FERTILITY PRESERVATION

Construction and use of artificial ovaries

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Abstract

Increasing numbers of patients are now surviving previously fatal malignant diseases, so for women of childbearing age, fertility concerns are paramount once they are cured. However, the treatments themselves, namely chemo- and radiotherapy, can cause considerable damage to endocrine and reproductive functions, often leaving these women unable to conceive. When such gonadotoxic therapy cannot be postponed due to the severity of the disease or for prepubertal girls, the only way to preserve fertility is cryobanking their ovarian tissue for future use. Unfortunately, with some types of cancer, there is a risk of reimplanting malignant cells together with the frozen-thawed tissue, so it is not recommended. A safer approach involves grafting isolated preantral follicles back to their native environment inside a specially created transplantable artificial ovary for their protection. This bioengineered ovary must mimic the natural organ and therefore requires an appropriate scaffold to encapsulate not only isolated follicles, but also autologous ovarian cells, which are needed for follicles to survive and develop. Here we review the indications for use of this artificial ovary and advances in the field that are bringing us ever closer to clinical implementation.

Introduction

Cancer remains one of the most pressing health issues worldwide and a leading cause of death across the globe. Thanks to early diagnosis and great improvements in cancer treatments, 5-year survival rates have now reached 65% in adults and 83% in children (Siegel et al. 2018). On the other hand, these very treatments have given rise to other issues, like premature ovarian insufficiency (POI) and infertility. Because of the gonadotoxic nature of cancer therapies, fertility preservation prior to cancer treatment is becoming a clinical and moral duty in oncological practice.

For adult women, a number of different fertility preservation strategies can be proposed (Donnez & Dolmans 2017). For prepubertal girls and women who require immediate chemo and/or radiotherapy, however, ovarian tissue cryopreservation (OTC) is the only alternative at present, since it does not require controlled ovarian stimulation. Frozen-thawed ovarian tissue can be transplanted back to the pelvic cavity once the patient is cured. This procedure leads to restoration of ovarian activity in more than 95% of cases (Donnez et al. 2015). Transplantation of cryopreserved ovarian tissue has been steadily increasing all over the world and more than 130 live births have been reported to date, yielding live birth rates in the range of 23–77% (Donnez et al. 2015, Meirow et al. 2016, Van Der Ven et al. 2016, Donnez & Dolmans 2017, Jensen et al. 2017, Diaz-Garcia et al. 2018, Silber et al. 2018).

The most frequent indications for OTC and subsequent transplantation in adults are hematological malignancies (Hodgkin's and non-Hodgkin's lymphoma and leukemia) and breast cancer (Dolmans et al. 2013a), while in pediatric patients, leukemia and myeloproliferative or myelodysplastic diseases top the list followed by sarcoma (Armstrong et al. 2018). A serious concern that must nevertheless be addressed is the risk of reimplanting malignant cells together with the grafted tissue, especially in patients with leukemia (Dolmans et al. 2010), which remains the most common hematological cancer in women under 20 years of age. The risk is particularly high in women with acute leukemia and cannot be completely eliminated, even if the biopsy destined for cryopreservation is taken from patients in complete remission (Dolmans 2012, Greve et al. 2012, Dolmans et al. 2013b).

For this reason, different research teams worldwide have been working on alternative strategies, including (i) in vitro (ex vivo) culture of follicles for review, see (Telfer...
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& Zelinski 2013, Fabbri et al. 2018, Fisch & Abir 2018) and (ii) *in vivo* (post-transplantation) growth of isolated preantral follicles inside a transplantable artificial ovary (for review, see (Amorim & Shikanov 2016, Fisch & Abir 2018)), with the main objective of safely restoring reproductive function in patients who cannot undergo ovarian tissue transplantation. Since the latter is our area of expertise, we will focus on development of a bioengineered artificial ovary for further transplantation and discuss the latest advances and applications of this technology based on our experience.

**Indications for an artificial ovary and risk of reimplanting malignant cells**

In order to ensure the safety of OTC and transplantation, it is crucial to take into account the risk of tumoral involvement in the ovaries and detect the possible presence of cancer cells in cryopreserved ovarian tissue. These patients will benefit most from advances in the field of artificial organ bioengineering.

Risks should be weighed up according to cancer type (Table 1). They are considered to be high (>11%) in case of leukemia, neuroblastoma and Burkitt lymphoma, and moderate (0.2-11%) in case of advanced breast cancer, colon cancer, cervical adenocarcinoma, non-Hodgkin’s lymphoma and Ewing sarcoma. The risk is considered to be very low (<0.2%) in all other pathologies (Dolmans & Masciangelo 2018).

**Leukemia**

Leukemia is a blood-borne disease and the most common cancer in children. Climbing survival rates among young leukemia patients highlight the potential for possible long-term complications, such as chemotherapy-induced POI and infertility. For this reason, an important element in the management of these patients is fertility preservation, which is challenged by considerations specific to leukemia (Shapira et al. 2018a). As treating recurrence often requires high-dose chemotherapy with alkylating agents and bone marrow transplantation, relapsing leukemia patients are at high risk of POI and infertility, which is why fertility preservation should be offered. Moreover, leukemia occurs most commonly in children and chemotherapy needs to be started as soon as possible, so OTC is the only option available at present for these patients.

Malignant leukemic cells are found in the bloodstream, so the risk of ovarian involvement in these patients is high. Ovarian tissue taken for cryopreservation purposes could harbor malignant cells that might lead to disease recurrence after tissue reimplantation (Dolmans et al. 2013b). Detection of leukemic cells in ovarian tissue was first described in 2008 (Meirion et al. 2008). The *BCR-ABL* gene was evidenced by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) in the ovarian tissue of a chronic myeloid leukemia (CML) patient. In 2010, Dolmans et al. evaluated the presence of leukemic cells in ovarian tissue frozen during the active phase of the disease in a series of 16 patients. Six CML patients and ten acute lymphoblastic leukemia (ALL) patients were included in the study, and malignant cells were identified in ovarian tissue from 2 (33%) and 7 (70%) patients respectively by RT-qPCR. Frozen-thawed ovarian tissue was also grafted to severe combined immunodeficient (SCID) mice for 6 months. Neither macroscopic (visible to the eye) nor microscopic (histologically detected) disease was observed in mice grafted with ovarian tissue from CML patients, while 5 out of 12 mice grafted with ovarian tissue from ALL patients appeared to have been invaded by the disease (Dolmans et al. 2010). In an analogous study, ovarian tissue from leukemia patients in complete remission was analyzed (Rosendahl et al. 2010, Greve et al. 2012). Although RT-qPCR evidenced malignant cells in two out of four patients in frozen-thawed ovarian tissue, it did not reveal the presence of any leukemic cells in ovarian tissue xenografted for 20 weeks (Greve et al. 2012). Hence, leukemic cells were detected in >50% of cryopreserved ovarian tissue specimens during the active phase of disease, while ovaries from leukemia patients in complete remission did not appear to contain a sufficient number of viable malignant cells to transmit the disease upon transplantation.

Numerous studies investigating ovarian tissue from leukemia patients discourage its transplantation, but a very recent publication (Shapira et al. 2018a) reported the first live birth in an acute myeloid leukemia patient. Ovarian tissue was frozen when this 32-year-old patient was in complete remission, prior to undergoing hematopoietic stem cell transplantation (HSCT). Three fragments were analyzed before transplantation.

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<tr>
<th>Low risk</th>
<th>Moderate risk</th>
<th>High risk</th>
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<tr>
<td>Breast cancer (stage I–II infiltrating ductal subtype)</td>
<td>Breast cancer (stage IV infiltrating lobular subtype)</td>
<td>Leukemia</td>
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<td>Squamous cell carcinoma of the cervix</td>
<td>Colon cancer</td>
<td>Neuroblastoma</td>
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<td>Hodgkin’s lymphoma</td>
<td>Adenocarcinoma of the cervix</td>
<td>Burkitt lymphoma</td>
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<td>Soft tissue sarcoma</td>
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<td>Rhabdomyosarcoma</td>
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<td>Renal tumors</td>
<td>Contralateral ovarian cancer or borderline ovarian tumor</td>
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**Table 1** Presence of malignant cells in the ovary according to cancer type (adapted from Dolmans & Masciangelo 2018).

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using a panel of techniques (light microscopy, cytogenetic analysis, next-generation sequencing and xenotransplantation for 6 months). None of the analyses detected any leukemic cells in the frozen-thawed ovarian tissue, so transplantation went ahead. The patient conceived twice, once after IVF and once spontaneously and remains disease-free 28 months after transplantation (Shapira et al. 2018b).

**Neuroblastoma**

Neuroblastoma (NB) is the most common extracranial solid tumor in infants, with considerable metastatic potential. Treatment includes high-dose chemotherapy and H SCT, leading to a significant risk of infertility (>80%). As these patients are mostly prepubertal, OTC is the only fertility preservation option available to them (Imbert et al. 2014). In the literature, a number of cases of ovarian involvement in metastatic NB have been reported (Meyer et al. 1979, Mchugh et al. 1999). Indeed, NB is the childhood tumor that most frequently spreads to the ovaries, with more than 60% of metastatic NBs showing circulating malignant cells at the time of diagnosis. In these circumstances, patients are at risk of primary disease recurrence upon transplantation of ovarian tissue.

In a study by Greze et al. (2017), healthy ovarian tissue from 20 patients was *in vitro* contaminated with human NB cell lines and subsequently tested for minimal residual disease (MRD) with RT-qPCR for the relevant genes. The results showed PHOX2B (paired-like homeobox 2b) to be a reliable and sensitive marker of NB cells contaminating ovarian tissue, as it was able to detect as few as ten neoplastic cells present in the ovarian fragments. Cryopreserved ovarian tissue from two prepubertal patients with NB was also tested, but the PHOX2B gene was not detected by RT-qPCR, suggesting an absence of NB cells in these samples (Greze et al. 2017).

In high-risk NB, the clinical significance of long-term MRD monitoring using RT-qPCR for NB mRNAs was investigated in four patients over the course of their disease (diagnosis, remission and relapse) and treatment (Tchirkov et al. 2018). The findings proved the stability of mRNA marker expression after different treatments and demonstrated their ability to predict relapse and assess therapeutic response. This opens up the possibility of long-term molecular monitoring of MRD. Indeed, evaluating these markers prior to retransplantation (of tissue or the transplantable artificial ovary) will undoubtedly be of great value in the clinical management of our oncological patients.

**Burkitt lymphoma**

Burkitt lymphoma is a rare but highly aggressive B-cell form of non-Hodgkin’s lymphoma. It frequently involves the lymph nodes, but can also affect the jaw, central nervous system, bowel, kidneys, ovaries or other organs. There are three main types of Burkitt lymphoma.

Endemic Burkitt lymphoma most often occurs in children living in malaria-rife regions of the world, where it is still the most common childhood cancer. Sporadic Burkitt lymphoma is found all over the world and is the most prevalent variant in places where malaria is not holoendemic. Immunodeficiency-related Burkitt lymphoma is most frequently encountered in people with AIDS and those with inherited immune deficiencies, but can also occur in patients taking immunosuppressive medication to prevent rejection after organ transplantation.

The Epstein-Barr virus has been shown to be linked to the development of Burkitt lymphoma, with the strongest association usually seen in case of the endemic form. Translocation of the MYC gene is a hallmark of Burkitt lymphoma, making this a key finding for diagnosis of the disease. Although extremely aggressive and often constituting a medical emergency, Burkitt lymphoma is generally very responsive to high-dose chemotherapy, with high cure rates (Molyneux et al. 2012).

Burkitt lymphoma is the most rapidly growing tumor in children, with a doubling time of approximately 24 h (Miyazaki et al. 2013). The ovary is the most frequent site of non-Hodgkin’s lymphoma in the female genital tract and Burkitt lymphoma has been reported to account for approximately 19% of adnexal lymphomas (Kosari et al. 2005, Perlman et al. 2005). It is also among the differential diagnoses for adnexal masses discovered during pregnancy (Magloire et al. 2006). Burkitt lymphoma occurring during pregnancy has a tendency to involve organs stimulated by sex hormones, like the breast and ovaries, with the most common site being the breast. Moreover, there are cases of primary breast disease with ovarian involvement diagnosed during pregnancy (Testa et al. 2013). Ovarian tissue transplantation is not considered safe in patients with Burkitt lymphoma due to its high aggressiveness and frequent ovarian involvement (Sonmezer & Oktay 2004, Dolmans et al. 2013b).

To conclude, currently accepted methods of screening ovarian tissue for cancer cells before ovarian tissue transplantation include histology, identification of immunohistochemical markers specific to the disease in question, testing for disease-specific markers by fluorescence in situ hybridization (FISH) and/or PCR and 6-month follow-up of SCID mice transplanted with fragments of thawed ovarian tissue. Some key principles, like preservation of a sample of the original tumor in a tissue bank for the purposes of cancer detection tests, are strongly emphasized by Shapira et al. (2018b). No official guidelines exist as yet, but at this stage in our understanding, we would only recommend ovarian tissue transplantation if the available analyses proved negative, with sufficient levels of sensitivity.
How to avoid reimplanting malignant cells when the risk is real: the advent of the bioengineered transplantable artificial ovary

Bioengineering a transplantable artificial ovary

The main objectives of an artificial ovary are (i) to safely transplant isolated primordial and primary follicles, avoiding inadvertent contamination by malignant cells possibly present in ovarian tissue that could potentially lead to the recurrence of the primary disease (Dolmans et al. 2013b) and (ii) to support follicle survival and development after transplantation, ensuring secretion of sex hormones and production of fertilizable mature oocytes. Based on these goals, a number of key conditions must be met in order to bioengineer an artificial ovary. First of all, we need to ensure safe isolation of a maximum number of primordial and primary follicles (Fig. 1). Since follicles are enclosed in a basement membrane that avoids direct interaction between follicular cells and capillaries, white blood cells and nerve processes (Rodgers et al. 2003), their isolation will guarantee that no malignant cells are returned back to patients. Interest in these small follicles is high because they represent more than 90% of the total follicle population in the ovary and are the most cryoresistant class of follicles (Shaw et al. 2000). While complete follicle isolation is required to safeguard patient health, follicle survival and growth depend on cross-talk between follicles and surrounding ovarian cells and the ovarian extracellular matrix (ECM). For instance, ovarian stromal cells are involved in activation of primordial follicles and some will be recruited to differentiate into theca cells (Parrott & Skinner 2000, Oktem & Urman 2010). On the other hand, endothelial cells are responsible for vascularization in ovarian tissue, essential for transportation of paracrine factors, oxygen and nutrients, as well as metabolic waste removal. The ovarian ECM maintains 3D follicle structure and plays an important role in folliculogenesis as it has the ability to bind growth factors, including those found in follicles (Rodgers et al. 2003). In other words, to build an artificial ovary, it is necessary to recreate an environment similar to the natural human ovary.

Isolation of primordial and primary follicles: quality and quantity

As mentioned earlier, the first requirement for the creation of a bioengineered artificial ovary is isolation of a large number of intact preantral follicles. This procedure aims to dissociate as many intact follicles as possible from the surrounding ovarian stroma. Due to the fibrous nature of human ovarian cortex where the majority of primordial and primary follicles reside, the most effective way of obtaining a high follicle yield and quality is usually based on a combination of mechanical and enzymatic tissue digestion (Chiti et al. 2018b). An optimal isolation protocol should also take into account all conceivable variations that may be encountered in different types of ovarian tissue. With this in mind, we optimized our isolation protocol (Vanacker et al. 2011) by inactivating the enzymatic digestion process every 30 min and filtering the suspension, so that fully isolated follicles would not be continuously exposed to enzymes, which could damage their basement membrane and result in their death. The remaining fragments of ovarian tissue left behind in the filter would then be digested in fresh enzymatic solution in order to increase the number of isolated follicles (Chiti et al. 2017a). This filtration-digestion step was repeated until all the ovarian fragments were completely digested. We found that by subjecting the remaining fragments to fresh enzymatic solution every 30 min, we were able to successfully digest any type of ovarian tissue, from the softest to the hardest. This ensured that all the tissue was processed without affecting the quality of the isolated follicles, as demonstrated by our findings after encapsulation in fibrin and xenografting (Chiti et al. 2017a). This new protocol allows personalization of ovarian follicle isolation according to individual tissue properties and should be implemented on a case-by-case basis to make best use of precious ovarian follicles and maximize the chances of pregnancy after artificial ovary transplantation.

It is important to stress that in order to allow its application in patients, reagents used in isolation protocols must fully comply with good manufacturing practice (GMP) guidelines. We therefore developed protocols associating two GMP-produced enzymes: Liberase DH and DNase (Vanacker et al. 2011, Chiti et al. 2017a).

Isolation of primordial and primary follicles: safety

Another important concern regarding the safety of the follicle isolation procedure is possible contamination of the suspension by malignant cells. After isolation, both follicles and cells can be found in the suspension, so during pick-up of isolated follicles to embed inside the artificial ovary, malignant cells may also...
be inadvertently retrieved (Fig. 2). In an experiment involving a suspension of digested ovarian tissue artificially contaminated with marked fluorescent leukemic cells (BV-173), Soares et al. (2015a) indeed detected their presence in medium droplets containing isolated follicles (Fig. 2). As the goal of the artificial ovary is to avoid reintroduction of malignant cells that could potentially reside in transplanted ovarian tissue, it is essential to prevent any possible contamination during follicle pick-up and embedding. Soares et al. (2015a) therefore devised a washing step whereby isolated follicles were transferred three times from one medium droplet to another in order to separate them from surrounding isolated cells. Such a small modification to the original protocol was enough to slash the levels of leukemic cell contamination from 196 cells to just one (Soares et al. 2015a). Results from this experimental model were subsequently confirmed in a further study using frozen-thawed ovarian tissue from 12 leukemia patients (Soares et al. 2017). PCR identified leukemic cells in 66% of the ovarian tissue, with malignant cells also detected in digested ovarian suspensions. After washing the suspensions according to the previously described protocol (Soares et al. 2015a), however, none of the follicle samples (>2300 follicles tested) showed any malignant cell presence at all. All isolated and washed follicle suspensions tested negative for leukemic cells, giving leukemia patients genuine hope of fertility restoration. This provides further reassurance about the safety of the bioengineered transplanted artificial ovary.

**Addition of isolated ovarian stromal cells**

It is well known that early follicle development is controlled by ovarian autocrine/paracrine regulators secreted by follicles and the ovarian stroma. Stromal cells have been shown to release various factors that positively regulate primordial-to-primary follicle transition (Knight & Glister 2006). After activation of primordial follicles, primary follicles secrete other factors to recruit fibroblast-like precursor cells or stem cells from the ovarian stroma to differentiate into functional theca cells (Orisaka et al. 2006, Honda et al. 2007). These play a supportive structural role for growing follicles, but also provide androstenedione and testosterone for estrogen biosynthesis by granulosa cells. Integration of ovarian stromal cells into the artificial ovary might therefore re-establish natural communication between follicles and surrounding stromal cells, potentially increasing follicle development.

Follicle survival and growth also require vascularization, so vascular development is essential after grafting. For this, it is vital to promote angiogenesis in the hypoxic environment of the artificial ovary. Endothelial cells are crucial in this context, aiding survival of different types of cells by enhancing the quality of revascularization after grafting (Liu et al. 2017).

Ovarian cells can also be isolated from a second fresh ovarian biopsy after the patient has completed chemo/radiotherapy, which would consequently avoid any potential contamination by malignant cells (Fig. 3). In order to assess the impact of previous chemotherapy on
isolated ovarian cells, Soares (2015) conducted a study involving isolation of ovarian cells from fresh ovarian tissue of three patients who were previously subjected to chemotherapy. These cells were then compared with fresh ovarian cells from healthy patients in terms of survival and proliferation. The results showed that previous chemotherapy did not impact cell behavior (Soares 2015), so fresh ovarian tissue obtained from patients after cancer treatment may be considered a legitimate source of ovarian cells.

Regarding isolated endothelial cells, the first study to demonstrate the importance of co-transplanting them along with stromal cells was performed by Dath et al. (2011). After 1 week of xenografting isolated human ovarian cells to mice, we observed well vascularized and structured ovary-like tissue (Dath et al. 2011). Vascularization in grafted tissue can therefore be boosted with higher concentrations of isolated endothelial cells, which can be easily obtained from the medullary part of the ovary (Soares et al. 2015b).

3D matrix to encapsulate isolated preantral follicles and ovarian cells

The choice of which 3D matrix to use for grafting of isolated follicles and cells is one of the most challenging and critical elements in the development of a bioengineered artificial ovary (Amorim 2011). Since its conception, both synthetic (Kim et al. 2016, Day et al. 2018, Mendez et al. 2018) and natural (Dolmans et al. 2007, 2008, Luyckx et al. 2014, Vanacker et al. 2014, Paulini et al. 2016, Chiti et al. 2016a, 2018a) polymers have been tested for the creation of an artificial ovary prototype. While synthetic materials (produced with chemical processes that do not commonly occur in nature) are more predictable in terms of degradation rate and mechanical properties (Asti & Gioglio 2014), studies have shown that natural polymers (biologically derived materials) constitute the matrix of choice for an artificial ovary (Amorim & Shikanov 2016).

One of the greatest advantages of synthetic polymers is the possibility of tailoring the mechanical properties according to the specific requirements of clinical application. These matrices can also be manufactured in large and uniform batches and have a long shelf life (Faulk et al. 2014). However, synthetic polymers do not contain molecules essential for cell adhesion, but bioactive factors can be incorporated to stimulate this aspect (Yoon & Fisher 2009, Kim et al. 2016).

To our knowledge, the only synthetic polymer that has been utilized to graft isolated preantral follicles is poly(ethylene glycol) (PEG) (Kim et al. 2016). PEG is a linear-chained polymer consisting of a simple oxygen-carbon-carbon repeating unit. Shikanov’s group has successfully used PEG gels modified with arginine, glycine and aspartate (RGD) to graft mouse preantral follicles, showing that this polymer allows follicle survival and development (Kim et al. 2016). Whether different types of PEG can support survival of isolated human preantral follicles nevertheless remains to be determined.

Unlike their synthetic counterparts, natural polymers show superior interaction with cells, thanks to the presence of biofunctional molecules – they play a useful role in cell adhesion, migration, proliferation and differentiation. Conversely, natural matrices also have some disadvantages, such as the lack of sufficient mechanical strength and difficulty modifying their composition because of their complex structure (Yoon & Fisher 2009).

The first natural matrix ever used to graft isolated preantral follicles was collagen (Telfer et al. 1990). This pioneering study found that when encapsulated in collagen, isolated mouse follicles were able to survive and grow after transplantation to the kidney capsule. However, the authors also reported oocyte atresia in antral follicles and granulosa cell luteinization (Telfer et al. 1990).

Published the same year as the collagen study, Gosden’s report (1990) documented the birth of pups after transplantation of isolated follicles in plasma clots. In this experiment, isolated murine ovarian follicles and cells were encapsulated in autologous plasma clots and this matrix could then be restructured into effective ovarian tissue after allografting, resulting in ovulation and delivery of normal offspring (Gosden 1990). These findings were further corroborated when murine follicles were isolated from cryopreserved ovarian tissue (Carroll & Gosden 1993).

Based on the successful results reported with mouse follicles, Dolmans et al. (2007, 2008) encapsulated isolated human ovarian preantral follicles in autologous plasma clots and xenografted them to immunodeficient mice for 1 week or 5 months. After short-term grafting, the follicles were able to grow to the secondary stage, while after long-term transplantation, antral follicles were also found in the grafts (Dolmans et al. 2007, 2008). In spite of these promising results, plasma clots have an inconsistent composition and degrade rapidly after grafting, which can lead to follicle loss and variable outcomes.

To replace plasma clots, our team decided to test fibrin, a similar natural polymer. Commercially available sources of fibrin may contain high concentrations of fibrinogen and thrombin, which can be further diluted to obtain fibrin formulations with different mechanical properties, including degradation rate, rigidity, morphology and fiber network (Dietrich & Lelkes 2006).

Short-term (7 days) grafting of isolated mouse preantral follicles in fibrin matrices with very low concentrations of fibrinogen and thrombin resulted in survival and growth of follicles and yielded recovery rates of 28–35% (Luyckx et al. 2014, Chiti et al. 2016a, 2017b). After a longer (3 weeks) grafting period, isolated
mouse preantral follicles were able to develop to the antral stage in a stiffer fibrin matrix (Smith et al. 2014). Ovulation and restoration of hormone cyclicity were evidenced by the presence of corpora lutea and a decrease in follicle-stimulating hormone levels.

Fibrin has also been combined with other natural polymers (fibrin-collagen and fibrin-alginate) and loaded with growth factors (vascular endothelial growth factor (VEGF)) in attempts to enhance follicle survival (Kniazeva et al. 2015). Surprisingly, after grafting isolated murine preantral follicles inside these different matrices, pups were only obtained in fibrin-VEGF clots (Kniazeva et al. 2015). Fibrin has also been associated with different concentrations of platelet lysate, a great source of growth factors (Lang et al. 2018), indicating that such supplementation can improve recovery rates after 14 days of isolated mouse follicle transplantation (Rajabzadeh et al. 2015).

A few studies have demonstrated the ability of this polymer to support survival and growth of human follicles. However, fibrin formulations need to be much stiffer than those tested for mouse follicles (Paulini et al. 2016, Chiti et al. 2017a). Indeed, fibrin matrices containing 50 mg/mL fibrinogen most closely resemble human ovarian cortex in terms of ultrastructure (Fig. 4) and rigidity (Chiti et al. 2018a). When using this matrix, follicle recovery rates after 1 week of xenografting to immunodeficient mice were between 22 and 35%, very similar to the results obtained after xenografting of human ovarian tissue (Nisolle et al. 2000).

Alginates is another natural polymer widely applied in tissue engineering, which was tested for grafting of isolated preantral follicles. A number of studies have used alginate to in vitro-culture preantral follicles from different animal species (for review, see: Vanacker & Amorim 2017), but only a few have exploited alginate in the context of isolated follicle transplantation (Vanacker et al. 2012, 2014, Rios et al. 2018). Following isolation and encapsulation in a 1% alginate matrix, isolated murine preantral follicles were able to survive and develop after 1 week of autotransplantation (Vanacker et al. 2014). Interestingly, using a lower alginate concentration (0.5%), Rios et al. (2018) were able to obtain metaphase II oocytes after allotransplantation.

Other natural matrices, such as gelatin and decellularized ovarian tissue, have also yielded successful outcomes in isolated mouse follicle transplantation (Laronda et al. 2015, 2017, Hassanpour et al. 2018, Pors et al. 2018). Recently, Pors et al. (2018) reported promising results with human preantral follicles seeded inside decellularized human ovarian tissue; isolated follicles were able to survive for 3 weeks after xenografting to mice.

Despite these promising results with decellularized ovarian tissue matrices, it is important to bear in mind that preantral follicles are of different sizes (30–150 µm) and it is very challenging to find an exact fit for them in the pores of the matrix. One alternative is to transform this matrix into a thermosensitive hydrogel. This approach allows perfect encapsulation of isolated follicles and ovarian cells, while retaining the composition of the decellularized ovarian tissue. Our preliminary in vitro experiments using this approach demonstrated that isolated mouse preantral follicles can successfully survive in this matrix (Chiti et al. 2016b). Table 2 summarizes the main findings obtained after grafting isolated follicles inside the above-mentioned matrices.

**Functionality of the transplantable artificial ovary**

Like ovarian tissue, a functional transplantable artificial ovary should be able to restore both endocrine and reproductive functions after grafting. Several prototypes have indeed been shown to support follicle development and ovulation, promote synthesis of female hormones and produce embryos and healthy offspring in murine

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**Figure 4** Ultrastructure of human ovarian cortex and different fibrin formulations. Scanning electron microscopy image of human ovarian cortex and fibrin matrix composition at different magnifications (×2000; ×12,000; ×90,000). F, fibrinogen; T, thrombin. Reprinted with permission from Chiti et al. (2018a) © Springer Link (2018).

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models (Gosden 1990, Telfer et al. 1990, Carroll & Gosden 1993, Smith et al. 2014, Kniazeva et al. 2015, Kim et al. 2016, Laronda et al. 2017, Rios et al. 2018). One could hypothesize that such findings may be extrapolated to human follicles inside these same matrices, but it is important to bear in mind that mouse and human ovary microarchitecture and structure are quite distinct, and requirements for a transplantable artificial ovary for human follicles can differ greatly from those of a prototype for mouse follicles (Paulini et al. 2016, Chiti et al. 2018a).

Although results with human follicles are still far behind those obtained with their murine counterparts, and despite not being able to assess their reproductive function for ethical reasons, studies have nevertheless demonstrated the feasibility of this approach. Dolmans et al. (2008) showed that 5 months after xenografting, human follicles were able to reach the antral stage. Follicle recovery rates in fibrin and decellularized human ovarian matrices after 1 week of xenografting were found to be comparable to those reported for human ovarian tissue in similar grafting experiments (Nisolle et al. 2000, Paulini et al. 2016, Chiti et al. 2017a, Pors et al. 2018). Formation of an ovary-like structure, which is necessary for further follicle development, was also demonstrated in several studies (Dolmans et al. 2007, 2008, Paulini et al. 2016, Chiti et al. 2017a). Future research testing a transplantable artificial ovary prototype for human follicles should now focus on follicular morphometry, growth rates, hormone secretion, molecular markers and the epigenetic status of oocytes, in order to understand how the selected matrix can impact follicle survival and development.

Conclusion

In conclusion, cancer patients who cannot undergo transplantation of frozen-thawed ovarian tissue could...
benefit enormously from a transplantable artificial ovary to restore their fertility. It is also important to stress that this technology could have other important uses. For instance, it could aid in the understanding of folliculogenesis, and its application in toxicological studies could elucidate the effect of different drugs on reproduction. Moreover, development of a matrix to encapsulate isolated follicles and ovarian cells could be extended to creation of other artificial organs to replace different reproductive tissues. Finally, it could also provide a suitable environment to investigate oogonial stem cells and assess their ability to reinitiate human gametogenesis. We firmly believe that development of a bioengineered artificial ovary would represent a revolutionary step in the field of reproductive tissue engineering.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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