nature reviews urology

https://doi.org/10.1038/s41585-023-00723-4

Review article

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Human in vitro spermatogenesis as a regenerative therapy — where do we stand?

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Abstract

Spermatogenesis involves precise temporal and spatial gene expression and cell signalling to reach a coordinated balance between self-renewal and differentiation of spermatogonial stem cells through various germ cell states including mitosis, and meiosis I and II, which result in the generation of haploid cells with a unique genetic identity. Subsequently, these round spermatids undergo a series of morphological changes to shed excess cytoplast, develop a midpiece and tail, and undergo DNA repackaging to eventually form millions of spermatozoa. The goal of recreating this process in vitro has been pursued since the 1920s as a tool to treat male factor infertility in patients with azoospermia. Continued advances in reproductive bioengineering led to successful generation of mature, functional sperm in mice and, in the past 3 years, in humans. Multiple approaches to study human in vitro spermatogenesis have been proposed, but technical and ethical obstacles have limited the ability to complete spermiogenesis, and further work is needed to establish a robust culture system for clinical application.

Sections

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Key points

- Spermatogenesis includes a complex and highly regulated series of steps required for the production of mature, motile sperm. Efforts to recreate this process in vitro are ongoing to support the treatment of male factor infertility, particularly non-obstructive azoospermia (NOA).
- Advances in the ex vivo production of mature spermatids in animal models have provided new insights for the successful achievement of these results in humans.
- Several approaches to achieve human in vitro spermatogenesis have been described, such as 2D and 3D cultures including scaffold, organoid-based and bioprinted systems, which are supported by a variety of media and biomaterials.
- Complete spermatid differentiation in vitro has been shown in some studies, although this process frequently occurred at an accelerated rate compared with the expected course in vivo; moreover, these results still need to be independently replicated by other research groups.
- The future perspective of using gene editing to investigate and rescue NOA phenotypes is promising, but is limited by regulatory and ethical considerations.

Introduction

Spermatogenesis is the foundation of male reproduction. Following the pubertal transition, males typically produce tens to hundreds of millions of motile sperm[1]. During natural conception, these sperm will navigate the female reproductive tract to find and fertilize the oocyte[2]. However, ~15% of couples globally experience challenges with natural conception, and approximately half of these instances can be ascribed to male factors[3]. Advances in assisted reproductive technologies have enabled the reproductive community to overcome most forms of male infertility, and pregnancies and live births can be achieved with techniques such as in vitro fertilization–intracytoplasmic sperm injection (IVF-ICSI) using as few as one viable and functional sperm[4,5]. However, IVF-ICSI is not a suitable solution in patients with no detectable sperm, a condition known as azoospermia.

Azoospermia is diagnosed when a complete absence of sperm is observed in at least two semen samples, although microscopic evaluation of a centrifuged sample is performed. Azoospermia ascribed to abnormalities in sperm production is termed non-obstructive azoospermia (NOA). Amongst European men, NOA with an intrinsic aetiology (due to genetic abnormalities) occurs in <25% of patients[6,7]. Therapeutic options in these patients are limited to microdissection testicular sperm extraction, which leads to surgical sperm retrieval in ~50% of patients and, in combination with IVF-ICSI, might result in a live birth in 10–25% of couples[8–10]. However, the remaining patients have no therapeutic options to father a biological child. NOA might also be ascribed to extrinsic factors such as gonadotoxic therapies including chemotherapy and radiotherapy[11,12]. Sperm banking might be offered to patients who undergo these iatrogenic insults after puberty[13]. With regard to patients with prepubertal iatrogenic insults, some centres offer testicular biopsy and cryopreservation, with the hope that future technologies will be developed to facilitate complete differentiation of spermatogonial stem cells (SSCs) into mature sperm capable of producing offspring[14]. Thus, regenerative treatment strategies for patients with NOA are strongly needed, particularly for patients with no retrievable sperm or prepubertal cancer survivors[15–22]. A possible approach is in vitro spermatogenesis and spermiogenesis (IVS), in which spermatozoa are generated from SSCs in a laboratory setting. Considering ethical concerns and scarcity of human fetal tissue, animal models have provided an invaluable tool to optimize technologies that can be applied to support human spermatogenesis. Approaches developed in animal models have laid the foundation for the potential use of in vitro spermatogenesis for infertility treatment. Successful ex vivo production of mature spermatids with fertilization capacity from immature germ cells using mouse testicular organotypic culture was first described in 2011 (ref. [23]). In this study, fertile, haploid sperm was obtained with similar efficiency from fresh and cryopreserved testicular samples from neonatal mice. Moreover, using a similar culture method, differentiated spermatogonia were produced from testis tissue obtained from adult mice with spermatogenic defects[24].

In this Review, we comprehensively discuss the approaches used to date in developing human IVS, emerging technologies that might be integrated to advance this field, and safety considerations necessary before implementing future IVS technologies into regular clinical use.

Human spermatogenesis

Human spermatogenesis is a complex process in which precise temporal and spatial events are coordinated by local support from somatic cells through direct contact, and juxtacrine and paracrine signalling[25–28] (Fig. 1). The entire process of spermatogenesis occurs in cycles of 74 days, with new waves of SSCs entering differentiation every 16 days[29]. Undifferentiated spermatogonia can typically be divided into A dark, A pale and type B[30–32]. However, results from single-cell RNA sequencing (scRNA-seq) of testicular cell transcriptomes indicated the existence of five discrete, sequential spermatogonial states based on the transcriptional state[33,34]: state 0 and 1, probably corresponding to A dark spermatogonia, which constitute the quiescent cells reserve population[33,34]; state 2 and 3, corresponding to the A pale cells, which are actively differentiating or self-renewing and, after puberty, start the process of differentiation and undergo mitosis to produce type B spermatogonia; and state 4, probably corresponding to type B spermatogonia, which commit to differentiation[33–35].

Spermatocytes are produced from type B spermatogonia through a final mitotic division, after which spermatocytes cross the blood–testis barrier (BTB), becoming immunologically isolated from the rest of the body[28,36,37], and proceed to meiosis I, in which chromosomes line up in pairs and exchange DNA segments to produce unique combinations, a crucial process contributing to genetic diversity[38]. After meiosis I, two secondary spermatocytes are produced and enter into meiosis II to produce four haploid round spermatids, each possessing one copy of each chromosome[39]. Subsequently, round spermatids mature into spermatozoa, a process known as spermiogenesis, after which no further cellular divisions occur and nuclear and cytoplasmic changes progress to produce mature sperm. The Golgi apparatus supports acrosomal biogenesis with the production of proteolytic and hydrolytic enzymes that contribute to the formation the acrosomal granule[40,41], the nucleus condenses and migrates to an eccentric position, and somatic histones undergo histone-to-protamine transition to enable chromatin compaction into supercoiled structures[42–44]. The sperm's tail arises from the centriole, which coincides with the gradual elongation of the spermatid's shape[45]. Any residual cytoplasm is then

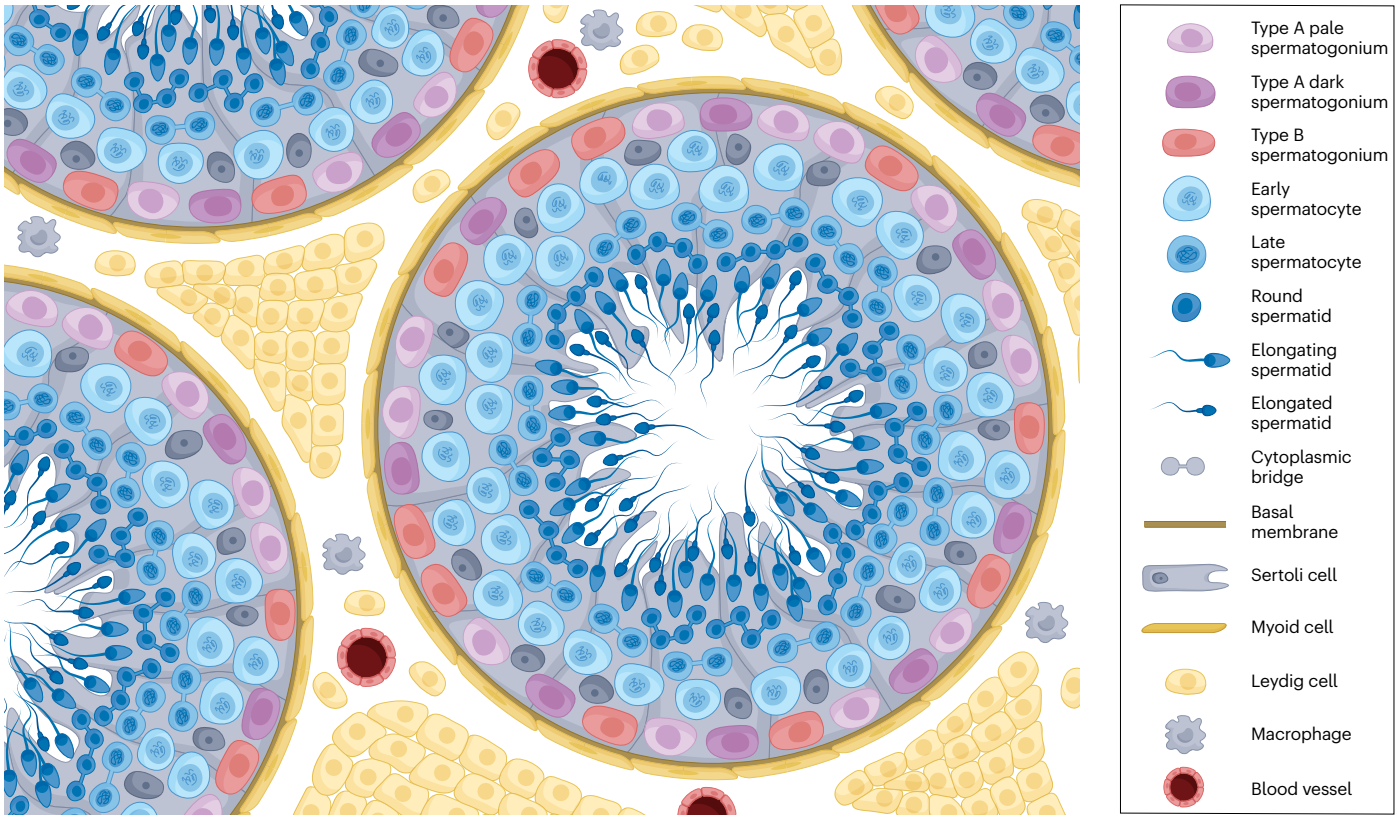

Fig. 1 | Human spermatogenesis. Simplified schematic cross-section of a seminiferous tubule and surrounding interstitial space. Reported cell–cell signalling pathways involved in the process of spermatogenesis are also shown. Notably, germ cells are not isolated as depicted, but are instead interconnected by intercellular bridges, enabling cytoplasmic exchange of contents until subsequent post-meiotic stages, in which these intercellular bridges disappear.

removed and phagocytosed by Sertoli cells[46]. At this point, spermatozoa detach into the seminiferous tubule lumen and migrate to the epididymis for storage and maturation.

Approaches for human in vitro spermatogenesis

IVS requires spermatogenesis and spermiogenesis to occur outside the body. Preliminary findings have suggested that partial differentiation can occur in vitro[47–51], but the entire process has been shown to be challenging. To date, several approaches have been attempted, spanning 2D and 3D culture systems (Fig. 2), and a variety of media and biomaterials (Table 1). These approaches have been tested in numerous animal models with impressive results[17,23,52–73]. Owing to the increasing breadth of research in model systems, this Review focuses on human IVS.

2D culture systems

2D culture systems consist of cell monolayers, which often include feeder cells to provide growth factors to support cell growth[74] (Fig. 2a,b). The first successful in vitro maturation of germ cells from patients with NOA occurred in 2D monolayer cultures, with the ultimate goal to facilitate the use of this technique in assisted reproduction[47–51].

Sertoli cell feeder systems. In a series of ground-breaking studies published in 1998 and 1999, a monolayer culture system in which autologous Sertoli cells were used as feeders for mature spermatocytes obtained from patients with obstructive azoospermia (OA) and NOA with maturation arrest were described[47–49].

In the first study, spermatogonia at varying stages of spermatogenesis and Sertoli cells were isolated from 18 patients with OA by mechanical dissociation into a cell suspension, and subsequently cultured in Gamete-100 medium in the presence or absence of recombinant follicle-stimulating hormone (rFSH)[47]. The proportion of germ cells at individual stages of spermatogenesis was assessed throughout the study by visualization through microscopy on the basis of cell size and morphological features. rFSH addition induced twofold and threefold increases in the proportions of secondary spermatocytes and spermatids, respectively. However, a concomitant increase in the incidence of abnormal elongating spermatid morphologies was also observed in the presence of rFSH (10.4–12.7% and 1.0–4.8% in cultures with and without rFSH, respectively). The authors observed an accelerated rate of spermiogenesis in this system, which occurred within 24 h, compared with the reported 20–22 days in vivo, and hypothesized that mechanisms coordinating in vivo morphogenetic and molecular transformations during differentiation from primary spermatocytes to elongated spermatids might be lost in vitro.

In a follow-up study by the same group, the effects of rFSH in combination with testosterone on the maturation of germ cells from patients with OA were measured over 24–48 h in vitro[48]. Similar to previous observations[47], rFSH supplementation at concentrations of 10–50 IU/l caused a dose-dependent increase in the percentage of

haploid spermatids, from 47.6% in the absence of FSH to 64.1% in the presence of FSH at the maximal effective concentration. This phenomenon was associated with an expected decrease in spermatocytes, which was potentiated by the addition of testosterone. Independently of rFSH addition, supplementation with 1 µmol/l testosterone reduced cellular apoptosis from 70.9% to 20.3%, which was hypothesized to be responsible for the FSH-dependent potentiating effect observed on spermatid development. Normal and abnormal morphological changes were observed during spermatid progression from round to elongated states and, also in this study, an increase in abnormal spermatid morphologies was observed.

Another study was subsequently conducted by the same group to assess the reproductive potential of spermatids derived in vitro from men with maturation arrest at the primary spermatocyte or round spermatid stage[49]. In cells obtained from five men with primary spermatocyte arrest, both normal and abnormal spermatid forms were found in samples from two men after 48 h of culturing. Injection into partners' oocytes resulted in fertilization and embryo cleavage in both women, and in live birth of phenotypically normal twins at 36 weeks gestation in one woman. Cells from two of four patients with maturation arrest at the round spermatid stage showed round spermatid elongation into both normal and abnormal forms. Oocyte injection of in vitro-derived spermatids resulted in two pregnancies: one ectopic pregnancy and one live birth at 39 weeks gestation. These findings showed that in vivo pre-meiotic arrest of spermatogenesis can be overcome by in vitro spermatocyte culture in some individuals. However, these findings generated some controversy. The authors of a published letter highlighted that genetic abnormalities associated with NOA could have been passed on to the sons, and that no clinical or genetic information was provided as evidence of the infants' normalcy[75]. The authors of the IVS studies[43,44] responded to the letter arguing that the system should be safe, as pachytene spermatocytes are specifically targeted for rapid differentiation[75]. Specifically, the authors hypothesized that the in vivo checkpoints that were absent in the culture system, which could have been responsible for the accelerated spermatogenesis observed in vitro, seemed to be limited to late-pachytene checkpoints

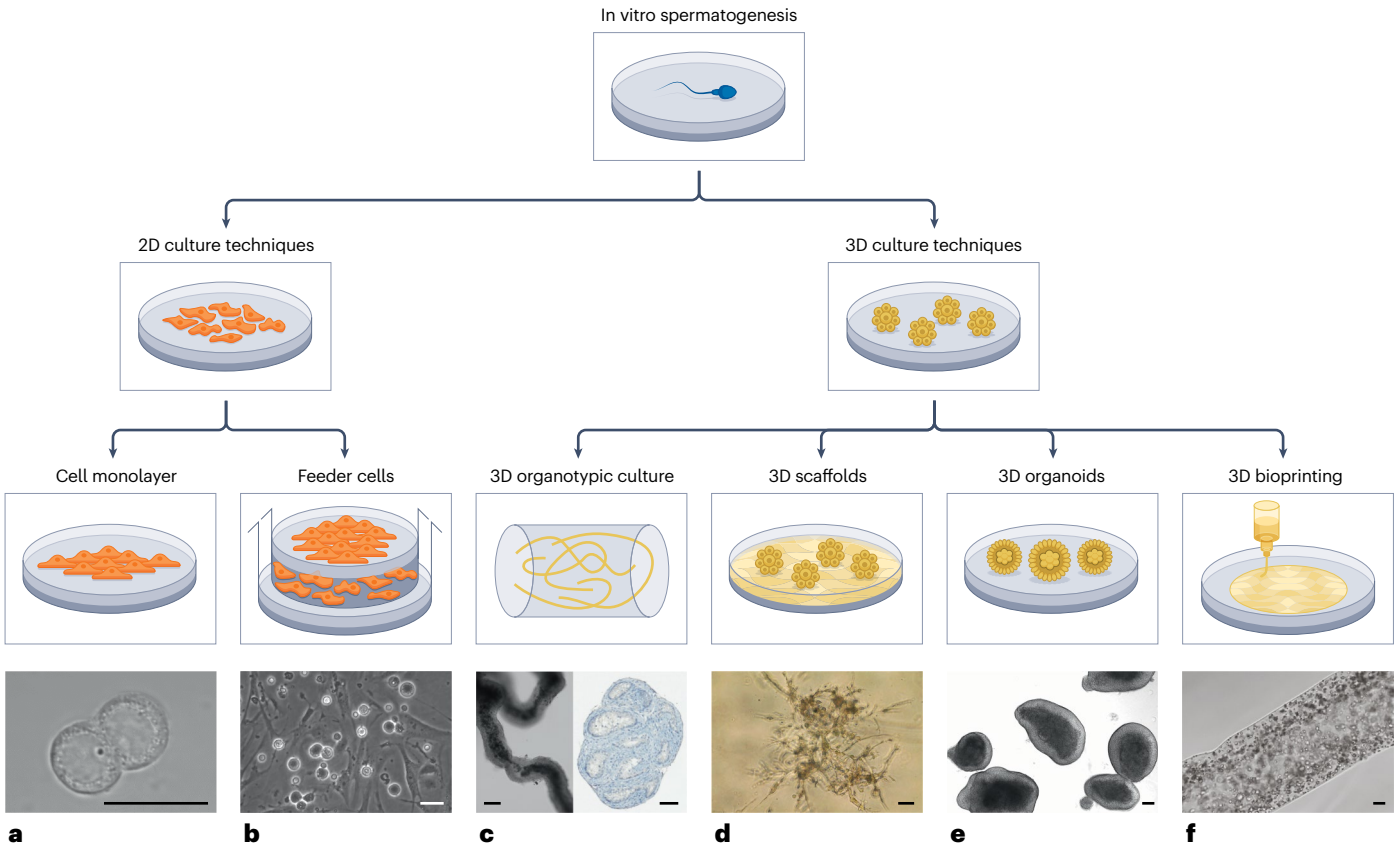

Fig. 2 | Culture techniques for supporting in vitro spermatogenesis. **a**, Example of 2D monolayer culture. Confocal brightfield image of freshly isolated human germ cells cultured on a 2D laminin-coated dish. Scale bar is 25 µm (R.F., unpublished work). **b**, Example of 2D feeder cell culture. Confocal brightfield image of human germ cells cultured on human testicular somatic feeder cells. Germ cells are rounded and have large nucleus to cytoplasm ratios, whereas somatic feeder cells are flat and fibroblast-like, with small nucleus to cytoplasm ratios. Scale bar is 100 µm (R.F., unpublished work). **c**, Example of 3D organotypic culture. Left: confocal brightfield image of freshly isolated human seminiferous tubules. Right: haematoxylin and eosin-stained section of a small fraction of human seminiferous tubules after organotypic culture. Scale bars are 100 µm (R.F., unpublished work). **d**, Example of 3D scaffold culture. Colour microscope image of human testicular cells cultured in a 3D Matrigel scaffold. Scale bar is 100 µm (R.F., unpublished work). **e**, Example of 3D human testicular organoid culture. Confocal brightfield image of organoids generated in microwells from human testicular cells after 4 weeks in suspension culture. Scale bar is 100 µm (R.F., unpublished work). **f**, Example of 3D bioprinted culture. Confocal brightfield image of a hollow tubular bioprint laden with human testicular cells. Scale bar is 100 µm (R.F., unpublished work).

Table 1 | Human germ cell culture for IVS or in vitro germ cell maturation

Starting cells	Tissue source (number of patients)	Culture method	Basal medium	Serum	Hormones	Supplements	Time in culture	Results	Fertilization capacity	Refs.
Pachytene spermatocytes	Patients with obstructive azoospermia (18)	Sertoli cell feeder	Gamete-100	NA	10, 25, 50 and 100 U/l recombinant follicle-stimulating hormone, 10^{-6} M testosterone	NA	1–2 days	Sd2 elongating spermatids (44%)[a]; 19% Scp abnormal[b]	NA	48
Pachytene spermatocytes	Patients with maturation arrest (9)	Sertoli cell feeder	Gamete-100	NA	25 U/l recombinant follicle-stimulating hormone, 10^{-6} M testosterone	NA	2 days	Normal and abnormal round and elongating spermatids	Three pregnancies with no abnormalities	49
Round spermatids	Patients with maturation arrest (2), patients with complete spermatogenesis <1% (1), patients with total globozoospermia (1)	Vero cell feeder	Mineral oil	NA	NA	NA	5 days	Single normal-headed spermatozoa from globozoospermia-derived round spermatid	NA	50
Pachytene spermatocytes	Patients with maturation arrest (5)	Vero cell feeder	MEM	Human serum or boar rete testicular fluid or synthetic oviduct fluid	50 U/l recombinant follicle-stimulating hormone, 10^{-6} M testosterone	NA	5 days	Round spermatids (4.3–9.8%)	NA	51
Pluripotent stem cells (induced and embryonic)	H1 human embryonic stem cells (WA01, WiCell) and HFF1 parent fibroblasts (ATCC, iPS derived internally)	Monolayer	αMEM	0.2% bovine serum albumin	NA	5 µg/ml insulin, 10 µg/ml transferrin, 60 µM putrescine, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 1 ng/ml basic fibroblast growth factor, 20 ng/ml glial cell-derived growth factor, 30 nM sodium selenite, 2.36 µM palmitic acid, 0.21 µM palmitoleic acid, 0.88 µM stearic acid, 1.02 µM oleic acid, 2.71 µM linoleic acid, 0.43 µM linolenic acid, 10 mM HEPES, and 0.5× penicillin/streptomycin	10 days	Round spermatids (3.9–4.5%)	NA	84
Spermatogonial stem cells	Prepubertal patients (3)	Organotypic air–liquid interface	DMEM/F12	10 mg/ml human serum albumin	From day 2, 50 U/l recombinant follicle-stimulating hormone and 7×10^{-9} M testosterone propionate or 1 U/l human chorionic gonadotropin; testosterone doubled on day 16	10 µg/ml insulin transferrin selenium, 10 µg/ml gentamicin, 0.35 mg/ml L-glutamine, 0.0025 M pyruvate, 10^{-6} M retinol, 0.05 mg/ml vitamin C	139 days	NA	NA	88
Spermatogonial stem cells	Prepubertal patients (3)	Organotypic air–liquid interface	DMEM/F12	10% knockout serum replacement	5 U/l follicle-stimulating hormone, 1 U/l human chorionic gonadotropin, 5×10^{-12} M triiodothyronine, 5 ng/ml prolactin	10 ug/ml gentamicin, 3 mg/l ceftazidime, 0.35 mg/ml L-glutamine, 10^{-6} M retinol, 0.05 mg/ml vitamin C, 0.0025 M pyruvate, 20 µM 22(*R*)-hydroxycholesterol, 10 ng/ml glial cell-derived neurotrophic factor	139 days	Round spermatids	NA	89,90

Table 1 (continued) | Human germ cell culture for IVS or in vitro germ cell maturation

Starting cells	Tissue source (number of patients)	Culture method	Basal medium	Serum	Hormones	Supplements	Time in culture	Results	Fertilization capacity	Refs.
Spermatogonial stem cells	Prepubertal patients (9)	Organotypic air–liquid interface	αMEM	10% knockout serum replacement	3 U/l follicle-stimulating hormone, 3 U/l luteinizing hormone, 10^{-7} M melatonin	1% penicillin–streptomycin, 10^{-6} M retinoic acid	35 days	NA	NA	94
Primordial germ cells	Fetal tissue: 12–19 weeks (44)	Organotypic air–liquid interface	αMEM	10% knockout serum replacement	10×10^{-3} M testosterone, 200 ng/ml follicle-stimulating hormone, 50 mg/ml bovine pituitary extract	20 ng/ml bone morphogenetic protein 4/7, 20 ng/ml stem cell factor, 20 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 20 ng/ml glial cell-derived neurotrophic growth factor, 100 ng/ml activin A	50 days	Round spermatids	Blastocyst stage embryo	95
Spermatogonial stem cells	Infants: 0.5–1.4 years old (4)	Organotypic air–liquid interface	αMEM	10% knockout serum replacement, 2% human umbilical cord plasma	10×10^{-6} M testosterone, 10 IU/l follicle-stimulating hormone	20 ng/ml bone morphogenetic protein 4/7, 20 ng/ml stem cell factor, 20 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 20 ng/ml glial cell-derived neurotrophic growth factor, 100 ng/ml activin A, with or without 10×10^{-6} M retinoic acid	60 days	Spermatocytes	NA	96
Spermatogonial stem cells	Hormone-treated patients (gender-affirming) (5)	Organotypic chitosan bioreactor	DMEM/F12	NA	1 ng/ml follicle-stimulating hormone, day 5 10^{-7} M testosterone	15 mM HEPES, antibiotics, 1.2 g/l $NaHCO_3$, 10 µg/ml insulin, 10 µg/ml transferrin, 10^{-4} M vitamin C, 10 µg/ml vitamin E, 3.3×10^{-7} M retinoic acid, 3.3×10^{-7} M retinol, 10^{-3} M pyruvate	55 days	Spermatozoa	NA	98
Spermatogonial stem cells	Patients with SCO syndrome (11), maturation arrest (2)	Alginate	DMEM/F12	10% fetal bovine serum	2.5×10^{-5} U/l recombinant follicle-stimulating hormone, 10^{-7} M testosterone	10 µg/ml insulin–transferrin–selenium, 10^{-4} M vitamin C, 10 µg/ml vitamin E, 3.3×10^{-7} M retinoic acid, 3.3×10^{-7} M retinol, 10^{-3} M pyruvate, 1× antibiotic–antimycotic	28 days	Round spermatids	Cleaved embryos failed to implant	106
Spermatocytes	Patients with maturation arrest (8)	Collagen–Matrigel	DMEM/F12	10% fetal bovine serum	0.1 U/l recombinant follicle-stimulating hormone, 10^{-7} M testosterone, 10^{-7} M dihydrotestosterone	10 mg/ml insulin–transferrin–selenium, 10^{-4} M vitamin C, 10 mg/ml vitamin E, 3.3×10^{-7} M retinoic acid, 3.3×10^{-7} M retinol, 10^{-3} M pyruvate, 10% antibiotic–antimycotic	12 days	Round spermatids	NA	108
Spermatogonial stem cells	Patients with obstructive azoospermia (60)	Matrigel	DMEM/F12	10% knockout serum replacement	10^{-6} M testosterone	2×10^{-6} M retinoic acid, 100 ng/ml stem cell factor, 100 ng/ml bone morphogenetic protein 4	20 days	Round spermatids	Eight-cell stage[c]	109
Elongated spermatids	Patients with complete spermatogenesis (14), maturation arrest (1), SCO syndrome (1)	Organoid	DMEM	15% knockout serum replacement	NA	0.1×10^{-3} M 2-mercaptoethanol, 2 mM L-glutamine, 0.1×10^{-3} M, 1× MEM non-essential amino acids, 1% penicillin–streptomycin, 40 ng/ml epidermal growth factor, 20 ng/ml fibroblast growth factors 1/2/9, 100 ng/ml glial cell-derived neurotrophic factor, 10 ng/ml insulin-like growth factor 1	14 days	Elongated spermatids	NA	110

Table 1 (continued) | Human germ cell culture for IVS or in vitro germ cell maturation

Starting cells	Tissue source (number of patients)	Culture method	Basal medium	Serum	Hormones	Supplements	Time in culture	Results	Fertilization capacity	Refs.
Spermatogonial stem cells	Prepubertal patients (2)	Organoid	DMEM/F12	NA	NA	10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml selenium, 20 ng/ml epidermal growth factor, 1% penicillin–streptomycin	5 days	NA	NA	111
Spermatogonial stem cells	Patients with complete spermatogenesis (patients after brain death) (3)	Organoid	StemPro-34	1% fetal bovine serum, 5 mg/ml bovine albumin	2.5×10^{-5} U/l follicle-stimulating hormone	25 µg/ml insulin, 100 µg/ml transferrin, 60×10^{-6} M putrescine, 30×10^{-9} M sodium selenite, 6 mg/ml D-(+)-glucose, 30 µg/ml pyruvic acid, 1 µl/ml DL-lactic acid, 2×10^{-3} M L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1× MEM vitamin solution, 1× MEM non-essential amino acid solution, 10^{-4} M vitamin C, 10 µg/ml D-biotin, 30 ng/ml β-oestradiol, 60 ng/ml progesterone, 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor, 10 ng/ml glial cell-derived neurotrophic factor, 2×10^{-6} M retinoic acid, 100 ng/ml recombinant stem cell factor, 1 µg/ml solubilized human testis extracellular matrix extract	23 days	Spermatids	NA	112
Spermatogonial stem cells	Patients with SCO syndrome (1)	Bioprinted	StemPro-34	1% fetal bovine serum, 5 mg/ml bovine albumin	100 ng/ml follicle-stimulating hormone, 100 ng/ml luteinizing hormone, 10^{-6} M metribolone	25 µg/ml insulin, 100 µg/ml transferrin, 60×10^{-6} M putrescine, 30×10^{-9} M sodium selenite, 30 µg/ml pyruvic acid, 1 µl/ml DL-lactic acid, 2×10^{-3} M L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1× MEM vitamin solution, 10^{-4} M vitamin C, 10 µg/ml D-biotin, 30 ng/ml β-oestradiol, 60 ng/ml progesterone, 10×10^{-6} M retinoic acid, 100 ng/ml recombinant stem cell factor, 100 ng/ml bone morphogenetic protein 4, 20 ng/ml epidermal growth factor, 10 ng/ml leukaemia inhibitory factor	12 days	NA	NA	117

ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFF1, human foreskin fibroblast 1; iPS, induced pluripotent stem cells; IVS, in vitro spermatogenesis and spermiogenesis; MEM, minimum essential medium; NA, not available; SCO, Sertoli cell only. [a]Sd2: last stage of elongating spermatid as defined by morphological criteria, that is spermatozoon. [b]Scp: elongating spermatid with abnormal form of flagellum and round head. [c]Mouse oocytes were used in this study.

and, therefore, would not have affected normal meiotic function, promoting the development of safe, albeit morphologically abnormal, spermatids[75]. Moreover, the authors provided data about the karyotype analyses of the three babies born after fertilization with in vitro-cultured spermatozoa, which were normal[75].

Vero cell feeder systems. Concomitantly with Sertoli cells feeder cultures, successful IVS results were obtained using another feeder monolayer system. This feeder system can support maturation of pachytene spermatocytes from patients with NOA into complete spermatozoa using Vero cells, an immortalized cell line derived from African green monkey kidney epithelial cells, which can be grown under serum-free conditions[51]. However, the use of Vero cells as feeders is not clinically translatable owing to the risk of xeno-transmission of infectious disease[76].

In 1999, Vero cell feeders were shown to be able to rescue spermatogenesis arrest in round spermatids obtained from patients with NOA[50]. In this study, testicular cells were isolated from two patients with NOA with maturation arrest and from one patient with <1% tubules showing

complete spermatogenesis. A patient with total globozoospermia (in which all spermatozoa in the ejaculate show complete spermatogenesis but with abnormally round-shaped heads) was included as a control. Round spermatids were selected manually and cultured on Vero cell monolayers covered by light mineral oil. Round spermatids without flagella from all patients with NOA differentiated into spermatids with signs of elongation within 5 days, and one spermatid grew a flagellum and matured through the elongation step to become a mature spermatozoon. Surprising results were also observed in cells from the patient with globozoospermia, as one of six round spermatids from this patient matured into normal-headed spermatozoa after 5 days in culture. These findings are preliminary, but suggest that in vitro maturation might also be used to overcome abnormal spermiogenesis.

In a subsequent study published in 2003, Vero cell feeders were used to differentiate pachytene spermatocytes isolated from five patients with NOA with maturation arrest at the round spermatid stage[51]. Spermatocytes were manually selected and cultured with Vero cells or with supplemented minimum essential medium (MEM) alone. Overall, 4.3–9.8% spermatocytes cultured with Vero cells matured into haploid spermatids over a 2–5-day period, as assessed by direct visualization of induced metaphase chromosome spreads and fluorescence in situ hybridization analysis. No difference was observed after including additives in the culture medium. Conversely, none of the spermatocytes cultured without Vero cell feeders showed maturation, indicating that Vero cell feeder cells were the main drivers of meiotic division of spermatocytes into round spermatids, potentially acting as a substrate for cellular attachment, as well as through the release of growth factors and synthesis of extracellular matrix proteins[77].

Pluripotent stem cell-derived systems. The discovery of human induced pluripotent stem cells (hiPSCs) in 2007 started a new era of personalized regenerative medicine in which adult cells can be reprogrammed back to a stem cell state and used to regenerate new cells and tissues[78,79]. hiPSCs have the potential to make IVS accessible to patients who lack SSCs, including men with Sertoli cell only (SCO) histology[80] and survivors of paediatric cancer who did not bank tissues before spermatotoxic therapy[81,82].

In 2012, human embryonic pluripotent stem cells (hESCs) and hiPSCs were successfully differentiated into SSCs and then haploid spermatids[83]. hiPSCs cultured in a monolayer in supplemented αMEM yielded germ cells (40–60%) after 10 days. Round spermatids constituted up to 3.9–4.5% of the population, with a peak observed on day 10, but eventually disappeared by day 20. Glial cell-derived neurotrophic factor (GDNF) was indispensable for germ fate acquisition in the culture matrix. Maternal and paternal genomic imprints are removed at the primordial germ cell stage and re-established during spermatogenesis[84]; thus, the authors assessed methylation status of the imprinting control region for paternally and maternally imprinted genes in the hESC and hiPSC-derived spermatids. Epigenetic modifications such as methylation govern chromatin structure and reversibly regulate gene expression. The timing of this imprinting influences the expression of genes involved in cell cleavage and determination and is therefore crucial[85]. Spermatids differentiated from hESCs showed typical imprinting, whereas spermatids generated from hiPSCs showed slightly elevated methylation around the maternally imprinted genes. These results show that SSCs can efficiently differentiate from a pluripotent state; however, similar to feeder cell culture systems[47–51,86], spermatogenesis was accelerated for reasons that are not yet understood. The downstream implications of spermatozoa derived from hiPSCs or hESCs on fertilization potential or offspring development and health are currently unknown.

2D IVS systems — considerations

In summary, results from studies in which 2D IVS systems were used have shown that with Sertoli or Vero cell feeder supports, spermatocytes from patients with NOA can be differentiated into functional spermatids[47–49,51], and round spermatids into elongated spermatids[50], which, in rare instances, can generate mature spermatozoa[50]. Both feeders accelerated spermatocyte maturation, but Sertoli cell feeders induced morphological abnormalities[47–49]. Interestingly, Vero cells were able to correct the abnormal spermatozoa morphologies caused by underlying globozoospermia, a result that requires further validation and research[50]. Accelerated spermatogenesis up to the round spermatid stage was also observed for hiPSC-derived SSCs using defined medium supplements, and GDNF was identified as a factor involved in germ cell fate acquisition, but not necessarily in spermatogenesis[83].

Overall, to date, 2D culture systems have been shown to induce incomplete or abnormal spermatogenesis; however, identifying the signalling mechanisms responsible for this accelerated maturation observed in 2D systems could provide valuable insights for developing fully defined stepwise 2D IVS systems for clinical use.

Organotypic culture systems

A possible approach to IVS is in vitro culture of the complete testicular niche (known as organotypic culture (Fig. 2c)), with the idea that spermatogenesis can resume in vitro with the support of the natural microenvironment. A major challenge with this approach has been to keep the tissues viable and functional without bodily support, including delivery of oxygen, vitamins, nutrients and trophic factors through diffusion from the local vascular system.

Air–liquid interface

In a series of studies published from 2016 to 2018, medium conditions to support testicular organotypic cultures were investigated[87–89]. In these studies, cryopreserved testicular tissues obtained from prepubertal patients (aged 2–12 years) were diced into 1 mm^3 fragments and cultured at the air–liquid interface, a technique introduced with the rationale to reduce hypoxia and the consequent oxidative stress by improving oxygen diffusion[53,90–92]. In all medium conditions, the percentage of tubules maintaining integrity in culture dropped to ~50% by day 139, and only tubules deemed to be intact were included in the analyses. In the first study, supplemented Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12) was used[87,88]. On day 2 of culture, rFSH in combination with either testosterone or human chorionic gonadotropin (hCG) was added. Testosterone concentrations were doubled on day 16 to mimic the physiological peripubertal increase. Cell death showed a prominent spike on day 1 of culture, and settled by day 10. SSCs were observed until day 139 of culture through immunohistochemical analysis of spermatogonial survival and proliferation markers, but the number of SCCs was drastically reduced. Supplementation with testosterone versus hCG made little difference in supporting differentiation or cell survival. Sertoli cells showed a steady loss of anti-mullerian hormone (AMH) expression, which is indicative of maturation. Sertoli cells began to establish a BTB by day 16 as highlighted by the expression of tight junction proteins; however, this expression pattern did not seem to be mature, and the authors suggested that this effect might be ascribed to a lack of hormone triiodothyronine (T3) in the medium. Testosterone production by Leydig

cells began by day 10 and lasted throughout maturation, although gradually dropping to ~75% by day 139, which the authors ascribed to insufficient cholesterol in the medium. No differences in Leydig cell activity was observed after supplementation of testosterone or hCG. The investigators concluded that the testicular fragments could be successfully maintained in culture for a long period and, importantly, cells showed signs of maturation; however, germ cell loss needed to be addressed.

In a follow-up study, DMEM/F12 supplemented with knockout serum replacement (KSR) and FSH with or without additional supplements including T3 and hCG was used[89]. Similar to what was observed in the previous study by the same group[87], Sertoli cells showed a reduction in AMH expression over time, indicating maturation. BTB tight junction proteins were not assessed, although T3 was added to the culture medium. Testosterone production by Leydig cells peaked at day 16 but was lost over time, and medium supplements did not affect this parameter. SSC numbers dropped drastically, but spermatogenesis was observed, with spermatocytes and round spermatids appearing in culture as early as day 16. The authors suggested that successful spermatogenesis might have been a direct consequence of a positive response of Sertoli cells to the adjustment of FSH amount in the medium, which was reduced tenfold to mimic physiological levels. However, no elongated spermatids were observed, indicating that Sertoli cell function was still impaired. The authors hypothesized that the accelerated rate of differentiation and the presence of round spermatids on day 16, compared with 22–24 days in vivo, might have been related to the substantial loss of SSCs. Thus, the authors suggested that the balance between SSC self-renewal and differentiation that occurs in vivo needs to be recreated in vitro.

In 2019, a study was carried out to assess whether fresh explanted prepubertal testicular tissues could be successfully cultured in vitro[93]. In this study, cryopreserved or fresh testicular tissue was collected from nine prepubertal patients (aged 6–14 years). Tissue fragments of 1 mm^3 were cultured for 5 weeks at the air–liquid interface in αMEM with or without the addition of FSH and luteinizing hormone (LH). In accordance with the results of previous studies[87,89], a considerable SSC loss was observed, which was monitored through the identification of proliferative spermatogonia by co-staining with markers of spermatogonia (MAGEA4) and proliferation (PCNA). In this study, no differences were observed in SSC loss between fresh extracted and cryopreserved tissues, showing that cryopreservation was not the cause of tubular deterioration and germ cell loss. The authors concluded that Sertoli cell maturation was incomplete as, although AMH expression declined, androgen receptor (AR) expression was absent, raising the interesting question of whether the loss of AMH expression on Sertoli cells observed in previous studies[87,88] should be interpreted as a sign of maturation or a sign of dysfunction.

In an interesting study published in 2020, IVS from prospermatogonia up to the round spermatid stage was performed using human fetal gonad tissue[94]. In this study, fetal testicular tissue organization and maturation occurred in vitro, generating functional round spermatids after 50 days. Gonadal tissue from 44 male fetuses (aged 12–19 weeks) were included in the study, and consisted of a mixture of early to late primordial germ cells. Tissue fragments of 3 mm^3 were cultured at the gas–liquid interface with supplemented αMEM. Overall, 21 tissue samples underwent apoptosis, whereas 23 samples formed organized tubules and began spermatogenesis. The authors ascribed the substantial apoptosis to variations in sample quality. The appearance of spermatocytes and spermatids in the viable tissue samples occurred by day 10 and day 30, respectively, and was monitored through immunostaining of cell-type-specific markers, observation of morphological features and flow cytometry. Seminiferous tubules formed from day 10 to day 50, and the maturation of Sertoli cells (defined by loss of AMH and gain of AR, as well as tight junction protein expression) occurred by day 30. Leydig cells also began to express enzymes related to testosterone production by day 30. After 50 days in culture, up to 7.24% of the population consisted of spermatids. The timeline of tissue morphogenesis, somatic cell maturation and spermatogenesis in this study seems to have been accelerated, and contrasts with the progression of events as expected in vivo, suggesting abnormal spermatogenesis. The authors hypothesized that, in this study, pro-spermatogonia bypassed the prepubertal quiescent period and underwent meiosis I and II to form spermatids in 30–50 days, which corresponds to the duration of the first and second meiosis in vivo. To assess genome integrity and copy number variations of the spermatids, single-cell sequencing of spermatid genomes was performed, and diploid fetal skin cells were used as a control. A total of ten spermatids were sequenced, of which nine had no abnormalities and one had a duplication event. The spermatid short tandem repeat (STR) profile was used to assess genetic variations resulting from chromosomal recombination, with the aim of assessing the meiotic function of spermatocytes; STR combinations in spermatids were different from those observed in the corresponding fetal somatic cells, confirming that chromosomal recombination occurred. The assessment of maternal and paternal gene imprinting in spermatids showed a comparable methylation status to that observed in vivo (paternal, 98.57% in vitro versus 95.71% in vivo; maternal, 0.0% in vitro versus 0.0% in vivo). Lastly, in this study, the fertilization capacity of round spermatids generated in vitro was tested through injection into oocytes. Among 16 injected oocytes, one embryo developed to the eight-cell stage and one to the blastocyst stage. Genetic parental analysis of the eight-cell stage embryo confirmed the presence of a single paternal and maternal allele, excluding the hypothesis of self-fertilization. In this study, the final stages of organogenesis followed by maturation and spermatogenesis up to the round spermatid stage occurred for the first time in vitro; however, the reason for the accelerated rate of spermatogenesis observed in this work is not completely understood and requires further investigation. Importantly, in this study, evidence of genetic diversity resulting from in vitro meiosis was also shown for the first time, and the normal genetic and epigenetic status as well as fertilization ability of the generated spermatids were confirmed.

In 2022, a study was carried out in testis tissue samples from four infant patients with cryptorchidism (aged 0.5–1.4 years) to assess whether medium conditions previously reported to support spermatogenesis in vitro[88] could support spermatogonia and somatic cell development[94,95]. In this system, testosterone and FSH levels in the medium were reduced to more physiological levels than previously obtained[88] and bovine pituitary extract (BPE) was replaced with human umbilical cord plasma to provide a xeno-free formulation; the medium formulation was tested with or without retinoic acid (RA). After 60 days, maturation of tubules, as indicated by increased diameter (although to subnormal levels), was observed both with and without RA; Sertoli cell maturation, as evidenced by increased AR and tight junction protein expression as well as decreased AMH expression, also occurred. Tight junction protein expression was indicative of BTB formation, but the pattern was disorganized. To our knowledge, this is the first study in which an increase in AR expression in testicular tissue obtained from a prepubertal patient has been shown. The authors hypothesized

that this phenomenon might be ascribed to differences in medium composition as well as to the young age of the tissue donors included in this study compared with the donors in previous work. Leydig cell steroidogenic function was maintained, and peritubular myoid cells began to appear in the cultures of tissue from three of the four patients, and with a higher percentage in tissue cultured without RA than with RA. However, spermatogonia diminished over time at a similar rate to that observed in previous organotypic culture studies of prepubertal tissue, and the furthest stage of spermatogenesis reached by day 60 was spermatocytes, in contrast to the observations in a previous study in which haploid germ cells were generated from immature testicular tissue after 16 days of culture[89]. Spermatocytes were identified through the expression of boule-like RNA-binding protein (BOLL), a late spermatocyte marker known to be decreased in patients with spermatogenic failure[96], which was observed in tissue from one of four patients cultured in RA-supplemented medium. Notably, in this study, germline and somatic cell maturation did not seem to be abnormally accelerated, differently to what was observed in a previous study[88], suggesting that 60 days in culture might have simply been a too short time to detect complete maturation and spermatogenesis. The authors concluded that further maturation is required to support spermatogenesis, and suggested that dynamic growth factor supplementation might be necessary to balance Sertoli cell proliferation and maturation in this process. Whether in previous studies[88], high testosterone and FSH concentrations or the use of BPE were responsible for prepubertal IVS remains unknown and deserves further investigation.

Bioreactors

In a 2016 study, adult human seminiferous tubule segments were cultured in a bioreactor system, and seemed to undergo complete spermatogenesis from the SSC stage to spermatozoa[97]. The tissues were obtained from five patients receiving gender-affirming treatment (aged 25–31 years) who had undergone treatment with oestrogens and antiandrogens leading to early spermatogenic arrest and inhibition of further spermatogonial differentiation. The tissues were not cultured in the typical air–liquid interface culture, but in a hydrogel bioreactor formed from a hollow chitosan cylinder. Seminiferous tubules, either freshly cultured or cryopreserved, were isolated by mechanical dissociation, and 20–50 mm^3 fragments were loaded into the internal luminal space of chitosan tubes and cultured in supplemented DMEM/F12 for 60 days. Testosterone supplementation was delayed until day 5. Chitosan is a natural polysaccharide derived from crustaceans, which possesses biocompatible, antimicrobial, antioxidant and tuneable sol-gel properties and, therefore, is often useful in tissue engineering applications[98,99]. In freshly prepared tubules isolated from one patient, spermatids appeared in culture as early as day 25, and spermatozoa by day 55 at a rate of 2–3.8%. Notably, on day 55, a new wave of spermatids was observed, indicating continuous spermatogenesis. Maturation to spermatids was also observed by day 24 using cryopreserved tubules isolated from another patient; however, spermatozoa appeared by day 34, earlier than observed in cultures from fresh tissues (day 55). The number of spermatozoa counted per bioreactor ranged from 5 to 20. The appearance of spermatozoa by day 55 could be expected according to the natural progression of spermatogenesis in vivo, whereas the appearance of spermatozoa by day 34 was surprisingly early, and might be explained by individual variances in the duration of the spermatogenic cycle. However, the possibility that spermatocytes or round spermatids were present on day 0 of culture cannot be excluded[100]. To date, the completion of spermiogenesis using bioreactors has not been replicated by independent research teams, which might be related to the patented nature of this approach[101]. Nevertheless, to date, this organotypic culture approach is the only system that has provided compelling evidence of spermatid elongation and maturation to spermatozoa (originating from SSCs, spermatocytes or round spermatids) outside 2D feeder systems. A possible explanation for the success obtained with this system might be the ability of the chitosan hydrogel to limit medium-based nutrient and cell-secreted growth factor exchange by reducing diffusion through the pores of the crosslinked matrix[102]. Owing to the presence of a porous scaffold, hydrogel-based bioreactors achieve close-to-physiological distribution and, in turn, activity of serum nutrients and hormones such as LH, FSH and testosterone, as well as cell-secreted growth factors that modulate the activity and differentiation of testicular cells[103].

Organotypic systems — considerations

To date, organotypic IVS systems have been used to culture tissues from prepubertal patients, who lack germ cells that have committed to differentiation[87–89,93]. One obstacle inherent to using IVS for prepubertal tissues is the induction of somatic cell maturation in vitro. Methods to reproduce the physiological testis microenvironment during the pubertal transition period remain elusive, although addition of bone morphogenetic protein 4 (BMP4) and BMP7, stem cell factor (SCF), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF) and activin A in the culture medium seems to have some benefit on somatic cell maturation[94,95]. Supplementation with gonadotropins and RA (also at physiological levels) failed to reliably induce meiosis and enable germ cell differentiation in organotypic cultures[87,89,93]. Passive molecule diffusion from the medium into the tissues might be ineffective, or perhaps traditional basal media lack metabolites, ions or nutrients required to support human testis function. Results from single studies showing long-term survival and complete spermatogenesis using adult hormone-treated or fetal gonad tissues have provided some insights into the conditions required for successful organotypic IVS[94,97], but whether these conditions can translate to culture of prepubertal tissues has yet to be investigated. Further independent investigation of these successful IVS models is warranted in order to replicate these findings and to identify the contribution of specific culture conditions to the maturation of germ cells and somatic cells to elucidate the requirements for IVS, with the aim of translating that knowledge to clinically relevant contexts, such as cultures of tissue from prepubertal individuals or patients with NOA.

3D scaffolds, 3D organoids and 3D bioprinted systems

An alternative approach to organotypic systems is to bioengineer the testicular niche (Fig. 2d–f). With this method, physiological tissue structures can be recreated in vitro, as intercellular signalling, diffusion and local concentration gradients of cell-secreted factors are recapitulated in the 3D histoarchitecture[104]. Thus, this approach could mimic in vivo tissue organization, but, differently from organotypic culture systems, cellular alterations or treatments can be performed before establishing the 3D cytoarchitecture. Moreover, the issue of cell viability is solved by diffusion of nutrients from the medium and oxygen through porous scaffolding.

3D scaffolds

In 2006, round spermatids were differentiated from SSC within 3D alginate gels, and showed fertilization capacity[105]. In this study, testicular

tissues from 11 patients with SCO and two patients with maturation arrest at the spermatocyte level were enzymatically digested and propagated in supplemented DMEM. After 2–4 weeks, colonies of SSCs appeared in all cultures of tissue from both patients with maturation arrest, and in four of 11 cultures of tissue from patients with SCO. To generate spermatids, >200 SSC colonies and somatic cells were collected from these cultures and mixed at high density into 1% alginate, a polysaccharide hydrogel derived from brown seaweed. Alginate is a bio-inert material with good diffusion and is therefore often used to encapsulate cells for the purposes of 3D culture or transplant[106]. After 4 weeks in a supplemented DMEM/F12 culture, alginate-encapsulated testis cells yielded both spermatocytes and spermatids. The functional capacity of spermatids was then evaluated by injection into human oocytes. Round spermatids were injected into 51 mature oocytes collected from six patients: 13 oocytes were injected with spermatids from patients with maturation arrest and 38 from patients with SCO, respectively. Overall, three cleaved embryos from the maturation arrest group (23%) and seven from the SCO group (18%) underwent uterus transfer, but all failed to implant. The authors ascribed this failure to incomplete spermiogenesis of the in vitro-derived spermatids.

In 2007, collagen, which was proposed as being a more biomimetic 3D matrix than alginate, was used to support IVS using testicular cells from patients with NOA[107]. Collagen is a natural component of seminiferous tubule basement membrane and was therefore hypothesized to be able to enhance germ cell maturation in vitro. A total of ten patients with complete spermatogenesis, seven with arrest at the spermatocyte stage and one with arrest at the round spermatid stage were included in the study. Collagen was mixed with fetal bovine serum, Matrigel, DMEM/F12 and testicular cells to create 3D cultures, which were grown in supplemented DMEM/F12 for 12 days. The presence of round and elongating spermatids was confirmed on day 12. An increase in the number of spermatids was shown in two of the samples from patients with maturation arrest, from 4.43% and 4.93% on day 0 to 28.02% and 30.87% on day 12, respectively (increases of 6.33-fold and 6.26-fold, respectively), with concomitant decreases in spermatocytes. Overall, these results suggested that collagen matrix holds promise as a 3D biomaterial for IVS. Notably, results from studies in which both alginate and collagen 3D scaffolds have been used to support IVS[96,97] have yet to be independently replicated by other research groups.

In 2018, another biomimetic Matrigel culture system was used to differentiate SSCs into round spermatids with fertilization potential[108]. In this study, cells were isolated from testicular tissue biopsies obtained from 60 patients with OA (aged 13–47 years), and differential plating was used to enrich SSCs and enable somatic cell attachment, followed by magnetic activated cell sorting using an antibody against adhesion G protein-coupled receptor A3 (GPR125), a membrane receptor expressed by SSCs[65]. Somatic cells were treated with mitomycin, a cytotoxic compound that inhibits DNA synthesis, to block proliferation. To create the 3D cultures, cells in DMEM/F12 were mixed with Matrigel at an SSC to somatic cell ratio of 1:3 and cultured for 20 days in supplemented DMEM/F12. Generation of spermatocytes and haploid spermatids occurred after 10–15 days and 20 days, respectively, and the proportion of haploid cells continued to increase from day 10 (1.6%) to day 20 (17.9%). A small proportion of spermatids (3%) had abnormal chromosomes (both X and Y chromosomes), whereas Y chromosome microdeletions were absent. Global gene expression profiling of human SSC-derived spermatids showed a 74.28% similarity with fresh round spermatids from patients with OA, whereas DNA methylation profiles showed similarity of 64% and 81% with fresh spermatocytes and round spermatids, respectively. The fertilization capacity of the generated spermatids was tested by injection into mouse oocytes: 50% of spermatids generated pro-nuclear embryos and developed to the two-cell stage, 17.86% of which further developed to the four-cell and eight-cell stages. Global gene expression profiles of these embryos showed a 41.7% transcriptional activation of the human paternal genome, indicating a marked expression of the human genome in the hybrid embryos generated from human SSC-derived spermatids. Positive staining of the hybrid embryos for human nuclear antigen (HumNuc) and H3k9 trimethylation (H3K9me3) further confirmed human-specific gene activation. These findings are promising, but are limited to patients with OA; moreover, the reason behind the accelerated development of spermatids (appearing within 20 days) is still unaddressed. Similar to the observations using a bioreactor[97], the presence of spermatocytes in cultures on day 0 cannot be ruled out; thus, whether these spermatids matured from SSCs or spermatocytes is debatable.

3D organoids

Some bioengineered systems exploit the innate regenerative qualities of testicular cells to regenerate the physiological spermatogenic niche. In a 2018 study, adult human testicular cells were shown to be able to spontaneously generate de novo multicellular tissues[109]. In this study, testis biopsies from 14 patients with complete spermatogenesis, one patient with maturation arrest and one patient with SCO were enzymatically digested into cell suspensions and cultured for 14 days in supplemented DMEM. Using time-lapse microscopy, cells were shown to have clustered into aggregates by day 5, which then fused into larger aggregates. Multicellular configuration and morphologies of the aggregates observed through transmission electron microscopy imaging were similar to those observed in vivo, and SSC round and elongating spermatids, peritubular myoid cells, endothelial cells, Sertoli cells, Leydig cells and macrophages were identified. No tissue-like organization was achieved, but these results provided evidence that human adult testicular cells retain strong cell–cell morphogenic signalling activity, suggesting that tissue-like self-organization of these cells might be possible using appropriate in vitro culture conditions.

In 2019, prepubertal human testicular cells from two patients (aged 6 months and 5 years) were shown to form organoids complete with basement membrane[110]. To generate organoids, testicular cells were placed into arrays of 400 µm diameter V-shaped microwells and centrifuged to produce uniformly sized aggregates of ~500 cells each. These aggregates were cultured for 5 days in supplemented DMEM/F12. The organoids seemed to assemble in an 'inside-out' manner, with basement membrane near the surface of the spherical organoids to separate peritubular myoid cells from Sertoli cells, but with peritubular myoids aggregated in the organoid cores and Sertoli cells on the surface. However, this study provided the only reported evidence of the ability of human testicular cells to regenerate organized basement membrane cytoarchitecture in vitro.

The generation of human testicular organoids from testicular cells expanded in vitro was first reported in 2017 (ref. [111]). In this proof-of-concept study, testicular tissues supporting active spermatogenesis from three adult donors after brain death (aged 56–61 years) were sorted into SSC, Sertoli cell and Leydig cell populations and propagated in vitro. Leydig and Sertoli cells were immortalized to ensure prolonged ability to divide using a lentivirus expressing human telomerase

reverse transcriptase (*hTERT*), a gene that supports the elongation of telomeres, enabling dividing cells to replicate indefinitely. To generate organoids, SSCs, Sertoli cells and Leydig cells were combined at a ratio of 8:1:1 with solubilized human decellularized testicular matrix collected from the same patients. Droplets were suspended in 96-well hanging drop plates facilitating aggregation by gravitational forces. After 48 h, organoids were cultured in non-adherent U-bottom plates for 23 days in a medium optimized for SSC culture. Over the course of organoid formation, SSCs gathered in the cores of the spherical organoids, but no tissue-like organization was observed. Spermatids accounted for 0.2% of cells, whereas SSCs survived and accounted for 52% of cells, perhaps owing to the use of optimized SSC growth medium. After 23 days, the authors observed increased expression of genes specific to the spermatid stage through digital PCR, but a similar increase in the expression of genes specific to meiotic spermatocytes (such as *SYCP3*) was not observed, and this limitation needs to be further investigated.

3D testicular organoids generated from testicular cells in vitro include functional, viable and steroid-producing somatic cells that model the in vivo testicular microenvironment. These organoids have clinical applicability both as a screening tool to monitor the effects of cytotoxic therapies and as instruments to support germ cell differentiation.

3D bioprinting

Organoids are inherently smaller than seminiferous tubules, and are spherical rather than tubular, limiting the potential to regenerate the testicular niche. 3D bioprinting is a new tool to regenerate human tissues with increasingly physiological architectures, promoting complexity and functionality similar to those observed in vivo[112–115].

In 2022, a 3D bioprinted niche was obtained for the first time[116]. This study was a proof of principle of the feasibility of bioprinting human adult testicular cells obtained from patients with NOA and expanded from a single biopsy. Using a microfluidic 3D bioprinter fitted with a coaxial printhead, adult testicular cells obtained from a patient with NOA could be propagated in vitro without immortalization and printed in alginate–collagen bioink without losing viability, basic phenotypes or spermatogenic potential. In extrusion bioprinting, biomaterials and cells are combined and extruded by pressure through a nozzle to create a continuous fibre, which is deposited into a desired 3D design. Graphic software converts a 3D image into lines and layers, which are then translated into computer code that instructs the printer arm. An adaptation of this form of printing is microfluidic extrusion printing, in which microfluidic nozzles, named printheads, are used instead of conventional cone-shaped nozzles[117]. Printheads are made of biocompatible polymers with embedded microchannels that enable the extrusion of multiple biomaterials simultaneously and the creation of fibres with complex composition[117]. The coaxial printhead used in this study[116] incorporated separate microchannel inlets for shell and core biomaterials, which converged into a single microchannel in which velocities were precisely controlled to enable the shell biomaterial to incorporate the core biomaterial and produce a core–shell fibre. Before printing, SSCs were enriched by differential plating, and the resulting cultures enriched in somatic cells and SSCs were combined at an SSC to somatic cell ratio of 1:4 into an alginate–collagen bioink and printed into ~500 µm coaxial fibres. Bioink was used as a core material and rinsed away after printing. Printed tubules were cultured for 12 days in supplemented StemPro-34 serum-free medium. The printing process did not substantially affect cell viability (93.4 ± 2.4% viable cells) or phenotypic protein markers for Sertoli, Leydig, peritubular myoid and germ cells[116]. Differentiation was attempted (although over a short period (12 days)) to assess the ability of the bioprinted cells to functionally respond to RA and the Sertoli-cell-specific growth factors SCF and BMP4, as these factors are known to induce meiosis in differentiating spermatogonia. The cell responses were measured comparing gene expression levels of spermatogenesis markers (assessed through relative quantitative PCR) on day 0 of culture with levels observed on day 12 and in non-bioprinted organoids generated in parallel[116]. Targeted genes were stage-specific spermatogenic markers for undifferentiated spermatogonia, differentiating spermatogonia, differentiating spermatogonia entering into meiosis, meiotic spermatocytes, round spermatids and elongating spermatids. Post-meiotic gene expression has been reported to cease beyond the round spermatid stage, as histones are swapped for protamines to enable genome condensation; thus, transcripts for subsequent stages of spermiogenesis are produced early and stored for late translation[118,119]. Similarly, results from in vitro studies showed that the expression of meiotic and post-meiotic transcripts can be upregulated as early as at the differentiating spermatogonia stage in preparation for subsequent events[120,121]. This evidence suggests that gene expression precedes differentiation events in human germ cells. Thus, in this study[116], expression of meiotic and post-meiotic genes in response to RA stimulation was presented as evidence of the onset of germ cell differentiation. Meiotic spermatocytes were observed on day 12 through positive immunofluorescence staining of the meiotic protein SYCP3. Some spermatocytes are known to exist in tissues from patients with NOA; thus, the presence of spermatocytes was not presented as definitive evidence of differentiation within the bioprints, but is promising evidence in combination with the substantial upregulation of meiotic and post-meiotic genes over the 12-day culture period. Moreover, functional genes considered specific markers of undifferentiated and differentiating spermatogonia were upregulated, indicating the presence of functional spermatogonia populations. However, all cells seemed suspended and rounded in the alginate–collagen bioink, indicating that new biomaterials are needed to promote cell–matrix interactions. These results are preliminary and limited to one patient with NOA; however, this study provides promising evidence for the culture of 3D bioprinted human testicular tissues and establishes a baseline for future optimization of bioink components and bioprinting techniques.

3D scaffolds, 3D organoids and 3D bioprinted IVS systems — considerations

To date, 3D systems have been focused on recreating cell–matrix or cell–cell relationships to reinitiate germ cell maturation in vitro. Notably, regardless of patient age, human testicular cells expanded in vitro with 3D systems show functions necessary for tissue morphogenesis, such as chemotaxis and growth[109–111]. A major challenge remains to identify the correct conditions to support de novo organization of these cells into tissue-like architectures. Another major challenge will be to improve techniques or controls for tracking differentiation from adult tissues to provide reliable proof of in vitro differentiation and identify cell stages originally present when cultures are established. Thus, 3D systems in combination with advances in tissue engineering techniques have the potential to support the realization of complete in vitro reconstruction of testicular tissues, which could in turn facilitate a personalized medicine approach to IVS in which patient tissues are separated into cell components, dysfunctional cell types are rescued or replaced, and functional tissues are rebuilt in vitro.

Lessons on successful IVS from animals

Success in animal IVS systems has informed study design choices in human IVS systems. The first complete IVS from mouse testicular organotypic culture was achieved in 2011 (ref. [23]). In this study, the addition of KSR to the medium was crucial to mouse IVS, and promoted spermatogenesis maintenance for 2 months and the generation of fertilization-competent sperm. AlbuMAX was identified as the main active component of KSR and was alone sufficient to promote mouse IVS. Several lipids and antioxidants in AlbuMAX were subsequently identified as crucial for spermatogenesis, such as the combination of vitamins A and E with glutathione, and lysophospholipids[55,56]. T3 was identified as the most important hormonal supplement, as T3 was shown to be necessary to induce AR expression in Sertoli cells and acrosin in spermatids[56]. Authors from the same group also explored the use of microfluidic flow to provide improved realistic nutrient–waste exchange in vitro[53]. Results from this study showed that seminiferous tubules function better when placed adjacent to flow than in direct flow, with nutrient and waste exchange occurring through passive diffusion. Mouse seminiferous tubules cultured in the presence of KSR remained functional for 6 months and generated sperm yielding healthy offspring. Translation of this technique in other species was unsuccessful until the first report was published in 2021. In this study, spermatogenesis could be maintained for 70 days in rat seminiferous tubules by lowering oxygen concentration in culture[54]. Indeed, in vivo, seminiferous tubules are distant from blood vessels; thus, diffusion and oxygen consumption by interstitial cells create an oxygen concentration gradient resulting in a hypoxic intratubular environment[122], which might directly stimulate seminiferous tubule function. This hypothesis is supported by results from transcriptomic profiling of mouse Sertoli cells, which showed that two of the three identified potential master regulators of Sertoli cells are hypoxia-induced transcription factors[123]. Similarly, in another study, rat IVS using a chitosan bioreactor system was successful, and effectively reduced diffusion and, in turn, the exchange of factors and oxygen between the medium and the tubules. Notably, in this system, IVS was completed without the use of KSR, indicating that KSR in this context might not be as crucial to IVS as in larger mammals. Using the same chitosan bioreactor-based system, IVS was successful in human cultured tubules from patients undergoing gender-affirming therapy, but with substantially reduced efficiency, suggesting that optimization of diffusion kinetics might be crucial for human IVS.

Using a mouse model system, mouse embryonic stem cells (mESCs) were successfully directed to form functional differentiated spermatozoa in a time frame that was consistent with expected in vivo programming[124]. Mouse primordial growth cell-like cells (mPGCLCs) induced from mESCs were cultured using an optimized neonatal testis somatic feeder culture system (rTestis), through which a robust yield of mouse spermatogonium-like cells was generated. The germline stem cell-like cells (GSCLCs) propagated from spermatogonia were ultimately transplanted into testes of infertile mice and showed efficacious rates of spermatogenesis; ICSI using these spermatozoa generated karyotypically normal offspring[124]. Importantly, in this study[124], GSCLCs produced under optimized culture conditions showed a methylome profile similar to that of native germline stem cells, particularly amongst promoter regions known to influence spermatogenic function and meiosis. The authors hypothesized that this hypermethylation contributes to impaired spermatogenesis, highlighting the crucial role of epigenetic modifications in appropriately directing spermatogonial differentiation. Specifically, in this study[124], the use of nearly completely demethylated mPGCLCs was shown to be crucial for achieving proper differentiation. This epigenetic blank state was accomplished through culturing mPGCLCs with the small molecules forskolin, rolipram and cyclosporin A for 5 days before culture in the rTestis system. Future work is needed to determine whether the induction of this epigenetic blank state might correct the abnormally accelerated rate and subsequent spermatid loss observed during hiPSC and hESC differentiation in currently available studies[83].

Bioengineered animal systems supporting IVS have not been described to date. However, results from two publications showed in vitro recreation of a niche-like cytoarchitecture from prepubertal piglet testicular cells with very high similarity to in vivo testis[70,71]; in one of these studies, vascularization and capsulation were also achieved[71]. In the first study, organoids were generated in collagen or decellularized extracellular matrix, and cultured at the air–liquid interface in supplemented medium[70]. In the other study, organoids were generated in the absence of biomaterials and were grown in supplemented medium either at the air–liquid interface or fully submerged[71]. This work showed that air–liquid interface culture is necessary for organoid organization[71]. Together, the results of these studies suggest that prepubertal tissues have unique properties amenable to organoid generation, which might explain why in the only study in which tissue-like reorganization in a human organoid system was reported, prepubertal tissues were used[110]. However, germ cell production did not occur in either these studies[66,67], although organoids had a niche-like appearance. The authors hypothesized that this evidence might be ascribed to failure of SSCs to migrate into the tubules during organoid morphogenesis, with consequent apoptosis[70].

Medium considerations

In most human IVS studies, KSR[89,93,94,108,109] and antioxidants[83,87–89,97,105,107,109,111,116] are added to the culture medium, considering the success in achieving spermatogenesis obtained using these factors in mouse model systems[23,53]. The preferred energy substrates of spermatocytes and spermatids[125–127] — pyruvate[87–89,97,105,107,111,116] and lactate[111,116] — were also added to several medium formulations to support spermatogenesis and spermiogenesis. Sertoli cell-derived growth factors such as FGF2 (refs. [83,109,111]), GDNF[83,89,94,109,111], SCF[108,111,116] and BMPs[94,108,116] are frequently added to culture media to maintain SSC survival and self-replication. In a few studies in which high SSC numbers in vitro were observed, putrescine was used[83,111,116], which is required by type B SSCs for DNA replication during mitosis, but might also inhibit meiosis if added in excessive amounts to preleptotene spermatocytes[128]. Retinol and its derivative RA are known to stimulate SSC entry into meiosis[129–131] and to regulate BTB function[129,130,132] and are therefore frequently added to culture media[87–89,93,95,97,105,107,108,111,116]. However, RA addition to fetal organotypic cultures caused cell death and tubular deterioration[94]. Notably, meiosis was able to initiate in the absence of RA, although at an abnormally accelerated rate, suggesting that RA might not be necessary in this process. Similarly, in another study, RA and retinol were unnecessary to achieve spermatid differentiation from hiPSCs, which, however, occurred at an accelerated rate[83]. Conversely, when RA was added to organotypic cultures of infant tissue, no degenerative effects were observed, and RA seemed to be necessary for the induction of meiosis in this context[95]. These conflicting results indicate that the action of RA might differ based on the age of the tissue.

Studies in which organotypic cultures of fetal and infant tissue were used provided compelling evidence for in vitro tubule maturation

induced by the addition of the growth factors activin A, BMPs, EGF, basic FGF (bFGF) and SCF to the culture medium[94,95]. These factors in combination with hormones at physiological levels promoted only partial tubule maturation in organotypic cultures of infant tissue[95]; however, successful maturation was observed in organotypic cultures of fetal tissue supplemented with an unusually high amount of FSH and testosterone[94], suggesting that the levels of hormones to be added in vitro might be higher than previously assumed. In organotypic cultures of prepubertal tissue not supplemented with activin A, BMPs, EGF, bFGF and SCF, hormonal supplementation had no effect on maturation[87–89,93]. This ineffectiveness was hypothesized to be a consequence of receptor desensitization, as Leydig and Sertoli cells are known to undergo dose-dependent loss of FSH and LH activity and expression following stimulation[133–137], which leads to a period of insensitivity[89], suggesting that prolonged supplementation of hormones in cultures of prepubertal tissue might be ineffective. Taken together, these studies point towards a need to optimize hormonal concentration and timing as well as growth factor composition to have an improved understanding of the role of these factors in supporting in vitro germ cell maturation from prepubertal tissues.

Overall, analysis of medium factors tested to date (Box 1) shows that molecular requirements for human IVS are not completely understood, and further examination and refinement of medium supplementation for human IVS are warranted. Together with medium factors, basal media should also be optimized towards physiological formulations to improve cell function support. MEMs are widely used in cell culture, but were originally formulated to support mouse embryonic cells and therefore do not resemble human plasma in terms of inorganic salt, amino acid, metabolite or nutrient content. Thus, the ineffectiveness of medium supplementations observed to date in promoting human IVS might be in part ascribed to cell function impairment caused by the standardized use of non-physiological media.

Box 1

Tested medium components to promote human in vitro spermatogenesis

Base media
- MEM
- αMEM
- DMEM/F12
- StemPro-34

Vitamins
- Retinoic acid
- Retinol
- Vitamins C and E
- MEM vitamin solution
- D-Biotin

Hormones
- FSH
- LH
- Testosterone
- T3
- DHT
- hCG
- Metribolone
- Prolactin
- Melatonin
- Oestradiol
- Progesterone
- BPE

Growth factors
- FGF2
- GDNF
- BMP4
- BMP7
- SCF
- EGF
- Activin A
- IGF1
- LIF

Energy substrates
- Glucose
- Pyruvate
- DL-Lactic acid

Other biomolecules
- Putrescine
- Insulin
- Transferrin
- Selenium
- Glutamine
- Solubilized dECM
- HEPES
- Lipids (palmitoleic acid, palmitic acid, oleic acid, stearic acid, linoleic acid)
- 22(*R*)-Hydroxycholesterol
- Solubilized dECM
- MEM NEAA

BMP, bone morphogenetic protein; BPE, bovine pituitary extract; dECM, decellularized extracellular matrix; DHT, dihydrotestosterone; DMEM/F12, Dulbecco's modified Eagle medium/nutrient mixture F12; EGF, epidermal growth factor; FGF, fibroblast growth factor; FSH, follicle-stimulating hormone; GDNF, glial cell-derived neurotrophic factor; hCG, human chorionic gonadotropin; HEPES, hydroxyethyl piperazineethanesulfonic acid; IGF, insulin-like growth factor; LH, luteinizing hormone; LIF, leukaemia inhibitory factor; MEM, minimum essential medium; MEM NEAA, MEM with non-essential amino acids; SCF, stem cell factor; T3, triiodothyronine.

Future directions

An impressive body of work has been dedicated to developing complete human IVS; however, the establishment of a fully characterized system for clinical use remains an open challenge. Additional research is required to generate a robust model to recreate the native testicular microenvironment and spermatogenic functionality.

3D-bioprinting, bioinks and microspheres

The most convincing evidence for complete spermatogenesis to date was perhaps provided by experiments using organotypic cultures[94,97], suggesting that the cytoarchitecture of the testicular niche is important for function. However, the inability to manipulate specific cells renders organotypic cultures unsuitable to rescue a spermatogenic phenotype in patients with NOA; results from organoid studies showed that testicular cells have de novo assembly ability[109–111], but, to date, regeneration of a disrupted tissue cytoarchitecture has never been shown. Tissue reconstruction using 3D bioprinting technology might help overcome this barrier, as 3D bioprinters have the ability to build biomaterials into desired geometries with micrometre resolution[138–140] and single-cell precision[141–143]. Using this technology, hiPSCs could simultaneously differentiate into multiple pre-programmed cell types upon induction[112]. Additionally, the use of smart bioinks might be essential to create sustained or targeted signalling necessary for complex tissue regeneration[144]. For example, the results from a series of studies by a single group showed that sustained local release of signalling factors from drug-loaded particles embedded within 3D bioprinted constructs including neural progenitors generated more complex neural tissues than those obtained through conventional cultures[145–148]. Depending on the drug, mature dopaminergic or spinal cord neural tissues were generated and completed with non-neuronal support cells such as astrocytes and oligodendrocytes, which are known to enhance neural maturation, plasticity and electrophysical properties[145,148]. The shape, size and surface properties of drug-releasing particles can prolong local

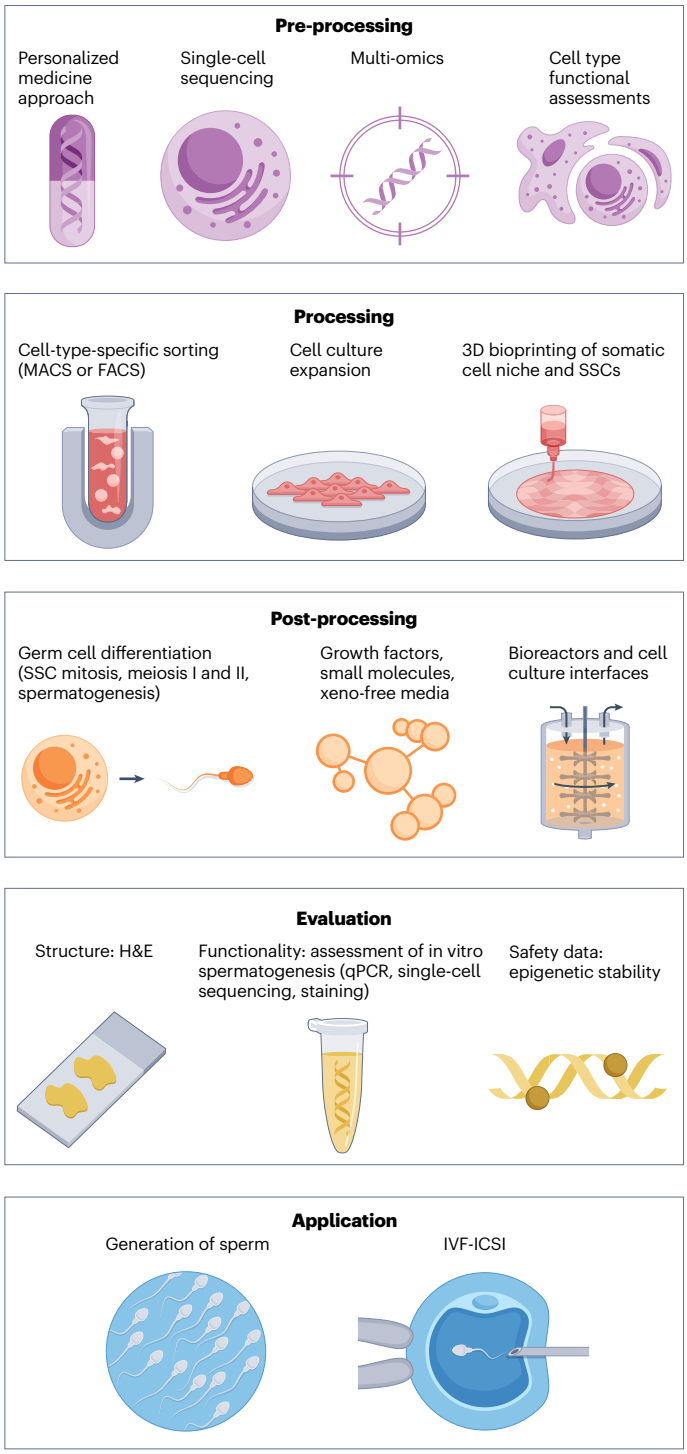

Fig. 3 | Proposed personalized and precision medicine workflow for restoring sperm production using IVS. Sequential phases of development describing the methodological components necessary to achieve in vitro spermatogenesis (IVS) and to translate this technology into clinical practice; these phases include pre-processing, processing, post-processing, evaluation and application. During pre-processing, cellular function and dysfunction within the testicular niche need to be understood through a personalized and precision medicine framework in which single-cell RNA and epigenetic-based sequencing techniques can be used to characterize patient-specific and testicular-cell-type-specific function and dysfunction. The processing phase consists of sorting specific testicular cell types from a patient's testis biopsy, and expanding these cells in vitro to potentially recreate a functional niche using 3D organoids, 3D bioprinting or other culture systems. During post-processing, culture conditions must be optimized by selecting the necessary growth factors and establishing culture system arrangements to facilitate survival and progression of germ cell differentiation into haploid, elongated spermatids. For the evaluation phase, the states of spermatogenesis and spermiogenesis must first be assessed through techniques such as haematoxylin and eosin (H&E) staining, analysis of cell-type-specific protein and RNA markers, and single-cell RNA sequencing; spermatid functionality might then be evaluated using sex-chromosome-specific fluorescent in situ hybridization probes and flow cytometry with nuclear stains to confirm the haploid state, and single-cell DNA sequencing to assess short tandem repeat (STR) and confirm the success of homologous recombination during meiosis[94]. Bisulfite sequencing might also be considered to assess the epigenetic methylation status of the resultant spermatids. Copy number variants and STRs in the resultant embryo can be used to validate the occurrence of fertilization of the oocyte with paternal and maternal DNA contributions. The application phase includes the selection and use of spermatozoa for in vitro fertilization–intracytoplasmic sperm injection (IVF-ICSI). FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting; qPCR, quantitative PCR; SSCs, spermatogonial stem cells. Contextual framework adapted for human spermatogenesis from ref. [172], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

drug bioavailability, and can also be tailored to enable targeted drug transport and delivery through tight junction barriers, cell membranes or specific cell receptors[149]. Similarly, particle properties can be chosen to introduce unique characteristics to the bioink microenvironment to support tissue function. For example, cardiac bioinks can be made electrically conductive through the addition of gold nanorods to improve synchronization of regenerated cardiac tissue[150], whereas addition of silver nanoparticles can give bioinks antimicrobial activity for wound healing applications[151,152].

In vitro rescue of somatic cell functions in patients with NOA

The aetiology of NOA is heterogeneous, but results from ongoing research suggest that defects in somatic cell functions are involved in NOA in many patients[153]. Thus, IVS success will depend on recovery of the tissue architecture, but also on the rescue of somatic cell function in testicular cells derived from patients with NOA. Amongst all somatic cell types, Sertoli cell dysfunction contributes the most to NOA[154]. Sertoli cells derived from testes of patients with NOA show abnormal morphology[155] and reduced expression of SCF and GDNF[156–159]. Aberrant differentiation, function and number of Leydig cells have also been implicated in infertility: in the pathogenesis of Klinefelter syndrome, stalled maturation and hyperplasia of Leydig cells contribute to the creation of a microenvironment with reduced capacity to support spermatogenesis[160–162].

Considering that ~25% of patients with NOA harbour an identified genetic abnormality, gene editing through CRISPR–Cas9 technology is a promising tool to investigate and rescue genetic dysfunctions in patients with NOA[163,164]. In combination with in vitro models, CRISPR–Cas9 technology can provide a means to identify transcriptional dysfunction in NOA cells through repression, activation or epigenetic modification of specific genes. For example, CRISPR–Cas9 was used to delete microRNA 202 (miR-202) in mouse SSCs, highlighting the role of this microRNA in SSC self-renewal[165]. In another study, CRISPR–Cas9 was used in mice to correct point mutations in the gene *Kit* and restore

fertility[166]. In these mice, impaired Kit function resulted in infertility owing to low SSC numbers and impaired BTB assembly[166]. Following repair of the *Kit* point mutations by CRISPR–Cas9, SSCs were transplanted back into the mice and fertility was restored[166]. Importantly, no off-target mutations were detected. These results are promising, but genome editing of human germ line cells is currently limited by the lack of regulatory guidelines addressing ethical concerns inherent to genetic modifications of the human germline, as well as by the need for technical improvements, such as the elimination of mosaicism and off-target effects[163]. A short-term clinically translatable solution might be CRISPR–Cas9-based correction of dysfunctional somatic cells to create a supportive testicular microenvironment in IVS systems derived from patients with NOA. Furthermore, scRNA-seq analyses might help identify transcriptional regulators and molecular pathways that control cell-type-specific dysfunctions, and might provide novel opportunities to rescue NOA cell-type-specific phenotypes through in vitro modulation of these identified transcriptional regulators and molecular pathways with different methods including CRISPR–Cas9-based gene editing or small-molecule agonists and antagonists[167]. For example, results from scRNA-seq experiments have shown heterogeneity in somatic cell function among testes from patients with NOA compared with testes from healthy individuals, with the highest difference in terms of gene expression profiles observed in Sertoli cells[154]. The transcriptome of Sertoli cells obtained from men with idiopathic NOA was then shown to be similar to that of immature tissues in which stem cell maintenance signalling (such as the WNT–β-catenin pathway) is aberrantly hyperactive[154]. Considering these data, WNT–β-catenin pathway inhibitors were hypothesized to be able to rescue the dysregulated Sertoli cell phenotype observed in patients with NOA; indeed, monolayer cultures of Sertoli cells from patients with idiopathic NOA exposed to a WNT–β-catenin pathway inhibitor showed decreased proliferation and expression of mature Sertoli cell transcription factors[154]. Thus, the inclusion of a personalized and precision medicine approach to regenerate sperm production might be required in future workflows for IVS (Fig. 3).

Ethical and safety measures

The promising applications of in vitro gametogenesis for research and clinical use in reproductive technologies must be carefully balanced with the ethical concerns and safety measures related to reproductive tissue engineering. First, media and biomaterials will require xeno-free components before clinical translation in humans to avoid variation in quality as well as the risk of transmitting animal-borne pathogens[168–170]. Additionally, the current understanding of cellular mechanisms and developmental checkpoints that occur during spermatogenesis in vivo is incomplete; thus, experiments in vitro carry the risk of producing abnormal sperm[163,164], and the confirmation of sperm normalcy is a necessary step before potential clinical trials can be considered. Thus, guidelines involving the use and destruction of human zygotes must be introduced to provide a framework for ethical practices[171].

Conclusions

Restoring sperm production as a potential therapeutic strategy for patients with infertility is urgently needed. To date, several approaches have been used to model human IVS. Organotypic and organoid cultures highlighted the importance of the testicular niche and, perhaps, have been the most promising approaches to support testicular microenvironment assembly and spermatogenesis[94,97,111]. However, in most studies to date, robust completion of spermiogenesis was not shown, and was limited in part by the need to optimize medium factors for delivery of physiological nutrients in vitro, and to recreate the testicular histological architecture and cell–cell signalling required for differentiation. Several promising technologies such as 3D organoid and bioprinted IVS systems are emerging with the potential to bring IVS technology close to clinical translation. However, numerous safety considerations and evaluations including the use of xenofree materials and the establishment of safety checkpoints to confirm the generation of genetically, epigenetically and morphologically normal sperm will be necessary to achieve direct application of these technologies to the clinical sphere.

Published online: 07 February 2023

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Acknowledgements
The authors thank A. Piechka for her illustrations used in this manuscript.

Author contributions
The authors contributed equally to all aspects of the article.

Competing interests
The authors declare no competing interests.

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Peer review information *Nature Reviews Urology* thanks H. Sadri, S. Schlatt and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.