human iPS cells	Standardized mouse SSC culture condition	Dazl, Vasa, Cxcr4, Piwil1, Nanog	DAZL, VASA, NANOG, PLZF, UTF1, CDH1, HILI, HIWI, ACROSIN, PROT1, TP1, RET, GFRA1	Easley <i>et al.</i> (2012)
Human iPS cells	A 3-step differentiation protocol	Oct4, Nanog, Sox2, Klf4, c-Myc, Dppa4, Dnmt3b, Rex-1, Sall2, Lin28, SSEA3, Tra-1–81, Vasa, Stra8, Lin28, Phlda2, Cdkn1c, Mest, Igf2, Nnat	VIMENTIN, VASA, NESTIN, 3β-HSD, SCP3, γH2AX	Eguizabal <i>et al.</i> (2011)
Human iPS cells	Overexpression of VASA and/or DAZL	Vasa, Fragilis, Dazl, Blimp1, Gcnf, c-Kit, Pelota, Scp3, Gdf3, Gdf9, Mlh1, Dmc1	VASA, CENPA, SCP3, ACROSIN	Medrano <i>et al.</i> (2012)
Human iPS cells	Co-culture with human fetal gonadal stromal cells	Sry, Cyp19, Amh, Vasa, Prmd1, Dppa3, Dazl, Hoxa2, Hoxc5	VASA, c-KIT, PLAP, SSEA1, AMH	Park <i>et al.</i> (2009)

(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 PGCLCs 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⁺, CD49f⁺, CD90⁺ and SSEA4⁺, 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 clinic 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 spermatogonial 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 spermatogonial 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* (Minaee 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 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.

Sources of cells	Methods	RNA markers	Protein markers	Functional assays	References
Rat testicular cell mixture	Direct culture	Tp1, Tp2	Not determined	Not determined	Staub <i>et al.</i> (2000)
Rat testis issues	3D cell culture	Tp2	TP2, PRM2, 3βHSD	Not determined	Lee <i>et al.</i> (2006)
Mouse type A spermatogonial cells	Induced by SCF and co-cultured with supportive cells	Oct4, Protamine-2	c-KIT, DAZL, SP-10, SCP3, LDH-C4	Not determined	Feng <i>et al.</i> (2002)
Mouse testis issue	Cultured with serum-free medium and induced by KSR	Not determined	SYCP3, SYCP1	Round spermatid injection (ROSI) and ICSI	Sato <i>et al.</i> (2011a,b)
Mouse SSCs	Co-cultured with Sertoli cells and in presence of hormones and vitamins	Dazl, Stra8, H2A, Th2b, Scp3, Ube1y, Tp1, Tp2, Prm1	α6-integrin, β1-integrin	Not determined	Minaee Zanganeh <i>et al.</i> (2013)
Mouse SSCs	Testes fragments cultured with injection of <i>Acr</i> -germline stem cells	Not determined	SYCP3, SYCP1	Round spermatid injection (ROSI) and ICSI	Sato <i>et al.</i> (2011b)
Human CD49f+ cells	Co-culture with Sertoli cells	Thy-1, c-Kit, Dazl, Vasa, Stella, Scp3, Piwill2, Boule, Dcm1, Prm1, Tnp2, Blimp1	CD49f, THY-1, GPR125, SCP3, CREST, MLH1	Not determined	Riboldi <i>et al.</i> (2012)
Human testicular cell mixture	3D cell culture	Not determined	PRM2	Not determined	Lee <i>et al.</i> (2007)

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 marker 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 tissues 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, Minaee 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, $\alpha 6$ -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 spermatozoa 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 iPS cells 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.

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