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# Synthetic human gonadal tissues for toxicology

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ABSTRACT

The process of mammalian reproduction involves the development of fertile germ cells in the testis and ovary, supported by the surrounders. Fertilization leads to embryo development and ultimately the birth of offspring inheriting parental genome information. Any disruption in this process can result in disorders such as infertility and cancer. Chemical toxicity affecting the reproductive system and embryogenesis can impact birth rates, overall health, and fertility, highlighting the need for animal toxicity studies during drug development. However, the translation of animal data to human health remains challenging due to interspecies differences. *In vitro* culture systems offer a promising solution to bridge this gap, allowing the study of mammalian cells in an environment that mimics the physiology of the human body. Current advances on *in vitro* culture systems, such as organoids, enable the development of biomaterials that recapitulate the physiological state of reproductive organs. Application of these technologies to human gonadal cells would provide effective tools for drug screening and toxicity testing, and these models would be a powerful tool to study reproductive biology and pathology. This review focuses on the 2D/3D culture systems of human primary testicular and ovarian cells, highlighting the novel approaches for *in vitro* study of human reproductive toxicology, specifically in the context of testis and ovary.

## 1. Introduction

The process of mammalian reproduction begins with the development of germ cells in the reproductive organ, namely the testis and ovary. These germ cells mature with the support of surrounding cells in the gonad, becoming functional, and capable of fertilization. Following fertilization, the embryo undergoes development within the mother's womb, eventually resulting in the birth of a baby who inherits the parental genome information. The germ cells of this offspring then undergo development in the gonad, perpetuating the reproduction cycle. This process is conserved across species and involves cooperation among various cells and functions within the body, and any disturbance in this process may cause several disorders, such as infertility and cancer.

Reproductive toxic agents that have detrimental effects on the

*Abbreviations:* FDA, Food and Drug Administration; 2D, Two-dimensional; 3D, Three-dimensional; BPE, Bovine pituitary extract; ITS, Insulin-Transferrin-Selenium; OSE, Ovarian surface epithelium; EGF, Epidermal growth factor; HCG, Human chorionic gonadotropinh; ECM, Extracellular matrix; FCS, Fetal calf serum; FF, Follicular fluid; LH, Luteinizing hormone; CAMP, Cyclic adenosine monophosphate; IGF1, Insulin-like growth factor-1; SCF, Stem cell factor; SSCs, Spermatogonial stem cells; LCs, Leydig cells; FBS, Fetal bovine serum; BTB, Blood-testis-barrier; SLCs, Stem Leydig cells; GDNF, Glial cell line-derived neurotrophic factor; LIF, Leukemia inhibitory factor; TGF-β, Transforming growth factor beta; BFGF, Basic fibroblast growth factor; PSCs, Pluripotent stem cells; IPSCs, Induced pluripotent stem cells; HPGCLCs, Human primordial germ cell–like cells; BMP4, Bone morphogenetic protein 4; RA, Retinoic acid; PCOS, Polycystic ovary syndrome; SAG, Smoothened agonist; DbcAMP, Dibutyryl cAMP; DHH, Desert hedgehog; PDGF, Platelet-derived growth factor; MII, Meiotically competent metaphase II; Cs, Chitosan; SF, Silk fibroin; DECM, Decellularized extracellular matrix; SERPINB2, Serpin Family B Member 2; CYP450, cytochrome P450; EDCs, Endocrine disrupting chemicals.

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reproductive system and embryo development can significantly impact the birth rate, healthy life and infertility, and ultimately leading to a decline in the number of new babies, which is recently concerned as one of the global issues [1]. It has been estimated that preclinical toxicity is responsible for approximately 20 % of drug attrition prior to clinical trial [2]. These attritions were mainly due to the toxicity on cardiovascular (27.3 %) and liver (14.8%), though the developmental and reproductive toxicity issues account for more than 10 % of preclinical toxicology-related attrition [2]. This emphasizes the value of animal safety studies in reducing risks to patients and avoiding extra cost in clinical trials. However, despite the clear evidence on the benefit of animal toxicity studies, drug companies still face significant challenges, with high compound attrition rates (resulting in over 1 billion U.S. dollars invested per marketed drug). Reports suggest that only approximately 10% of phase 1 candidates are expected to gain approval from the U.S. Food and Drug Administration (FDA) [3–5]. These statistics highlight the significant unpredictable problems existing in the current process of drug discovery and development.

The study of the human reproductive system largely relies on the knowledge gained using animal models. While animal models offer many opportunities for translational discoveries, animal models only partially model human-specific features including receptor expression [6], metabolism [7], ovulation rate and cycle length [8]. Moreover, developmental differences also exist among mammals [9,10]. For example, a well-known environmental chemical, bisphenol A, works as a chemical agonist against an estrogen receptor so that the extent of its effect highly depends on the level of estrogen receptor expression. In addition, the difference of developmental timing between animal models and human alter the response of reproductive toxicants *in vivo* depending on the timing of exposure.

Since the introduction of the principles of the 3Rs (reduction, refinement, and replacement) for regulatory risk assessment involving animals, several organizations have emerged with the aim of promoting humane science and advocating for the utilization of alternatives to animals in research, for instance product safety testing. However, studying the human reproductive tissues raises another ethical concerns. Fetal development, fertilization, spermatogenesis, and ovulation, all these are only open for a certain limited window only with aborted fetal materials, thereby highly inaccessible in humans. Even when *in vivo* samples are obtainable, they are mostly collected for clinical purposes (*e.g.,* diagnosis) and may not represent suitable traits for studying natural physiology due to potential genome mutations or functional disorders. As a result, there is a growing desire for alternative experimental models that can recapitulate human reproductive physiology and assess chemical compound toxicity in the human body.

The *in vitro* culture approaches used to model human reproductive biology have expanded from primary cell cultures and tissue or organotypic culture to more recent organoid or 3D tissue culture from human stem cells (Summarized in Table 1). Traditionally, researchers have also utilized immortalized cell lines to understand the role of genetic and molecular alterations in various diseases. These cell line-based two-dimensional (2D) culture system elucidated key insights in understanding molecular signals to dissect cell intrinsic processes of human reproduction [11,12] (Table 2). Alternatively, to study more complex cell-cell communication among different types of cells, rapid advancements in the three-dimensional (3D) bioengineering have broaden the opportunity to model their intricate physiological multicellular topology (Table 3) [13]. This emerging principle offer a wide range of potential applications and approaches for the study of human reproduction [14]. Current bioengineering strategies can be discussed around three key components: matrices, culture method and device (Fig. 1). Combinatorial usage of these components can be applied to advance reproductive tissue culture in 3D. Among these components, organoid culture are particularly noteworthy as they can replicate microarchitectural components of native organ systems *in vitro*. The most fundamental and essential characteristic of organoids is their self-organizing potential, enabling them to naturally instruct collective cell behaviors to build a 3D structure [15]. The complexity and heterogeneity of organoids have progressed to induce vascularization [16,17] across multiple tissue types [18]. These features are powerful for understanding reproductive biology, for example, maintenance of blood perfusion is important for maintaining follicular health, implantation, thereby, the development of functional vasculatures is crucial in modeling ovulation. In addition, there is an evidence that the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) induces male sexual dysfunction, and it is most likely associated with the endothelial/vascular disintegrity, destruction, and dysfunction in the male reproductive system [19]. Given the world-wide spread of SARS-CoV-2 infection, it is urgent to synthesize vascularized human gonadal organoid to investigate how SARS-CoV-2 infection affects the function of male reproductive system.

Organoids can be derived from healthy or diseased tissues, constituting new avenues to investigate the normal biology and pathology of the reproductive system with the tissue environments that were previously not explored in single-layer culture. These solutions will advance the understanding of human reproduction *in vitro* and help fill knowledge gaps by providing effective models for drug screening and toxicity testing. There are numerous reviews on *in vitro* culture of reproductive organ-derived cells [13,14,20–22]. This review aims to summarize the 2D/3D culture systems of primary or stem cell-derived reproductive tissues, and discuss the emerging toxicology approach using human cells *in vitro*, with a particular focus on the testis and ovary, the organs where germ cells originally develop.

## 2. 2D model of human gonadal cells (Table2)

The 2D culture method has been widely employed to maintain cells outside of the body, and it has been successfully applied to various types of human gonadal cells.

### 2.1. Ovary

The ovary, an essential reproductive organ, is a meticulously

**Table 1**
The pros and cons of 2D/3D culture for toxicity testing.

| | | The pros | The cons |
|---|---|---|---|
| **2D culture** | | Scalability and Reproducibility | Limited biological relevantce |
| | | Cost effectiveness | Lack of cell extrinsic interaction |
| | | Less laborius | Cellular heterogeneity |
| | | High throughput screening | |
| **3D culture** | **Organoid** | Inter-cellular communication | Irreproducibility |
| | | Microanatomical feature | Expensive |
| | | Inclution of cell extrinsic factor (e.g., ECM) | More complex culture and analysis system |
| | | | Time and labor |
| | **Organ-on-a-chip** | Perfused network | Suboptimal for stem cell and organoids |
| | | Optimized for immortal cell lines | Material drug absorbability |
| | | More physiological relevant model | |

**Table 2**
The summary of culture protocol for 2D gonadal systems.

| 2D model of human gonadal cells | Cell type | Basal medium | FBS, Cytokines, etc | Cel line | coating | Reference |
|---|---|---|---|---|---|---|
| **Ovary** | **Ovarian Surface epithelium (OSE)** | 1:1 MCDB202 and M199 | 15% FBS, 20 ng/ml EGF, 0.4 μg/ml HC | Primary OSE, immortalized OSE lines, ovarian carcinoma line | | 26 |
| | | 1:1 MCDB105 and M199 | 15% FBS, 10 ng/ml EGF, 0.5 μg/ml HC, 5 μg/ml insulin, and 34 μg protein/ml Bovin pituitary extract (BPE) | Primary OSE | | 27 |
| | | 1:1 RPMI and Ham's F-12 | 10% FCS | Primary OSE | | 28 |
| | | Waymouth's medium 752/1 | 25% FBS | Primary OSE | collagen gel | 36 |
| | **Granulosa Cells** | Krebs-Ringer bicarbonate buffer (KRB) | 5.5 mM glucose and 0.1%BSA | Primary granulosa cells | | 34 |
| | | McCoy's 5 A medium supplemented by 10 mM HEPES at pH 7.4 | 5% FCS, 2 mM L-glutamine, and 10 U/ml mycostatin | Primary granulosa cells | ECM pro-duced by bovine corneal endothelial cells | 35 |
| | | DMEM | 20% huma male serum, 4 mM L-glutamine | Primary granulosa cells | | 36 |
| | | DMEM | 10% FCS and 2 mM L-glutamine | Primary granulosa cells | | 38 |
| | | DMEM/F12 | 2% FCS, 10 mg/ml ascor-bic acid, 2 mM L-glutamine, 0.05 μM dexamethasone, 20 ng/ml EGF and 50 ng/ml bFGF | Primary granulosa cells | | 39 |
| | | 1st week: BIO-AMF™-1, 2nd week: DMEM/F12 | 2nd week: 4% L-glutamine and 5% FCS | Primary granulosa cells | | 40 |
| | | DMEM/F12 | 2% FCS, 10 mg/ml ascor-bic acid, 2 mM L-glutamine, 0.05 μM dexamethasone, 20 ng/ml EGF, 50 ng/ml bFGF and FSH | Primary granulosa cells | | 41 |
| | **Theca Cells** | McCoy's 5 A medium | 2 mM L-glutamin, 1 mg/ml BSA, 100 nM Insulin, 30 nM IGF-1 | Primary theca cells | | 43 |
| | | DMEM/F12 | 10% FBS, 2 mM L-glutamine, 20 ng/ml EGF, 10 ng/ml bFGF, 20 ng/ml leukemia inhibitor factor, and 10 ng/ml glial cell line-derived neurotrophic factor | Theca stem cells | | 45, 46 |
| **Testis** | **Sertoli Cells** | EMEM | 5% FCS, 92 mg/l D-valine and, with or without 5 ug/ml NIH-FSH-S12 | Primary sertoli cells | | 54 |
| | | DMEM/F12 | 5% FCS | Primary sertoli cells | | 55, 56 |
| | | DMEM/F12 | 5% FCS | Primary sertoli cells | Fibronectin | 57 |
| | **Leydig Cells** | DMEM/F12 | 15 mM NaHCO3, 20 mM HEPES, and 0.1% BSA | Leydig cell mesenchymal precursors | | 62 |
| | | DMEM/F12 | 2% fetal calf serum and 10 μg/ml transferrin | Leydig cells | | 63, 64 |
| | | DMEM/F12 | 10% FBS | Leydig cells | | 65 |
| | | DMEM/F12 | 10 ug/ml Insulin, 10 ug/ml transferrin, 0.1 mM Vitamin C, 10 ug/ml Vitamin E, and 10 U/ml Nystatin | Leydig cells | Collagen, fibronectin, and laminin | 66 |
| | | DMEM/F12 | 15 mM NaHCO3, 15 mM HEPES, 10 ug/ml Insulin, 10 ug/ml transferrin, 0.1 mM Vitamin C, 10 ug/ml Vitamin E, and 10 U/ml Nystatin | Leydig cells | ECM pro-duced by bovine corneal endothelial cells | 67 |
| | **Spermatogonial Stem Cells (SSC)** | StemPro-34 SFM | 20 ng/ml EGF, 10 ng/ml GDNF, and 10 ng/ml LIF | SSC | Laminin | 72, 73 |
| | | StemPro-34 SFM | 20 ng/ml EGF, 100 ng/ml GDNF, 300 ng/ml GFRA1-Fc fusion protein, 10 ng/ml NUDT6, 10 ng/ml LIF, 30 ng/ml TGFB, and 100 ng/ml Nodal | SSC | Gelatin | 74 |
| | | StemPro-34 SFM | StemPro supplement, B27 supplement minus vitamin A, 6 mg/ml d-(+)-glucose, 30 mg/ml pyruvic acid, 1 μl/ml DL-lactic acid, 2.5 mg/ml lipid-rich bovine serum albumin, 1% FBS, 2 mM L glutamine, 50 μM β-mercaptoethanol, 1 × minimal essential medium, non-essential amino acids, 1 × MEM vitamins, 50 ng/ml GDNF, 20 ng/ml EGF 10 ng/ml human bFGF, and 10 ng/ml LIF | SSC | Hydrogel | 75 |

organized tissue composed of germ cells (oocytes) and somatic cells (surface epithelium, granulosa cells, and theca cells) [23,24]. Among these cell types, granulosa and theca cells work together to support the growth of ovarian follicles, eventually leading to the release of a mature oocyte from the ruptured ovarian surface epithelium (OSE) during ovulation (Fig. 2). Thus, oocyte maturation depends on highly orchestrated endocrine events involving multiple ovarian tissues. Single-layer culture of human ovarian cells has been demonstrated using either

**Table 3**
The summary of culture protocol for 3D gonadal systems.

| 3D model of human gonadal cells | Cell type | Basal medium | FBS, Cytolines, etc | Cell line | Form | Reference |
|---|---|---|---|---|---|---|
| **Ovary** | **Ovary Follicle** | DMEM | 1 % Insulin-Transferrin-Selenium (ITS), 15 mM HEPES, 0.5 % BSA, and w/ or w/o FSH | Preantral follicle | Agar sandwich | 99 |
| | | αMEM | 5 % Human serum, 1.5 U/ml FSC, and 2.5 ng/ml LH | Preantral and small follicles | | 100 |
| | | Ham's F10 | 15 % Fetal cord serum, 0.15 IU/ml HMG, 40 % hFF and 6 ng/ml EGF | Preantral follicle | | 101 |
| | | McCoy's 5 A medium with bicarbonate | 20 mM HEPES, 0.1 % BSA, 3 mM glutamine, 2. 5 ug/ml transferrin, 4 ng/ml selenium, 10 ng/ml insulin, and 50 ug/ml ascorbic acid | Cortical ovarian tissue | | 111 |
| | | | 100 ng/ml Activin A | Preantral follicle | | |
| | | αMEM | 6 % Serum protein substitute supplemented, 1 mg/ml bovine fetuin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 0.1 IU/ml FSH | Secondary follicle | Alginate beads or matrigel | 112 |
| | | Waymouth's media | 3 mg/ml Human serum albumin (HSA), 0.5 mg/ml bovine fetuin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 64 μg/ml ascorbic acid, and 10 mIU/ml FSH | Primordial follicle | Alginate bead | 113 |
| | | αMEM | 3 mg/ml HSA, 0.5 mg/ml bovine fetuin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 10 mIU/ml FSH | Secondary follicle | Matrigel | |
| | | αMEM | 1 % HSA, 1 % insulin-transferrin-selenium, 50 mg/ml ascorbic acid, 50 mIU/ml FSH, and 200 ng/ml bFGF | Early follicle | Alginate bead | 114 |
| | | 1:1 αMEM glutamax and F-12 glutamax | 3 mg/ml HSA, 10 mIU/ml FSH, 1 mg/ml bovine fetuin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 μg/ml selenium | Secondary follicle | Alginate bead | 115 |
| | | | | Preantral follicle | released form aliginate bead | |
| | | αMEM | 10 % BSA, 1.5 IU/ml hCG, 10 ng/ml EGF, and 10 mIU/ml FSH | Antral follicle | | |
| | | McCoy's 5 A medium with bicarbonate | 20 mM HEPES, 0.1 % HSA, 3 mM glutamine, 2.5 ug/ml transferrin, 4 ng/ml selenium, 10 ng/ml insulin, and 50 ug/ml ascorbic acid | Cortical ovarian tissue | | 117 |
| | | | 100 ng/ml Activin A and 1 ng/ml FSH | Secondary follicle | | |
| | | | | Antral follicle<100uM | Nucleopore membrane | |
| | | SAGE IVM medium | 75 mIU/ml FSH and 75 mIU/ml LH | Antral follicle>100uM | | |
| | **OSE** | 1:1 MCDB105 and M199 | 15 % FBS, 10 ng/ml EGF, 0.5 μg/ml HC, 5 μg/ml insulin, and 34 μg protein/ml BPE | Primary OSE | polyHEMA | 118 |
| | | 1:1 MCDB105 and M199 | 10 % FBS and 2 % matirgel | | Matrigel | 119 |
| | **Ovarian Stroma Cells** | DMEM/F12 | 10 % FBS | Primary ovarian stormal cells | Chitosan-silk fibroin hydrogel | 121 |
| | **Reconstituted Ovary** | SAGE IVM medium | 10 % albumin,75 mlU/ml FS, and 100 mlU/ml hCG | Primary theca cells and cumulus granulosa-oocyte complex | Agarose mold containing 18 honeycomb-shaped wells | 123 |
| | | DMEM | 5 % FBS, 1 % ITS, 50 mg/ml ascorbic acid, and 1 % BSA | Primary ovarian stormal cells and preantral follicle | Decellularized human ovarian tissue | 125 |
| | | NutriStem | 10 % KSR | Dissociated embryonic gonad tissue | Matrigel with hanging insert | 126 |
| | **Organ-on-a-chip with Ovarian Organoids** | DMEM | 10 % FBS | Ovarian chamber: primary theca and granulosa cells, endometrial chanber: endometorial stem cells | PDMS baased organ-on-a-chip | 128 |
| **Testis** | **Reconstituted Testis** | StemPro-34 SFM | 1 μg/ml solubilized human testis ECM, 2 μM Retinoic acid, 2.5 × 10^5 IU FSH, and 100 ng/mLSCF | Primary SSC, and immortalized leydig and sertoli cells | hanging drop | 131, 132 |
| | | DMEM/F12 | 10 ug/ml insulin, 5.5 ug.ml transferrin, 6.7 ng/ml selenium, and 20 ng/ml EGF | Bulk testicualr cells | aggregation | 133 |
| | | Knockout DMEM | 10 % CTS KnockOut SR XenoFree Medium, 1× GlutaMAX, 5 IU/L hCG, 5 IU/LFSH | Bulk testicualr cells | hanging transwell insert with decellularized testis matrix | 137 |

(*continued on next page*)

**Table 3** (*continued*)

| 3D model of human gonadal cells | Cell type | Basal medium | FBS, Cytolines, etc | Cell line | Form | Reference |
|---|---|---|---|---|---|---|
| | **Organ-on-a-Chip with Testicular Organoids** | William's Medium E | 10 % CTS™ KnockOut™ SR XenoFree Medium, 5 μg/ml human insulin, 2 mM L-glutamine, 5×10^5 M hydrocortisone hemisuccinate | Liver chamber: HepaRG spheroid, testis chamber: primary testicular organoid | PDMS baased organ-on-a-chip | 139 |
| | | 1:1 EGM media and StemPro-34 | 0.5 μg/ml solubilized human testis ECM | Liver, cardiac, lung, blood vessel, brain, and testis organoids | adhesive film-based microfluidic chip | 140 |

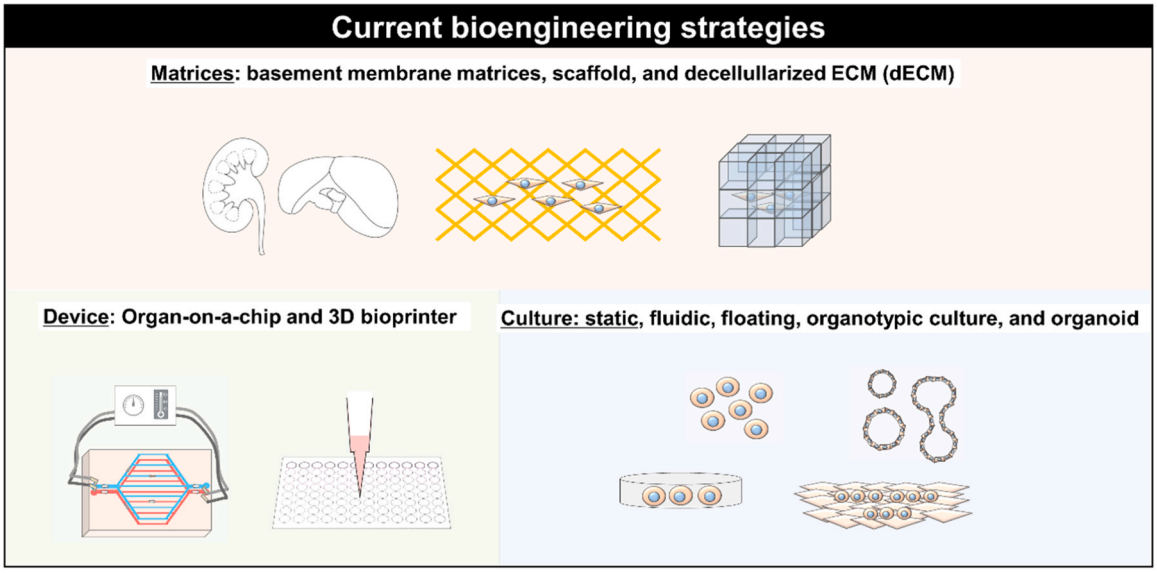


**Fig. 1.** 2D/3D bioengineering technology.

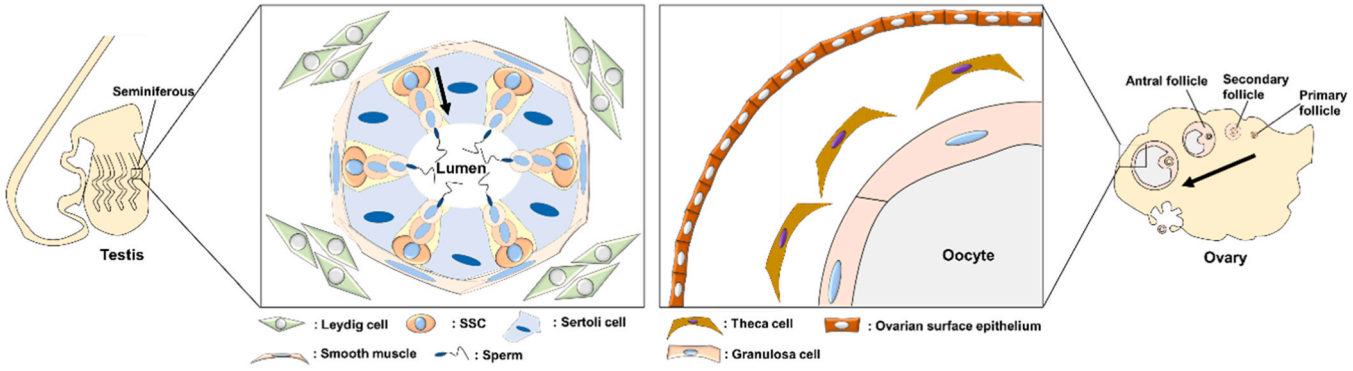


**Fig. 2.** The location and development of functional cells in the testis and ovary.

immortalized/cancer cell lines or primary cells, although many studies have been focused on ovarian carcinoma cells [25]. The extensive research on ovarian cancer therapy has driven the use of these cell lines, which offer unlimited proliferation and are valuable for developing *in vitro* culture systems that require extensive testing [26]. However, immortalized or cancer cell lines, often harbor genomic mutations and are under supra-physiological conditions *in vitro* to assess the toxicity of chemicals. In this section, we summarize the 2D culture of primary ovarian cells, with a focus on the functional cell types: ovarian surface epithelium, granulosa cells, and theca cells.

### 2.1.1. *Ovarian surface epithelium*

Various approaches have been employed to replicate the physiology of the ovary *in vitro* using 2D culture systems. These culture systems have been predominantly applied to ovarian surface epithelium to predict the efficacy of chemotherapy [27–29]. This is because approximately 90 % of malignant ovarian tumors are epithelial and believed to originate from the OSE [30–32]. Since the first attempt at OSE cultures, techniques for cell collection and culture media have been optimized to extend the lifespan of these cells [33]. Characterization of cultured human OSE cells has been performed to distinguish phenotypic variations of normal cells from early (pre)neoplastic changes [27,29]. For instance, 2D cultured human OSE expressed androgen receptors in

consistent with *in vivo* counterparts and addition of synthetic androgen in the culture medium stimulate DNA synthesis of human OSE resulting in increase of cell proliferation. Human normal OSE cells have a limited lifespan, which has hindered their practical use in biological manipulation. This growth limitation has been overcome by using modified media containing bovine pituitary extract, epidermal growth factor (EGF), insulin and hydrocortisone, that significantly improve the growth of human normal OSE cells *in vitro* while preserving their epithelial cell characteristics [28]. Long-term culture of OSE facilitates the investigation of its characteristics, rendering it suitable for large-scale screening of drugs or chemical toxicants.

#### 2.1.2. Granulosa cells

Granulosa cells play a vital role in oocyte maturation and follicular development [34]. The characteristics of granulosa cells, such as steroidogenesis, responsiveness to human chorionic gonadotropin (hCG), and the effect of the bovine corneal endothelial cells-derived extracellular matrix (ECM) on their responsiveness to hCG, have been investigated through 2D culture techniques [35–39]. Optimized medium containing 2 % fetal calf serum (FCS) and follicular fluid (FF) have been established for long-term cultivation of granulosa cells, and recently, a more feasible and cost-effective method for culturing granulosa cells has been developed using BIO-AMFTM media followed by 5 % FCS medium culture [40–42]. Granulosa cells surround oocytes and provide crucial supports for oogenesis within ovarian follicles in a 3D arrangement. Consequently, it is imperative to explore the relationship between granulosa and germ cells in order to comprehend the role of granulosa in oogenesis. Hence, the implementation of 3D cellular structures, such as organoids, composed of granulosa cells and oocytes, holds promise in facilitating *in vitro* oocyte development and serving as a platform to assess reproductive toxicity associated with granulosa-oocyte interactions.

#### 2.1.3. Theca cells

Theca cells have diverse functions in the growth and maturation of ovarian follicles. They provide structural support to the growing follicle and collaborate with granulosa cells and oocytes in synthesizing androgens during development [43]. Long-term culture of human theca cells was first achieved using serum-free medium supplemented with forskolin, hCG, luteinizing hormone (LH), or cyclic adenosine monophosphate (cAMP) analogs. Several growth factors, including insulin-like growth factor-1 (IGF1), insulin, and stem cell factor (SCF), have been observed to stimulate proliferation in primary theca cell cultures [44,45]. Recently, the characterization of human theca stem cells and their differentiation into human theca progenitor cells has been demonstrated using isolated theca layers from small antral follicles [46]. The same research group investigated the direct role of human theca progenitor cells in the growth and development of human primordial follicles using stem cell-derived progenitor cells [47]. Theca stem cells may have potential for differentiating into three subtypes of human theca cells, which have recently been identified through single-cell multi-omics analysis of primary cell populations purified from human antral stage follicles [48]. Consequently, theca stem cells are attractive cell sources for *in vitro* assay of theca cells, and they have potential to evaluate the effect of reproductive toxicity on the stem cell or progenitor lineage of theca cells.

### 2.2. Testis

The main role of the testis is spermatogenesis and androgen production. Spermatogenesis, the complex process by which spermatogonia undergo a series of divisions to produce mature spermatozoa, occurs within the seminiferous epithelium [49]. Human spermatogenesis involves the differentiation of adult spermatogonial stem cells (SSCs) into mature sperm, a process regulated by the testis niche [50]. Sertoli cells within the seminiferous tubules support spermatogenesis by serving as "nurse cells" and maintaining the testis niche through various functions. In the interstitium, Leydig cells (LCs) support the development of SSCs by secreting testosterone, an indispensable hormone for sperm production (Fig. 2) [51]. Accessing *in vivo* germ cell samples has been challenging because sampling itself may damage the reproductive ability of donors: it emphasize the need for *in vitro* culture systems to study the male reproductive system in humans. In this context, we summarize the current 2D culture techniques for functional cells derived from primary human testicular samples to focus on studying the behavior of chemical hazards in a physiological context.

#### 2.2.1. Sertoli cells

Sertoli cells are a unique cell type that remains quiescent during puberty but remains active throughout the reproductive lifetime of males, undergoing cyclic changes in morphology and gene expression patterns[52]. They play an essential role in spermatogenesis at different developmental stages. Initially, they support fetal germ cell commitment to the male pathway and contribute to the development of germ cells during both the prenatal and postnatal periods, from maintaining the spermatogonial stem cell niche to the production of functional spermatids [52–54]. Emerging evidence in the past few decades has revealed their critical role in other testicular somatic cell functions [54]. The first demonstration of human Sertoli cells in monolayer culture was performed in 1982 to investigate their characteristics, such as the expression of androgen-binding protein. Human Sertoli cells were maintained for up to 45 days in this study, but the proliferation of these cells was not investigated because Sertoli cells were known to be terminally differentiated cells at puberty [55]. Later on, the proliferation of adult human Sertoli cells was demonstrated in a medium containing 5 % fetal bovine serum (FBS). Human Sertoli cells resumed proliferation in culture and even repaired DNA damage, unlike terminally differentiated cells [56]. This finding allowed researchers to perform a detailed characterization of human Sertoli cells, and proliferative human Sertoli cells were used as a model to monitor toxicant-induced junction disruption and study the mechanism of toxicant-induced testicular dysfunction [57,58]. Blood-testis-barrier (BTB) between adjacent Sertoli cells, serves as a gatekeeper to prohibit harmful substances from reaching developing germ cells [59]. Since cultured human Sertoli cells form tight junction barrier *in vitro*, 3D assembly of these Sertoli cells may recapitulate BTB *in vitro*, and contribute to understand how toxicants penetrates BTB.

#### 2.2.2. Leydig cells

LCs are a cell population located in the interstitium of the testis. Their most critical function is the secretion of androgens, particularly testosterone, which is essential for the development and maintenance of the male reproductive system [60,61]. The dynamics of testosterone production are regulated by two generations of LCs: fetal LCs and adult LCs. Fetal LCs initially produce high levels of testosterone during the embryonic period, which are required for the differentiation of the male genitalia. Testosterone production declines as the number of fetal LCs decreases, but it resurges with the development of adult LCs from neonatal testicular stem cells. *In vitro* culture of human LCs has been attempted in a few reports using the medium supplemented with hydrocortisone, and vitamin E with hCG, and mesenchymal progenitors of LCs, known as stem LCs, have been established as a new source of LCs [62–64]. The first isolation and purification of human LCs were performed using percoll gradients after collagenase digestion of the human testis. Testosterone production in response to hCG was analyzed in human LCs [64]. The same method was used to isolate human LCs for investigating the morphological features of these cells *in vitro* [65]. *In vitro* culture technology has enabled researchers to explore the effect of radiation on human LCs, revealing that higher doses of radiation impair LC steroidogenesis by affecting LH signal transduction [66]. Enhancement of testosterone secretion by adult human LCs was observed *in vitro* when these cells were co-cultured with adult human Sertoli cells [67]. A further study reported by the same group suggests that this effect is

associated with an enhancement of the expression of several steroidogenic enzymes [68].

Stem Leydig cells (SLCs) are a specific type of mesenchymal stem cells with self-renewal ability and the capacity to differentiate into multiple cell lineages. What sets SLCs apart from other mesenchymal stem cells is their potency to differentiate into LCs, making them a promising candidate for testosterone replacement therapy in patients with androgen deficiency. Although not initially defined as SLCs, mesenchymal progenitors of human LCs were first isolated from the testicular interstitium of two patients with androgen insensitivity syndrome by removing hCG form the medium components [63]. Following this report, several studies have demonstrated the *in vitro* culture of SLCs [62,69]. SLCs offer a valuable avenue for studying *in vitro* development of LCs and examining the detrimental impact of chemical hazards on SLCs within the testis.

#### 2.2.3. Spermatogonial stem cells

SSCs are a group of adult stem cells in the testis that are capable of self-renewal to maintain the stem cell pool of spermatogenesis and differentiation to produce mature spermatozoa [70,71]. Despite recent progress in cell culture technology, long-term culture of human SSCs remains challenging. Therefore, successful culture of human SSCs would enable us to design a platform for analyzing the harmful effects of chemicals or toxicants on SSCs and may provide novel stem cell-based therapies for assisted reproduction. Although the culture of human diploid spermatogenic cells was initially demonstrated in the 1970s, a long-term culture method was not developed until 2009, using StemPro-34medium supplemented with 10 % FCS, human EGF, glial cell line-derived neurotrophic factor (GDNF), and leukemia inhibitory factor (LIF), that was already being used for culturing human hematopoietic stem cells [72,73]. In this medium, human SSCs were propagated for up to 28 weeks, and the same authors succeeded in expanding SSCs harvested from prepubertal boys [74]. The isolation and purification of SSCs is critical since contaminated cells from the testis may have adverse effects on SSC culture. Isolation of the SSC population was achieved by sorting them based on the presence of cell surface markers (*e.g.,* CD49f) and further culturing them with the addition of cytokines, such as GDNF family receptor alpha 1-Fc fusion protein, nudix hydrolase 6, transforming growth factor beta (TGF-β) and Nodal, into the previously reported medium [73,75]. The same authors established a defined culture system containing human GDNF, EGF, basic fibroblast growth factor (bFGF), LIF and a hydrogel to expand human SSCs for an extended period while maintaining their undifferentiated status [76]. Sertoli cells, which are the orchestrators of spermatogenesis and have an essential role in germ cell development, were used in an *in vitro* co-culture system with CD49f positive SSCs, promoting meiosis induction and resulting in the generation of haploid cells, suggesting the potential usage of this method for clinical translation [77]. This result reminds us of the importance of cell-cell interaction among different types of cells, and multicellular organoids technology may help to develop SSCs toward functional sperm.

## 3. Pluripotent stem cell-derived gonadal cells

Human pluripotent stem cells (PSC) including embryonic stem cells and induced pluripotent stem cells (iPSC) are attractive cell sources for their unique characteristics, self-renewal potential and differentiation ability to three germ layers [78]. Human PSCs have been used for modelling human genetic diseases. As demonstrated in the preceding section, gonadal adult stem cells are appealing candidates for *in vitro* research, however, acquiring these cells using tissue biopsy puts significant burden on patients. On the other hand, iPSCs can be derived from readily accessible cell sources like skin or blood cells. Therefore, utilizing PSCs to derive gonadal cells presents a promising technology that not only avoids the risks associated with biopsies but also provides ample quantities of cells for *in vitro* studies. The detailed protocols for PSC-derived gonadal cells have been described in this review (Fig. 3).

### 3.1. Germ cells

There has been significant research in the past decade focused on reconstituting germ cell development *in vitro* using human PSCs [22]. Two reports have demonstrated the induction of human primordial germ cell-like cells (hPGCLCs) from PSCs cultured in either bFGF based conventional medium or medium containing 4 chemical inhibitors, MEK, GSK3β, p38, and c-Jun N-terminal kinase inhibitors. While PSCs cultured in the conventional medium are first induced into incipient mesoderm-like cells and then produce hPGCLCs in response to bone morphogenetic protein 4 (BMP4) signaling, the PSCs cultured in 4 inhibitors condition directly produce hPGCLCs by BMP signaling. [79,80]. Subsequently, an aggregation culture of hPGCLCs with mouse embryonic ovarian somatic cells was used to further develop hPGCLCs into human germ cells [81]. Within the aggregation, hPGCLCs matured and differentiated into oogonia or gonocyte-like cells, expressing key factors such as DAZL. Continuous culture of these cells resulted in the generation of retinoic acid (RA)-responsive female germ cells. Similarly, when hPGCLCs were aggregated with mouse embryonic testicular somatic cells, they differentiated into gonocytes and matured into prospermatogonia [82]. Although PSC-derived human oogonia or prospermatogonia are induced within the aggregation of mouse somatic cells, use of a xenogeneic system would not be ideal for the safety assessment of drug or chemical substances. No xenogeneic system is required to recapitulate human gem cell development *in vitro*. Besides, both oogonia and prospermatogonia are still immature germ precursor cells at the embryonic stage, and cannot be used for adult germ cell toxicological study. Further development of maturation protocol is required to apply PSC-derived germ cells for toxicity assay.

### 3.2. Ovarian cells

Regarding ovarian cells, the differentiation of human PSCs into granulosa-like cells has been reported using a multistep differentiation protocol, via embryonic body formation followed by attachment culture. These granulosa-like cells expressed the granulosa cell-specific forkhead transcription factor *FOXL2* and were biologically functional as they were able to aromatize testosterone to estradiol [83,84]. In addition, these granulosa-like cells expressed *CYP19A1*, *AMHR2*, and/or *FSHR*, providing further evidence of the presence of mature granulosa cells. The same method was used to generate granulosa-like cells from patients with polycystic ovary syndrome (PCOS). The PCOS granulosa-like cells exhibited persistent hyperactivation of the cAMP response element-binding protein signaling pathway, which may be involved in the pathogenesis of PCOS [85]. Interestingly, overexpression of two transcription factors, *NR5A1* and either *RUNX1* or *RUNX2*, was shown to be sufficient for generating functional granulosa-like cells from PSCs, which could recapitulate key ovarian phenotypes including follicle formation and steroidogenesis [86]. This overexpression strategy may provide clues for deriving other ovarian cells from PSCs, and PSC-derived ovarian cells can be used for the large screening of drug or chemical toxicity, which cannot be done with the primary cells due to the limited number of cells.

### 3.3. Testicular cells

In the case of testicular cells, there have been several reports on PSC-derived testicular cells. Human LC-like cells were first derived from PSCs by overexpressing steroidogenic factor-1 at the mesodermal lineage stage [87]. A more efficient and transgene-free protocol was established using small compounds, such as smoothened agonist (SAG), to induce human PSC-derived LC-like cells. These cells acquired testosterone synthesis capabilities, exhibited similar gene expression profiles to LCs, and expressed LC lineage-specific protein markers [88]. A chemically

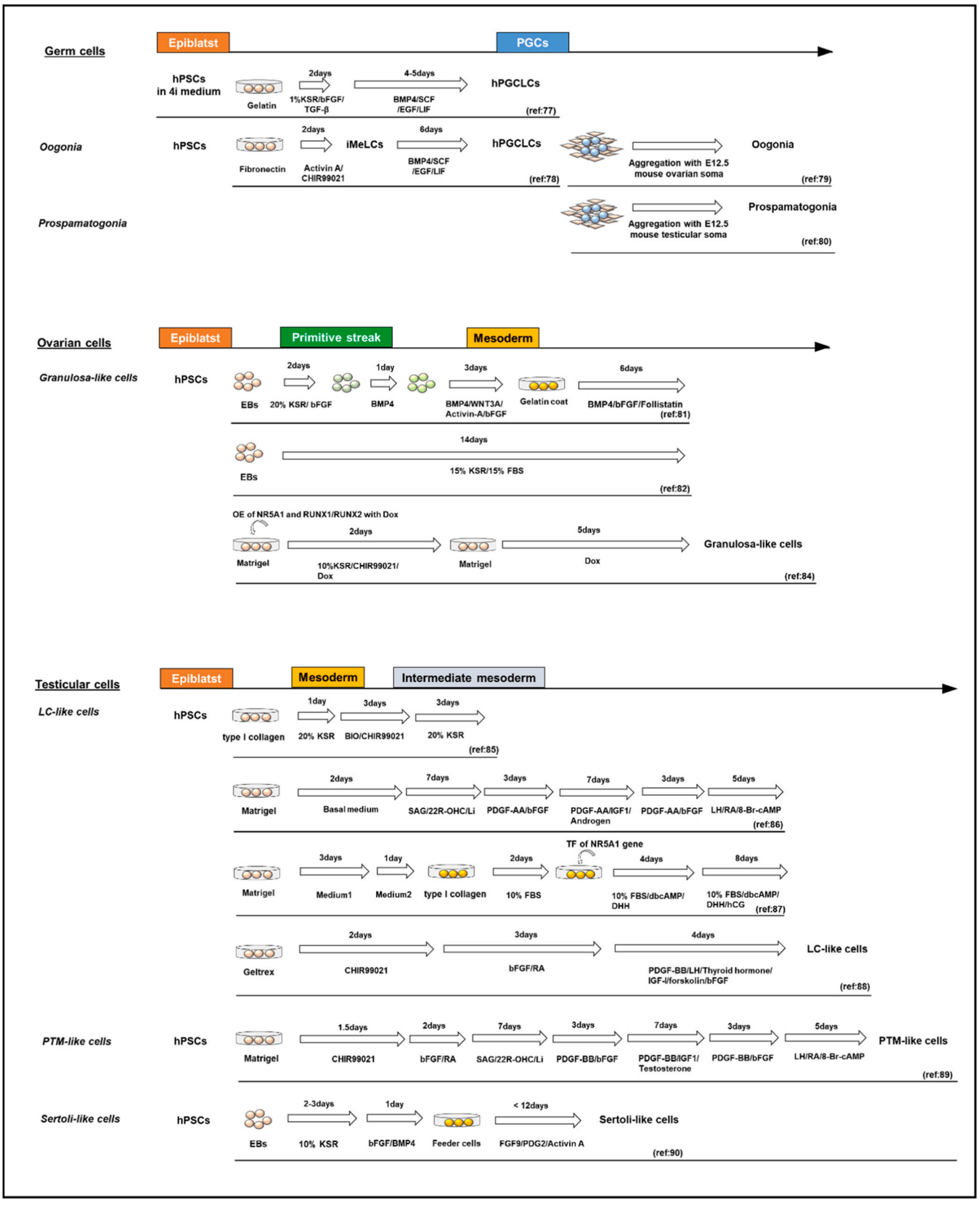


**Fig. 3.** Protocols for deriving gonadal cells from human PSCs. KSR: Knock out serum, CHIR99021: GSK3β inhibitor, BIO: GSK 3 Inhibitor, SAG: Hedgehog pathway activator, Li: Lithium chloride.

defined medium supplemented with dibutyryl cAMP (dbcAMP), desert hedgehog (DHH) agonist, and hCG was further developed to generate either human LC cells or adrenal-like cells, which produced testosterone and cortisol, respectively [89]. Recently, another group reported a rapid and efficient procedure using GSK3β inhibitor for mesoendoderm differentiation, bFGF and RA for intermediate mesoderm induction, and platelet-derived growth factor (PDGF)-BB, LH, thyroid hormone, IGF1, forskolin, and bFGF for LC induction. This protocol allows for the differentiation of human PSCs into LCs within nine days [90]. Although there are fewer reports, the induction of human peritubular myoid-like cells or Sertoli-like cells from PSCs has also been demonstrated [91,92]. PSC-derived LC-like cells showed similar gene expression pattern to primary LCs, though the level of gene expression and the amount of testosterone synthesis are not equivalent to that of primary LCs. Additional methodological components such as organoid culture may be required to generate matured or functional PSC-derived LCs.

### 3.4. Modeling peri-implantation embryo development

The study of human peri-implantation development is severely limited due to ethical reasons [93]. To challenge this issue, researchers have first developed synthetic embryos comprising mouse PSCs and extraembryonic trophoblast stem cells, which give rise to the placenta [94]. This construct cultured in matrigel exhibits a significant resemblance to peri-implantation normal embryos, including the presence of mesoderm and primordial germ cells at the boundary between the embryonic and extraembryonic compartments, though it lacks the extraembryonic endoderm lineage, which plays a critical role in early embryogenesis [95]. This novel technology was further attempted to generate human stem-cell derived synthetic embryos to mimic peri-implantation embryo development.

Some research groups have successfully generated stem cell-derived synthetic embryos, called blastoids, that contain all three lineages and resemble blastocyst stage embryos. They accomplished this by utilizing naïve human pluripotent stem cells, which exhibit totipotency in the N2B27 based specific medium supplemented with MEK, WNT, PKC inhibitors and human LIF, called PXGL medium [96–98]. These blastoids exhibit a similar transcriptome profile to human blastocyst-stage embryos and have demonstrated an induced implantation reaction when cultured on human endometrial tissue *in vitro* [96]. Another research group has demonstrated the assembly of all three lineages, including extraembryonic endoderm derived from PSCs, which mimics several aspects of post-implantation human embryos [99]. This research field has been progressing rapidly and bioengineering approaches have been utilized for further development (see the Future perspective section).

## 4. 3D model of human gonadal cells(Table3)

Recent progress in the field of bioengineering has facilitated the development of complex human 3D culture methods. Integration of these technologies with organoids allows the generation of more intricate *in vitro* systems that resemble human reproductive physiology.

### 4.1. Ovary

The multiple cell layers surrounding the oocyte play critical roles in the development of ovarian follicles and oocytes. Therefore, it is natural to incorporate these complex structures into the culture system in order to replicate the physiological processes of the ovary *in vitro*. 3D culture methods have primarily been employed to culture ovarian follicles using different types of biomaterials, with the goal of developing functional oocytes from immature follicles. Organoid technology has also been utilized to culture epithelium, stroma, and even reconstituted ovaries.

#### 4.1.1. Ovarian follicle

For several decades, attempts have been made to achieve *in vitro* growth and maturation of human ovarian follicles for the purpose of developing assisted reproductive technologies, preserving fertility, and studying folliculogenesis. Currently, ovarian tissue cryopreservation and transplantation of thawed tissue are the only available options for preserving the fertility of pre-pubertal girls and young women who are unable to delay radiation or chemotherapy for cancer treatment. However, this technology cannot be applied to patients diagnosed with leukemia or cancers originating in the ovary due to the risk of reintroducing malignant cells when the ovarian tissue is transplanted. *In vitro* culture of human ovarian follicles is a desired alternative approach for preserving the fertility of such patients, and the successful development of functional oocytes *in vitro* would be useful for modeling reproductive toxicity of chemicals or reproductive failure. The isolation and culture of human preantral follicles were initially performed using a simple medium for a duration of five days in agar [100]. The culture period was extended to several weeks by incorporating a medium with a low concentration of LH [101]. The diameter of preantral follicles increased from 100 μm to approximately 300 μm with the use of a more complex medium composition with human menopausal gonadotrophin, FF, and EGF [102]. The culture of ovarian tissue, including follicles, has been employed to grow and mature primordial/primary follicles [103–111]. A two-step culture method in combination with tissue culturing was developed to mature primordial follicles toward the late pre-antral/early antral stage [112]. Human secondary follicles, isolated from ovarian tissues, were encapsulated in 0.5 % alginate beads and grown *in vitro* for 30 days, while primordial follicles directly encapsulated in alginate did not survive in culture. This indicates that the early stages of *in vitro* human follicle development require the support of the native ovarian cortex [113,114]. The addition of bFGF improves the growth, survival, and viability of human early follicles during *in vitro* growth [115]. Secondary follicles could be grown from the preantral to antral stage using an alginate bead-based bioengineered condition, although the development of meiotically competent human oocytes from preantral follicles has not yet been achieved. Alginate bead culture of preantral follicles, followed by 2D culture, has produced meiotically competent metaphase II (MII) oocytes [116]. The composition of fibrin matrix was investigated to mimic the architecture of human ovarian tissue for encapsulation and grafting of human ovarian follicles [117]. Finally, a serum-free multi-step culture system using organotypic culture followed by the culture of isolated COC on polycarbonate film has successfully achieved the development of human oocytes from the primordial stages to MII, with the emission of a polar body. However the polar bodies were larger than that observed from *in vivo* grown oocytes, indicating abnormal development of these oocytes [118]. These 3D culture system can be applied to validate the adverse effect of reproductive toxic agents on human oocyte development, though further improvement is required to recapitulate human oocyte maturation *in vitro*.

#### 4.1.2. Ovarian surface epithelium

There are limitations to culturing OSE cells as 2D monolayers, as they tend to lose some of their epithelial characteristics [28]. Epithelial cells are surrounded by a complex ECM and spatially communicate with each other. Therefore, 3D culture of OSE would more closely resemble the phenotypes observed *in vivo* compared to traditional 2D culture. OSE cells cultured as multicellular spheroids on polyHEMA-coated plastics displayed defined internal architecture and expressed AE1/AE3, laminin, and vimentin, which were undetectable in 2D culture [119]. An organoid model of OSE cultured on a matrigel matrix recapitulated the epithelial inclusion cysts observed in the human ovarian cortex, where ovarian epithelial dysplasia occurs. In this organoid model, a precancerous phenotype was induced by the treatment of tumor necrosis factor α [120]. The 3D model of OSE represents *in vivo* phenotype that is not observed in monolayer culture, so that use of this model may facilitate to improve the accuracy for the safety assessment of reproductive toxicity.

#### 4.1.3. Ovarian stroma cells

The majority of the ovarian stroma is composed of a mixed population of incompletely characterized cells commonly referred to as stromal cells [121]. A double network hydrogel composed of chitosan (Cs) and silk fibroin (SF) was used to maintain the proliferation and viability of human ovarian stromal cells *in vitro* [122]. The ovarian stromal cells were encapsulated into the Cs-SF hydrogels and cultured for 7 days to validate cell survival and proliferation. The 1 % Cs in addition to 1 % SF hydrogel yielded a higher number of viable cells compared to the other ratio of hydrogels, demonstrating the positive effect of Cs-SF hydrogel at proper ratio on viability and proliferation of ovarian stromal cells. However, more investigation, for instance function assay, is required to certify the advantage of these scaffolds.

#### 4.1.4. Reconstitution of human ovary by organoid or decellularized ECM Technology

Despite the development of *in vitro* culture of human ovarian follicles, successful generation of human offspring from these follicles has not yet been achieved. This is particularly crucial for patients who desire to have a baby but cannot undergo current cryopreservation strategies due to the risk of malignant cell contamination in the ovary [123]. Therefore, the creation of an artificial ovary is an attractive approach to preserve fertility for such patients. The self-assembly of isolated theca and granulosa cells in micro-molded gels resulted in the reconstitution of an artificial ovary, allowing the maturation of early antral follicles (<10 mm) into MII oocytes [124]. Decellularization, the process of removing cells and their components from the ECM, has been used to generate a natural matrix with preserved mechanical integrity [125]. The reconstitution of a human ovary has been attempted using a decellularized ovarian tissue restored with stromal cells, and isolated human follicles have been shown to survive in this human decellularized (dECM) scaffold [126]. Organoids derived from human embryonic gonadal tissues have been utilized to explore gonadal organogenesis [127]. Ovarian organoids generated from dissociated female gonad cell suspensions formed loosely organised cords after 7 days culture and DDX4- and POU51-positive germ cells appeared comparable to the situation *in vivo* in organoids generated from dissociated female gonads, suggesting ovarian organoids provide an environment capable of supporting germ cell survival [127]. Altogether, reconstituted ovary has potential to mature early stage human oocytes toward MII stage *in vitro*, though the effect of this method remains unclear due to the limited number of experiments. Further investigation is necessary to ascertain the benefit of reconstituted ovary on *in vitro* oocyte maturation.

#### 4.1.5. Organ-on-a-Chip with ovarian organoids

The current progress in 3D culture systems and animal experiments facilitate to make more accurate predictions regarding the efficacy and toxicity of newly developed drugs. However, pharmaceutical companies still face challenges, and there are many instances of drug failure due to safety concerns before reaching the clinical stage [128]. Organ-on-a-chip platforms using human-derived materials provide preclinical insights into the systemic functioning of different body parts. In particular, multi-organ chips, which consist of interconnected chambers where organoids or tissues are cultured, closely simulate the activity of multiple human organs in their physiological context. An organ-on-a-chip system was developed to culture both human uterine endometrium and ovarian cells (granulosa and theca cells), reflecting the bidirectional endocrine cross-talk between these cells [129]. The viability of these cells within each chamber of the chip platform was improved, and a reproductive toxicity marker, Serpin Family B Member 2 (SERPINB2), showed a significant increase in response to dioxin in this uterus-ovary chip. This result suggests that current system can be used to predict the reproductive toxicity of various hazardous materials [129].

### 4.2. Testis

While *in vitro* culture technology has advanced for testis-derived cells, long-term maintenance of human SSCs has only been achieved when the SSCs were transplanted into the testes of mice *in vivo* [130]. Additionally, cryopreserved testicular tissue obtained from prepubertal boys maintained spermatogonia and generated haploid germ cells *in vitro* for up to 16 days [131]. These findings indicate that physiological relevant conditions, composed of ECM or multiple cell types, are required to recapitulate the physiological conditions of spermatogenesis *in vitro*. A more reliable *in vitro* model system for the testis is essential for studying cell-cell interactions, tissue development, and accurately assessing the gonadotoxic potential of drugs before progressing to clinical trials. Based on this concept, several 3D culture systems for human spermatogenesis, including organoids, dECM, and organ-on-a-chip, have been demonstrated.

#### 4.2.1. Reconstitution of human testis by organoid and dECM Technology

The reconstitution of the human testis using organoid and dECM technology represents a crucial first step towards more accurate and rapid drug and toxicity screening on testicular tissues. These multicellular organoids not only recapitulate the functionality of native tissue but also provide a platform for studying testicular function, morphogenesis, and cell-cell interactions. Human testicular organoids composed of SSCs, Sertoli, Leydig and peritubular cells were created and evaluated for the capable of responsive to androgen stimulation and spermatogenesis [132]. These 3D organoids were then exposed to four chemotherapeutic drugs (cisplatin, etoposide, doxorubicin, and Busulfan) and exhibited significantly higher IC50 values compared to 2D cultures [132]. This pioneering human testicular organoid holds promise for drug screening purposes, although it should be noted that the presence of immortalized Sertoli and Leydig cells in the organoid may influence its structure and function. The same organoid model was also employed to assess the susceptibility of testicular organoids to Zika virus infection, a virus known to cause brain defects, including microcephaly and other severe brain abnormalities, during pregnancy [133]. Through the use of a microwell centrifugal forced aggregation approach, researchers were able to generate reproducible, size-defined organoids [134]. These testicular organoids, composed of primary pre-pubertal testis cells, formed a seminiferous epithelium and interstitial compartment separated by a basement membrane, a feature that was not observed in the previously reported organoid model [132]. Additionally, it was found that these organoids exhibited an increase in germ cell autophagy when treated with mono phthalate, and the ablation of primary cilia on the original testicular cells inhibited organoid formation [134]. CRISPR Cas9–mediated gene editing was utilized to investigate the role of primary cilia and Hedgehog signaling in Sertoli cells on testicular organoid morphogenesis [135].

The ECM plays a crucial role in maintaining homeostasis in the testis, particularly in terms of spatial arrangements between Sertoli cells and germ cells. Therefore, it is evident that the dECM of testicular tissue can support the reorganization and physiological processes of the derived cells [136]. Researchers have successfully demonstrated the decellularization of human testicular tissue and restoration of the testicular dECM using isolated suspensions of testicular somatic and germ cells [137–139]. However, despite the fact that the restored cells populated both the tubular and interstitial compartments, the dECM organoid did not exhibit the typical cytoarchitecture of testis due to the remodeling of the ECM by the testicular cells [138]. Reconstituted testis provide a platform to validate the effect of toxic substances or to model the pathology of infectious diseases *in vitro*, though the testicular function of these organoids has been poorly developed. For example, the frequency of differentiation of SSC to postmeiotic germ cells was very low approximately 0.2 % in testicular organoids. Optimized protocol need to be developed to recapitulate spermatogenesis *in vitro*, and to utilize the organoid technology for the assessment of testicular toxicity.

#### 4.2.2. Organ-on-a-chip with testicular organoids

Multi-organ-chips developed with co-culture of human liver and primary testicular cell organoid were maintained at a steady-state for at least 1 week [140]. Cyclophosphamide is an extensively used anticancer agent, which is administered as an inactive prodrug but it is activated afterward in the liver by cytochrome P450 (CYP450) enzymes. In multi-organ-chips, cyclophosphamide activated in the liver organoid channel circulated into the testicular organoid channel, and showed considerable germ cell loss in the organoids, that was not observed in single-testicular organoids systems [140]. A similar concept was further expanded to a more integrated system composed of six channels, where liver, cardiac, lung, endothelium, brain, and testes organoids were cultured [141]. Ifosfamide is a structural isomer of cyclophosphamide that has demonstrated efficacy in treating a wide range of tumors. Among these tumors, the FDA has approved the drug only for germline tumors of the testis. Following administration into the chips, ifosfamide was metabolized into chloroacetaldehyde in the liver organoids, resulting in decrease of cell viability in the brain organoids [141]. Given that encephalopathy is a common adverse effect of ifosfamide, this result indicates the multi-organ-chips can reflect the toxicity of anti-cancer drug *in vitro*. However, in this experiment the toxicity of ifosfamide was only investigated by the cell viability assay. To evaluate the reproductive toxicity, the other factors, such as germ cell loss, need to be investigated.

## 5. Toxicological assessment of chemical compounds

Exposure to chemical substances from the environment has detrimental effects on human reproduction [142]. The National Institute of Environmental Health Science states that environmental chemicals such as lead, mercury, diethylstilbestrol, and endocrine-disrupting chemicals (EDCs) contribute to human reproductive disorders. Among them, EDCs are commonly found in everyday items including plastic food containers, personal care products, and food, raising significant concerns about their potential toxicity [143]. Therefore, it is crucial to employ toxicity tests to ensure adequate protection for human health and the environment against these various toxic substances [144]. In this regard, *in vitro* culture technology has been utilized for decades to evaluate the effects of chemical substances on human reproductive organs [20,21]. Recently, these assessments have been updated using more physiologically appropriate systems that can investigate the effects of chemicals at the inter-tissue or organ level. For instance, a model derived from PSCs that mimics many aspects of human spermatogenesis has revealed that exposure to chemicals such as 2-bromopropane or 1,2-dibromo-3-chloropropane, known to affect male fertility, leads to a reduction in germ cell viability through apoptosis caused by the formation of reactive oxygen species [145–147]. In addition to environmental substances, the IC50 values of chemotherapy drugs have been re-evaluated using human testicular organoids. Interestingly, these organoids maintained significantly higher IC50 values than 2D cultures, indicating that the human testicular organoid system presents a more drug-resistant phenotype than equivalent cell mixtures cultured in 2D platform. This could be explained by the altered drug uptake kinetics, and germ cell protection provided by the compact structure within the organoid [132].

A liver-on-a-chip device, coupled with modeling and simulation techniques, has been developed for quantitative drug metabolism studies. This innovative approach allows for monitoring pharmacokinetics, investigating the hepatic disposition characteristics of drug candidates, and exploring the toxicogenic and metabolic interactions between the liver and other organs. One crucial aspect of this system is the quantitative assessment of metabolism and transporter processes within the chips. Unlike direct exposure of metabolites to the system, the presence of medium flow and different system compartments leads to non-homogeneous drug concentrations, resembling dynamics of drug metabolism in the body [148].

Cyclophosphamide is an anticancer agent, which is administered as an inactive prodrug but it is activated afterward in the liver by CYP450 enzymes to generate 4-hydroxycyclophosphamide. The 4-hydroxycyclophosphamide circulates and enters other cells to be converted to the principle metabolite, phosphoramide mustard which exert its chemotherapeutic effect by binding an alkyl molecule to DNA, forming an adduct that leads to inhibition of DNA replication and cell apoptosis [149]. Metabolic axis between the liver and testis has been investigated using organ-on-a-chip technology, enabling the evaluation of the secondary effects of metabolites synthesized by liver organoids on the testis and other organoids *in vitro* [140,141]. In multi-organ-chips where live and testicular organoid were incubated, cyclophosphamide activated in the liver organoid circulated into the testicular organoid channel, and showed considerable germ cell loss in the organoids, that was not observed in single-testicular organoids systems [140]. A liver-on-a-chip system was investigated using a more complex system where liver, cardiac, lung, endothelium, brain, and testes organoids were cultured [141]. Ifosfamide is a structural isomer of cyclophosphamide that need to be metabolized in the liver to be an effective form, isophosphoramide mustard, that been used for treating a wide range of tumors. It is known that Ifosfamide can be activated in liver by other metabolic pathway, called side-chain oxidation, and produce chloroacetaldehyde that has neural toxicity [150]. Following administration into the chips, isofamide was metabolized into chloroacetaldehyde in the liver organoids, resulting in downstream neurotoxicity in the brain organoids [141].

In the last two decades, there has been growing awareness of the potential adverse effects on human health from exposure to endocrine-disrupting chemicals (EDCs) that can interfere with the endocrine system [151,152]. EDCs disrupt homeostasis by interfering with the function of natural hormones. These disruptions can occur in various ways: some chemicals have a similar chemical structure to natural hormones and bind to their receptors, causing an exaggerated or inappropriate response; some block the effects of natural hormones; and others directly stimulate or inhibit the endocrine system, leading to increased or reduced hormone production. Many EDCs have been extensively studied using animal models, *in vitro* models, predictive or computational methods, and it has been demonstrated that a wide range of EDCs have negative effects on human reproductive function in certain experiments [151,152]. However, the evidence on the reproductive consequences of human exposure to EDCs is not clear and often conflicting in the scientific literature. The effects of EDCs on human reproduction have been assessed using current *in vitro* culture systems. For example, human testicular organoids treated with mono phthalate demonstrated an increase in germ cell autophagy in a dose-dependent manner [134]. In addition, a dual reproductive organ-on-a-chip system, where uterus and ovarian organoids were cultured in separate chambers, was used to validate the expression of the reproductive toxicity marker SERPINB2 in response to dioxin, a well-known EDC [129].

## 6. Toxicological assessment of viral infection

Recent report showed that the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) induces male sexual dysfunction, and it is most likely associated with the endothelial/vascular disintegrity, destruction, and dysfunction in the male reproductive system [19]. Given the world-wide spread of SARS-CoV-2 infection, it is urgent to synthesize vascularized human gonadal organoid to investigate how SARS-CoV-2 infection affects the function of male reproductive system. Although SARS-CoV-2 infection presents acute male reproductive disorder, the underlying mechanism on this dysfunction is unclear. There is a hypothesis that severe immune responses and inflammatory reactions caused by SARS-CoV-2 infection may disrupt vascular function in the male reproductive system, eventually inducing sexual dysfunction as seen in other blood vessels [153]. Synthetic human gonadal tissues-on-a-chip incorporated with immune or vascular system would enable us to model SARS-CoV-2 infection *in vitro* and to interrogate its mechanism on male sexual dysfunction.

## 7. Future perspective

Emerging *in vitro* technology allows us to re-evaluate the toxicological effects of chemical substances on human reproductive organs, although the information obtained *in vitro* is still limited and not directly comparable to *in vivo* studies. For example, immune cells, neural tissue, and endocrine circuit, those have important role for maintaining the physiology of gonadal tissues, are not exhibited in the current model of human reproductive system. Recently, organ-on-a-chip equipped with these constructs has been used for understanding how immune, neural, and endocrine factors are associated with tissue damage, inflammation, and endocrine disruption by EDCs [154–157]. Combination of these components with gonadal organoids would provide more feasible platforms for evaluating reproductive toxicity.

So far, chemical toxicity has been evaluated in a natural physiological state *in vitro*, but experiments in disease conditions have not been performed. Environmental chemicals, particularly EDCs, disrupt natural endocrine function. Therefore, diseases that interfere with endocrine function may alter the effects of these hazards in the body. Patient-derived PSC organoids may be useful to elucidate unexplored phenomena in the body of patient, and helpful in predicting the behavior of chemicals in the bodies of patients.

Although there is currently no *in vitro* human model that accurately mimics post-implantation embryo development, animal models have been successfully demonstrated using whole embryo culture systems to develop rodent embryos and blastoids from the peri-implantation to mid-developmental stage [158–161]. Emerging bio-engineering technology introduces additional cellular complexity into organoids in a controlled manner with a possibility to study higher-order functions such as directed perfusion [15]. Thus, organoid and organ-on-a-chip system provides more physiological relevant model to advance predictive drug toxicology. Accounting missing important factors, such as tissue shape, size, and compositional control, may improve variability in toxicity testing. These combinatorial technology provides a valuable tool for investigating the sequential events of embryo development after implantation.

While the analysis of human embryonic development at the post-implantation stage is prohibited, the International Society for Stem Cell Research has announced that human blastoids, synthetic embryos, can be used for post-implantation studies under specialized supervision and a rigorous review process (https://www.isscr.org/guidelines/#guidelineskeytopics) [162]. Consequently, the cultivation of human blastoids using a whole embryo culture system may pave the way for a new field of research, shedding light on human embryo development and enabling the exploration of human embryonic toxicity.

## 8. Conclusion

In this review, we have summarized the advancements in 2D and 3D *in vitro* technologies for culturing and maintaining testicular or ovarian cells. We have introduced the current advances in stem cell-based technologies, organoids, and microphysiological systems that partially mimic physiological conditions. Since 3D culture method is generated fundamentally based on 2D culture technology, the further development of both 2D and 3D technologies will be crucial in generating more sophisticated systems.

## CRediT authorship contribution statement

**Toshiya Nishimura:** Writing – original draft, Project administration, Investigation, Conceptualization. **Takanori Takebe:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

No data was used for the research described in the article.

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