

## REVIEW ARTICLE

Insights into in vitro spermatogenesis in mammals:  
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Considering the self-renewal and differentiation ability of pluripotent stem cells, some studies have pointed out the possibility of stem cell-derived sperm production. Most studies that test this hypothesis have been conducted on rodents, with some promising results; however, studies on humans are progressing slowly, and have encountered technical and ethical hurdles. Established methods to differentiate stem cells—including embryoid bodies, co-culturing, and various feeder cells—may provide a niche that is similar to in vivo conditions and resolve epigenetic abnormalities, but a gonadal-like three-dimensional structure is still required to produce germ cells with the correct imprinting. In the last few years, sperm-like cells with fertilizing capacity were produced from mouse embryonic stem cells, and the resulting embryos from these cells yielded live offspring. Future research should move towards the use of adult stem cells, however, owing to the unavailability of embryonic cells in adults. More intensive research and techniques are required since in vitro spermatogenesis provides hope to individuals without mature sperm who cannot be treated, and may be a useful system to study the precise mechanism of spermatogenesis. In this review, we describe recent studies of in vitro spermatogenesis mechanisms and related techniques in mammals. We also discuss the possible cell surface markers and culture conditions that might improve in vitro spermatogenesis.

**KEYWORDS**

gametogenesis, germ cell differentiation, in-vitro derived gametes, spermatogenesis, stem cell

*[The] possibility of obtaining patient-specific sperm, derived from stem cells, has drawn much attention in recent years.*

**Abbreviations:** BMP, bone morphogenetic protein; BMSCs, bone marrow stem cells; DDX4, DEAD-box helicase 4 (or Vasa); EGF, epidermal growth factor; ESC, embryonic stem cell; FGF, fibroblast growth factor; GDNF, Glial cell-derived neurotrophic factor; GFP, green fluorescent protein; INHBA, Inhibin beta A (or activin A); LIF, Leukemia inhibitory factor; PGC, primordial germ cell; PRM1, Protamine 1; SSC, spermatogonial stem cell; STRA8, Stimulated by retinoic acid 8.

**1 | INTRODUCTION**

About 10–15% of all couples are infertile, with male infertility accounting for half of these cases (Gu, Liu, & Belmonte, 2012, Massart, Lissens, Tournaye, & Stouffs, 2012). About 200,000 babies are born every year using assisted reproductive technologies, which has helped many couples with fertility problems (Zegers-Hochschild et al., 2009); unfortunately, patients without gametes cannot benefit from the existing approaches. For example, normal sperm collection in 10% of infertile men with obstructive azoospermia and non-obstructive azoospermia is not possible—especially in non-obstructive azoospermia patients, for whom even testicular extraction of sperm is not helpful (Su et al., 1999). Other causes of male infertility are associated with impaired spermatogenesis due to genetic deficiency or environmental factors, or result from cancer treatments, including

radiotherapy and chemotherapy (Gu et al., 2012). The only currently available method for treating such male infertility is through donor sperm. Owing to the demand for biological offspring as well as for legal and ethical concerns about using donated sperm, the possibility of obtaining patient-specific sperm, derived from stem cells, has drawn much attention in recent years.

In vitro-derived sperm from males without viable sperm can be used in assisted reproductive methods (Hunter, Anand-Ivell, Danner, & Ivell, 2012). In vitro spermatogenesis has the potential to create a large number of sperm, which provides the opportunity to select the best genetic and morphologically sperm-like cells for fertilization (Bourne, Douglas, & Savulescu, 2012). In addition, an ex vivo sperm production system could help reveal the physiological mechanisms and factors involved in sperm production, such as hormones and growth factors as well as epigenetic modifications, owing to the difficulty to access and manipulate the testis environment in vivo. For example, in vitro spermatogenesis provides a valuable model for elucidating mechanisms underlying idiopathic male infertility (Newson & Smajdor, 2005) and provides the possibility of evaluating the effects of various drugs and toxins on spermatogenesis, which could help identify new drugs for treating male infertility. Some researchers also suggest that in vitro spermatogenesis would be useful in the production of transgenic animals and in the conservation of endangered animals (Cai et al., 2013; Nayernia, Nolte, 2006).

Stem cells are undifferentiated cells with self-renewing ability, but can be differentiated into many cell types (Marques-Mari, Lacham-Kaplan, Medrano, Pellicer, & Simón, 2009) as well as various tissues, such as the liver, brain, and pancreas (Aejaz et al., 2007). In general, stem cells are divided into embryonic stem cells (ESCs) and non-embryonic or adult stem cells, depending on their origins: ESCs are specifically obtained from an embryo at the blastocyst stage (Attar & Attar, 2008; Nagy et al., 1990), whereas adult stem cells can be isolated from bone marrow, dental pulp, hair follicles, skin, adipose tissue, blood, liver, and even the testes (Aejaz et al., 2007).

Recent studies documented the possibility of producing sperm-like cells from stem cells; however, a proper in vitro system for the production of mature sperm cells was not established (Miryounesi et al., 2014; Silva et al., 2009). Nevertheless, in vitro production of mammalian sperm cells from various stem cells will eventually be possible and standardized, based on the reported production of live pups from in vitro spermatogenesis (Nayernia, Nolte, 2006). Proper in vitro production of sperm requires the replication of in vivo spermatogenesis, although perfectly good sperm can develop in vitro along a time course that differs from in vivo development.

Spermatogenesis in mammals is a complex process involving various cells, such as Sertoli cells, Leydig cells, and peritubular myoid cells (Hess, Cooke, Hofmann, & Murphy, 2006). During embryogenesis, primordial germ cells (PGCs) emerge from proximal epiblast. These cells then migrate to the genital ridge, where they are enclosed by Sertoli cell precursors and become gonocytes (Zhao & Garbers, 2002). Gonocytes remain in the G0/G1 phase until birth, and mature to spermatogonial stem cells (SSCs) after birth (Nayernia, Li, & Engel, 2004). SSCs asymmetrically divide into a daughter stem cell and spermatogonia, with the latter differentiating into spermatozoa

(Nayernia, Li, & Engel, 2004). Therefore, in vitro-spermatogenesis systems must first differentiate true stem cells into PGCs, induce meiosis, and then differentiate PGCs into mature sperm in order to simulate in vivo sperm production.

In this review, we describe recent studies on in vitro spermatogenesis, focusing on their techniques and outcome. We also discuss the possible cell-surface markers and culture conditions that could improve in vitro spermatogenesis.

## 2 | SOURCE OF STEM CELLS FOR IN VITRO SPERMATOGENESIS

### 2.1 | ESCs as a source of in vitro spermatogenesis

ESCs are pluripotent cells, and numerous studies have used them to produce male gametes (Table 1). These cells are suitable for in vitro sperm production because they can differentiate into various somatic cells and form embryoid bodies (Toyooka, Tsunekawa, Akasu, & Noce, 2003). Some labs have succeeded in coaxing ESCs to form embryonic germ cells, based on the similarity of their gene and protein expression patterns to those of PGCs. The first reported differentiation of human ESCs into embryonic germ cells was by Clark and co-workers, who confirmed that the cultivation of human ESCs into embryoid bodies formed embryonic germ cells that express germ cell-specific genes, such as *DAZL* (deleted in azoospermia-like), *DDX4* (DEAD\_box helicase 4, also known as *VASA*), *CTDSP1* (Carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 1), and *GDF9* (Growth and differentiation factor 9) (Clark et al., 2004). These scientists only proceeded to the production of PGCs, without continuing to the production of haploid sperm-like cells (Clark et al., 2004). In another study, germ cell-specific *DDX4* expression was observed in human ESCs cultured in the presence of Bone morphogenetic protein 4 (BMP4) (Kee, Gonsalves, Clark, & Pera, 2006), which is consistent with the reported production of PGCs from monkey ESCs in the presence of BMP4, retinoic acid, or KIT ligand (KITLG, also known as Stem cell factor) (Yamauchi, Hasegawa, Chuma, Nakatsuji, & Suemori, 2009). In 2009, Kee, Angeles, Flores, Nguyen, and Pera (2009) reported that the overexpression of the *DAZ* gene family could differentiate human ESCs into haploid embryonic germ cells.

A hybrid in vitro-in vivo approach—the transplantation of in vitro-produced PGCs into the testis to foster their differentiation into mature sperm—was initially used to overcome the inability to produce sperm-like cells from ESCs exclusively by in vitro approaches (Hayashi, Ohta, Kurimoto, Aramaki, & Saitou, 2011; Toyooka et al., 2003). Toyooka and co-workers cultured ESCs, derived from a *Ddx4*-green fluorescent protein (GFP) knock-in reporter mouse, to embryoid bodies in the presence of BMP4-producing feeder cells, and reported the production of GFP-positive cells, which implied the expression of the germ cell-specific marker *Ddx4* (Toyooka et al., 2003). After flow cytometric collection and co-culturing these GFP-positive embryonic germ cells with gonadal cells, they transplanted the cells beneath the testis capsule of mice and reported the production of morphologically normal sperm—unfortunately, no information was provided on the

**TABLE 1** In-vitro spermatogenesis studies using ESC

Studies	ESC source	Culture method	PGCs	Haploid cell	Embryo	Offspring	Differentiation-inducing factor(s)
Toyooka et al. (2003)	Mouse	Embryoid body/ Transplantation	Yes	n/a	n/a	n/a	BMP4
Clark et al. (2004)	Human	Embryoid body	Yes	n/a	n/a	n/a	Spontaneous
Geijsen et al. (2004)	Mouse	Embryoid body	Yes	Yes	Yes	n/a	Retinoic acid
Nayernia, Nolte (2006)	Mouse	Monolayer	Yes	Yes	Yes	Yes	Retinoic acid
Kee et al. (2006)	Human	Embryoid body	Yes	n/a	n/a	n/a	BMPs
Kerkis et al. (2007)	Mouse	Embryoid body	Yes	Yes	n/a	n/a	Retinoic acid
Teramura et al. (2007)	Monkey	Embryoid body	Yes	n/a	n/a	n/a	Spontaneous
Yamauchi et al. (2009)	Monkey	Embryoid body	Yes	n/a	n/a	n/a	BMP4/SCF/retinoic acid
Aflatoonian et al. (2009)	Human	Embryoid body	Yes	Yes	n/a	n/a	BMP4/retinoic acid
Silva et al. (2009)	Mouse	Embryoid body	Yes	n/a	n/a	n/a	Retinoic acid/testosterone
Kee et al. (2009)	Human	Monolayer	Yes	Yes	n/a	n/a	Overexpression of DAZ, DAZL, and BOULE + BMPs
West et al. (2008, 2010)	Human	Monolayer	Yes	Yes	n/a	n/a	bFGF
Easley et al. (2012)	Human	Monolayer	Yes	Yes	n/a	n/a	SSCs differentiation medium (contains bFGF, GDNF)
Zhou et al. (2016)	Mouse	Monolayer	Yes	Yes	Yes	Yes	Activin A/BMPs/retinoic acid

bFGF, basic Fibroblast growth factor; BMP, bone morphogenetic proteins; GDNF, Glial cell-derived neurotrophic factor; PGCs, primordial germ cells; SCF, Stem cell factor; n/a, not assessed.

functionality of the obtained sperm. Hayashi and co-workers, however, initially induced differentiation of epiblast-like cells from mouse ESCs, and used them for the production of the embryonic germ cells, based on the *in vivo* origin of germ cells formed from the embryonic epiblast during embryogenesis (Hayashi et al., 2011). They then transplanted the embryonic germ cells into a germ cell-deficient mouse testis, and reported production of functionally normal sperm that could fertilize the oocytes and create fertile offspring (Hayashi et al., 2011). Therefore, the differentiation of epiblast cells from ESCs followed by the production of PGCs from these cells could sufficiently mimic the early *in vivo* process, with the caveat that the final stage of differentiation into sperm-like cells from PGCs requires the *in vivo* testicular environment.

Some laboratories attempted to conduct all differentiation processes of ESCs into sperm using *ex vivo* techniques. Geijsen and co-workers, for example, reported that culturing mouse ESCs as embryoid bodies, with addition of retinoic acid in the medium, leads to their spontaneous differentiation into PGCs (Geijsen et al., 2004). They sorted the obtained germ cells based on the marker FUT4 (Fucosyltransferase 4; also known as SSEA-1), and showed that the methylation pattern of FUT4-positive cells was similar to that of normal germ cells. Furthermore, their continued differentiation into post-meiotic cells and use for intracytoplasmic sperm injection of eggs resulted in a 20% blastocyst rate. Unfortunately, these researchers did not transfer these blastocysts to track the development of the fetus or the possibility of obtaining offspring (Geijsen et al., 2004). In another study, Kerkis and co-workers reported the production of female and male germ cells from murine ESCs via embryoid bodies, and that retinoic acid supplementation of the culture medium and the duration of the culture period are influential in the production of male germ cells

(Kerkis et al., 2007). The obtained male germ cells were able to fertilize eggs and produced blastocyst-like structures.

In 2006, Nayernia, Nolte (2006) first reported the production of fertile sperm with the ability to yield live offspring. These scientists used a monolayer-culture method in the presence of retinoic acid, as a proliferation- and differentiation-inducing factor. The novelty of this study was its use of a two-step sorting method for isolating well-differentiated cells: First isolating PGCs using STRA8 (Stimulated by retinoic acid 8) as a marker, followed by the isolation of post-meiotic cells using PRM1 (Protamine 1). This approach allowed them to track the progression of murine ESCs differentiation as well as enrich for differentiated male germ cells. Isolated sperm-like cells were injected into eggs, and the resultant blastocysts were transferred into the uterus of pseudopregnant mice, which led to the birth of seven live pups; however, the pups died a few months after birth due to imprinting defects (Nayernia, Nolte, 2006). In 2016, Zhou and co-workers reported the successful generation of functional spermatids that produced viable fertile offspring (Zhou et al., 2016). This group differentiated murine ESCs first into epiblast-like cells and then into PGCs, based on a previously described procedure (Hayashi et al., 2011). The PGCs were subsequently co-cultured with neonatal testicular somatic cells and exposed to Inhibin beta A (INHBA, also known as Activin A), BMPs, and retinoic acid to simulate *in vivo* differentiation. They suggested that BMPs and INHBA are necessary for the proliferation of PGCs, whereas retinoic acid induces meiotic entry and differentiation (Zhou et al., 2016). They showed that such step-wise differentiation produces functional sperm-like cells with attributes in accordance with the “gold standards” of meiosis, such as erasure of imprints, synapsis, and recombination (Handel, Eppig, & Schimenti, 2014).

Mammalian *in vitro* gametogenesis using ESCs has been reviewed in depth elsewhere (Zhou et al., 2010). According to the outcomes of the studies highlighted above, the differentiation of ESCs into sperm may be a good model to study spermatogenesis and the effects of various genetic and environmental factors on this process, although more research is required to develop a robust protocol for differentiating ESCs into sperm with normal epigenetic status.

## 2.2 | Non-ESCs as a source of *in vitro* spermatogenesis

Studies on the differentiation of ESCs into sperm have shown promising results, but difficulties related to full and controlled germ cell differentiation and the unattainability of ESCs from infertile adults have led to the evaluation of adult stem cells as a source for *in vitro* sperm production (Table 2). Bone marrow stem cells (BMSCs) are one appropriate source, and most studies have used them for gametogenesis studies (Drusenheimer et al., 2007; Mazaheri, Movahedin, Rahbarizadeh, & Amanpour, 2011; Nayernia, Lee, 2006; Shirazi et al., 2012). Bone marrow contains various stem cells, including hematopoietic stem cells, endothelial stem cells, and mesenchymal stem cells, which can be differentiated into multiple cell types such as bone, cartilage, fat, muscle, tendon, skin, liver, kidney, heart, and brain cells (Jiang et al., 2002; Pittenger et al., 1999). Human umbilical cord Wharton's jelly-derived mesenchymal stem cells can also be utilized to produce germ cells, although few studies have examined the possibility of using these cells to produce male germ cells (Huang et al., 2010).

In a study by Nayernia and co-workers, the expression of *Ddx4* was demonstrated in mouse BMSCs in the presence of BMP4 (Nayernia, Lee, 2006). Transplantation of mesenchymal stem cell-derived germ cells into the sterile mouse testis, however, resulted in their pre-meiotic arrest (Nayernia, Lee, 2006). Although this study showed the possibility of generating germ cells from mesenchymal stem cells, other approaches and additional growth factors are clearly needed to produce sperm-like cells. These scientists suggested that their incomplete meiosis could be related to their inability to form embryoid bodies, which are thought to mimic the microenvironment of early embryos, and thus are blocked from complete reprogramming to the germ line (Nayernia, Lee, 2006). In a similar study, the expression of *Ddx4* was reported in BMSCs treated with BMP4 (Mazaheri et al., 2011). Pluripotent, FUT4-positive stem cells from bone marrow were reported to differentiate into PGCs and germ cells (Shirazi et al., 2012). Another study by Hua and co-workers reported that human BMSCs express markers of PGCs and male germ cells in the presence of retinoic acid, and could subsequently differentiate into sperm-like cells (Hua et al., 2009). They also demonstrated that treating human BMSCs with testicular extracts could increase the expression of several germ cell-specific genes, such as *DDX4*, *STRA8*, *CTDSPL* (CTD small phosphatase-like; also known as *SCP3*), and *PRM1*, and facilitate the differentiation of mesenchymal stem cells into germ cells (Hua et al., 2009). However, the competency of the obtained cells to complete meiosis and undergo spermatogenesis was not determined. Curiously, Lue and co-workers reported that testicular somatic cells could be directly isolated from BMSCs fractions (Lue et al., 2007).

Some researchers have attempted to use induced-pluripotent stem cells (iPSCs), generated from somatic cells by the transduction of transcription factors (Takahashi & Yamanaka, 2016; Takahashi et al., 2007; Yu et al., 2007), to produce germ cells. Park and co-workers, for example, generating PGCs by reprogramming human fetal somatic cells to iPSCs, which were then differentiated into germ cells (Park et al., 2009). Two years later, Panula and co-workers produced haploid male gametes from human fetal and adult-derived iPSCs by overexpressing proteins of the DAZ family (Panula et al., 2011). Other groups reported the production of haploid cells from various iPSCs derived from cord blood or keratinocytes (Eguizabal et al., 2011). Recently, Medrano and co-workers showed that meiosis can be induced in differentiating human iPSCs by overexpressing *DAZL* and/or *DDX4* (Medrano, Ramathal, Nguyen, Simon, and Reijo Pera, 2012). In 2015, Sasaki and co-workers differentiated two independent male human iPSCs lines into incipient mesoderm-like cells, which were then robustly converted into human PGC-like cells (Sasaki et al., 2015). These scientists suggested that INHBA and WNT-signaling agonist are essential for this process, whereas both BMP4 and Fibroblast growth factor 2 (FGF2, also known as basic FGF [bFGF]) block such induction (Sasaki et al., 2015). However, the absence of reports on the fertilization capacity and the possibility of producing living offspring from these non-ESC-derived germ cells dampens the enthusiasm for this approach. Use of these iPSCs to treat infertility also encounters the obstacle associated with the requisite gene manipulation currently used to generate these stem cells.

The possibility of producing sperm-like cells from embryonal carcinoma cells, derived from teratocarcinomas, has also been shown (Nayernia et al., 2004). Embryonal carcinoma cells, like ESCs, can form embryoid bodies and be differentiated into spermatogonia in the presence of retinoic acid (Marques-Mari et al., 2009; Nayernia et al., 2004). Spermatogonia obtained by this method can be converted into mature sperm after transplantation into the testes; these gametes can fertilize eggs; and the resultant zygotes reach the six- to eight-cell embryo stage. However, these sperm have abnormal morphology and motility, and the harvested embryos are not able to develop beyond early cleavage stages (Nayernia et al., 2004). A less-risky endeavor is the production of PGCs from skin-derived stem cells (Linher, Dyce, & Li, 2009). This latter study using endogenous stem cells demonstrated the potential of adult stem cells to produce male germ cells without genome modification, providing a path where autologous gametes could be generated for the treatment of infertile patients.

## 2.3 | SSCs as a source of *in vitro* spermatogenesis

Male cancer patients undergoing radiotherapy or chemotherapy often choose to cryopreserve their sperm before treatment, thereby ensuring a supply for later *in vitro* fertilization, but this method is not an option for pre-pubescent individuals (Jahnukainen & Stukenborg, 2012). Furthermore, cryopreservation has an associated risk of damaging and reducing the fertilizing capacity of the sperm (Omnes et al., 2013). An alternative option for patients is the transplantation of autologous SSCs after treatment, although this too has the risk of tumorigenesis. Thus, the cryopreservation of a testicular biopsy that contains SSCs, followed by later *in vitro* differentiation of these SSCs

**TABLE 2** In-vitro spermatogenesis studies using non-EsCs

Studies	Source	Culture method	PGCs	Haploid cell	Embryo	offspring	Differentiation inducing factor(s)
Nayernia et al. (2004)	Mouse TCSCs	Monolayer/ transplantation	Yes	Yes	Yes	n/a	Retinoic acid
Nayernia, Lee (2006)	Mouse BMSCs	Monolayer/ transplantation	Yes	n/a	n/a	n/a	Retinoic acid
Drusenheimer et al. (2007)	Human BMSC	Monolayer	Yes	n/a	n/a	n/a	Retinoic acid
Hua et al. (2009)	Human BMSC	Monolayer	Yes	n/a	n/a	n/a	Retinoic acid/testicular extracts
Huang et al. (2010)	Human umbilical MSCs <sup>a</sup>	Monolayer	Yes	n/a	n/a	n/a	Retinoic acid/testosterone
Hayashi et al. (2011)	Mouse iPSCs	Monolayer/transplantation	Yes	n/a	Yes	Yes	BMP4/LIF/SCF/BMP8b/EGF/KSR
Panula et al. (2011)	Human iPSCs	Monolayer	Yes	Yes	n/a	n/a	BMP-4, -7, and 8b
Eguizabal et al. (2011)	Human iPSCs	Monolayer	Yes	Yes	n/a	n/a	Retinoic acid
Shirazi et al. (2012)	Mouse BMSCs	Monolayer	Yes	n/a	n/a	n/a	BMP4
Cai et al. (2013)	Mouse iPSCs	Monolayer/embryoid body/transplantation	Yes	n/a	n/a	n/a	bFGF/SCF/retinoic acid
Li, Pan, et al. (2014)	Human umbilical MSCs <sup>a</sup>	Monolayer/embryoid body	Yes	n/a	n/a	n/a	BMP4
Li, Wang, et al. (2014b)	Mouse iPSCs	Monolayer/transplantation	Yes	n/a	n/a	n/a	Activin A/bFGF/RA/BMP-4 & -8b/SCF
Ghasemzadeh-Hasankolaei, Sedighi-Gilani, and Eslaminejad (2014)	Ram BMSC	Monolayer	Yes	n/a	n/a	n/a	TGFb1
Kashani et al. (2014)	Mouse BMSCs	Embryoid body	Yes	n/a	n/a	n/a	retinoic acid
Sasaki et al. (2015)	Human iPSCs	Monolayer	Yes	n/a	n/a	n/a	BMP4/SCF/EGF/LIF/Activin A/WNT agonist
Yan, Fan, Li, Zhang, and Wang (2015)	Goat BMSC	Monolayer	Yes	n/a	n/a	n/a	Retinoic acid/BMP4

bFGF, basic fibroblast growth factor; BMSCs, bone marrow-derived mesenchymal stem cells; BMP, Bone morphogenetic protein; EGF, Epidermal growth factor; iPSCs, induced pluripotent stem cells; KSR, Knock out Serum Replacement; LIF, Leukemia inhibitory factor; MSCs, mesenchymal stem cells; PGCs, primordial germ cells; SCF, Stem cell factor; TCSCs, teratocarcinoma stem cells; n/a, not assessed.

<sup>a</sup>Human umbilical cord Wharton's jelly derived (mesenchymal stem cells).

into mature sperm, may be a better option. Indeed, testicular sperm extraction is routinely performed to provide therapy for azoospermic patients, although this method is not useful for patients with non-obstructive azoospermia, for whom isolation and maturation of autologous SSCs for *in vitro* sperm production is suggested (Kanatsu-Shinohara et al., 2003, 2005).

SSCs are the precursors of sperm in the testes, and they possess self-renewing and differentiation capabilities that ensure spermatogenesis can be maintained over the lifespan of an adult male (Meistrich & Van Beek, 1993; Rooij & Russell, 2000). SSCs reside in a microenvironment, or “niche,” in conjunction with Sertoli cells, which provide the factors necessary for differentiation and self-renewal (Chiarini-Garcia, Raymer, & Russell, 2003). The number of SSCs in the testes are very low—one in every 3,000–4,000 cells in the mouse testis (Tagelenbosch & De Rooij, 1993)—so most studies have been conducted on primary culture of spermatogonia cells, of which the SSCs are minor portion.

The self-renewing capability of SSCs can only be maintained in an appropriate microenvironment. Addition of Glial cell-derived neurotrophic factor (GDNF) to culture medium can maintain self-renewal of mouse and bovine SSCs for a long time (Izadyar et al., 2003; Kanatsu-Shinohara et al., 2003; Kubota, Avarbock, & Brinster, 2004b). Various feeder cells, such as fibroblasts and Sertoli cells, or serum plus other supplements in the culture medium also induced SSC proliferation and maintenance in cultures of testicular cells (Nagano, Ryu, Brinster, Avarbock, & Brinster, 2003). Yet, the ideal culturing method would utilize serum-free medium and no feeder cells to avoid cross-contamination. While this xeno-free approach is possible, its efficiency is poor and long-term culturing of spermatogonia is difficult (Creemers, Den Ouden, Van Pelt, & De Rooij, 2002; Kubota, Avarbock, & Brinster, 2004a).

Complete *in vitro* differentiation of SSCs into mature sperm with fertilizing ability also faces challenges. Kanatsu-Shinohara and co-workers cultured spermatogonia cells from neonatal mouse testes on a feeder layer of mouse embryonic fibroblasts, in the presence of various factors—such as GDNF, FGF2, Epidermal growth factor (EGF), Leukemia inhibitory factor (LIF), and fetal calf serum—but could only produce mature sperm cells when they transplanted the obtained cells into the testes of infertile mice (Kanatsu-Shinohara et al., 2003). In another study using testicular cells from sheep, melatonin was used to induce differentiation of SSCs (Deng et al., 2016). Melatonin is synthesized in the testes and plays a role in testis function by increasing testosterone levels and modifying the morphology of spermatogenic cells (Sanchez-Barcelo et al., 1991; Tsantarliotou, Kokolis, & Smokovitis, 2008). Deng and co-workers reported that melatonin increased the rate of SSC differentiation twofold and up-regulated the expression of differentiation-related genes (Deng et al., 2016). Furthermore, the functional haploid sperm-like cells produced could activate eggs, and yielded a high frequency of blastocysts—but no information about the ability of embryos to produce healthy offspring was provided.

Future studies utilizing SSCs need to identify an appropriate culturing method that maintains their proliferation, and then find novel ways to induce the *in vitro* differentiation of these cells into mature sperm. SSCs are a minor fraction of testicular cells, so isolation of these

cells for pure culture is a major challenge. Most studies to date have attempted to optimize SSCs culturing and *in vitro* differentiation without first enriching for these cells, resulting in low efficiency methods that could be contaminated with Leydig and peritubular cells (intermediate and Type B spermatogonia and somatic cells) (Creemers et al., 2002; Deng et al., 2016; Kanatsu-Shinohara et al., 2003; Nagano et al., 2003). Such contamination in the culture system probably provided cell-to-cell communication and paracrine signaling that would be extremely difficult to parse out for replication of positive results. Therefore, further studies are needed to establish more robust methods for the isolation and purification of SSCs from the testes; this topic was recently reviewed (Komeya & Ogawa, 2015; Yokonishi & Ogawa, 2016).

### 3 | CULTURING METHODS FOR IN VITRO SPERMATOGENESIS

Many methods were reported for culturing, expansion, and differentiation of stem cells for *in vitro* spermatogenesis, but appropriate and standard protocols have not yet been introduced. Ultimately, the approach most likely to achieve an *in vitro* model of spermatogenesis will mimic the highly complex niche in the testis where this process occurs naturally. Different approaches have tried to simulate the testicular niche.

#### 3.1 | Embryoid bodies

The simplest approach to create this niche *in vitro* is by allowing stem cells, such as ESCs, to aggregate and form three-dimensional embryoid bodies (Geijsen et al., 2004; Toyooka et al., 2003). In the early mammalian embryo, PGCs arise from the proximal epiblast, and spermatogonia arise from PGCs (McLaren, 2000; Zhao & Garbers, 2002). Given the similarities between embryoid bodies and the early embryo (Itskovitz-Eldor et al., 2000), these artificial structures could be leveraged to generate sperm-like cells by mimicking the types of intercellular interactions that are crucial for PGC differentiation. Strategically, the self-contained nature of embryoid bodies might prove beneficial for spermatogenesis and the production of haploid male germ cells with the capacity to fertilize an egg (Geijsen et al., 2004). Indeed, embryoid bodies are able to produce steroid hormones (Aflatoonian et al., 2009) and their surface is suitable for the production of male germ cells (Kerkis et al., 2007), both of which should foster spermatogenesis. Yet, the embryoid body-derived germ cells obtained to date do not undergo normal meiosis and lack an intact synaptonemal complex (Clark et al., 2004), implying that additional exogenous factors and more somatic cell interactions are required to induce normal meiosis within the embryoid body environment.

#### 3.2 | Monolayer culturing

Unlike embryoid bodies, two-dimensional monolayer culturing is simpler than the embryoid body approach, allowing for more control of the microenvironment as well as detailed analysis of spermatogenesis

in vitro. A critical benefit of this method is the ability to control growth conditions by removal of pluripotency-inducing factors, such as LIF and FGF, or addition of differentiation- and meiosis-inducing factors, such as retinoic acid. Furthermore, monolayer culturing can be applied to stem cells that are not able to aggregate into an embryoid body. Although the production of germ cells occurs slower in monolayer culture than in embryoid bodies (Ko, Huebner, Mueller-Keuker, & Schoeler, 2009) and oocyte production is more likely than sperm (Daley, 2007), the most promising in vitro spermatogenesis results as well as live offspring production were obtained from monolayer cultures (Nayernia, Nolte, 2006; Zhou et al., 2016).

### 3.3 | Culture supplements

Step-by-step intervention and addition of growth factors are required to differentiate any cell type from stem cells. The in vitro differentiation of germ cells from stem cells, for example, requires at least two distinct phases: (i) the production of PGC-like cells followed by (ii) induction of meiosis. Most studies have reported spontaneous differentiation of ESCs into PGCs, although obtaining the proper enrichment or quantity of PGCs without using appropriate feeder cells and growth factors is not possible (Chen et al., 2007; Clark et al., 2004). Inducing factors—such as BMPs, EGF, forskolin, and GDNF—are commonly employed to increase the production efficiency of PGCs (Eguizabal et al., 2011; Richards, Fong, & Bongso, 2010; West et al., 2008, 2009).

BMPs, as ligands of the TGF superfamily, function as one of the first factors for PGC production in embryos, so many studies use these signaling factors so supplement in vitro differentiation from stem cells to PGCs (Kee et al., 2006; Toyooka et al., 2003). For instance, BMP4, BMP8a, and BMP8b, have regulatory roles in the specification of PGCs in the embryo (Ying, Liu, Marble, Lawson, & Zhao, 2000; Ying & Zhao, 2001), and play pivotal roles in the regulation and maintenance of spermatogenesis in vivo (Hu et al., 2004; Zhao, Deng, Labosky, Liaw, & Hogan, 1996; Zhao, Liaw, & Hogan, 1998). Indeed, addition of BMP 4, 7, and 8b reportedly increases the differentiation of stem cells into PGCs (Kee et al., 2006). Treatment of murine stem cells with BMP4 can also increase the expressions of PGC-specific genes such as *Dppa3* (also known *Stella*) and *Ddx4* (Nagano, 2007). The effect of BMP4 on the proliferation and differentiation of BMSCs into PGCs is dose-dependent, and, at a low concentration (<5 ng/ml BMP4), the number of *Ddx4*-positive cells is reduced (Mazaheri et al., 2011). BMPs can also differentiate ESCs into germ cells, but they are insufficient by themselves to induce meiosis and gamete maturation (Kee et al., 2006).

GDNF secreted by Sertoli cells in the testes plays a pivotal role in the self-renewal and maintenance of SSCs (Hofmann, 2008), and its expression persists throughout life (Chen et al., 2005) in response to regulation by Follicle-stimulating hormone, cytokines, and growth factors (Simon et al., 2007; Tadokoro, Yomogida, Ohta, Tohda, & Nishimune, 2002). GDNF can also induce spermatogonia differentiation or self-renewal at low and high levels, which is supported by the diminished self-renewal and proliferation of spermatogonia in *Gdnf*<sup>+/-</sup> mice and by the accumulation of undifferentiated spermatogonia in

seminiferous tubules of transgenic mice overexpressing *Gdnf* (Meng et al., 2000). Considering the above-mentioned evidence, supplementing the culture system with GDNF may have a positive influence on the maintenance and differentiation of SSCs in vitro.

EGF is a regulator of germ-cell development used by the testes of human and other mammals, making it a likely factor to enhance in vitro spermatogenesis (Nakazumi, Sasano, Maehara, & Orikasa, 1996; Niederberger, Shubhada, Kim, & Lamb, 1993; Yan, Sun, Zhang, & Koide, 2009). Previous studies reported changes in testicular EGF concentration during spermatogenesis (Bartlett, Spiteri-Grech, & Nieschlag, 1990), and that it is involved in fetal testis development as well as steroidogenesis and spermatogenesis in adults (Levine, Cupp, Miyashiro, & Skinner, 2000; Wong et al., 2000; Yan et al., 2009).

FGF2 is expressed in Sertoli cells as well as pre-meiotic and post-meiotic germ cells (Cancilla, Davies, Ford-Perriss, & Risbridger, 2000; Mayerhofer, Russell, Grothe, Rudolf, & Gratzl, 1991). The role of FGF2 in gamete production and steroids synthesis in the testis is well documented (Laslett, Mcfarlane, Hearn, & Risbridger, 1995; Van Dissel-Emiliani, De Boer-Brouwer, & De Rooij, 1996), and it may directly contribute to gametogenesis by inducing germ cell proliferation and spermatogenesis (Wahlgren, 2003).

Feeder cells alone or in combination with growth factors have been used in some in vitro gametogenesis systems (Kanatsu-Shinohara et al., 2005; Nayernia, Nolte, 2006; Park et al., 2009; West et al., 2008). In addition to providing a physical support, feeder cells likely aid in proliferation and differentiation of the cells by producing various factors. For example, culturing human ESCs on mouse embryonic fibroblast feeders increases the efficiency of PGC differentiation (West et al., 2008). Culturing of spermatogonia on mouse embryonic fibroblasts with GDNF, LIF, EGF, FGF2, and fetal calf serum in the media may improve the survival of the cells (Kanatsu-Shinohara et al., 2003). The presence of a proper feeder layer may not be required, however, since spermatogonia cultured on plates coated with an extracellular matrix were also reported to facilitate in vitro spermatogenesis by providing structural and functional support (Akbarinejad et al., 2015). Given that culturing pure SSCs has not yet been achieved (see section 2.3), the outcomes of optimization studies could be affected by non-SSC contaminants.

### 3.4 | Inducing meiosis and spermatogenesis

The crucial phase of in vitro sperm production is the induction of meiosis and production of haploid sperm-like cells that are functional and have normal epigenetic features. Removal of LIF from culture medium can direct cells towards meiosis (Bowles et al., 2006; Farini, Scaldaferrri, Iona, La Sala, & De Felici, 2005; Koshimizu, Watanabe, & Nakatsuji, 1995).

Addition of retinoic acid is frequently used as an inducer of meiosis, and has resulted in the production of haploid (Bowles et al., 2006). Retinoic acid-synthesizing enzymes are expressed in Sertoli cells (Vernet et al., 2006), suggesting that retinoic acid produced by these somatic cells act on spermatogonia via nuclear receptors to initiate meiosis in the postnatal testis (Akmal, Dufour, & Kim, 1997; Bowles and Koopman, 2007; Chen et al., 2012; Eskild, Ree, Levy,

Jahnsen, & Hansson, 1991). Based on these documented effects by retinoic acid, our group and others use this molecule to induce meiosis during in vitro spermatogenesis (Nayernia, Nolte, 2006; Nourashrafeddin, Aarabi, 2014; Nourashrafeddin, Ebrahimzadeh-Vesal, 2014). The optimal concentration of retinoic acid needed to achieve this outcome, however, depends on the type of stem cells studied. In human fetal tissue, retinoic acid increases the expression of pre-meiotic and meiotic genes (Chen et al., 2012). Retinoic acid also facilitated the production of germ cells from ESCs and BMSCs (Hua et al., 2009; Nayernia, Nolte, 2006).

Successful in vitro differentiation of sheep SSCs into haploid sperm-like cells was achieved using melatonin (Deng et al., 2016). This outcome is consistent with the crucial function of melatonin in differentiation along the spermatogenic lineage (Deng et al., 2016; Sanchez-Barcelo et al., 1991; Tsantariotou et al., 2008).

Serum contains many factors, including Follicle-stimulating hormone, testosterone, albumin, and EGF—any of which may be involved in meiotic induction (Cui et al., 2006; Hashimoto, Minami, Takakura, & Imai, 2002; Smits, Cortvrindt, & Hu, 1998, Tesarik, Guido, Mendoza, & Greco, 1998). Indeed, fetal bovine serum can enhance meiosis in PGC-like cells (West, Mumaw, Gallegos-Cardenas, Young, & Stice, 2010), possibly through its content of retinoic acid (Marques-Mari et al., 2009), although testosterone may also contribute to this phenotype given that this hormone play an important role in in vivo spermatogenesis (Walker & Cheng, 2005).

### 3.5 | Defined culture conditions

Although the use of feeder cells and serum help maintain and differentiate cells, employing such options can make the system uncontrollable and inappropriate if the purpose of in vitro spermatogenesis is to study the mechanisms and factors driving this process. One efficient approach to produce sperm-like cells from stem cells in vitro utilized a stepwise differentiation protocol (Zhou et al., 2016). First, mouse ESCs were differentiated into epiblast-like cells, and then into PGCs, based on the method described by Hayashi et al. (2011), in order to increase the efficiency of PGC production. Next, the obtained cells were mixed with mouse testicular cells at a ratio of 1:1, and cultured for 6 days in alpha minimum essential media ( $\alpha$ MEM) supplemented with knockout serum replacement (KSR), BMPs, retinoic acid, and INHBA. The mixture of testicular cell was used to partly mimic in vivo conditions, while BMPs, INHBA, and retinoic acid were used to support the proliferation and meiotic induction of the PGCs. During the final phase (Days 7–14), the cells were cultured in  $\alpha$ MEM containing KSR, testosterone, Follicle-stimulating hormone, and bovine pituitary extracts, resulting in strong expression of haploid spermatid markers by the cells (Zhou et al., 2016). Although Zhou and colleagues reported a robust protocol for fertile and fully differentiated sperm-like cells, testicular cells are still needed to establish an in vivo-like niche.

Another strategy to create the optimal niche involves the overexpression of PGC-specific genes, such as *DAZL*, *DAZ*, and *BOLL* (also known *BOULE*), in the stem cells, which resulted in the conversion of PGCs to haploid cells (Kee et al., 2009). Unfortunately,

this process is not efficient (Kee et al., 2009), and is not suited for studying spermatogenesis or for infertility treatment owing to the required genetic manipulations.

The testis produces all of the growth factors and cytokines needed for the differentiation of the SSCs into sperm. Considering the inability of studies to induce complete meiosis in PGCs, researchers have transplanted in vitro-derived PGCs into the testes as a proof-of-concept that their precursors can complete meiosis (Kanatsu-Shinohara et al., 2005; Kita et al., 2007; Toyooka et al., 2003). This transplantation approach consistently yielded mature sperm with normal morphology and function (Kanatsu-Shinohara et al., 2005).

The next-closest system to the testis itself is testicular tissue culture, which is superior to cell culture at producing sperm (Komeya et al., 2016; Sato, Katagiri, Kubota, & Ogawa, 2013; Yokonishi et al., 2014), but requires autologous tissue and poses a complex and uncontrollable model for studying spermatogenesis. The optimum temperature (37°C) and addition of Triiodothyronine and KITLG to the culture media enhances the in vitro differentiation of sperm from testis tissue culture more than twofold (Kim et al., 2015). Triiodothyronine, induces androgen receptor expression in Sertoli cells (Arambepola, Bunick, & Cooke, 1998) and stimulates DNA synthesis in germ cells by increasing IGF1 secretion from Sertoli cells (Palmero et al., 1990). This thyroid hormone may also increase germ cell proliferation and regulate the pre-meiotic phase of spermatogenesis (Jannini et al., 1993; Marchlewska et al., 2011). KITLG is also produced by Sertoli cells (Rossi et al., 1993), and exerts its physiological effects through KIT, a tyrosine kinase receptor, that exists on various testicular cells, including Leydig cells, spermatogonia, spermatocytes, and round spermatids (Albanesi et al., 1996; Dym et al., 1995; Manova, Nocka, Besmer, & Bachvarova, 1990). Previous studies identified potential roles for KITLG in PGC maintenance as well as SSC proliferation (Prabhu et al., 2006; Rossi, Sette, Dolci, & Geremia, 2000). Thus, supplementing cultures with Triiodothyronine and KITLG would be a reasonable approach to enhance in vitro spermatogenesis using testis tissue culture system (Kim et al., 2015).

Future effort should focus on developing robust culture methods that produce sperm-like cells with normal function and epigenetic features (see also reviews by Hunter et al. (2012), Huleihel, Nourashrafeddin, and Plant (2015)). Based on results obtained from organ culture and transplantation of in vitro-derived PGC-like cells into the testis, further studies on in vivo spermatogenesis and the factors utilized therein will be informative to solve the current problems underlying in vitro spermatogenesis. A stepwise, three-dimensional culturing system that includes the appropriate cell-cell interactions in the testis-like microenvironment could be the solution.

## 4 | MARKERS FOR DIFFERENTIATED MALE GERM CELLS

Identification, isolation, and enrichment of in vitro-differentiated PGCs are needed before any subsequent stage of spermatogenesis can be completed. Identification of this cell population requires knowledge of



PGC-specific gene expression to distinguish them from stem cells (reviewed by Kashir, Jones, Child, Williams, and Coward (2012)). A second issue associated with the differentiation process is determination of the spermatogenesis stage of cells differentiated cells in culture.

Expression of stage-specific genes is commonly used to characterize the progressive differentiation of germ cells. Although a variety of marker genes have been studied (Kashir et al., 2012), appropriate markers specific to germ cells—as opposed to more pluripotent stem cells—have not been found. Another issue is the overly sensitive method of detecting gene transcription, wherein polymerase chain reaction amplification can detect low-abundance transcripts even if they do not approach the levels of expression needed to drive differentiation. Therefore, meiotic hallmarks, such as the pairing of homologous chromosomes and tetrad formation as well as DNA recombination, should be considered as markers because they are germ cell-specific (Handel et al., 2014).

The multi-stage aspect of spermatogenesis requires a means to distinguish each step of differentiation during the culture period to appropriately adjust culture conditions. Various genes—*DPPA3*, *POU5F1* (also known as *OCT4*), *KIT*, and *NANOG*—have been used to detect and purify differentiated PGC-like cells from ESCs, although each of these genes is also expressed in ESCs (Aflatoonian et al., 2009; Clark et al., 2004; Geijsen et al., 2004; Nayernia, Nolte, 2006). In 2011, Hayashi and co-workers showed that *Fut4* and *Itgb3* (Integrin subunit beta 3) may be appropriate markers for mouse PGCs with spermatogenic capacity (Hayashi et al., 2011). *Fut4* encodes a cell-surface marker (SSEA-1), so it can also be used for sorting PGCs. *STRA8* is a possible pre-meiotic marker of male germ cells (Oulad-Abdelghani et al., 1996), and previous studies showed that male germ cells obtained from *Strat8*-positive cells could fertilize mouse eggs (Nayernia et al., 2004; Nayernia, Nolte, 2006), so we used this marker to sort differentiated pre-meiotic male germ cells (Nourashrafeddin, Aarabi, 2014; Nourashrafeddin, Ebrahimzadeh-Vesal, 2014). *DDX4* is another germ cell marker expressed throughout the pre-meiotic to post-meiotic phases as well as from the spermatogonia to spermatid stages in the human and mouse testis (Castrillon, Quade, Wang, Quigley, & Crum, 2000; Fujiwara et al., 1994; Yamauchi et al., 2009). Indeed, *Ddx4*-positive cells derived from mouse embryoid bodies were equivalent to in vivo-derived, post-migratory to post-meiotic germ cells (Yamauchi et al., 2009), and that transplantation of ESCs-derived *Ddx4*-positive cells into testes yields mature sperm cells (Toyooka et al., 2003). Thus, *DDX4* is a good candidate for isolating differentiated male germ cells.

Meiotic and post-meiotic markers are likely more specific and reliable for detection of germ cells than early stage markers for PGCs, but not enough of these markers have been studied from the in vitro perspective because induction of meiosis remains so challenging. Some potentially useful markers of meiotic/post-meiotic male germ cells are *CTDSP1* to 3, *DDX4*, *PRM1*, *BOLL*, *TNP2* (Transition protein 2), *DMC1* (DNA meiotic recombinase 1), *TEKT1* (Tektin 1), *ACR* (Acrosin), *TRIM36* (Tripartite motif-containing 36), *TEP1* (Telomerase-associated protein 1), and *MLH1* (MutL homolog 1) (Edelmann et al., 1996; Hua et al., 2009; Kee et al., 2009; Nayernia, Nolte, 2006; Teramura et al., 2007; West et al., 2010; Xu, Moore, & Pera, 2001.). *Tekt1*, for example,

was reported as a marker for the identification of adult mouse spermatids (Larsson et al., 2000). Expression of *PRM1* (Protamine 1) would also be reliable to identify post-meiotic male germ cells because it is used to replace histones during spermiogenesis (Nayernia, Nolte, 2006; Peschon, Behringer, Brinster, & Palmiter, 1987). Phospholipase C zeta (PLCZ) protein, which plays a crucial role in egg activation, was suggested as a marker of sperm competence (Kashir et al., 2010).

Gene expression-based reporters are commonly used for sorting and enriching for viable, differentiated cells. Such enrichment reduces the inhibitory effects of undifferentiated neighbouring cells from a mixed population, such as testicular preparations. This approach requires a reporter gene (e.g., *GFP* or *LacZ*) expressed by a germ-cell-specific gene promoter that is either genetically encoded or transfected into the stem cells to be differentiated. Thus, only the cells that express the specific gene of interest are visualized for collection. Toyooka and co-workers were the first to use a *Ddx4-GFP* gene reporter with fluorescence-activated cell sorting for the detection and isolation of differentiated PGCs from cultures (Toyooka et al., 2003). Researchers then expanded on this concept by incorporating two fusion genes to isolate differentiated cells in various stages of in vitro spermatogenesis. For example, Nayernia, Nolte (2006) simultaneously used a pre-meiotic reporter (*Strat8-GFP*) and a post-meiotic reporter (*Prm1-DsRed*) to purify differentiated cells based on their stage of maturation during the differentiation of ESCs into sperm cells. We used the same dual-reporter system and fluorescence-activated cell sorting to study the various stages of spermatogenesis (Nourashrafeddin, Aarabi, 2014; Nourashrafeddin, Ebrahimzadeh-Vesal, 2014). Although such a method allows for separating those differentiated cells with more potential to become mature sperm, the genetic manipulation prevents the translation of these harvested sperm to clinical infertility treatment. Instead, new and reliable cell-surface markers and appropriate sorting methods are needed (reviewed by Kashir et al. (2012)).

## 5 | IMPRINTING AND EPIGENETIC FEATURES OF IN VITRO-DERIVED MALE GCS

Epigenetic modifications are crucial for regulating gene expression during embryonic development (Güneş & Kulaç, 2013; Kierszenbaum, 2002). Many epigenetic marks are acquired during mammalian spermatogenesis (described in detail by Khalil and Wahlestedt (2008)), and improper modification of the epigenome are thought to underlie cases of infertility, including low sperm count and poor morphology, chromosomal aneuploidies, and DNA fragmentation (Stuppia, Franzago, Ballerini, Gatta, & Antonucci, 2015). Abnormal epigenetics are also linked to disorders like neuron-developmental syndromes (Beckwith–Wiedemann syndrome, Prader–Willi syndrome and Angelman syndrome), metabolic syndromes (transient neonatal diabetes mellitus), psychiatric behavioral disorders (schizophrenia, bipolar, and autism), and cancers (retinoblastoma) (Arnaud & Feil, 2005; Luedi, Hartemink, & Jirtle, 2005; Nicholls, 2000).

Production of in vitro-derived sperm with an abnormal epigenetic pattern has been demonstrated (Nayernia, Nolte, 2006), but how these specific abnormalities might affect embryo growth and offspring health are uncertain. Animal studies report the premature death of offspring due to the abnormal epigenetic patterns (Nayernia, Nolte, 2006), but the degree of deviation from the normal epigenetic pattern and which genes need to be affected to cause damage must be

determined. Other concerns over epigenetic patterning include what causes these abnormalities and which in vitro culturing system would foster normal patterns in sperm and in the obtained embryo. One likely source of such abnormalities is using the somatic stem cells, which carry somatic imprinting instead of germ-line stem cell imprinting. Another source could be inability to provide an in vivo-like environment within the culturing systems (Pannetier & Feil, 2007).

**TABLE 3** In vitro spermatogenesis studies that utilize “gold standard” metrics

Studies	DNA content (applied technique)	Chromosome content (applied technique)	Recombination (detected protein, applied technique)	Viable euploid offspring?
<b>ESCs</b>				
Toyooka et al. (2003)	n/a	n/a	Yes (SYCP3, immunohistochemistry)	n/a
Geijsen et al. (2004)	Yes (flow cytometry)	n/a	n/a	n/a
Clark et al. (2004)	n/a	n/a	No (SYCP3 & MLH1, immunohistochemistry)	n/a
Nayernia, Nolte, (2006)	Yes (flow cytometry)	n/a	n/a <sup>a</sup>	Yes <sup>c</sup>
Kee et al. (2006); Teramura et al. (2007)	n/a	n/a	n/a <sup>a</sup>	n/a
Kerkis et al. (2007)	n/a	Yes (FISH)	n/a <sup>a</sup>	n/a
West et al. (2008)	n/a	n/a	Yes (SYCP3 & MLH1, immunohistochemistry)	n/a
Silva et al. (2009)	n/a	n/a	Yes <sup>b</sup> (SYCP3, immunoblot)	n/a
Aflatoonian et al. (2009)	Yes (flow cytometry)	n/a	n/a	n/a
Yamauchi et al. (2009)	n/a	n/a	n/a	n/a
West et al. (2010)	Yes (flow cytometry)	n/a	Yes (SYCP3 & MLH1, immunohistochemistry)	n/a
Easley et al. (2012)	Yes (flow cytometry)	Yes (FISH)	Yes (SYCP3, immunohistochemistry)	n/a
Zhou et al. (2016)	Yes (flow cytometry)	Yes (Giemsa banding)	Yes (SYCP1, SYCP3, SPO11, & RAD51, immunohistochemistry)	Yes
<b>Non-ESCs</b>				
Drusenheimer et al. (2007); Huang et al. (2010); Shirazi et al. (2012); Cai et al. (2013); Ghasemzadeh-Hasankolaei et al. (2014); Kashani et al. (2014); Sasaki et al. (2015); Yan et al. (2015)	n/a	n/a	n/a	n/a
Nayernia et al. (2004)	Yes (DNA imaging, Feulgen reaction)	n/a	n/a <sup>a</sup>	n/a
Nayernia, Lee, 2006	n/a	n/a	No (SYCP3, immunohistochemistry)	n/a
Hua et al. (2009); Li, Pan, et al. (2014)	n/a	n/a	n/a <sup>a</sup>	n/a
Hayashi et al. (2011)	n/a	n/a	n/a	Yes
Eguizabal et al. (2011)	Yes (flow cytometry)	Yes (FISH)	Yes (SYCP3, immunohistochemistry)	n/a
Panula et al. (2011)	Yes (flow cytometry)	Yes (FISH)	Yes (SYCP3, immunohistochemistry)	n/a
Li, Wang, et al. (2014b)	n/a	n/a	Yes (SYCP3, immunohistochemistry)	n/a

FISH, fluorescence in situ hybridization; MLH1, mutL homolog 1; RAD51, RAD51 recombinase; SPO11, Initiator of meiotic double stranded breaks; SYCP3, Synaptonemal complex protein-3.

Yes, the standard was assessed and confirmed; No, the standard was assessed, but was not confirmed; n/a, not assessed.

<sup>a</sup>The mRNA expressions of SYCP-1 or -3 were detected, but recombination itself was no confirmed.

<sup>b</sup>Existence of SYCP3 protein confirmed using immunoblot, but nuclear and chromosomal localization of the protein was unclear.

<sup>c</sup>The obtained offspring were viable but died prematurely.

The best solution would involve developing an *in vivo* mimetic of the testes—possibly including blood vessels, Sertoli cells, myoid cells, Leydig cells, and macrophages as well as the provision for paracrine signals for the regulation of spermatogenesis—but this is technically difficult (Cheung & Rennert, 2011; Oatley & Brinster, 2008). Nevertheless, Zhou and co-workers described the first stepwise differentiation method for producing sperm-like cells from mouse ESCs with hallmarks of erasure of imprints, synapsis, and recombination (Zhou et al., 2016). Analysis of the epigenetic profile of these *in vitro*-derived PGCs showed that they were similar to *in vivo*-derived PGCs (Seki et al., 2005; Zhou et al., 2016), so complete meiotic induction and appropriate epigenetic modification can be achieved during *in vitro* spermatogenesis (Zhou et al., 2016).

Inclusion of mix cells from the testis in an *in vitro* culture system is compatible with the desire to mimic a process as complex as spermatogenesis (Cheung and Rennert, 2011; Lo; Zhou et al., 2016). Melatonin was already mentioned as an epigenetic regulatory factor that has important roles in modulating genome methylation status during spermatogenesis (Deng et al., 2016), but other potential culture media supplements that might govern the epigenetic remodeling of *in vitro*-derived sperm are not known. Clarification of the factors that possibly affect epigenetic—including stem cell- and Sertoli cells-secreted factors, steroid hormones, and growth factors (Cheung and Rennert, 2011; Laurentino, Borgmann, & Gromoll, 2016; Nugent et al., 2015)—offer new options to help manipulate culture conditions in favor of epigenetically normal sperm development.

## 6 | PROSPECTS AND CONCLUSION

Several obstacles still need to be overcome before *in vitro*-derived sperm can be used for infertility treatment. First, personalized sperm must be reliably produced using autologous cells, such as mesenchymal stem cells or paternal germ line stem cells, for a patient to obtain biological offspring. Second, the epigenetic state of *in vitro*-derived sperm should be assessed prior to use to ensure the production of healthy offspring. Most studies have used cells that retained part of their original somatic imprinting, whereas germ line stem cells need to be used as the primary source to achieve the desired results. Third, a suitable microenvironment is essential to produce fully functional sperm cells; the application of feeder cells, embryoid body intermediates, addition of growth factors, and/or the inclusion of testis mix cells to the culture are all viable means to achieve complete gamete differentiation.

In conclusion, achieving proper *in vitro* spermatogenesis requires the demonstrated expression of germ-cell-specific markers and completion of meiosis, which are suggested as “gold standard” metrics of differentiation (Handel et al., 2014). The meiotic metrics can be measured by flow cytometry or quantitative cytology for the correct nuclear DNA content during pre-meiotic stage (2C/2N), after meiotic S phase (4C/2N), end of the first (2C/1N), and the second (1C/1N) meiotic division—with a chromosome count, especially for X and Y chromosomes, to confirm the 1N count (Handel et al., 2014). Yet, some

studies limit their analysis of DNA and chromosome content to the existence of haploid cells (Table 3). A second assessment of meiosis involves detection of DNA recombination, which could be accomplished by assessing proteins involved in homologous synapsis and recombination, including synaptonemal complex protein and mutL homolog 1 (Handel et al., 2014). Of the handful of studies that evaluated these meiotic recombination markers, most have confirmed their mRNA expression, which is simply a measure of the gene activity, but could not confirm the protein or occurrence of any recombination (Table 3). However, the ultimate standard by which *in vitro* spermatogenesis needs to be measured is the production of healthy and euploid offspring, which has been achieved by two studies (Hayashi et al., 2011; Zhou et al., 2016). Nevertheless, future *in vitro* spermatogenesis studies should pay attention to the reviewed criteria and checklists that include “gold standard” metrics to ensure that their method is robust. Only then might *in vitro* spermatogenesis be a viable option for the clinical treatment of male infertility.

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