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# Recent Developments in In Vitro Spermatogenesis and Future Directions

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**Abstract:** Recent developments in stem cell technologies have made significant advancements in the field of in vitro gametogenesis. In vitro gametogenesis (IVG) is a promising technology where functional gametes (sperm or egg cells) can be generated from stem cells. Scientists have made continuous advancements in the field and successfully derived fully functional sperm from stem cells in mice. Two recent papers generated excitement in IVG by generating bi-maternal and bi-paternal mice from embryonic stem cells (ESCs) and pluripotent stem cells (PSCs). IVG is a promising technology with potential applications that include infertility treatment, fertility preservation, same-sex reproduction, bypassing oocyte depletion in women with advanced age, conservation biology, genetic disorder prevention, and research into human germ cell development. In vitro spermatogenesis (IVS) is the attempt to recreate the process of spermatogenesis in a culture system. Spermatogenesis is essential for male fertility and reproductive health, but it can be impaired by various factors such as genetic defects, environmental toxicants, infections, aging, or medical therapies. Spermatogenesis is a complex and highly regulated process involving multiple cell proliferation, differentiation, and maturation stages. The main challenges of IVS are to provide a suitable microenvironment that mimics the testis in vivo, to support the survival and development of all the cell types involved in spermatogenesis, and to achieve complete and functional spermatogenesis. Therefore, there is a great interest in developing methods to study spermatogenesis in vitro, both for basic research and clinical applications. This review covers recent developments in in vitro spermatogenesis in the past two years. Advances in tissue engineering and regenerative medicine have introduced techniques like ex vivo tissue culture and technologies such as bioreactors, microfluidic systems, and organoids. Bioreactors and microfluidic systems replicate physiological conditions for tissue and cell cultivation, while organoids model organ functionality. Meanwhile, scaffolds, made from various materials, provide essential structural support, guiding the growth and organization of cells into functional tissues.

**Keywords:** in vitro spermatogenesis; spermatogonial stem cells; tissue engineering; regenerative medicine; bioreactors; microfluidic systems; organoids; ex vivo tissue culture; scaffolds



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## 1. Introduction

Recent developments in stem cell technologies, especially induced pluripotent stem cells (iPSCs) derived from patient-specific cell sources, have brought many promises and potential applications in many research fields. There are many applications of iPSCs, such as disease modeling, drug discovery and development, regenerative medicine, personalized medicine, gene therapy, and developmental biology, to name a few. Although iPSCs hold immense promise, some obstacles and concerns must be addressed, such as improving reprogramming efficiencies, epigenetic carryovers from the cell source, and ethical and regulatory matters. However, the field of stem cell research keeps progressing. One of the most promising fields is regenerative medicine, where cells, tissues, or organs can be generated by reprogramming cells into iPSCs from patient-specific (i.e., allogeneic) sources.

In vitro gametogenesis (IVG) is a promising technology where functional gametes (sperm or egg cells) can be generated from pluripotent stem cells (PSCs). Although it is still in development, potential applications include infertility treatment, fertility preservation, same-sex reproduction, bypassing oocyte depletion in women with advanced age, genetic disorder prevention, and research into human germ cell development. Efforts to derive functional gametes in vitro have been made since the 1920s [1], with early efforts mainly focused on ex vivo organ cultures to produce mature gametes. Success in deriving fully functional sperm from stem cells in mice [2] and haploid spermatid-like cells from human PSCs [3] and primates have been reported [4]. The successful derivation of testicular sperm was reported in rodents, whereby rhesus spermatid-like haploid cells derived from rhesus PSCs successfully fertilized oocytes and triggered blastocyst development [4]. The protocol has been independently replicated in other studies [5–9], demonstrating the potency and efficacy of in vitro spermatogenesis in more relevant systems to humans. Two recent papers on mice generated excitement in the field of IVG by generating bi-maternal and bi-paternal mice from ESCs and PSCs [10,11].

In vitro spermatogenesis (IVS) is the attempt to recreate the process of spermatogenesis in a culture system. The main challenges of IVS are to provide a suitable microenvironment that mimics the testis in vivo, to support the survival and development of all the cell types involved in spermatogenesis (germ cells, Sertoli cells, Leydig cells, and peritubular myoid cells), and to achieve complete and functional spermatogenesis. Spermatogenesis produces male gametes (spermatozoa) from spermatogonial stem cells (SSCs) in the testis of mammals. It is a complex and highly regulated process involving multiple cell division, differentiation, and maturation stages. Spermatogenesis is essential for male fertility and reproductive health, but it can be impaired by various factors such as genetic defects, environmental toxicants, infections, aging, or medical therapies. Therefore, there is a great interest in developing methods to study spermatogenesis in vitro, both for basic research and clinical applications. Different methods have been utilized for IVS, such as cell cultures, ex vivo organ cultures, bioreactors, microfluidics, organoids, and scaffold methods. In vitro spermatogenesis is a crucial research area due to its potential in treating male infertility by artificially inducing sperm maturation outside the body. It can aid young cancer patients who risk sterility from treatments by using previously harvested spermatogonial stem cells to produce sperm later in life. The study of this process also offers valuable insights into developmental biology and genetic disorders. Additionally, it provides a platform for drug and toxicology testing without live subjects. Lastly, there is potential for treating genetic diseases by modifying genes during the process before conception.

Two recent publications have covered extensively the topic of in vitro spermatogenesis (IVS) in mice and human [12,13], and two publications on IVG [14,15]. Therefore, this review will only cover the recent developments in in vitro spermatogenesis in the past two years and focus on nonmurine studies.

## 2. Male Germ Cell Development

In mammals, male germ cell development begins during embryogenesis: conserved germ cell developmental markers are presented in Figure 1. Primordial germ cells (PGCs) [16] form from the epiblast [17]. Bone morphogenetic protein 4 (BMP4) released from extraembryonic ectodermal cells [18] induces neighboring extraembryonic mesodermal cells to begin to express PGC-specific markers such as *Blimp1/Prdm1* [19], *Tfap2C/AP2γ* [20], *Prdm14* [21], and *Stella* [22]. A cluster of PGCs develop on the amniotic membrane and start to migrate to the genital ridge [23], where they colonize the embryonic gonads [24]. During embryo development, PGCs undergo extensive epigenetic reprogramming, such as resetting genomic imprinting, DNA methylation, chromatin remodeling, and global re-methylation later in the embryo development [25–27]. In developing seminiferous tubules, Sertoli cells and the extracellular testicular environment signal sex specification to occur by expressing *sex-determining region y* (*SRY*) and *SRY-box 9* (*SOX9*) mediated by fibroblast growth factor 9 (FGF9) and retinoic acid (RA) and differentiate into prospermatogonia while slowly

losing mitotic activity and establishing the paternal epigenome [16,28,29]. The mitotic activity ceases about 2.5 and 6 months after birth, and prospermatogonia differentiate into spermatogonial stem cells (SSCs). The male gonad arrests development until puberty. The spermatogenesis activity restarts at puberty when juxtacrine and paracrine signaling cause cells to rapidly divide/develop into haploid gametes (Figure 2). Every 16 days, a new batch of SSCs enters the differentiation process, which takes about 64 to 74 days in humans [30]. The first stage of spermatogenesis is slow self-renewing spermatogonia (stages 0–1) [31,32]. The second stage is actively differentiating or self-renewing spermatogonia (stages 2–3). After puberty, spermatogonia cells enter the committed differentiation process (stage 4), followed by spermatocyte and spermatid states [33]. Spermatocytes cross the blood–testis barrier (BTB) and enter the lumen, where they are immunologically separate [34–36]. Spermatocytes undergo meiosis to produce two genetically unique secondary spermatocytes, and after undergoing meiosis II, secondary spermatocytes create four haploid spermatids. Spermatids undergo spermiogenesis, where cytoplasmic changes produce mature spermatozoa via spermiogenesis. During this process, an acrosome is formed, the nucleus is condensed, and a histone-to-protamine transition occurs [37–41]. The flagellum arises from the centriole, and residual cytoplasm is absorbed by Sertoli cells [41,42]. The fully matured spermatozoa travel through the epididymis, where they undergo further maturation via spermiation and are stored until ejaculation.

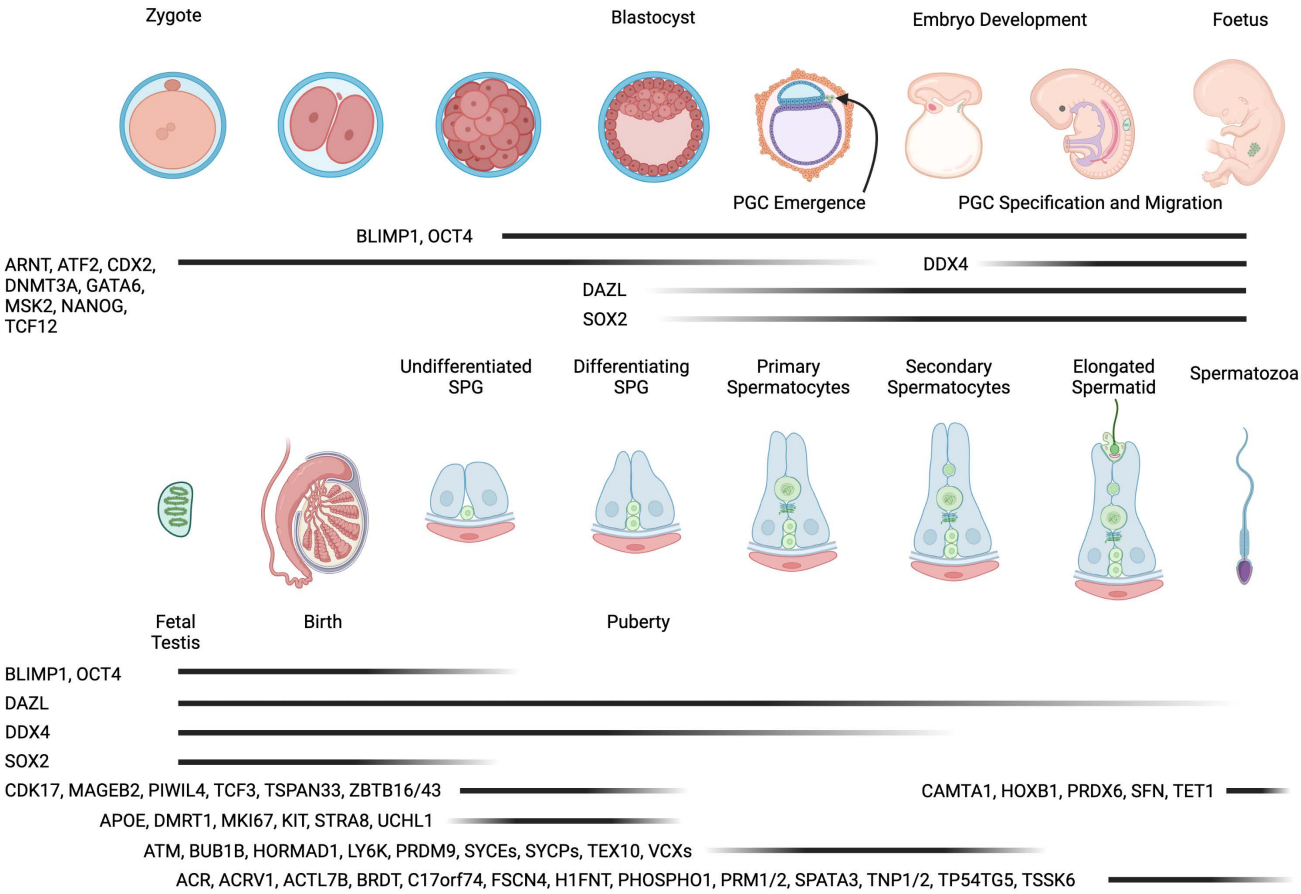


**Figure 1.** Conserved expression markers during male germ cell development from zygote to mature spermatozoa in vivo. After fertilization, cells undergo a differentiation process where primordial germ cells (PGCs) develop in the embryo and migrate toward the genital ridge, where early PGCs arrest and differentiate into gonocytes. Gonocytes mature during development and eventually develop into spermatogonial stem cells. Spermatogenesis resumes at puberty. Complex orchestrated stage-specific gene expressions regulate the development of gonocytes, and markers and relative expression levels are depicted [43–49].

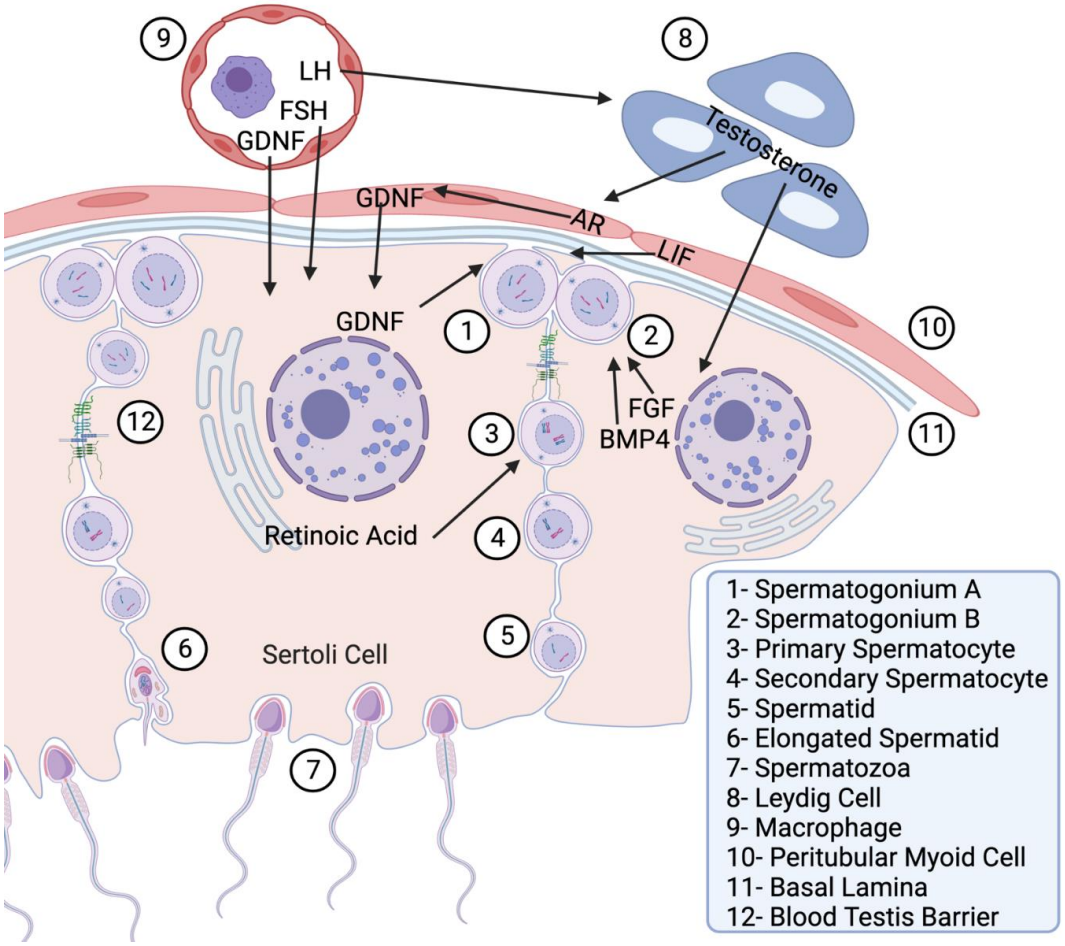


**Figure 2.** Spermatogenesis is orchestrated by juxtacrine signaling from Leydig cells (8) and myoid cells (10), and paracrine signaling causes cells to divide/develop into haploid gametes. The first stage of spermatogenesis is slow self-renewing spermatogonia (1). The second stage is actively differentiating or self-renewing spermatogonia (2). Spermatogonia cells enter the committed differentiation process (3), where meiosis starts developing the primary spermatocytes (3), secondary spermatocytes (4), and spermatids (5). Spermatocytes cross the blood–testis barrier (BTB) (12) and enter the lumen. Spermatids undergo spermiogenesis, where cytoplasmic changes produce elongated spermatids (6) and eventually mature spermatozoa (7) [31–33,37–41].

Spermatogenesis is an evolutionarily conserved process that is similar in different vertebrate species. While the general outline of male germ cell development is conserved, several differences in spermatogenesis in vertebrate species exist, such as developmental, epigenetic, genetic, transcriptional, physio-temporal, signaling, and metabolic signatures [45,50–56].

Rodents have the most transit-amplifying division, with 12–13 divisions between the spermatogonial stem cell and the mature sperm, while nonhuman primates have 8–9 divisions, and humans have only 5–6 divisions [51]. The transitional 2 ($T_2$) prospermatogonia state, an intermediate precursor state to SSC, is unique to rodent species [57]. Another difference between rodent and primate spermatogenesis is the timing of meiosis [45,50–52]. In rodents, meiosis occurs continuously throughout adulthood, whereas in primates, meiosis is restricted to discrete periods during early postnatal development and adulthood [51–53]. In humans, for example, meiosis starts around puberty and continues throughout life, with the production of sperm cells taking about 64–72 days [52,54,55]. Also, the duration of spermatogenesis differs among rodents, primates, and humans, with 35 days, 45 days, and 74 days, respectively [51]. The hormonal regulation of spermatogenesis also differs in rodents, nonhuman primates, and humans. Rodents rely more on testosterone to regulate

spermatogenesis [52,53], while nonhuman primates and humans rely on testosterone and FSH to regulate spermatogenesis [58].

The process of spermiation, which is the release of mature sperm cells from the seminiferous tubules, also differs between rodents and primates. In rodents, spermiation is continuous, whereas in primates, it is episodic and coordinated with hormonal changes [56]. Additionally, the morphology of sperm cells can vary between species. For example, in rodents, geometric morphometrics of the head shape are slanted and sickle-shaped with long flagella, whereas, in primates, the heads are typically oval-shaped with a shorter flagellum [59].

Finally, there are some differences in the molecular regulation of spermatogenesis between rodents and primates. For example, some genes involved in spermatogenesis are expressed differently between the two groups, and specific signaling pathways that are critical for germ cell development may also differ [45,56]. *GFRA1*, *ITGA6*, *ZBTB16*, *GPR125*, *SALL4*, and *THY1* are expressed in undifferentiated spermatogonia in mice, monkeys, and humans, but, at odds with the mouse, *UTF1*, *MAGEA4*, and *FGFR3* are only expressed in type A spermatogonia in humans [60–62]. Recent single-cell studies have identified many conserved and divergent gene expressions among different cell types within testis [43,45,63]. For example, when gene expression profiles from human, macaque, and mouse testes were analyzed together, six spermatogonia (SPG) states, four meiosis states, ten round spermatids states, and five to six elongated spermatids states were identified [45]. SPG6 state cells represented type B of preleptotene spermatocytes in all three species and expressed *FMR1NB*, *ZCWPW1*, *DPEP3*, *IQBP1*, and *CALR*, but *BEND2*, *ZRANB2*, *PAGE4*, *ZNFX1*, *HLTF*, and *ZBED5* were only expressed in primates [45]. Moreover, their study showed that Sertoli and Leydig cells have the least interaction with SPG cell populations in mice [45], which highlights conserved pathways, but the timing of activation and the origin or target of the signal might have diverged during the evolutionary process. Some epigenetic regulations also differ among rodents, nonhuman primates, and humans. The percentage of histone switching for protamines during spermatogenesis varies among rodents, nonhuman primates, and humans. In rodents, about 98–99% of the histones are replaced by protamines, while in nonhuman primates and humans, about 80–96% are replaced by protamines [64]. Overall, methylation profiles are also different between humans and mice. About 80% of rodent and 75% of human genes are methylated, and the number of imprinted genes also considerably differs in rodents and humans (~125–252 and ~50–90) [65].

Overall, while the general process of spermatogenesis is conserved between rodents and primates, there are several differences in the timing, morphology, and molecular regulation between these groups. Understanding these differences is essential for developing spermatogenesis models and advancing research in male infertility and contraception.

## 3. Stem Cells and Regenerative Medicine

In vitro PSC differentiation into spermatogenic cells has become a promising approach for studying spermatogenesis and treating male infertility. At the same time, ex vivo organ cultures and tissue culture methods are still the most common approaches to derive/expand SSCs and sperm/spermatids. Multiple in vitro differentiation methods have been developed, each with advantages and disadvantages. This review will summarize the recently published protocols on the differentiation methods of in vitro stem cells (spermatogonial stem cells and pluripotent stem cells) into spermatogenic cells in the past two years, mainly focused on nonrodent studies.

## 4. Ex Vivo Organ/Tissue Culture

Ex vivo culture involves maintaining and studying cells or tissues outside their original organism in a controlled environment. Ex vivo tissue culture has been used extensively with various successes since the beginning of IVS. Researchers used frozen goat testis tissue to compare the efficacy of the hanging drop culture method and organ culture methods, where they were able to observe the development of elongated spermatozoa in ex vivo organ

culture on an agarose gel block with high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), AlbuXL, L-glutamine, nonessential amino acids (NEAA), and penicillin-streptomycin in 5% $CO_2$ in air [66]. The same group used a scaffold (agarose matrix) to culture prepubertal farming goat testis in α-MEM supplemented with nucleosides, 10% FBS, AlbuXL, and antibiotics [67]. After 60 days, researchers observed sperm-like cells with tails in the lumen of the tissue [67].

Isolated spermatogonial stem cells (SSCs) have been used to generate advanced spermatogenic cells. SSCs comprise a small cell population in testis (0.03% mice, 4–15% in monkeys, and 22% in humans) [51,68]. Human azoospermia patients' testicular tissues were cultured in a medium consisting of human glial-cell-line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and laminin with either 10% FBS or platelet-rich plasma (PRP) [69]. At the end of day 14, PRP-supplemented cells showed proliferation and differentiation of SSCs expressing PLZF, OCT4, and CKIT [69].

One of the applications of in vitro spermatogenesis is treating male factor infertility. A group from Denmark showed that the organotypic culture of human fetal testis from infant boys with cryptorchidism could grow and generate boule-like RNA-binding protein (BOLL)-positive spermatocytes [70]. The thawed testis tissue was placed on an agarose gel stand (0.35% *w*/*v*) with an air–liquid interface and cultured for 60 days with a medium composed of MEM-α, 2% human umbilical cord plasma, 10% KnockOut SR XenoFree CTS, GDNF (20 ng/mL), bFGF (20 ng/mL), EGF (20 ng/mL), SCF (20 ng/mL), BMP4 (20 ng/mL), Activin A (100 ng/mL), FSH (10 IU/L), testosterone (10 µM), and 0.5% penicillin-streptomycin [70]. More advanced spermatocytes, $BOLL^+$ cells, $SOX9^+$ Sertoli cells, and alpha-smooth muscle actin (ACTA)-positive peritubular myoid cells were observed in organotypic culture supplemented with retinoic acid (RA) [70].

Although genotypically and phenotypically closer to humans, nonhuman primate (NHP) models pose unique challenges. In recent studies, both rodent and human SSCs have been isolated using highly specific antibodies against SSC-specific surface antigens such as CD90.2 [71], ITGA6 [72], or GPR125 [73]. However, such methods sometimes work less effectively in NHPs. Utilizing the STA-PUT velocity sedimentation method, Percoll gradient, and differential plating methods, researchers were able to isolate highly pure (over 80%) spermatogonia ($UCHL1^+$) from rhesus tests [74]. Isolated cells were cultured on monkey fibroblast cells with defined serum-free mouse SSC culture medium supplemented [71] with 20 ng/mL GDNF, 1 ng/mL bFGF, 100 ng/mL GFRα1, 20 ng/mL EGF, 0.05 ng/µL BMP7, and $10^4$ U/mL LIF [74]. However, the isolated spermatogonia cells did not propagate but maintained testicular germ cells (*Dazl* and *Zbtb16*) with stem cell (*Fgfr3* and *Utf1*) characteristics for up to 3 weeks [74].

## 5. Bioreactor

A bioreactor is a device that supports the growth of cells and tissues in a biologically active environment by maintaining optimal temperature, pH, oxygen, and nutrient conditional for the cell growth while removing waste. The topic of utilizing microfluidic systems, scaffolds, and bioreactors used in in vitro spermatogenesis has been reviewed elsewhere [75]. In 2022, a group generated human 3D organoids in mini-spin bioreactors from hPSCs [76]. The human embryonic stem cell (hESC) line H1 was differentiated in large clumps in a maintenance medium supplemented with 8 µM CHIR 99,021 and 5 µM ROCK inhibitor Y-27632 and transferred to low-adhesion cell culture dishes for embryo body (EB) formation [76]. The medium was changed to a chemically defined medium (CDM) composed of DMEM/F12, 64 mg/L L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AA2P), 1 X insulin-transferrin-selenium (ITS), and 100 U/100 µg pen/strep [76]. The medium was supplemented with 8 µM CHIR on the first day, and 10 ng/mL of FGF9 was supplemented on day 3 [76]. On day 6, EBs were transferred into a mini-spin bioreactor, and 50 ng/mL of BMP4 and 50 ng/mL of SHH were added on day 10–16 [76]. On days 20–24 and 30–38, 10 IU/mL of hCG and 25 mIU/mL PMSG were added [76]. They ob-

served the development of mesonephros-like organoids in D10-16, nontubular progenitors in D16-22, regression of mesonephric tubules and ingression of progenitor cells in D22-30, and cord-like structures and distinct cell clusters in D30-40 [76]. The researchers reported the expression of PAX2 and WT1 in D16 EBs and DMRT1 in D26 and D35 EBs [76]. The gonadal ridge markers, GATA4 and WT1, were expressed in D16-20 EBs, and SOX9 was expressed in D20 [76]. They also observed AMH and Inhibin B expression in D26-40 EBs and the development of steroidogenic cells ($CYP11A1^+$, $StaR^+$, and $3\beta\text{-}HSD^+$) [76]. Their model showed vasculature development and coelomic vessel formation in EBs ($VE\text{-}Cad^+$ and $CD31^+$) [76]. However, the expression of early-stage primitive gametogenic marker (OCT4) and post-migratory germ cells (DAZL) were not observed in the EBs [76]. The authors of the study speculated that EBs generated in bioreactors might be able to provide microenvironments necessary for further development of in vitro-generated PGCLCs [76].

## 6. Microfluidic System

A microfluidic system is a system where multiple channels with a micrometer diameter control a culture condition by manipulating fluids at a microscale. The microfluidic system can closely mimic the in vivo condition by balancing hormones, growth factors, temperature, pH, pressure, and other requirements necessary for spermatogenesis. The most significant advantage is the ability to control the fluid flow rate within the system, which can help stimulate fluid movement, provide nutrients, and remove waste products. A recent study compared the efficacy of immature porcine testicular tissue culture via four culture methods: a static system with a polytetrafluoroethylene membrane, agarose gel, agarose gel with a polydimethylsiloxane chamber, and a microfluidic system [77]. The study showed that agarose gel with a polydimethylsiloxane chamber moderately improved the number of meiotic and post-meiotic germ cells [77]. The culture medium was based on a previous publication [78], and is only composed of DMEM/F-12, 10% Knockout Serum Replacement (KSR), FSH, and antibiotics [77].

## 7. Organoid

An organoid is a miniaturized and simplified version of an organ with micro-anatomy produced in vitro in a three-dimensional culture. In in vitro differentiation studies, organoids serve as a bridge between traditional two-dimensional cell cultures and whole-organ experiments. They can be derived from stem cells or dissociated tissues and can self-organize into structures that closely resemble the functionality and architecture of organs. In this context, organoids are utilized to study organ development, disease modeling, drug testing, and potentially regenerative medicine applications, offering a more accurate representation of in vivo conditions than traditional cell culture methods.

Spermatogonial stem cell isolation and later differentiation into advanced spermatogenic cells have significantly progressed. Isolated cell cultures involve the enzymatic dissociation of testis tissue into single cells or cell clusters cultured in various media and conditions. This method allows the effects of specific factors (such as hormones, growth factors, or feeder cells) on the proliferation and differentiation of germ cells or other testicular cells to be investigated. However, isolated cell cultures have several limitations, such as the loss of cell–cell interactions, tissue architecture, and physiological signals essential for spermatogenesis. Moreover, in most species, isolated cell cultures have yet to achieve complete spermatogenesis beyond certain stages (such as meiosis or spermiogenesis).

Fragment cultures involve the culture of small pieces of testis tissue that retain some of their original structure and cell composition. This method preserves some critical cell–cell interactions and tissue organization for spermatogenesis. However, fragment cultures also have drawbacks, such as difficulty controlling the oxygen and nutrient supply, accumulating waste products, and the heterogeneity of tissue fragments. Fragment cultures have supported partial or complete spermatogenesis in some species (such as mice or rats) but not in others (such as humans or monkeys).

Recent developments in testicular organoids have shown promising results in generating fully mature spermatids and sperm from pluripotent stem cells, at least in mice and pigs [2,79–84].

These involve culturing stem cells or dissociated testis tissue in a nonadherent environment that induces them to form 3D aggregates known as embryoid bodies. Three-dimensional cultures involve generating organ-like structures from testicular cells that self-organize into a seminiferous epithelium and an interstitial compartment separated by a basement membrane. These structures are called testicular organoids (TOs), aiming to mimic the testicular microenvironment in vitro. TOs can be formed via multiple methods, such as microwell aggregation, bioprinting, or microfluidics. TOs have several advantages over other IVS methods, such as maintaining the cell–cell interactions, tissue architecture, and physiological signals essential for spermatogenesis. Under specific culture conditions, TOs differentiate into germ cells, including spermatogenic cells. The efficiency of germ cell differentiation can be low, and the resulting cells often need proper maturation. TOs have also shown promising results in supporting partial or complete spermatogenesis in some species (such as pigs or mice). Still, more work is needed to optimize their generation and function.

While the generation of fully mature sperm cells from pluripotent stem cells and testicular organoids in humans is still a relatively new field of research, these advancements hold significant promise for developing new treatments for male infertility and other reproductive disorders. More research is needed to optimize the culture conditions and improve the efficiency and reproducibility of these techniques. Additionally, ethical considerations regarding using human fetal tissue and genetic manipulation must be carefully addressed. A recent review paper extensively covered the topic of testicular organoids [85,86], so this review will only focus on the recent developments in testicular organoids in the past two years.

In the past two years, only six studies have utilized 3D organoids in nonrodent studies, including one previously discussed in the bioreactor section. One study used bovine testes to generate testicular organoids in ultra-low attachment plates [87]. The cells formed organoids in 3 days when cultured with DMEM/F12, 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL amphotericin B [87]. From day three, the culture medium was supplemented with 100 ng/mL of BMP4 and 10 ng/mL of FGF2, and from day 7, with 15 ng/mL of GDNF [87]. Although no specific maturation of spermatogenic cells were observed, the study showed that 3D organoids could be generated from bovine testicular tissue. One recent study characterized pre-migratory primordial germ cells, derived iPSCs, and differentiated iPSCs into expandable cultures in 2D. It differentiated the early prospermatogonia-like state in an organoid culture system (xrTestis) in a common marmoset [88]. Reprogrammed peripheral blood mononuclear cells (PBMCs) and cjiPSCs were first induced into primordial germ cell-like cells (PGCLCs) on Mitomycin C-treated MEFs in the presence of DK20F20 medium (6–7 days). The cells were passed with Accutase into a low-cell-binding V-bottom 96-well plate at 3500 cjiPSCs per well in GK15 (GMEM, 15% KSR, 0.1 mM NEAA, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 25 U/mL penicillin-streptomycin) or aRB27 (Advanced RPMI 1640, 2xB27, 0.1 mM NEAA, 2 mM L-glutamine, and 25 U/mL penicillin-streptomycin) supplemented with 200 ng/mL of BMP4, 1000 U/mL of human LIF, 200 ng/mL of SCF, 100 ng/mL of EGF, and 10 mM o ROCK inhibitor (Y-27632) [88]. The aggregates were cultured for eight days, and the aggregates were used to establish extendable culture in 2D by platting on Mitomycin C (MMC)-treated STO cells in DMEM supplemented with 15% KSR, 2.5% FBS, 0.1 mM NEAA, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 25 U/mL penicillin-streptomycin, with supplements (10 µM forskolin, 200 ng/mL SCF, and 20 ng/mL bFGF) [88]. At day 10, $PDPN^+$ and $ITGA6^+$ cells were sorted with FACS, and cells were plated on a 24-well plate at a $1.0 \times 10^4$ cells/well density in 0.5 mL of medium supplemented with 10 µM Y27632 and 0.5 mL of medium without Y27632 on the following day [88]. Those cells were expandable for at least 30 days and expressed

early primordial germ cell markers without expression of late germ cell markers [88]. The authors of the study further matured the cells by generating xenogeneic reconstituted testes (xrTestes) by aggregating FACS-sorted $PDPN^+$ 5000 cjPGCLCs with 60,000 MACS-enriched $SSEA1^+$ mouse fetal testicular somatic cells harvested from E12.5 mouse embryos in a Lipidure-coated U-bottom 96-well plate in α-MEM supplemented with 10% KSR, 55 μM 2-mercaptoethanol, 100 U/mL penicillin/streptomycin, and 10 μM Y-27632 [88]. Aggregates were allowed to form organoids for two days and transferred onto Transwell-COL membrane inserts [88]. The cells were maintained in an air–liquid interface (ALI) culture for up to 30 days [88]. The authors of the study reported reconstitution of the testicular cord at day 15 surrounded by $NR2F2^+$ and the notable number of $TFAP2C^+$, $POU5F1^+$, and $NANOG^+$ cells [88]. They also reported $DAZL^+$, $DDX4^+$, $SOX17^+$, $POUF5F1^+$, $TFAP2C^+$, and $SOX2^-$ cells, suggesting progression into early prospermatogonia [88]. Their transcriptome analysis also showed upregulation of key germ cell genes (*DND1*, *NANOS3*, *PRDM1*, *SOX17*, and *TFAP2C*) with endodermal and mesodermal development genes (*EOMES*, *HAND1*, *MESP1*, *MIXL1*, *NODAL*, and *SNAL1*) [88].

Three studies have utilized the organoid approach in humans in the past two years, including one previously discussed in the bioreactor section. One group generated self-organizing compartmentalized human gonadal organoids from dissociated first-trimester embryonic male gonad tissues with the three-layer gradient system (LGS) 3D organoid generation technique [89]. This technique utilized a multilayer approach, where dissociated testicular cells (44,000,000 cells per mL of Matrigel) were embedded in Matrigel between cell-free Matrigel layers (5 μL cell-free Matrigel layer, 3 μL Matrigel–cell suspension, and 8 μL cell-free Matrigel layer) [89]. The hanging cell inserts were cultured in NutriStem, 10% KOSR, and 1% Pen/Strep for up to 14 days [89]. The resulting organoids showed testis-specific architecture and testis-specific somatic cell ($COL4^+$, $FN^+$, $SOX9^+$, $AMH^+$, $ACTA2^+$, $StAR^+$, $CYP17A1^+$, $AR^+$, and $HSD17B3^+$) differentiation [89]. Also, they were able to observe a small number of DAZL- and POU5F1-positive cells at day 7 and day 14 [89]. However, they found no DDX4-positive cells in testicular organoids [89]. In another study, a group utilized three-dimensional bioprinters, AGC-10 Bioink, and an RX1 bioprinter with a CENTRA coaxial microfluidic printhead from Aspect Biosystems to print 3D testicular organoids with in vitro expanded cells expanded from dissociated human testicular tissue from a single donor with nonobstructive azoospermia (NOA) [90]. After 12 days of culturing organoids in vitro with SSC expansion medium without GDNF (StemPro-34 SFM, 1× Insulin-Transferrin-Selenium Liquid Media Supplement, 30 μg/mL sodium pyruvate, 1 μg/mL sodium DL-lactic acid solution, 5 mg/mL BSA, 1% FBS, 1X Gluta-Max, $5 \times 10^{-5}$ M 2-mercaptoethanol, 1X Minimal Essential Medium Vitamin Solution, $1 \times 10^{-4}$ M L-ascorbic acid, 10 μg/mL biotin, 30 ng/mL of β-estradiol, 60 ng/mL progesterone, 20 ng/mL human recombinant EGF, and 10 ng/mL human recombinant LIF) with the addition of 100 ng/mL FSH, 10 ng/mL LH, 1 μM metribolone, 100 ng/mL BMP4, 100 ng/mL animal-free recombinant human stem cell factor, and 10 μM of all-trans retinoic acid, the authors confirmed the viability and cell specificity via immunofluorescence with SOX9, IL-3, ACTA2, and SYCP3, confirming the presence of Sertoli, Leydig, peritubular myoid, and meiotic germ cells [90]. When transcripts were compared between bioprinted organoids with nonbioprinted controls, organoids generated in AggreWell with 7000–8000 cells mixed in a 1:4 ratio of germ and somatic cells, the authors observed a significant increase in *ID4*, *FGF3*, *CKIT*, *STRA8*, *DAZL*, *SYCP3*, *ZPBP2*, *TP1*, and *PRM2* [90].

One study differentiated porcine iPSCs (piPSCs) into primordial-germ-cell-like cells (pPGCLCs) in nonadherent AggreWell plates [91]. To induce piPSCs into pPGCLCs, the iPSCs were first differentiated into epiblast-like cells (EpiLCs) by plating on a fibronectin (16.7 μg/mL)-coated plate and cultured with N2B27 culture medium supplemented with 20 ng/mL activin A, 12 ng/mL bFGF, and 1% KSR for two days [91]. The resulting cells, EpiLCs, were further differentiated into pPGCLCs on AgreeWell with GK15 medium supplemented with 500 ng/mL BMP4, 500 ng/mL BMP8a, mLIF, 100 ng/mL SCF, and 50 ng/mL EGF for four days [91]. The resulting organoid showed *SOX2*, *OCT4*, *NANOG*,

*STELLA*, *BLIMP1*, *DAZL*, *VASA*, and *PRDM14*, but only *PRDM14* was significantly different compared to the piPSCs [91]. Immunofluorescence showed that the pPGCLCs expressed STELLA, VASA, OCT4, and NANOG, and DAZL-positive cells were observed in the pPGCLCs derived from piPSCs maintained with bFGF and bFGF with mLIF [91]. Most pPGCLC cells were positive for H3k27me3 and H3k9me2, and a small population of cells were positive for 5hmC and 5mC [91].

## 8. Scaffolds

Scaffolds used in in vitro studies offer structural supports that mimic the in vivo environment, aiding cell attachment, proliferation, and differentiation. These scaffolds support three-dimensional cell interactions, guide cell growth, allow efficient nutrient and waste diffusion, and can be modified to release specific growth factors or drugs. In in vitro spermatogenesis research, the extracellular matrix (ECM) maintains the necessary complex interactions for germ cell maturation. Commonly utilized ECM components include collagen, which offers a natural environment for cell adhesion; laminin, which supports cell differentiation and migration; fibronectin, promoting cell attachment and growth; gelatin, often used as a hydrogel for cell encapsulation; and Matrigel, a protein mixture resembling the intricate extracellular environment in tissues. Thus, selecting suitable scaffold and ECM components is vital for replicating in vivo conditions and ensuring accurate differentiation studies.

Spermatogenesis requires spatiotemporal regulation of SSCs, which interact with the extracellular matrix (ECM), paracrine factors, hormones, and testicular somatic cells. To mimic such highly orchestrated interactions, various scaffolds have been developed to further enhance the in vitro maturation of SSCs.

In a recent study, Salem et al. decellularized human testicular tissue, and SSCs were cultured on the scaffold with platelet-rich plasma for four weeks [92]. The differentiation medium contained DMEM/F12, 5% KSR, 5% FBS, 1 µM Retinoic acid, 0.1 µM testosterone, 25 µU FSH, 100 IU/mL penicillin, and 100 µg/mL streptomycin [92]. Decellularized human testicular scaffold showed increased viability, $PLZF^+$ cells, and *PLZF* and *PRM2* expression after four weeks [92]. Another group used a multilayer agarose/laminin 3D soft agar culture system to support the maturation of SSCs [93]. The spermatogonial stem cells were collected from three brain-dead donors of 15, 21, and 26 years, and SSCs were isolated and expanded in 2D culture with DMEM/F12, 10% KSR, 10 ng/mL bFGF, 10 ng/mL LIF, 20 ng/mL GDNF, 100 IU/mL pen/strep, and 40 µg/mL gentamycin [93]. The 3D culture was composed of two layers: the bottom layer was formed of 1% ($w/v$) agarose, while the upper layer was composed of 0.5% ($w/v$) agarose and laminin (100 µg/mL) mixed with $1.6 \times 10^6$ cells [93]. For the first three weeks, cells were cultured with DMEM/F12, 10% KSR, 20 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF, and 100 ng/mL stem cell factor [93]. For the rest of the experiment, cells were cultured with DMEM/F12, 10% KSR, GDNF, SCF, 2 µM retinoic acid, $10^{-7}$ M testosterone, and 50 U/L FSH [93]. At 74 days, the cells expressed higher *PLZF*, *SCP3*, and *PRM2* and stained positive for PLZF, SCP3, PRM2, and acrosin [93]. The resulting cells also showed sperm-like structures, such as an elongated spermatid and tail, which were also observed for 9 + 2 pairs of the microtubular axoneme structure with a transmission electron microscope [93].

## 9. Future Direction

Despite progress in in vitro spermatogenesis and the development of testicular organoids, several challenges still need to be addressed. Different approaches and methods covered in this review are summarized in Table 1 and Figure 3. These include the need for more efficient culture methods, the ability to generate functional sperm cells, and the development of reproducible and scalable protocols. In addition, ethical and regulatory considerations are associated with using human testicular tissue for research.

Recent single-cell studies have discovered complex crosstalk among cells in seminiferous tubules and the importance of different factors during development and spermatogen-

esis. Therefore, it will be necessary to investigate further the coculture methods involving culturing stem cells with testicular somatic cells, such as Sertoli cells, which provide a supportive microenvironment for spermatogenesis. However, the use of testicular somatic cells can introduce variability in the resulting cells, and the regulatory requirements associated with using human testicular tissue can limit the applicability of this method. Still, various workarounds exist to purify advanced spermatogenic cells, such as round spermatids and spermatozoa.

Also, the step-wise differentiation method can provide accurate temporal regulation to enhance proper transcriptional and epigenetic regulations. The step-wise differentiation method involves the induction of pluripotent stem cells or somatic cells into primordial germ cell (PGC)-like cells, which can further differentiate into male germ cells. PGCs are the precursors of male and female germ cells and are responsible for forming gametes. The step-wise differentiation method uses several growth factors and culture conditions to mimic the in vivo developmental process. For example, adding BMP4 and bFGF to the culture medium can induce the differentiation of pluripotent stem cells into PGC-like cells, which can be further differentiated into spermatogonia by adding RA and Kit ligands. The spermatogonia can then differentiate into spermatocytes via the addition of RA and testosterone, which induce the initiation of meiosis. Finally, spermatids can be generated by adding FSH and hCG, which activate signaling pathways that promote the formation of mature sperm. For example, researchers in a recent rodent study developed functional spermatozoa from mouse embryonic stem cells using the step-wise differentiation method [80,94]. The generated spermatozoa could fertilize eggs and produce healthy offspring [80,94].

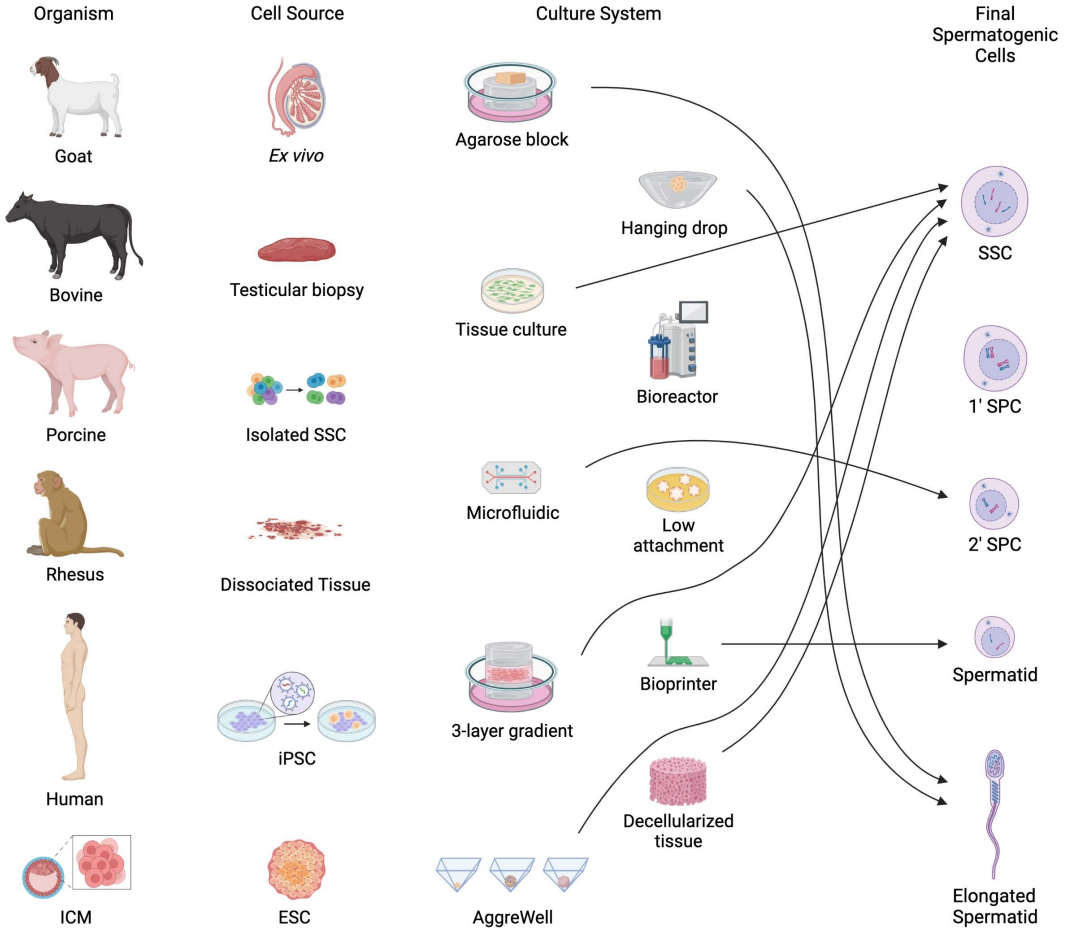


**Figure 3.** Summary of different approaches used to achieve in vitro spermatogenesis covered in this review.

**Table 1.** Summary of different in vitro spermatogenesis methods used in recent publications.

| Species | Cell Source | Culture System | Basal Medium | Growth Factors | Other Additive | Serum | Culture Duration | Final Spermatogenic Cells | Study |
|---|---|---|---|---|---|---|---|---|---|
| Goat | Ex vivo—testis | Hanging drop/organ culture | DMEM | | AlbuXL | 10% FBS | 14 Days | Elongated spermatozoa | [66] |
| Goat | Ex vivo—testis | Agarose block | MEM-α | | Nucleoside, AlbuXL | 10% FBS | 60 Days | Sperm-like cells with tails | [67] |
| Human | Isolated SSCs | 2D | DMEM/F12 | GDNF, bFGF, EGF, Laminin | | 10% FBS or 10% PRP | 14 Days | PLZF$^+$, OCT4$^+$, CKIT$^+$ | [69] |
| Human | Testicular tissue | Agarose gel stand | MEM-α | GDNF, bFGF, EGF, SCF, BMP4, Activin A, FSH, Testosterone, RA | 2% Human Umbilical Cord Plasma | 10% KSR XenoFree CTS | 60 Days | BOLL$^+$ | [70] |
| Rhesus | Isolated SSCs | 2D (on monkey fibroblast) | MEM-α | GDNF, bFGF, GFRα1, EGF, BMP7, LIF | 0.2% BSA | | 3 Weeks | *DAZL*, *ZBTB16*, *FGFR3*, *UTF1* | [74] |
| Human | hESC | Mini-spin bioreactor | DMEM/F12 | FGF9, BMP4, SHH, hCG, PMSG | AA2P, ITS, CHIR | | 40 Days | | [76] |
| Porcine | Testicular tissue | Various/Microfluidic | DMEM/F12 | FSH | | 10% KSR | 30 Days | VASA$^+$, SYCP3$^+$, and CREM$^+$ cells | [77] |
| Bovine | Testicular tissue | Organoid ultra-low attachment plates | DMEM/F12 | BMP4, FGF2, GDNF | | 10% FBS | 28 Days | | [87] |
| Marmoset | PBMC-derived iPSCs | On-feeder differentiation/V-bottom 96-well plate/Lipidure-coated U-bottom 96-well plate/Transwell-COL membrane with air–liquid interface | DMEM/GMEM/RPMI 1640/DMEM/α-MEM | BMP4, human LIF, SCF, EGF/forskolin, SCF, bFGF/ | Sodium pyruvate, β-mer/B27, Y-27632/ | 20% KSR, 20% FBS/15% KSR/15% KSR, 2.5% FBS/10% KSR | 30 Days | PDPN$^+$, ITGA6$^+$/NR2F2$^+$, TFAP2C$^+$, POU5F1$^+$, NANOG$^+$, DAZL$^+$, DDX4$^+$, SOX17$^+$. POUF5F1$^+$, TFAP2C$^+$, and SOX2$^-$, *DND1*, *NANOS3*, *PRDM1*, *SOX17*, and *TFAP2C* | [88] |
| Human | Dissociated embryonic gonad | Three-layer gradient system/Matrigel | NutriStem | | | 10% KOSR | 14 Days | DAZL$^+$, POU5F1$^+$ | [89] |
| Human | Dissociated human testis | Bioprinter | StemPro-34 SFM | Progesterone, EGF, LIF, FSH, LH, BMP4 SCF | ITS, sodium pyruvate, sodium DL-lactic acid, BSA, glutamax, β-mer, MEM vitamin, L-ascorbic acid, biotin, β-estradiol, metribolone, RA | 1% FBS | 12 Days | SYCP3$^+$, *ID4*, *FGF3*, *CKIT*, *STRA8*, *DAZL*, *SYCP3*, *ZPBP2*, *TP1*, *PRM2* | [90] |

**Table 1.** *Cont.*

| Species | Cell Source | Culture System | Basal Medium | Growth Factors | Other Additive | Serum | Culture Duration | Final Spermatogenic Cells | Study |
|---|---|---|---|---|---|---|---|---|---|
| Porcine | piPSCs | AggreWell | GK15 | BMP4, BMP8a, mLIF, SCF, EGF | | 1% KSR | 6 Days | *STELLA*, *VASA*, *DAZL*, *STELLA*, *BLIMP1*, *DAZL*, *VASA*, *PRDM14* | [91] |
| Human | Isolated SSC | Decellularized testicular tissue | DMEM/F12 | RA, Testosterone, FSH | Platelet-rich plasma | 5% KSR, 5% FBS | 4 Weeks | PLZF, *PLZF*, *PRM2* | [92] |
| Human | Isolated SSC | Agarose/Laminin 3D Agar | DMEM/F12 | GDNF, LIF, bFGF, SCF, Testosterone, FSH | RA | 10% KSR | 74 Days | *PLZF*, *SCP3*, *PRM2*, $PLZF^+$, $SCP^+$, $PRM2^+$, Acrosin, with sperm-like structures with 9 + 2 microtubular axoneme structure | [93] |

Similarly, another study demonstrated the generation of haploid germ cells from human pluripotent stem cells [3], which has been successfully adopted in primates for successful fertilization and blastocyst development [4]. Despite the progress made in the step-wise differentiation method, several challenges still need to be addressed in generating functional spermatozoa. These challenges include optimizing culture conditions and identifying specific signaling pathways in germ cell differentiation. In past years, studies in rodent models have significantly improved the development of spermatogenesis into functional spermatid/sperm in vitro, especially in coculture and organoid models [81–84]. Groundbreaking studies in germ cell developments led to elucidation of the intricate processes involved in specific developmental pathways to generate mature gametes, such as the role of various genes and proteins, the interaction of germ cells with surrounding somatic cells, the role of the spermatogonial niche, and the epigenetic modifications required for germ cell development [31–33,43–45,48,51,95,96]. The success in spermatogonial stem cells in rodents and monkeys demonstrated the efficacy of regenerative medicine in restoring male fertility [97–101]. The ethical concerns associated with using human pluripotent stem cells must be addressed before this technology can be translated into clinical applications.

Future studies will need to investigate the combinations of methods, which will provide more accurate spatiotemporal signals to bring us closer to successfully generating functional sperm in vitro.

## 10. Conclusions

The research on in vitro spermatogenesis holds transformative potential across various domains of medicine, social sciences, and ethics. Its most immediate application is in reproductive medicine, which could provide new avenues for treating male infertility. By creating mature sperm cells outside the body, men with low sperm counts, poor sperm quality, or even those rendered infertile due to medical treatments could have an alternative means to father biological children. Additionally, it could be invaluable for preserving the fertility of young men undergoing treatments like chemotherapy, which may compromise their reproductive capabilities. This could alleviate not just the physical, but also the emotional and psychological challenges of infertility, offering hope to millions of families. The research also has significant implications for drug discovery and toxicology testing. Generating sperm in vitro would allow for high-throughput screening of substances that could affect male fertility, speeding up the safety profiling of new drugs and identifying potential reproductive hazards among existing ones. The overall significance of in vitro spermatogenesis research is immense. It stands to revolutionize reproductive medicine, contribute significantly to genetic research, and offer new pathways in drug discovery.

In vitro spermatogenesis and testicular organoids are promising technologies that have the potential to revolutionize the field of male reproductive biology. While many challenges still need to be addressed, the progress in these areas provides hope for developing new treatments for infertility and other male reproductive disorders. So far, ex vivo organ/tissue culture has achieved complete spermatogenesis in vitro in nonrodent species (Figure 3). However, even with an ex vivo organ/tissue culture system, it is not certain that continuous spermatogenesis can be achieved in vitro. Since the ex vivo organ/tissue culture system is the oldest approach to in vitro spermatogenesis, the newest approaches, such as organoids, bioprinting, and microfluidics, will improve differentiation efficiency.

In conclusion, IVS is a challenging but promising research field with potential applications for fertility preservation, drug discoveries, infertility treatment, and a basic understanding of spermatogenesis. Among the different methods of IVS, TOs represent a novel and advanced model that can better simulate the testicular microenvironment in vitro. However, TOs are still in their early stages of development and require further refinement and validation before they can be widely used for research or clinical purposes.

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