A review on biomaterials for ovarian tissue engineering

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Abstract

Considerable challenges in engineering the female reproductive tissue are the follicle's unique architecture, the need to recapitulate the extracellular matrix, and tissue vascularization. Over the years, various strategies have been developed for preserving fertility in women diagnosed with cancer, such as embryo, oocyte, or ovarian tissue cryopreservation. While autotransplantation of cryopreserved ovarian tissue is a viable choice to restore fertility in prepubertal girls and women who need to begin chemo- or radiotherapy soon after the cancer diagnosis, it is not suitable for all patients due to the risk of having malignant cells present in the ovarian fragments in some types of cancer. Advances in tissue engineering such as 3D printing and ovary-on-a-chip technologies have the potential to be a translational strategy for precisely recapitulating normal tissue in terms of physical structure, vascularization, and molecular and cellular spatial distribution. This review first introduces the ovarian tissue structure, describes suitable properties of biomaterials for ovarian tissue engineering, and highlights recent advances in tissue engineering for developing an artificial ovary.

Keywords

Ovarian tissue engineering; follicle; *in vitro* culture; 3D printing; ovary-on-a-chip

1 Introduction

Ovarian follicles are the mammalian ovary's functional units, consisting of an oocyte surrounded by granulosa cells and different types of extracellular matrices (zona pellucida, antrum, basement membrane, and theca matrix) [1, 2]. The firmly connected granulosa cells surrounding the oocyte are responsible for its nourishment and development and mediate the external signals during its maturation process [3]. Consequently, the 3D architecture of follicles with tight maintenance of granulosa cell and oocyte interactions is crucial for successful oocyte growth and maturation [4, 5].

In the 90s, *in vitro* culture of rodent ovarian follicles [6] established the framework for the possible *ex vivo* follicle development, which could aid women or girls with fertility-threatening diseases or treatments [7]. However, the conventional 2D culture systems showed to be inefficient in preserving the 3D spatial arrangement required by human follicles. The 2D culture resulted in immature oocytes, as the follicles adhered to the flat plastic bottom of the culture dish and lost their 3D structure and the connections between granulosa cells and oocytes [8, 9]. For follicles from large mammalian species and humans, the 3D cell culture systems revealed a better choice to preserve cell-cell and cell-matrix interactions and maintain the spherical shape of follicles [10, 11], and provide proper steroid production [12, 13].

Tissue engineering offers a multitude of 3D cell-seeded/encapsulated scaffolds for *in vitro* culture. Some of these 3D scaffolds can also be implanted into the target site to restore function, repair, or replace the damaged tissue or organ [14-16]. In this aspect, the ovarian tissue engineering concept presents a 3D system for folliculogenesis resumption, supporting follicle survival and growth [17]. An engineered ovary can be fabricated by encapsulating

ovarian follicles and cells into a 3D biomaterial (Fig. 1). In addition to the general requirement of biomaterials, which should be biocompatibility, biodegradability, and being able to exchange nutrients and waste, the biomaterial for ovarian tissue engineering needs to have a right balance between rigidity and elasticity to maintain follicle spherical shape and provide an appropriate substrate for its radial growth [18]. Indeed, when the diameter of follicles increases, they receive compressive forces from the biomaterial surrounding them that the magnitude of the forces depends on the elasticity of the biomaterials and the follicle size changes. For example, when the diameter of murine secondary follicles changes from 120 μm to 400 μm, their volume increases 37-fold. This volume alteration is much more in human follicles, increasing 4.7 million-fold volume when they develop from secondary (120 μm) to the pre-ovulatory stage (20 mm) [19]. The shear stiffness (G') of the normal human ovary could be around 1-2.5 KPa [20], and they have an average strain of 23.05 με (± 10.74) in the response of axial compression of a square wave (500 Hz, 50% duty) [21]. Therefore, recapitulating the natural ovary's mechanical properties is crucial when developing a suitable scaffold for the survival and development of follicles.

The conventional tissue engineering methods such as seeding cells in decellularized scaffolds, nanofibers, or encapsulating them in hydrogels have been somewhat able to mimic biological tissues. However, precisely recapitulation of the natural tissue complexities, cellular architecture, mass transfer, and oxygen diffusion remains a challenge for the traditional scaffold fabricating methods [22-24]. Nowadays, 3D biofabrication methods have been developed to construct complex biomimetic structures with high control over the geometry, ability to pattern biomolecular cues, and suitable mechanical properties [25, 26], overcoming tissue engineering challenges, such as vascularization [22] and spatiotemporal

control of cell-cell and cell-extracellular matrix (ECM) interactions [27]. This article aims to review natural and synthetic biomaterials used to promote ovarian follicle growth and describe recent advances in ovarian tissue engineering in terms of the 3D printing approach, microfluidics for encapsulation of follicles, and ovary-on-a-chip.

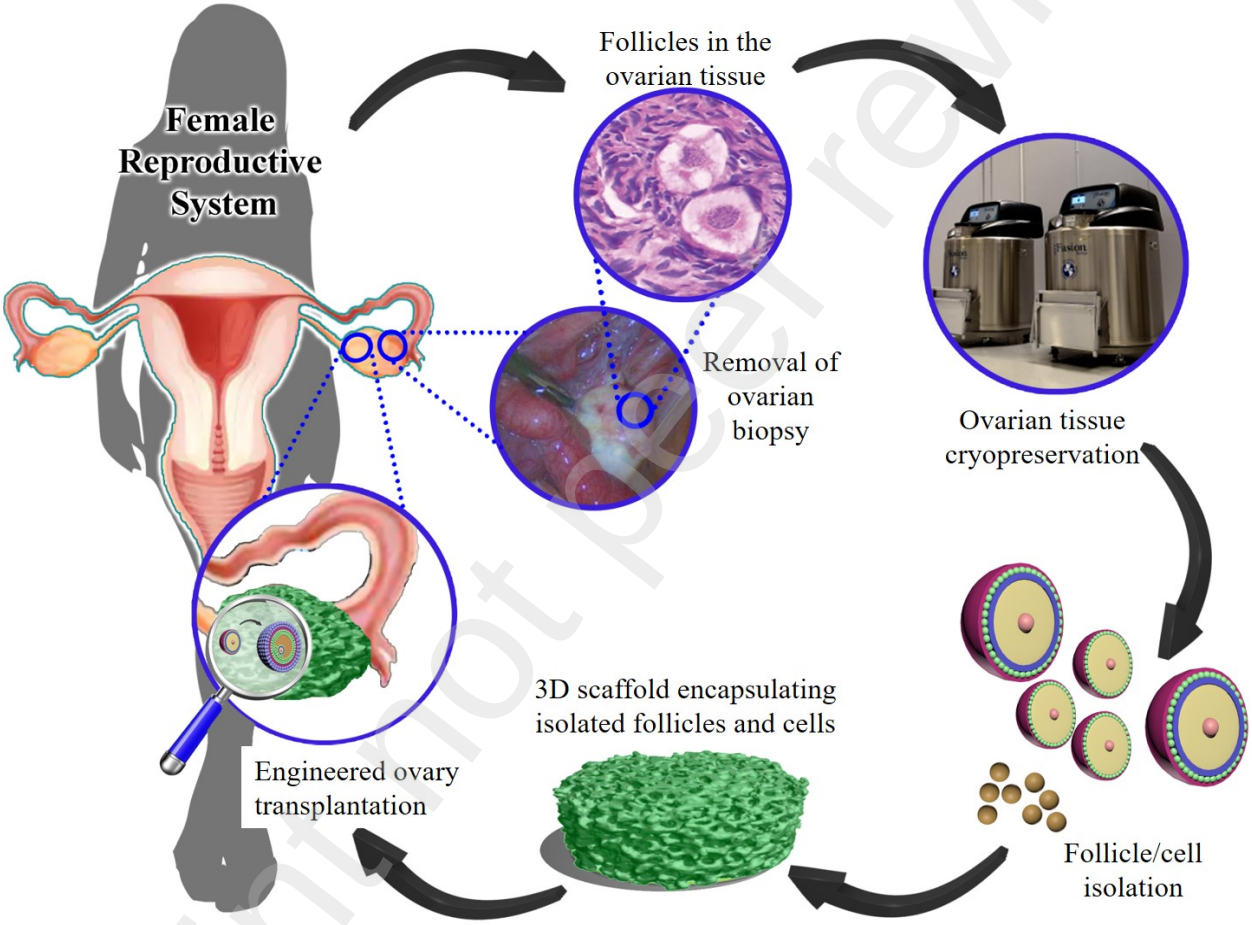

Figure 1. A schematic illustration of the fertility preservation strategy using ovarian tissue cryopreservation and ovarian tissue engineering. Before chemo- or radiotherapy, the ovarian tissue is removed and cryopreserved. Once the patient is cured, the tissue fragments are thawed, and their follicles and cells are isolated and seeded/encapsulated in a 3D bioengineered scaffold. Finally, the construct is orthotopically transplanted.

2 The ovary

Ovaries are reproductive glands located beside the uterus. They are responsible for gamete production and hormone secretion [28, 29]. The ovary consists of four layers from the outer to the central section, respectively: the germinal epithelium layer, the collagenous connective tissue, the cortex containing preantral follicles, the medulla containing loose connective tissue, blood vessels, and large follicles [30]. Follicles are the primary units of ovaries that consist of an oocyte surrounded by granulosa cells. A basal lamina separates granulosa cells from stromal cells in primordial and primary follicles or from the theca cells in the secondary and antral-staged ones [31, 32]. Follicle development can be affected by bidirectional signaling between the oocyte and granulosa cells, the granulosa and theca cells, and their interactions with the ECM and growth factors [31]. Moreover, one of the requirements for folliculogenesis is the presence of interactions between the oocyte and somatic cells [33], which mediate the action of external signals and nourish the oocyte during the maturation process [3]. Folliculogenesis (Fig. 2) is the process of follicle development from the primordial stage to either ovulation or death by atresia [34]. The growing of follicles occurs in two stages: the first slow stage that takes weeks in small rodents and months in large mammalian species is characterized by proliferation of granulosa cells and an increase in follicles and oocyte diameter; in the second stage, the follicle is responsive to follicle-stimulating (FSH) and luteinizing hormones (LH), forms antrum cavity, synthesizes steroid hormones and reaches the pre-ovulatory step [9].

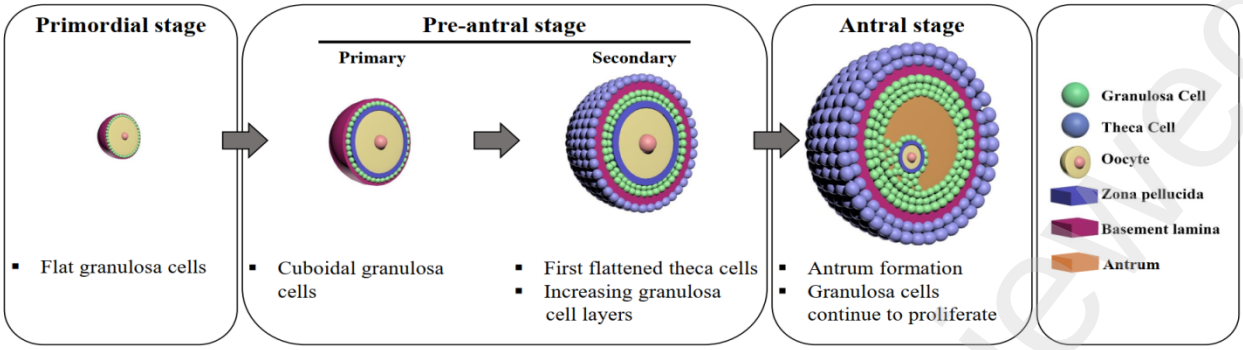

Figure 2. A schematic of follicle development from the primordial to the antral stage.

3 Development of follicle culture systems

The 2D *in vitro* system has been routinely used for follicle' *in vitro* culture [35-40]. Several approaches, such as multi-well plates, cell culture dishes, or coverslips coated by gels or ECM proteins such as collagen, fibronectin, and laminin, have been used in this procedure [3, 33, 41]. When isolated follicles are attached to a 2D culture surface, the somatic cells migrate to the bottom of the well, gradually losing their interaction with the oocyte, and consequently, the follicle 3D structure is destroyed [7]. Indeed, 2D culture systems are different from *in vivo* microenvironment conditions, where cells and tissues have complex connections with different adherent ligands in the 3D ECM [42]. Cell-cell and cell-matrix interactions are essential for follicle and oocyte development [43], and therefore, 3D culture approaches have been developed to maintain the original follicle structure [3, 44, 45].

In 3D systems, it is possible to preserve follicle architecture, which has positively impacted morphological follicle integrity, diameter [46], and oocyte maturation [7]. The 3D follicle culture systems have been demonstrated to yield higher follicle survival and growth rates, preserve follicle morphology, and reduce oocyte maturation genes' expression compared to

the 2D strategies. Moreover, using a 3D culture, it is possible to co-culture multiple types of cells to better mimic *in vivo* conditions [47]. Although the 3D culture systems have been widely investigated, there are still many challenges such as reproducibility, hypoxia probability, and insufficient nutrient supplying that must be overcome before its clinical applications [48].

4 Biomaterials used in ovarian tissue engineering

Compared to synthetic polymers, naturally-derived biomaterials have an interdependence of mechanical and biological properties. For instance, increasing collagen concentration alters the integrin-binding and protease-sensitive sites, which has been indicated by cell proliferation and mitochondrial activity and changes the mechanical behavior of this protein [49, 50]. On the other hand, polyethylene glycol (PEG) as an inert polymer has tunable physical properties independent of its bioactivity [49, 50]. Therefore, the combination of required biological (such as providing a substrate for cell attachment, differentiation, migration, remodeling, etc.) and mechanical properties resembling the natural organ or tissue is essential when choosing a biomaterial for ovarian tissue engineering [19, 50-52]. Biomaterials employed for this strategy (Fig. 3) must have a certain amount of elasticity (storage modulus between 1 and 2.5 KPa), as this is an essential requirement for granulosa expansion during follicular growth while supporting the spherical shape of follicles. Furthermore, comparing the development of follicles encapsulated in biomaterials with different stiffness could give an insight into finding the proper scaffold rigidity for follicle encapsulation.

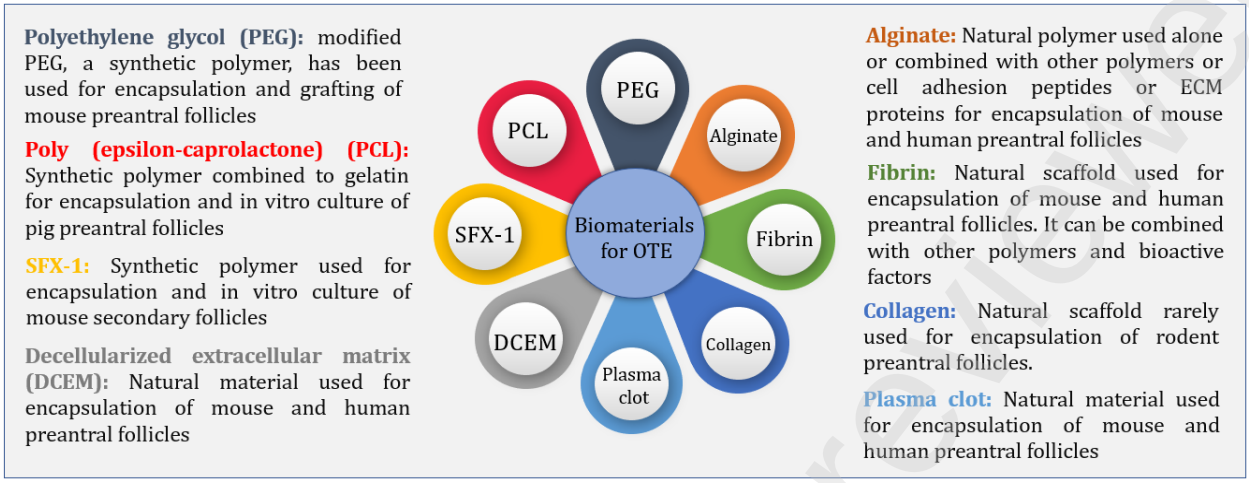

Figure 3. Biomaterials used for ovarian tissue engineering.

4.1 Natural polymers for ovarian tissue engineering

4.1.1 Alginate

Alginate is a linear polysaccharide derived from algae with biocompatible, readily available, bio-inactive, biodegradable, and non-immunogenic properties [53, 54]. It has FDA approval for clinical use [55] and has been widely used in tissue engineering applications [53-58]. Mechanical strength and gelation properties of alginate can be affected by its molecular weight, crosslinking degree, and the percentage of guluronic acids units available in its backbone [55, 59].

Alginate is the most used polymer for *in vitro* culture of isolated preantral follicles [4, 28, 46, 47, 60-69]. It has been applied as a matrix to promote follicle development *in vitro* and a tool to learn about folliculogenesis and test the impact of cryopreservation and chemotherapy on follicle population [4, 65, 68-71]. The effect of different bioactive factors, such as vascular endothelial growth factor (VEGF) [11], kit ligand [8], LH, and epidermal growth factor [72],

on follicle survival and development, has also been assessed with the aid of the alginate. Similarly, the effect of preantral follicle density on its growth and survival was evaluated using alginate [73]. Different numbers of mouse follicles (1, 5, and 10) were encapsulated in 0.5% alginate beads. A positive correlation was found between follicle development and their density in the alginate bead, demonstrating that crosstalk among follicles is necessary for their survival and growth [73].

Alginate was also applied to graft isolated follicles. Vanacker et al. [74] allografted mouse preantral follicles and ovarian cells encapsulated in 1% alginate. The results showed that alginate beads could successfully support the survival and development of follicles and the viability and proliferation of ovarian cells after one week of transplantation [74].

While several studies reported alginate as a suitable polymer for encapsulation of ovarian follicles [62, 68, 74], controlling the degradation rate of the alginate hydrogel to match with the follicle growth is challenging, and the polymer rigidity can negatively affect further development of the follicles [54]. For instance, mouse preantral follicles encapsulated in 0.125% alginate showed better follicle survival and antral formation than their counterparts encapsulated in 0.25% alginate [75]. Similarly, West-Farrell et al. [76] compared two different alginate concentrations (0.5 and 1.5%) to *in vitro* culture mouse secondary follicles and reported that the softer hydrogel could better support their development. On the other hand, in large animal species, the results were the opposite: compared to 1%, 2% alginate showed a better influence on the diameter of sheep primordial and primary follicles [77]. Such discrepancy among mammalian species could be due to the different follicle mechanical properties requirements. For instance, as regards the shear modulus of 1.5% alginate (approximately 1500 Pa) is much more than the alginate 0.5% (around 121 Pa) [76], it seems

that the follicles isolated from the mouse compared to the human isolated follicles prefer to be encapsulated in a softer biomaterial.

Alginate is inert and does not have cell-binding sites. However, the cell adhesion property of the alginate can be enhanced through the binding of cell adhesion peptides, such as the RGD (arginine-glycine-aspartic acid) sequence [54]. Indeed, Kreeger et al. [78] modified alginate with ECM proteins or RGD to encapsulate secondary follicles and showed that the modified alginate improved follicle development. Combining alginate with other polymers such as fibrin [79, 80], Matrigel [66], and gelatin [53] is another way to enhance its cell attachment properties and biodegradation rate. Jin et al. [7] encapsulated isolated mouse secondary follicles in a fibrin-alginate matrix, which proved to be superior to alginate alone to improve follicle growth and oocyte diameter, antrum formation, and increasing the percentage of competent oocytes for fertilization. On the other hand, Jamalzaei et al. [81] showed that alginate/hyaluronic acid hydrogel was superior to fibrin/alginate hydrogels for the *in vitro* culture of mouse preantral follicles, improving their development up to the antral stage, yielding metaphase II oocytes and higher expression of growth and differentiation genes.

4.1.2 Fibrin

Fibrin is the major component in blood coagulation, playing a temporary matrix role in supporting fibroblast and endothelial cell invasion [82]. FDA has approved commercial fibrin sealants, such as Tisseel®, EvicelTM, and CrossealTM, which have different compositions, methods of production, and formulations [17] for numerous clinical applications [83].

Fibrin is a biocompatible [84] and biodegradable biomaterial [85, 86], which has been studied as a cell carrier, drug delivery system, and scaffold [87]. It provides a biomimetic

ECM for cells, improving their interaction with scaffolds [88], adhesion, and proliferation [85, 89, 90]. Moreover, fibrin has been shown to support the formation of the capillary network *in vitro* [91].

Activated thrombin cleaves fibrinogen peptides A and B from the central domain of fibrinogen (named E domain). Then, the central domain of cleaved fibrinogen is linked to the end group (named D domain) of other cleaved fibrinogen, which this end group has an interaction with another end group (D-D). Then, the formed fibers are covalently linked by fibrin stabilizing factor (FXIII) to generate a stabilized fibrin network [17]. Combinations of different concentrations of fibrinogen and thrombin result in fibrin matrices with wide varieties of morphology and rigidity [92], which affect cell behavior. For instance, decreasing fibrinogen concentration from 50 mg/ml to 5 mg/ml changes human mesenchymal stem cells morphology from round to spindle-like shape and their higher proliferation was observed in dilute fibrinogen solutions. The spindle shape morphology, establishing cytoplasmic projections, and increasing cell proliferation allow the cells to form a 3D cellular structure [93]. On the other hand, changing thrombin concentration can affect fibrin mechanical strength and cell activity [92, 94]. Taking advantage of this plasticity, Luyckx et al. [92] encapsulated human isolated ovarian cells in nine combinations of fibrinogen (F; mg/ml) and thrombin (T; IU/mL) (F1/T4, F12.5/T1, F12.5/T20, F25/T0.1, F25/T4, F25/T500, F50/T1, F50/T20, and F100/T4) to report the best combinations to support cell proliferation and viability. They showed that two fibrin formulations (F12.5/T1 and F25/T4) yielded positive effects on cell density dynamic, which ranged from 94.0% to 96.6% and 94.2% to 98.9% with high proliferation indexes 4.45 ± 2.34 and 4.38 ± 4.81, respectively. Then, Luyckx et al. [95] used these two fibrin formulations to encapsulate and auto-

transplant isolated mouse preantral follicles. Both matrices were able to successfully support follicle survival and development up to the antral stage. However, when tested with isolated human preantral follicles, the follicles showed a recovery rate of approximately 2%. Hypothesizing that this may be due to the lack of rigidity of these matrices, Paulini et al. [96] increased both fibrinogen and thrombin concentrations (F50/T10), obtaining a stiffer fibrin matrix to graft isolated human preantral follicles. Their results showed a follicle recovery rate of 22% after one week of xenografting in immunodeficient mice [96]. Such results were comparable to Nisolle et al. [97] after xenotransplantation of human ovarian tissue during the same period.

Aiming to optimize fibrin matrix to transplant isolated human ovarian follicles, Chiti et al. [98] investigated four different fibrin formulations (F12.5/T1, F30/T50, F50/T50, and F75/T75). They observed that fibrin formulations of F50/T50, and F75/T75 yielded fiber thickness similar to the human ovarian cortex, which varied between 61.3 to 72.4 nm. Also, increasing fibrinogen and thrombin concentrations augmented G', and F50/T50 yielded a G' similar to the natural human ovary. Therefore, they concluded that F50/T50 could closely mimic natural human ovary properties in terms of mechanical properties and fiber thickness [98].

In another study, Chiti et al. [99] used fibrin hydrogel to investigate the correlation between the stages of mouse follicle development on its survival and matrix vascularization after grafting. They reported that secondary follicles had higher survival and recovery rates than primordial and primary ones. Also, a superior number of vessels was found in clots containing secondary follicles. It appears that the higher survival rate of these larger follicles may be due to their spatial distribution in fibrin and the larger vascularization observed in

this group. Another fibrin formulation (20mg/ml fibrinogen and 50 IU/ml thrombin) also showed to support mouse follicle development after transplantation, as observed by the increase in follicular diameter and restoration of hormone cyclicity [100].

Fibrin can also be enriched with bioactive factors to enhance its biological properties. For instance, fibrin was supplemented with VEGF for transplantation of mouse preantral follicles, resulting in resumption of estrous cycling in the mice and offsprings [101]. Also, Rajabzadeh et al. [102] used fibrin hydrogel enriched with different human platelet lysate concentrations (5, 10, 15, 20%) to transplant murine preantral follicles. They showed that fibrin plus 15% platelet lysate yielded a higher follicle recovery rate, indicating the positive effects of the growth factors of platelet lysate on follicle survival. In another study [103], this group showed that this fibrin supplemented with 15% platelet lysate increased vascularization and follicle survival and development compared to the fibrin matrix alone.

While fibrin is a promising hydrogel for ovarian tissue engineering [17, 95, 96, 99, 104], this matrix has rapid degradation and intrinsic instability, which leads to loss of implant volume within days and, consequently, loss of the physical protection of follicles and stromal cells [83, 86, 105-108]. However, the fibrin degradation rate can be controlled by combining fibrinogen with natural or synthetic polymers such as alginate [79, 80] or using enzyme inhibitors such as aprotinin [86, 109, 110]. In a fibrin-alginate matrix, fibrin provides cell adhesion properties, while alginate supports matrix stability. This hybrid hydrogel is an example of a dynamic construct for embedding follicles, allowing follicle growth while decreasing the tension of the scaffold on the follicles during *in vitro* culture [12]. The fibrin-alginate hybrid hydrogel has been shown to enhance follicle development [50, 52, 111]. Shikanov et al. [110] introduced the fibrin-alginate interpenetrating network as a dynamic

297 mechanical scaffold whose stiffness changes according to fibrin degradation during *in vitro*
298 culture. This network could allow follicle expanding by the degradation of fibrin and
299 decreasing the concentration of alginate from 0.5 % to below 0.25%. Moreover, they
300 reported that for extending the mechanical gradient of the scaffold, different concentrations
301 of aprotinin as a protease inhibitor could be added to the culture medium to change the fibrin
302 degradation rate. Xu et al. [112] investigated a matrix containing 25 mg/ml fibrinogen, 50
303 IU/ml thrombin, and 0.25% alginate to encapsulate macaque primary and secondary
304 follicles. The results indicated that the fibrin improved primary follicle development and had
305 no effect on secondary follicles, in contrast to the alginate hydrogels. In another study, Xu et
306 al. [113] tested a matrix containing 50 mg/ml fibrinogen, 50 IU/ml thrombin, 0.5% alginate,
307 and Matrigel for culturing isolated baboon preantral follicles. They showed that this hydrogel
308 yielded metaphase II oocytes.
309
310 4.1.3 Collagen
311 Collagen is the most widely distributed type of protein in the human body and the first
312 natural matrix used to graft isolated preantral follicles [114]. Collagen possesses several
313 advantages: biodegradability, biocompatibility, and great versatility [115, 116]. Torrance et
314 al. [114] showed that collagen is a promising hydrogel for encapsulation and in vitro culture
315 of mouse preantral follicles. Telfer et al. [117] encapsulated mouse follicles in collagen and
316 in vitro culture them for five days before transplantation to mouse kidney capsule for a
317 period between 2 and 21 days. Although they reported a suitable survival rate of the follicles
318 after transplantation, they also observed oocyte atresia in antral follicles, and granulosa cell
319 luteinization [117]. Recently, Joo et al. [118] studied cell survival, follicle growth, hormone

production, and oocyte maturation in rat ovarian follicles encapsulated in type I collagen hydrogels as a 3D culture system. The results demonstrated that varying collagen hydrogel density and elasticity could significantly affect follicle development regarding their phenotype, hormone secretion, and maturation. In another study, the transplantation of adipose-derived stem cells on soluble collagen scaffolds showed to contribute to long-term restoration of ovarian function and the fertility of rats after tripterygium glycosides-induced ovarian damage [119].

4.1.4 Plasma clots

Plasma clot is an autologous material with high biocompatibility and a rich in growth factors [120]. It has been successfully used for culturing different types of cells such as human mesenchymal stromal cells [121, 122], chondrocytes [123], carcinoma cells [124], and monocytes [125], as well as follicle encapsulation [126-129]. Compared to purified fibrinogen, plasma clots are stiffer primarily due to the platelet, which can be increased to enhance the matrix rigidity [130]. The use of autologous plasma clots to encapsulate isolated follicles was first reported in the 1990s. After transplantation of mouse ovarian follicles and cells in plasma clots, animals could ovulate and deliver normal offspring [126, 127]. Following these successful results, Dolmans et al. [128, 129] encapsulated freshly isolated human follicles in autologous plasma clots and xenografted them to the ovarian bursa of immunodeficient mice. Although the results demonstrated follicle development up to the secondary stage after one week [128] and even finding antral follicles after five months [129], plasma clots degraded rapidly, which could have led to follicle loss [120].

4.1.5 Decellularized extracellular matrix

In tissue engineering, decellularized extracellular matrix (DECM) has been shown considerable potential to promote regeneration in various organs, such as the kidney, liver, and heart. DECM from ovarian tissue for ovarian tissue engineering has also yielded promising outcomes in isolated follicle transplantation. Recently, Pors et al. studied human preantral follicles seeded in DECM from ovarian tissue [131]. The results indicated a high survival rate of follicles injected with Matrigel after three weeks of xenografting to mice [131]. Hassanpour et al. [132] evaluated bioengineered ovary construction using a 3D scaffold based on a sodium lauryl ester sulfate-treated DECM protocol. After 14-day transplantation in rats, the results showed that DECM could be an ideal scaffold for this regard due to the great bioactivity and viability of primary ovarian cells and the ability to reconstruct the primordial or primary follicle-like structures. Laronda et al. [133] decellularized bovine and human ovaries with sodium dodecyl sulfate and recellullarized them with primary ovarian cells. They reported that the decellularized scaffolds could preserve ovarian microstructure and provide estradiol hormone production *in vitro*. Recently, Alshaikh et al. [134] investigated the decellularization of mouse ovarian tissue using two different types of detergents: 0.5% sodium dodecyl sulfate and 2% sodium deoxycholate. While both protocols showed high biocompatibility, the latter produced DECM that appeared to be slightly more advantageous due to a higher recellularization efficiency. Moreover, Eivazkhani et al. [135] studied the effects of two different treatments of sodium dodecyl sulfate and NaOH for the decellularization of mouse, sheep, and human ovaries. The scaffolds created by using NaOH demonstrated better decellularization and support cell growth than sodium dodecyl sulfate. Liu et al. [136] used physical, chemical, and enzymatical

366 treatments for decellularization of the porcine ovary to shorten the sodium dodecyl sulfate
367 treatment time and consequently reduce its damaging effect on the tissues. They showed
368 that the three steps of decellularization (using Triton X-100, SDS, and DNAase I) could
369 successfully remove cell components, and the decellularized scaffolds were biocompatible
370 with minimum host immune responses, supported cell penetration, and enhanced the
371 estradiol production.
372
373 4.2 Synthetic hydrogels for ovarian tissue engineering
374 4.2.1 Polyethylene glycol (PEG)
375 PEG is a synthetic biocompatible polymer [137-139]. It is FDA-approved [25, 137] and
376 widely used in tissue engineering and regenerative medicine [137]. Pure PEG is biologically
377 inert and unable to support cell adhesion and proliferation [137]. However, it can be
378 modified with Arg-Gly-Asp (RGD) peptides for encouraging cell attachment using Michael-
379 type addition (MTA) chemistry. When crosslinked by protease-sensitive peptides, it can
380 induce cell migration and proliferation [50, 140-144]. Kim et al. [107] used PEG hydrogel
381 modified with RGD and crosslinked with matrix metalloproteinase-sensitive tri-functional
382 crosslinking peptides for ovarian tissue engineering and implanted it to mice for *in vivo*
383 culture. Their results demonstrated that this synthetic matrix could support the survival and
384 development of early-stage follicles in addition to graft remodeling and revascularization
385 [107]. This approach demonstrated the ability of scaffold engineering by adding susceptible
386 protease peptides to the PEG backbone to achieve the goal of synchronizing biodegradation,
387 which occurs in response to cell-associated proteolytic activities, with ECM remodeling and
388 supporting the tissue regeneration [145]. Furthermore, 8-Arm PEG-vinyl sulfone (PEG-VS)

supplemented with the MMP- and plasmin-sensitive crosslinker was investigated by Tomaszewski et al. [146] for encapsulating follicles with or without adipose-derived stem cells to evaluate their effect on follicles. They reported that this biomimetic matrix preserved multipotency of the cells and induced them to produce paracrine factors beneficial for enhancing follicle viability and development.

Recently, Tomaszewski et al. [147] modified PEG with the ECM-sequestering peptides, Heparin-binding peptide (HBP), ECM-binding region of placental growth factor 2 (RRR), laminin-derived peptide (AG73), and basement membrane binder (BMB) to mimic the natural ovarian ECM. They used two types of crosslinkers: fast-degrading GCYKNRGCYKNRCG (YKNR) and slow-degrading GCYKNSGCYKNSCG (YKNS) to optimize the proteolytic degradation of hydrogels. They encapsulated single isolated murine follicles in each hydrogel and showed that these modified hydrogels improved follicle viability, development, and maturation and caused the recreation of ECM molecules in terms of laminin, fibronectin, perlecan, and collagen I [147].

4.3 Other natural and synthetic scaffolds

Synthetic polymers have been widely used for tissue engineering and regenerative medicine applications, but they have been poorly explored in ovarian tissue engineering. Poly(epsilon-caprolactone) (PCL) is one of the most biodegradable and biocompatible polyesters with many applications in tissue engineering [119, 148]. Gelatin/PCL electrospun fibrous 3D scaffold [149] was studied for seeding isolated porcine preantral follicles. Gelatin, as a natural polymer, was used to enhance cell-biomaterial interactions, improving cell adhesion, migration, and differentiation. The gelatin/PCL scaffold was demonstrated to preserve

follicles morphology, increase their adhesion. Moreover, the amount of estradiol and progesterone produced by follicles seeded in gelatin/PCL was higher than PCL samples, which indicated better follicle growth in this scaffold. SFX-1, a synthetic polymer, which was produced from N-isopropylacrylamide (NIPAM) monomer has also been used for ovarian tissue engineering [150]. Murine secondary follicles were encapsulated in the 30 mg/ml SFX-1 and *in vitro* cultured for two days. Although the results demonstrated an increase in follicles diameter from 153 ± 28 μm to 201 ± 38, only 12.5% of follicles maintained their morphological integrity [150].

5 Recent trends

The 3D biofabrication strategies can manufacture complex cellular environments to recapitulate natural tissues by precise control of material geometry and properties and spatially molecular concentration [25]. Indeed, computer-aided technologies have been altering the concept of manufacturing in both industry and human life [151], such as 3D printing systems that aim to fabricate viable constructs by the following biomimicry, autonomous self-assembly, and mini-tissue approaches [152, 153]. The 3D printing technology can be an alternative for tissue engineering and regenerative medicine challenges [154-157], fabricating functional tissues by controlling precise cellular and biomaterial spatial distribution [158, 159] and recapitulating microstructures [160]. Despite the promising results, this technology is in the early stage of development, and only simple tissue constructs have been fabricated by the current 3D printers [159]. On the other hand, *in vitro* growth of ovarian follicles from large mammalian species remains a challenge

because of the long-term culture necessity and follicle size, which exponentially increases during folliculogenesis, and their complex metabolic conditions.

The innovative ovary-on-a-chip platforms using dynamic systems may overcome these difficulties. They can be a strategy to restore fertility in cancer patients and a valuable tool for preserving endangered mammalian species [161]. In the mouse model, Xiao et al. [162] showed that organ-on-a-chip platforms using microfluidic technology could maintain ovarian endocrine function, mimic menstrual cycle, and provide high stability and controllability of flow patterns for approximately 100 days. This study opened a door for improving investigations about tissue-tissue interactions, biological and pharmacological research by producing organs-on-a-chip models consisting of the ovary, fallopian tube, uterus, cervix, and liver for representing the human reproductive tract. Overall, organ-on-a-chip systems can replicate the natural structure and functions of organs and their dynamic flow conditions via controllable microfluidic fluids [163].

5.1 3D printing

Three-dimensional printing fabricates 3D complex tissue constructs mimicking natural tissues using robotic additive manufacturing technologies [154, 164, 165]. Three different types of 3D printing approaches have been developed: laser, inkjet, and extrusion-based 3D printing [153, 154, 166, 167] (Fig. 4), which provide cell viability of 95%, >85%, and 80%, respectively [153]. Different natural, synthetic, and hybrid biomaterials have been studied as 3D printing materials for encapsulating cells [159, 164]. In general, 3D printing materials include cell aggregates, cells encapsulated in hydrogels, viscous fluids, and microcarriers

involving cells [164]. They must also have printability, biocompatibility, biodegradability, biological activity, and appropriate physical, chemical, and mechanical properties [159].

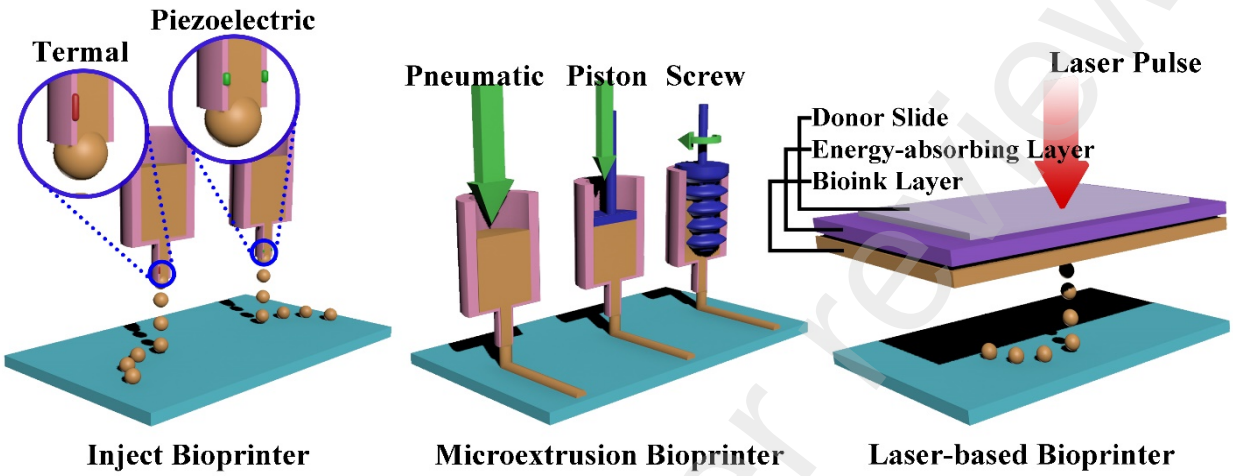

Figure 4. Classification of 3D printing technologies. Inkjet 3D printers are divide into thermal and piezoelectric setups, which use heat or mechanical stimulation, respectively, to generate droplets. Extrusion-based 3D printers extrude bioink using pneumatic, piston, or screw dispensing systems. In laser-based 3D printers, a laser pulse focuses on the donor slide coated by an absorbing layer and induces a vapor bubble that ejects the droplet onto the collection substrate.

Several functional tissues have been developed using this emerging technology, such as cartilage [165, 168-171], skin [172-176], vascular system [177-181], and liver [182-184]. However, ovary 3D printing is still in its infancy. Laronda et al. [185] developed a functional 3D printed follicle-seeded ovarian scaffold. In this study, by altering the orientation of printed layers, the authors assessed the effect of pore geometry on follicle viability. Interestingly, they found a positive correlation between the number of interactions between

scaffold and follicles and their survival rate. The follicles seeded in these partially crosslinked gelatin 3D-printed platforms accounted for increasing vascularization and restoring ovarian function in surgically sterilized mice and yielded healthy pups (Fig. 5) [185].

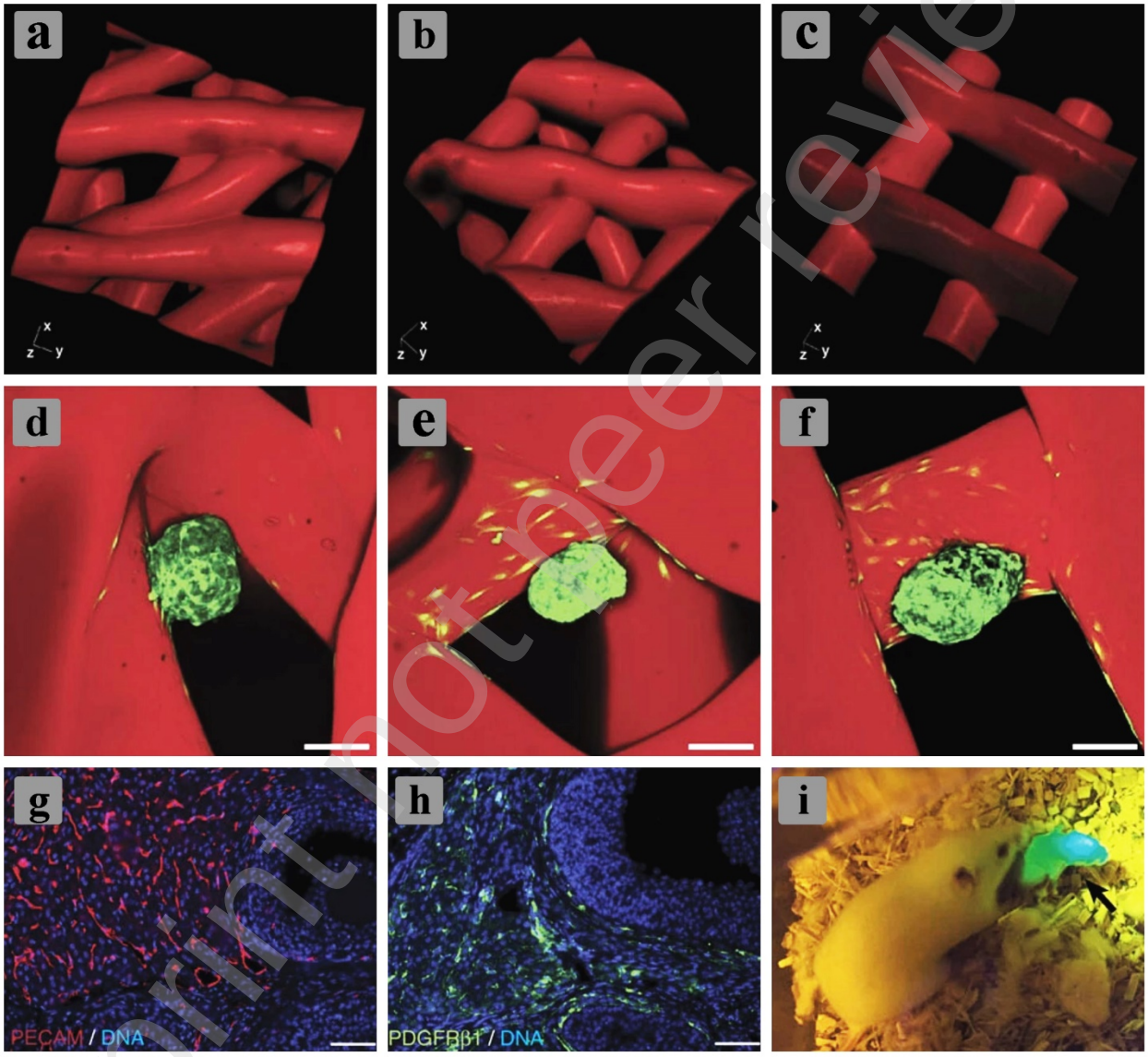

Figure 5. 3D printed construct for isolated follicles. a-c) 3D reconstructions of confocal fluorescence images of 30°, 60°, and 90° angle 3D printed scaffolds (a, b and c, respectively). d-f) green fluorescent protein-positive (GFP+) follicles seeded in pores after two days in

culture. Follicles in 30° and 60° pores (d and e, respectively) often resided in corners, whereas they tended to be on one strut in 90° pores. g and h) vascularization in 60° angle 3D printed scaffold with immunostaining for endothelial marker platelet endothelial cell adhesion molecule (PECAM) (red) or pericyte marker PDGFR β1 (green) and DNA (blue) in corpus luteum, antral follicles and interstitial space of bioprosthetic ovary removed after 8-10 weeks. i) a healthy pup after mating transplanted animals [185]. Reprinted with permission from [185] © Springer Nature (2021).

Wu et al. [186] investigated the gelatin-methacryloyl for 3D printing of ovarian tissue and reported that the isolated primary stromal cells lost their viability after printing, indicating more vulnerability of the primary cells to the printing process than ovarian tumor cell lines (COV434, KGN, ID8). Then, the authors evaluated isolated murine follicles seeded in scaffolds with 60° or 90° angles between the adjacent upper and lower strands. They observed that while 60° constructs successfully supported follicle viability (84%) after seven days of *in vitro* culture, most follicles in 90° constructs fell into the bottom of the culture plate and a few numbers of them attached to the strands [186].

5.2 Microfluidics for follicle encapsulation

A mammalian ovary is divided into stiffer and softer sections regarding the mechanical and structural properties, which are defined as cortex and medulla, respectively. Recapitulation of the ovary's mechanical heterogeneity has an essential role in folliculogenesis [187]. However, it is also important to take into account that the length of oxygen and nutrients penetration is less than 200 μm, and therefore thin microtissues should be constructed to

prevent cell death in the core of the substrates [42]. As one of the microfluidics technology applications is fabricating shape-controlled microgels [188], Choi et al. [72] simulated ovarian biomechanical structure by using two different hydrogels (alginate and collagen as hard and soft segments of the ovary, respectively) and microfluidics for encapsulating mouse early secondary preantral follicles into the microcapsules. They successfully biofabricated a core shell biomimetic ovary prototypes, in which alginate was placed in the shell and collagen in the core section. This biomimetic microtissue led to follicle development up to the antral stage after nine days of *in vitro* culture (Fig. 6). In another study [42], this group encapsulated mouse embryonic stem cells (mESCs) and ovarian preantral follicles in the core (collagen I at different concentrations containing 1% sodium carboxymethyl cellulose)-shell (alginate) microcapsules to evaluate cell proliferation and follicle development. To improve mechanical properties, 5 mg/ml alginate was added to some samples. Although the elastic modulus of microtissues had an increasing trend in cores from 0.5 to 3 mg/ml collagen, the expression of pluripotency genes from mESCs embedded in these microcapsules was decreased, which indicated the possibility of better E-cadherin mediated interactions between cells in a softer microenvironment [42]. Moreover, constructs with softer core (1 mg/ml collagen I) showed a significantly lower proportion of antral follicles compared to counterparts with the higher collagen concentration (5 mg/ml), which also produced higher estradiol concentrations (around 3 ng/ml at day 10). Interestingly, when stiffer cores contained additional alginate concentration (5 mg/ml), the proportion of antral follicles augmented but remained lower than those stiffer microgels without alginate.

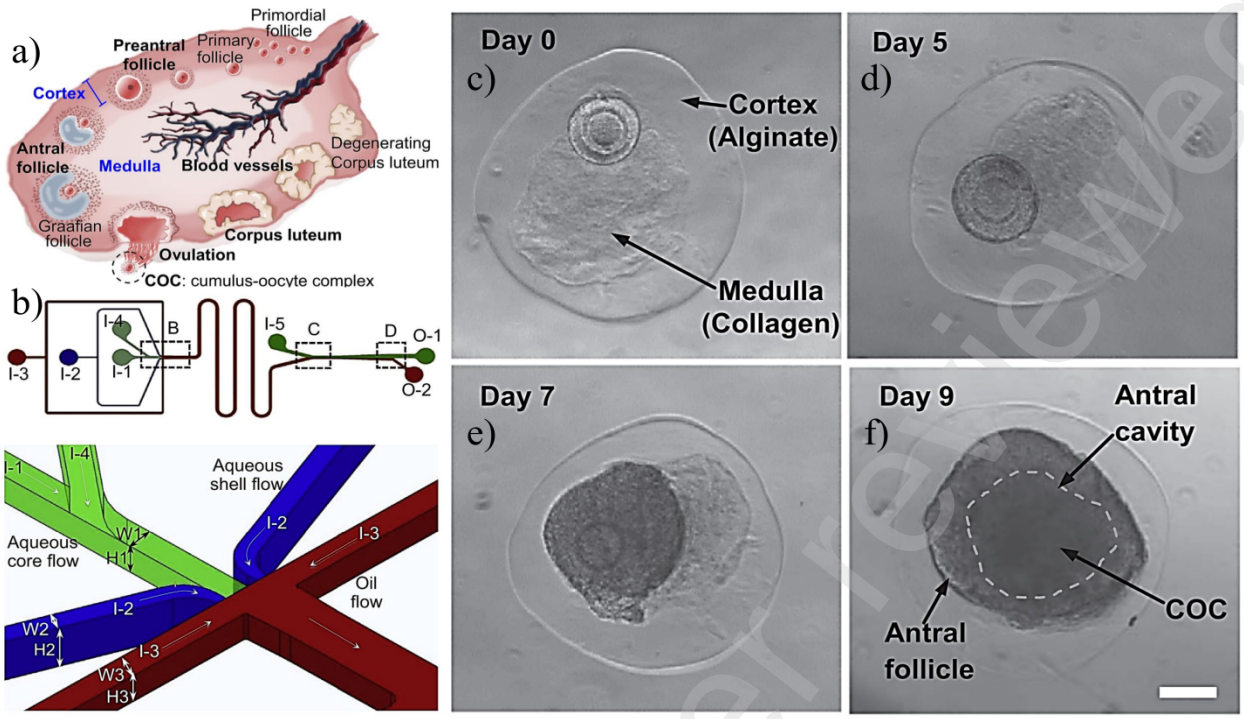

Figure 6. Design and materials of biomimetic ovarian microtissue. a) Ovary anatomy with two distinct layers: a more rigid cortex and softer medulla. b) A schematic view of microchannels system for encapsulating follicles (top) developed by Choi et al. [72] together with a zoom-in view of the nonplanar design of the flow-focusing junction (bottom) where W1= 200, H1=200, W2= 80, H2=300, W3=200, and H3=400 μm. c-f) follicle growth in the engineered microtissue on days 0, 5, 7, and 9, in which antral cavity and a cumulus-oocyte complex (COC) were observed on day 9 [72]. Reprinted and adapted from [72] © Elsevier (2021).

5.3 Organ-on-a-chip

The organ-on-a-chip system converges two research areas (tissue engineering and microfluidic technologies) to emulate key characteristics of a specific living organ, such as extracellular microenvironments and cell-cell interactions. The main goal of developing

organ-on-a-chip systems is to use them as a replacement for animal tests. They have numerous applications, including creating *in vitro* healthy/diseased models for tissue development studies, drug discovery and development, and evaluating the efficiency and safety of drugs [189-191].

An organ-on-a-chip platform can mimic the critical environment multifunction of a specific organ on a single chip. Its fabrication consists of designing a microchannel network, which allows for controlled loading, placement, diffusion, and permeation of microfluidic, a cell chamber to replicate the microenvironment of the organ, and a cell-retention filter that can also be used in connection sites with external tubing [192-194]. Despite the wide variety of organ-on-a-chip platforms, such as liver [195-200], lung [201-204], vessel [205-208], and kidney [209-212], which have been developed to improve studies on functional human organs, only a few studies are aiming to create the ovary-on-a-chip.

The first studies using the ovary-on-a-chip technology have shown promising results supporting follicle development [161, 162, 213, 214]. It has also been successfully applied to monitor ovotoxicity induced by microcystins [213], study anti-cancer drugs effects on ovarian follicles growth and function [215], and as a model for ovarian cancer [163, 216, 217] and a tool to characterize oocyte quality [218, 219]. However, this approach is still in its infancy, and it will surely be explored in the following years.

6 Conclusion

In this review, we have summarized the biomaterial requirements for ovarian tissue engineering, and the 2D and 3D culture systems for follicle development. Moreover, we have

discussed innovative biofabrication methods that have been employed for creating ovarian tissue. The 3D printing and organ-on-a-chip technologies, as biofabrication techniques, endeavor to recapitulate the complexity of natural organs and be a solution for convenient tissue engineering challenges. Indeed, they have the potential to create biomimetic functional systems, which can be used for tissue engineering and regenerative medicine applications. These emerging strategies can help the ovarian tissue engineering concept by precisely mimicking the ovarian microenvironment. This can allow follicle development to restore fertility in cancer patients and analyze and monitor the effects of biological and chemical agents such as hormones, growth factors, and drugs on folliculogenesis.

Credit author statement

Arezoo Dadashzadeh drafted the initial version of the manuscript together with Saeid Moghassemi. Amin Shavandi and Christiani A. Amorim reviewed and agreed on the final version of the manuscript.

Data availability

Not applicable.

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.

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