Establishment and applications of male germ cell and Sertoli cell lines

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Abstract

Within the seminiferous tubules there are two major cell types, namely male germ cells and Sertoli cells. Recent studies have demonstrated that male germ cells and Sertoli cells can have significant applications in treating male infertility and other diseases. However, primary male germ cells are hard to proliferate *in vitro* and the number of spermatogonial stem cells is scarce. Therefore, methods that promote the expansion of these cell populations are essential for their use from the bench to the bed side. Notably, a number of cell lines for rodent spermatogonia, spermatocytes and Sertoli cells have been developed, and significantly we have successfully established a human spermatogonial stem cell line with an unlimited proliferation potential and no tumor formation. This newly developed cell line could provide an abundant source of cells for uncovering molecular mechanisms underlying human spermatogenesis and for their utilization in the field of reproductive and regenerative medicine. In this review, we discuss the methods for establishing spermatogonial, spermatocyte and Sertoli cell lines using various kinds of approaches, including spontaneity, transgenic animals with oncogenes, simian virus 40 (SV40) large T antigen, the gene coding for a temperature-sensitive mutant of *p53*, telomerase reverse gene (*Tert*), and the specific promoter-based selection strategy. We further highlight the essential applications of these cell lines in basic research and translation medicine.

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Introduction

Spermatogenesis is a complex process including the mitosis of spermatogonia, meiosis of spermatocytes and spermiogenesis of spermatids. Infertility affects 10–15% of couples, of which male infertility contributes to half of the cases (Schlegel 2009). Azoospermia has been observed in 1% of the general population and it comprises 15% of male infertility (Wosnitzer *et al.* 2014). Notably, we have recently demonstrated that human spermatogonial stem cells (SSCs) from cryptorchid patients can be coaxed to differentiate into haploid spermatids with fertilization and developmental capacity (Yang *et al.* 2014a), suggesting that human SSCs have the significant potential to treat male infertility. Furthermore, SSCs can dedifferentiate to become embryonic stem (ES)-like cells that differentiate into numerous types of cells (Kanatsu-Shinohara *et al.* 2004, Guan *et al.* 2006, Seandel *et al.* 2007, Golestaneh *et al.* 2009, Ko *et al.*

2009), and they are able to directly transdifferentiate into the cells of other lineages (Simon *et al.* 2009, Zhang *et al.* 2013, Yang *et al.* 2015). These studies reflect the prospect of SSCs in regenerative medicine to treat human diseases.

As the only type of somatic cell within the seminiferous tubules, Sertoli cells play essential roles in regulating spermatogenesis, including physiological contact and structural, immunological and nutritional support for male germ cells (Hai *et al.* 2014). Sertoli cells can be reprogrammed to pluripotent stem cells (Sun *et al.* 2014), and significantly they are directly converted to morphologic, phenotypic and functional neural stem cells (Sheng *et al.* 2012) and Leydig cells (Zhang *et al.* 2015). Moreover, Sertoli cells are engineered to produce certain proteins that restore cell metabolic function (Kaur *et al.* 2014). Therefore, Sertoli cells can be utilized in disease modeling and regenerative medicine.

It is hard to expand primary male germ cells *in vitro*, and the number of spermatogonial stem cells is very limited. Thus, it is essential to immortalize male germ cells and Sertoli cells to obtain sufficient cells in order to enlarge their usage from the bench to the bed side. The spontaneous immortalization in nature is extremely uncommon as evidenced by the fact that rodent cells can be spontaneously immortalized with a maximal frequency of 10^{-5} and the chance of human cell spontaneous immortalization is less than 10^{-12} (Katakura *et al.* 1998). Several approaches have been used to induce cell immortalization, including overexpression of telomerase reverse transcriptase (*Tert*), virus (e.g. simian virus 40 (SV40) large T antigen, polyomavirus large T antigen (PylT), papillomaviruses E6 and E7, adenovirus E1A, and herpesvirus saimiri), oncogenes (e.g. *Myc*, *Ras*, *Jun*), and tumor suppressor genes (*p16*, *p53*, *Rb1*). *Tert* has certain advantages over other immortalized genes since immortalized cells by overexpressing *Tert* retain genomic stability and phenotypic properties of the cells with normal karyotype and growth rate without transformation. Based on recent progress, we address the establishment and characterization of male germ cell and Sertoli cell lines using various kinds of immortalization approaches. We also highlight the

potential and significant applications of these cell lines in mechanistic studies and clinic.

Establishment and characterization of Sertoli cell lines with different methods

In rodents, immature Sertoli cells start to divide at 15 days of age and express immature cell markers, such as anti-Müllerian hormone (*Amh*), *Krt8* (also known as *Ck18*), and M2A (Blagosklonova *et al.* 2002, Hai *et al.* 2014). Subsequently, Sertoli cells begin to differentiate and express mature cell hallmarks, e.g. androgen receptor (AR), and acquire the function of mature Sertoli cells to support spermatogenesis (Regadera *et al.* 2001).

As illustrated in Fig. 1, a number of rodent Sertoli cell lines have been established by different approaches for comprehensive understanding of the biology and function of Sertoli cells. The phenotypic characteristics of these Sertoli cell lines are summarized in Table 1. There are three categories of markers for Sertoli cells, namely immature, mature and whole-stage markers. As shown in Table 1, immature markers for Sertoli cells include AMH, KRT18 (CK18), M2A and NCAM (Neural cell adhesion molecule) (Blagosklonova *et al.* 2002, Sharpe *et al.* 2003, Hai *et al.* 2014), whereas AR, SGP2

Table 1 The phenotype of primary Sertoli cells and Sertoli cell lines.

Cell types	Markers	Undetected	Species	References
Primary Sertoli cells	Immature markers: AMH, KRT18, M2A, NCAM; mature markers: AR, SGP2, GATA1, p27 ^{kip1} ; whole-stage markers: WT1, GATA4, SOX9, VIM, GDNF, SCF	–	Rats and mice	Sharpe <i>et al.</i> (2003), Kanatsu-Shinohara <i>et al.</i> (2012)
TM4 cell line	Immature marker: <i>Amh</i> ; mature marker: <i>Sgp2</i> ; whole-stage markers: <i>Scf</i> , <i>Sox9</i> , other markers: <i>Sf1</i> , <i>Dhh</i>	<i>Wt1</i> , <i>Sry</i>	11-day-old mice	Mather (1980), Beverdam <i>et al.</i> (2003)
A31C4, C22A4, C24A4 cell lines	Immature marker: <i>Amh</i> ; mature markers: <i>Ar</i> , <i>Sgp2</i> ; whole-stage markers: <i>Wt1</i> , <i>Scf</i> , <i>Sox9</i> ; other markers: <i>Tf</i> (transferrin), <i>Fshr</i> , <i>Ocln</i> (Occludin), <i>Clu</i> (clusterin), <i>Inha</i> (inhibin alpha), <i>Tgfb</i> , <i>Fgf2</i>	<i>Shbg</i> (known as <i>Abp</i>)	6-week-old mice	Sato <i>et al.</i> (2013)
15P-1 cell line	Whole-stage markers: <i>Wt1</i> , <i>Steel</i> (ligand for <i>Kit</i> , known as <i>Scf</i>)	–	Young mice	Paquis-Flucklinger <i>et al.</i> (1993), Rassoulzadegan <i>et al.</i> (1993)
SMAT1 cell line	Immature markers: <i>Amh</i> ; mature marker: <i>Sgp2</i> ; whole-stage markers: <i>Wt1</i> , <i>Scf</i> ; other markers: <i>Sf1</i> , <i>Sgp1</i> , <i>Shbg</i> , <i>Inha</i> , <i>Inhb</i>	<i>Tf</i> , <i>Fshr</i>	6.5-day-old mice	Dutertre <i>et al.</i> (1997)
ASC-17 cell line	Immature marker: <i>Krt18</i> ; mature marker: <i>Sgp2</i> ; other markers: <i>Tf</i> , <i>Sgp1</i>	<i>Shbg</i>	120-day-old rats	Roberts <i>et al.</i> (1995)
42GPA9 cell line	Mature marker: <i>Sgp2</i> ; whole-stage marker: <i>Scf</i> ; other markers: <i>Tf</i> , <i>Fshr</i> , <i>Tjp1</i> (Zo1)	–	6-month-old mice	Bourdon <i>et al.</i> (1998)
TTE3 cell line	Mature marker: <i>Sgp2</i> ; whole-stage marker: <i>Scf</i> ; other markers: <i>Fshr</i> , <i>Tjp1</i> , <i>Tf</i> , <i>Inha</i>	<i>Krt18</i>	8-week-old mice	Tabuchi <i>et al.</i> (2002)
RTS3-3 cell line	Mature marker: <i>Sgp2</i> ; other marker: <i>Tf</i>	<i>Fshr</i>	27-week old rats	Tabuchi <i>et al.</i> (2003)
SerW3 cell line	Other marker: <i>Tf</i>	–	17-day-old rats	Pognan <i>et al.</i> (1997)
93RS2 cell line	Mature marker: <i>Sgp2</i> ; whole-stage marker: <i>Scf</i> ; other markers: <i>Tf</i> , <i>Sgp1</i>	<i>Fshr</i> , <i>Shbg</i> , <i>Gdnf</i> , <i>Bmp4</i>	16-day-old rats	Jiang <i>et al.</i> (1997)
SCIT-C8 cell line	Whole-stage marker: <i>Scf</i> ; other markers: <i>Tf</i> , <i>Clu</i>	<i>Ar</i> , <i>Fshr</i>	19-day-old rats	Konrad <i>et al.</i> (2005)
SK11 cell line	Immature marker: <i>Amh</i> ; mature markers: GATA1, AR and SGP2 induced by hormone treatment; whole-stage marker: <i>Scf</i> ; other markers: <i>shr</i> , <i>Sf1</i> , <i>Tf</i> , <i>Inha</i>	<i>Shbg</i> , <i>Cd68</i> , <i>Lhr</i>	10-day-old mice	Sneddon <i>et al.</i> (2005), Walther <i>et al.</i> (1996)
B6SC-2 and B6SC-3 cell lines	Immature marker: <i>Amh</i> ; mature marker: <i>Gata1</i> ; other markers: <i>Fshr</i> , <i>Tjp1</i> , <i>Shbg</i>	–	19-day-old mice	Chuang <i>et al.</i> (2007)

Figure 1 Schematic diagram showing the establishment of Sertoli cell lines. The immortalization methods of rodent Sertoli cell lines include spontaneity, SV40 transgenic animals, transfection with SV40 or *Tert*, and starvation.

(Sulfated glycoprotein 2), GATA1 and p27^{kip1} can be used as mature hallmarks for these cells (Regadera *et al.* 2001, Sharpe *et al.* 2003, Sato *et al.* 2013). In addition, WT1, GATA4, SOX9, VIM, SCF and GDNF serve as whole-stage markers for Sertoli cells.

Establishment of Sertoli cell lines without gene transfection and their characteristics and applications

TM4 cell line is an earlier Sertoli cell line developed spontaneously without gene transfection (Mather 1980). Sertoli cells were isolated from mouse testes and cultured with certain kinds of substances, such as retinoic acid (RA), luteinizing hormone (LH), follicle-stimulating hormone (FSH), somatomedin C, LH-B10, FSH-S11, [³H]-progesterone and [¹⁴C]-cholesterol. Eventually, TM4 cell line with Sertoli cell identity was established according to the morphology, metabolism of steroids and the response to insulin, transferrin, epidermal growth factor (EGF), growth hormone, FSH and RA (Mather 1980). TM4 cell line can be cloned several times and cultured for over 2 years. These cells possess epithelial appearance and form contact-inhibition monolayer at confluence. Moreover, no tumor is observed when these cells are injected into nude mice. Since the TM4 cell line was established from immature mice, its properties may be more similar to the cells from prepubertal rodents, which can serve as an excellent model for investigating the division of immature Sertoli cells.

TM4 cell line has been applied in many aspects. Although the function of AR in spermatogenesis is well known, its mechanisms in regulating Sertoli cells remain unclear. TM4 cell line has been used to identify *Hsf1* as a novel target of AR in mouse Sertoli cells (Yang *et al.* 2014b) and CLMP as a new tight junction transmembrane

protein (Sze *et al.* 2008). In addition, this cell line has recently been utilized to elucidate the pharmacological roles and ERK/Akt signaling pathway of diosgenin and offer novel therapeutic methods to treat male infertility (Wu *et al.* 2015). Starvation method has also been applied to generate three Sertoli cell lines, including A31C4, C22A4 and C24A4 (Sato *et al.* 2013). Similar to TM4 cell line, these Sertoli cell lines were created without inducing ectogenic oncogenes, and thus their behavior is close to primary Sertoli cells and they can be applied to basic research on the interactions among male germ cells and Sertoli cells. Furthermore, these cell lines without gene transfection are unique cells that express mature Sertoli cell markers (e.g. AR and SGP2) under usual culture condition and can respond properly to FSH.

Establishment of Sertoli cell lines from transgenic animals or by infecting primary Sertoli cells with oncogenes

Numerous Sertoli cell lines have been immortalized by large T agent (LTAg), PyLT, oncogenes, or from transgenic animals expressing LTAg gene. For example, 42GPA9 and 15P-1 Sertoli cell lines were derived from PyLT transgenic mice (Paquis-Flucklinger *et al.* 1993, Bourdon *et al.* 1998). The 42GPA9 cell line possesses the characteristics of mature Sertoli cells since it expresses many markers of primary mature Sertoli cells (e.g. AR), responds to FSH and forms tight junctions (Bourdon *et al.* 1998). The 15P-1 cell line retains the morphological and functional features of primary Sertoli cells. Both cell lines may be utilized to examine the self-renewal and differentiation of SSCs. SMAT1 cell line has been generated from mice with SV40

gene (Duterte *et al.* 1997) and its expression pattern is similar to the characteristics of Sertoli cells from prepubertal mice. SMAT1 cell line can produce AMH even at the 37th passage, suggesting that this cell line might maintain an immature status for a long period. The conditionally immortalized RTS3-3 and TTE3 Sertoli cell lines have been generated from transgenic rats or mice harboring temperature-sensitive simian virus 40 (tsSV40) large T antigen (Tabuchi *et al.* 2002, Tabuchi *et al.* 2003). Another Sertoli cell line 93RS2 was established by immortalizing primary rat Sertoli cells with SV40 tsA255 (Jiang *et al.* 1997). However, this cell line can form tumor although it has normal chromosome. Unlike the primary Sertoli cells, AR and FSHR are undetected in Sertoli cell lines immortalized by LTag. Some immature Sertoli cell lines (e.g. 93RS2) have no response to FSH, and most of them are unable to produce GDNF or BMP4. On the other hand, almost all of these Sertoli cell lines show epithelial attributes in appearance and have phagocytic activity. Since these cell lines have unique characteristics of Sertoli cells in response to changes of temperature, they can be used to explore the influence of epigenetic environment including temperature on spermatogenesis.

Sertoli cell lines immortalized by human telomerase reverse gene (Tert)

Two Sertoli cell lines B6Sc-2 and B6Sc-3 have been established from primary Sertoli cells of 19-day-old mice by immortalization with pBK-CMV-Tert (Chuang *et al.* 2007). These cell lines possess the characteristics of Sertoli cells since they express tyrosine-tubulin, vimentin and stem cell factor (SCF) (Chuang *et al.* 2007). Moreover, the expression of SCF is maintained in these cell lines for 20 passages, which promotes the proliferation and differentiation of SSCs via the KIT receptor (Sette *et al.* 2000). The expression of FSHR is also detected in both cell lines, which can respond to FSH and phosphorylate a number of proteins. B6Sc-2 cell line and TM4 cell line express similar levels of Sertoli cell-specific genes, including *Amh*, *Cldn11* (also known as *claudin11*) and *Tjp1* (also known as *Zo1*). Notably, the expression of AR can be induced by tumor necrosis factor- α (TNF- α) in both B6Sc-2 and B6Sc-3 cell lines. The morphology, cytoskeleton distribution, growth rate and phenotype of B6Sc-2 and B6Sc-3 cell lines are analogous to primary Sertoli cells, and thus they may provide a useful cell source for understanding Sertoli cell biology.

As described above, a number of established rodent Sertoli cell lines have unlimited proliferation potentials and possess biochemical characteristics of primary Sertoli cells. These cell lines can facilitate our understanding of the biology of Sertoli cells and their roles in mediating spermatogenesis. Nevertheless, human Sertoli cell lines are not yet available. Recently, we have shown that

human primary Sertoli cells can be cultured for 2 months and expanded with a remarkable increase of cell numbers while retaining their morphology, phenotype and AKT and SMAD1/5 signaling pathways (Guo *et al.* 2015). This study can provide a basis for establishing a human Sertoli cell line.

The establishment and applications of spermatogonial cell lines

We illustrate the generation of rodent spermatogonial cell lines in Fig. 2, and the immortalization approaches and phenotype of these cell lines are summarized in Table 2.

Immortalization of rat and human spermatogonial stem cell (SSC) lines with SV40 large T antigen

In 2002, rat SSC lines A303 and A304 were established with SV40 large T antigen (van Pelt *et al.* 2002). The starting cells were A_{sr} , A_{pr} and A_{al} spermatogonia, and both cell lines express *Hsp90a* and *Pou5f1* (also known as *Oct4*), which are markers for germ cells and SSCs (He *et al.* 2007, 2009); however, these cells are negative for KIT, a hallmark for differentiating A_1 – A_4 spermatogonia (He *et al.* 2007). In the *in vitro* study, these cell lines have been cultured for 3 years and passaged for more than 50 times without morphological or DNA content changes. For the *in vivo* transplantation, they can migrate to the basement membrane and colonize in the seminiferous tubules of recipient mice, and no tumor formation is observed. The findings indicate that these cell lines possess typical characteristics of primary SSCs, and therefore they could be utilized to unveil the mechanisms regulating self-renewal (proliferation and survival) of rodent SSCs. Since it is unclear if these cell lines can differentiate in the recipient mice, it remains to be defined whether they are suitable to be used to examine the differentiation of rat SSCs.

Most importantly, we have for the first time established a human SSC cell line by stably overexpressing human SV40 large T antigen (Hou *et al.* 2015). Molecular and cellular analyses reveal that this cell line expresses a number of human spermatogonial and SSC hallmarks, including VASA, DAZL, MAGEA4, GFRA1, RET, UCHL1, GPR125, PLZF and THY1 (Hou *et al.* 2015), indicating that these cells are human SSCs in phenotype. It is worth noting that this cell line can be cultured for 1.5 years and expanded with a significant increase of cell numbers, while maintaining high levels of PCNA and UCHL1. Moreover, this human SSC line is able to colonize and proliferate in the recipient mice (Hou *et al.* 2015). Neither Y chromosome microdeletions of numerous genes nor tumor formation has been observed in human SSC line. Therefore, this human SSC line can provide an adequate source of human SSCs for basic studies and translational medicine.

Table 2 The establishment and characterization of male germ cell lines.

Cell lines	Species	Method	Cell identity	Markers	Differentiation	References
GC-1spg cell line	10-day-old mice	SV40	Type B spermatogonia and preleptotene spermatocytes	<i>Ldhc4</i>	No differentiation	Hofmann <i>et al.</i> (1992)
GC-2spd cell line	6-week-old mice	SV40 and the gene coding for a temperature-sensitive mutant of <i>p53</i>	Preleptotene spermatocytes	<i>Ldhc4</i>	Differentiate into round spermatids; cultured with complete CMRjkiL-1066 medium	Hofmann <i>et al.</i> (1994)
GC-3spc cell line	6-week-old mice	SV40 and the gene coding for a temperature-sensitive mutant of <i>p53</i>	Preleptotene spermatocytes	<i>Ldhc4</i>	No differentiation	Hofmann <i>et al.</i> (1994)
GC-4spc cell line	Adult transgenic mice	SV40 and a spermatocyte-specific promoter for human PGK2	Preleptotene and early pachytene spermatocytes	<i>Pgk2</i> , TSPY	No differentiation	Tascou <i>et al.</i> (2000)
A303 and A304 cell lines	Vitamin A-deficient rats	SV40	A _{pr} , A _{pr} , A _{al} spermatogonia	<i>Hsp90</i> , <i>Pou5f1</i>	No differentiation	van Pelt <i>et al.</i> (2002)
C18-4 cell line	6-day-old mice	SV40	Type A spermatogonia	<i>Dazl</i> , <i>Pou5f1</i> , <i>Gfra1</i> , <i>Piwi12</i> , <i>Tex13</i> , <i>Tex14</i> , <i>Tex15</i> , <i>Tex16</i> , <i>Tex19</i>	No differentiation	Hofmann <i>et al.</i> (2005)
S4 cell line	6-day-old mice	<i>Tert</i>	Type A spermatogonia	<i>Dazl</i> , <i>Pou5f1</i> , <i>Kit</i>	Differentiate into sperm; cultured in DMEM with SCF	Feng <i>et al.</i> (2002)
Unnamed	Human	SV40	Spermatogonial stem cells	VASA, DAZL, MAGEA4, GFRA1, RET, UCHL1, GPR125, PLZF, THY1	Not determined	Hou <i>et al.</i> (2015)

Immortalization of mouse type A spermatogonia cell line C18-4 cells with the SV large T antigen gene

The C18-4 cell line has been established with type A spermatogonia from 6-day-old mice using the SV large T antigen gene under the control of an ecdysone-inducible promoter (Hofmann *et al.* 2005). After selection with

Figure 2 Schematic diagram revealing the establishment of male germ cell lines. SSC, spermatogonial stem cell; A_{pr}, type A paired spermatogonia; A_{al}, type A aligned spermatogonia; A₁₋₄, type A₁₋₄ spermatogonia; B, type B spermatogonia; Spc, spermatocytes; Spd, spermatids; phosphoglycerate kinase 2 (PGK2).

antibiotics zeocin and G418, the C18-4 cell line was obtained and its appearance was round or polygonal with a high ratio of nucleus to cytoplasm. Notably, this cell line possesses the phenotype of male germ cells and undifferentiated spermatogonia since it expresses *Dazl*, *Pou5f1*, *Gfra1*, *Piwi12*, *Tex13*, *Tex14*, *Tex15*, *Tex16* and *Tex19* (Hofmann *et al.* 2005); however, C18-4 cell line does not express *Kit* (a marker for differentiating spermatogonia) (He *et al.* 2007). Additionally, this cell line responds to GDNF rather than SCF, suggesting that it prefers to proliferate rather than differentiate. The C18-4 cell line has important applications in uncovering the molecular mechanisms of rodent SSCs. As examples, we have used this cell line to demonstrate that GDNF signals via the Ras/ERK1/2 pathway (He *et al.* 2008) and Nodal acts through the SMAD2/3 and *Pou5f1* pathway to stimulate the self-renewal of mouse SSCs (He *et al.* 2009). The GDNF/Ras signaling pathway in C18-4 cell line has been confirmed in mouse primary SSCs (He *et al.* 2008, Lee *et al.* 2009).

Immortalization of mouse type A spermatogonial cell line S4 cells using *Tert* gene

Since spermatogonial cell lines immortalized by SV large T antigen gene prefer to proliferate rather than differentiate, *Tert* gene seems to be a more useful approach to establish spermatogonial cell lines for both proliferation and differentiation. In fact, the S4 mouse

spermatogonial cell line has been developed by retrovirus vector containing mouse *Tert* (Feng *et al.* 2002). The S4 cells display the morphology and biochemical phenotype (e.g. *Dazl*, *Pou5f1* and *Kit*) of mouse type A spermatogonia. Notably, this spermatogonial cell line can be coaxed by SCF to differentiate into spermatocytes and eventually haploid round spermatids *in vitro* (Feng *et al.* 2002). However, it remains unclear whether these spermatids have fertilization and developmental capacity.

Spermatogonia after immortalization with SV40 large T antigen or *Tert* can be cultured for a long period. Compared to the SV40 large T antigen method, *Tert* can immortalize cells without transformation or tumor formation (Morales *et al.* 1999). Spermatogonial cell lines immortalized with *Tert* not only proliferate and survive for a long period but also differentiate into spermatocytes and spermatids, which can be used to probe genetic and epigenetic regulation of spermatogenesis. Unfortunately, no human spermatogonial cell line immortalized by *Tert* is available now.

The establishment and applications of spermatocyte lines

We also illustrate the generation of rodent spermatocyte lines in Fig. 2, and their immortalization methods and phenotype are summarized in Table 2.

Immortalization of spermatocytes using the SV40 large T antigen

The cell line GC-1spg has been characterized as a male germ cell line at a stage between type B spermatogonia and preleptotene spermatocytes (Hofmann *et al.* 1992). This cell line was established by transfection of SV40 large T antigen gene to all cell types in seminiferous tubule of 10-day-old mice, and it has been cultured successfully for 90 generations in 2.5 years. In morphology, GC-1spg cells are larger than somatic cells in testis and have round nuclei with numerous clumps of chromatin, which corresponds to the characteristics of type B spermatogonia and preleptotene spermatocytes. In phenotype, synaptonemal complexes are undetected in the nuclei of GC-1spg cells, which usually appear in the leptotene or later spermatocytes (West *et al.* 2010). In addition, GC-1spg cells are positive for lactate dehydrogenase-C4 (LDHC4) isozyme, a marker specific for meiotic and postmeiotic germ cells (Liang *et al.* 1986); however, these cells are negative for phosphoglycerate kinase 2 (PGK2), which is normally expressed in secondary spermatocytes (McCarrey *et al.* 1996). Based on its morphological and biochemical features, GC-1spg cell line corresponds to the characteristics of cells between type B spermatogonia and preleptotene spermatocytes. This cell line can be used to examine mechanisms underlying the onset

of meiosis of spermatocytes although it is unable to differentiate further *in vitro*.

Spermatocyte lines immortalized with the SV40 large T antigen gene and the gene coding for a temperature-sensitive mutant of p53

The GC-2spd(ts) and GC-3spc(ts) cell lines were established from mouse preleptotene spermatocytes using an alternative strategy with the SV40 large T antigen and the gene coding for a temperature-sensitive mutant of p53 (Hofmann *et al.* 1994). Both cell lines express LDHC4 isozyme and Cytochrome ct isoform, hallmarks for spermatocytes. Wild-type p53 is able to abolish the initiation and maintenance of SV40 transformation (Fukasawa *et al.* 1991), and p53 protein is inhibited at the nonpermissive temperature of 39°C, which allows SV40 large T antigen to exert its immortalization ability. The p53 gene can be activated at the permissive temperatures of 37°C or 32°C, which enables the cells to differentiate. Moreover, the GC-2spd line is able to generate cells with haploid DNA as well as morphological and phenotypic characteristics of round spermatids although the GC-3spc(ts) cell line cannot undergo meiosis. Therefore, the GC-2spd cell line has greater applications in uncovering the machinery underlying the differentiation of spermatocytes. Furthermore, using the GC-2spd line and the immortalized Sertoli cell lines, seminiferous tubules can be reconstituted *in vitro*, which could be used to identify novel autocrine and paracrine factors involved in meiosis and spermiogenesis. However, subsequent research is unable to confirm the original work by the same group (Wolkowicz *et al.* 1996) since LDHC4, *Acr*, *Prm2* and haploid chromosome peak were undetected in GC-2spd line and it might be transformed. In addition, there is currently no human spermatocyte line immortalized by the SV40 large T gene and/or other genes.

Spermatocyte line immortalized with a specific promoter-based selection strategy

Another male germ cell line GC-4spc has been established from adult male transgenic mice, using a specific promoter-based selection method, i.e. the SV40 early promoter and a spermatocyte-specific promoter for human *PGK2* (Tascou *et al.* 2000). Conventional method utilizing the oncogenes under the control of ubiquitous promoters might lead to contamination with other cells. In contrast, the promoter-based selection strategy can immortalize male germ cell lines at specific stages of spermatogenesis with unique promoters. The GC-4spc cell line has been identified as cells with the characteristics between those of preleptotene and early pachytene spermatocytes. This cell line has been cultured for a long period without morphological changes and can be transfected with other genes, and therefore it may be used to unveil the mechanisms of meiosis.

Applications and perspectives of male germ cell and Sertoli cell lines in basic studies and translation medicine

As illustrated in Fig. 3, male germ cell and Sertoli cell lines can be utilized in basic studies as well as in reproductive and regenerative medicine. First of all, these cell lines can provide substantial sources of various kinds of male germ cells (spermatogonia and spermatocytes) and Sertoli cells. This makes it feasible to examine the genetic and epigenetic regulators in controlling the mitosis of spermatogonia and meiosis of spermatocytes. Specifically, these cell lines can be employed for investigating gene regulation, signaling transduction pathways, and non-coding RNA (e.g. microRNA and lncRNA) and DNA methylation in controlling the self-renewal and differentiation of male germ cells (Yao *et al.* 2015). For example, using spermatogonial stem cell line C18-4, we have successfully demonstrated that Nodal plays an essential role in promoting the self-renewal of mouse SSCs via Smad2/3 pathway and *Pou5f1* activation (He *et al.* 2009) and GDNF stimulates the proliferation and survival of mouse SSCs by activating Ras/ERK1/2 signaling pathway and transcription factor *Fos* (He *et al.* 2008). In addition, we have shown that miRNA-133b is uninvolved in the regulation of the human SSCs using a human SSC line (Hou *et al.* 2015) and it specifically enhances the proliferation of human Sertoli cells (Yao *et al.* 2016). Sertoli cell lines, e.g. SerW3 cell line and 93RS2 cell line, can be utilized to examine toxicology in male urinogenital system (Pognan *et al.* 1997) and develop new drugs for treating testicular tumor (Jiang *et al.* 1997) respectively. Sertoli cell lines contribute to the long-term culture of SSCs and 2- or 3-dimensional (3D) culture system to support the differentiation of SSCs into mature spermatids. Additionally, the G5, A31C4, C22A4 and C24A4 Sertoli cell lines can secrete numerous growth factors, e.g. leukemia inhibitory factor (LIF), transforming growth factor- β (TGF- β) and

bFGF (Hofmann *et al.* 2003, Sato *et al.* 2013), which are extremely important for the survival and proliferation of SSCs. Therefore, these Sertoli cell lines might be used as an excellent feeder layer for long-term culture and expansion of SSCs. When the SF7 Sertoli cell line is cocultured with Leydig cells and spermatocytes cell line GC-1spg, seminiferous tubules are formed (Hofmann *et al.* 1992). Therefore, it is practicable to coculture Sertoli cell and male germ cell lines with 3-D biomaterials or use Sertoli cell lines as feeder cells for 3-D culture of SSCs to reconstitute the testicular tissues (Kanatsu-Shinohara *et al.* 2012) and explore the precise process of testis formation in the near future. Sertoli cell lines could also be employed for 2-D and/or 3-D culture to generate mature and functional spermatids from SSCs (Sato *et al.* 2011) and ES cells (Zhou *et al.* 2016).

The establishment of male germ cell and Sertoli cell lines brings new promise for reproductive and regenerative medicine due to the great plasticity of spermatogonia and Sertoli cells. Human SSCs including spermatogonia can differentiate into male gametes (Yang *et al.* 2014a), dedifferentiate to pluripotent ES-like cells (Golestaneh, *et al.* 2009), and transdifferentiate to functional and mature hepatocytes (Chen *et al.* 2016). Therefore, they may bring inspirations for new therapeutic strategies to cure male infertility patients with spermatogonia and other human diseases (e.g. end-stage liver disease). The MSC-Ehl-Zs Sertoli cell line can produce insulin consistently (Kaur *et al.* 2014), which offers new opportunities for treating diabetes. Sertoli cells can also be reprogrammed to generate functional neurons (Sheng *et al.* 2012) and other cell lineages (e.g. Leydig cells and iPS cells) (Zhang *et al.* 2015) and thus they can have potential clinical value in translation medicine. Furthermore, these cell lines could be exploited to uncover the underlying etiology of male infertility (e.g. genetic factors and epigenetic errors) and perform drug toxicity screening for environmental chemicals and hormones.

Summary

In conclusion, we have discussed various kinds of approaches for establishing a number of male germ cell and Sertoli cell lines and addressed their phenotypic characteristics. Although immortalizing cell lines with virus (e.g. SV40 large T antigen) can enable cells to proliferate forever, it results in transformation and inability to differentiate. In comparison, immortalization with *Tert* can lead to both proliferation and differentiation of the cell lines, and thus they have advantages over the cell lines by SV40 large T antigen in uncovering the differentiation mechanisms of male germ cells. In addition, the establishment of male germ cell lines using their specific promoters could lead to unique types of cell lines. It is worth noting that the development of male germ cell and Sertoli cell lines

Figure 3 Schematic diagram illustrating the applications of male germ cell and Sertoli cell lines in basic studies and translation medicine.

could have important applications for clinic and research fields. We have recently established a human SSC line with an unlimited proliferation potential and no carcinogenesis, which could offer adequate human SSCs for both mechanistic studies and clinical applications. Nevertheless, other human cell lines for male germ cells and Sertoli cells are unavailable. Since there were distinct identity and phenotype of male germ cells and Sertoli cells between rodents and human (He *et al.* 2010), it is essential to establish human testicular cell lines in order to better understand the mechanism of human spermatogenesis and develop therapeutic approaches to treat human diseases.

Declaration of interest

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

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