Trends in
Molecular Medicine

CellPress

Special Issue: Reproductive and Sexual Health

Review

# Fertility Preservation: The Challenge of Freezing and Transplanting Ovarian Tissue

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**Cancer treatments are increasingly effective, but can result in iatrogenic premature ovarian insufficiency. Ovarian tissue cryopreservation is the only option available to preserve fertility in prepubertal girls and young women who require immediate chemotherapy. Ovarian tissue transplantation has been shown to restore hormonal cycles and fertility, but a large proportion of the follicle reserve is lost as a consequence of exposure to hypoxia. Another crucial concern is the risk of reimplanting malignant cells together with the grafted tissue. In this review, the authors advance some challenging propositions, from prevention of chemotherapy-related gonadotoxicity to ovarian tissue cryopreservation and transplantation, including the artificial ovary approach.**

## Highlights

Chemotherapy triggers direct primordial follicle depletion by increasing apoptosis and disrupting signaling pathways involved in quiescence maintenance and indirect damage by injury to the vascular network and stromal cells.

Ovarian tissue cryopreservation and transplantation is an effective fertility preservation strategy, leading to endocrine restoration in more than 95% of cases and a pregnancy rate of 40%.

The avascular nature of ovarian tissue transplantation causes major follicle loss of 50–90%, occurring before complete graft revascularization.

The goal of a transplantable artificial ovary development is to be able to safely graft ovarian follicles, ensuring their survival and development, which can be achieved by use of a biocompatible scaffold implanted together with ovarian stromal cells.

## Fertility Preservation for Oncological Diseases: Where Are We?

In recent years, demand for fertility preservation for oncological diseases, benign conditions, and social reasons, coupled with age-related fertility decline, has increased dramatically [1,2]. Meeting this rising demand is challenging, despite endorsement of three methods of fertility preservation (embryo cryopreservation, oocyte cryopreservation after ovarian stimulation, and ovarian tissue cryopreservation) by the American Society for Reproductive Medicine [3]. Oocyte **vitrification** (see Glossary) provides the highest yield for women with benign diseases, those seeking fertility preservation for personal reasons, or women with cancer if their treatment can be postponed. Ovarian tissue cryopreservation, however, is indicated for young girls and women whose cancer treatment cannot be delayed (Table 1).

Fertility preservation remains a particular challenge in case of hematological cancers (Hodgkin's lymphoma, non-Hodgkin's lymphoma, and leukemia) and breast cancer. These cancers constitute the most common indications for fertility preservation, since chemotherapy (especially with **alkylating agents**), radiotherapy, and surgery, or indeed a combination of these treatments, can induce premature ovarian insufficiency in some circumstances.

As stressed in a recent review [1], there is a need for selection criteria to be implemented, the most important of which is age under 35 years (when the ovarian stockpile is still relatively high), a realistic chance of surviving for 5 years, and at least a 50% risk of experiencing premature ovarian insufficiency [4].

After reimplantation of ovarian tissue in the pelvic cavity (Figure 1) [5–9], ovarian activity is restored in more than 95% of cases [10,11]. The mean duration of ovarian function postgrafting is 2–5 years, but it can last for up to 7 years, depending on follicle density at the time of ovarian tissue cryopreservation [11,12]. The first pregnancy after this procedure was reported back in 2004 [9] and pregnancy and live birth rates have continued to climb steadily since then, showing an exponential increase (Figure 2). Indeed, taking into account the latest published series [12–17], the number of live births as of June 2017 exceeded 130 and is now well above 200 [18].

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Table 1. Indications for Fertility Preservation

| | |
|---|---|
| Malignant diseases requiring gonadotoxic chemotherapy, radiotherapy, or bone marrow transplantation | Hematological diseases (leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma) |
| | Breast cancer |
| | Sarcoma |
| | Some pelvic cancers |
| Benign conditions | Systemic diseases requiring chemotherapy, radiotherapy, or bone marrow transplantation |
| | Ovarian diseases (bilateral benign ovarian tumors, severe and recurrent ovarian endometriosis, possible ovarian torsion) |
| | Risk of premature ovarian insufficiency (family history, Turner's syndrome) |
| Personal reasons | Age |
| | Childbearing postponed until later in life |

In 2015, as the denominator (the number of reimplantations performed worldwide) was not known, the success rate of the technique was calculated based on patients from five major centers (a total of 111 patients), yielding a pregnancy rate of 29% and a live birth rate of 23% [10]. Since then, live birth rates have climbed further, first to 36% [1] and very recently to 41% in a series of 60 patients undergoing ovarian tissue reimplantation of their frozen-thawed tissue in three different centers [17]. The goal of this review is to analyze recent developments in the field, focusing specifically on the ovarian reserve as the core of our research (see Clinician's Corner).

## The Ovarian Reserve: The Core of Creation

The term ovarian reserve is typically used to refer to the population of **primordial follicles (PMFs)** [19]. Initiation of the resting follicle reserve commences in the fetus, when around 100–2000 primordial germ cells colonize the genital ridges and embark on a massive proliferation process that results in 7 million potential oocytes at mid-gestation. In the human ovary, 85% of these potential oocytes are lost before birth [19]. The decline in follicle numbers continues throughout reproductive life, during which time approximately 450 ovulatory cycles occur, with the majority of follicles undergoing atresia during their growth phase [19]. At menopause, around 1500 PMFs remain in the ovary.

This part will be divided into three distinct sections, covering new developments in this field:

(i) Chemotherapy-induced PMF loss.
(ii) Revascularization of transplanted human ovarian tissue: from hypoxia to restoration of blood supply.
(iii) The why and how of the artificial ovary.

## Chemotherapy-Induced PMF Loss

The effects of chemotherapeutic drugs on female reproduction began to be reported in the 1970s. The first accounts associated one particular alkylating agent, **cyclophosphamide (CPM)** [20,21], with premature ovarian insufficiency.

### CPM and Ovarian Follicles

CPM, inducing DNA crosslinking and ultimately preventing DNA replication [22], is used in the treatment of a wide range of cancers, particularly childhood cancers and other diseases. Among

## Glossary

**Alkylating agents:** chemotherapeutic drugs used in gynecological oncology (carboplatin, cisplatin, oxaliplatin, cyclophosphamide, ifosfamide, altretamine) to prevent cell division by crosslinking DNA strands.
**Burn-out hypothesis:** this theory is based on the observation of primordial follicle behavior in response to injury in the ovary, namely after both chemotherapy and ovarian tissue transplantation. According to this hypothesis, the ovarian reserve accelerates its activation, resulting in massive and rapid activation and growth of follicles, which leads to depletion of the ovarian reserve with detrimental effects on fertility potential.
**Cyclophosphamide (CPM):** a prodrug, its primary active metabolite is phosphoramide mustard. It is able to induce DNA crosslinking, as other alkylating agents, and moreover to prevent DNA synthesis, with consequent broader spectrum of activity.
**Electronic paramagnetic resonance:** this method allows repeated oxygen concentration measurements by insertion of lithium phthalocyanine into living tissues in the form of an external probe, which is implanted in the tissue and interacts with oxygen without consuming it.
**Follicle atresia:** the physiological and continuous controlled death of early-stage follicles in the ovary, occurring from birth and throughout the entire fertile life of women, until depletion of the ovarian reserve by the time of menopause.
**Hypoxia-inducible factor 1 (HIF1):** this transcription factor activates transcription of a number of genes in hypoxic conditions, known as hypoxia response elements (HREs), crucial to tissue adaptation.
**Microdialysis:** this established method measures metabolites *in vivo* in the extracellular space of target tissues, without altering their concentrations. A semipermeable probe is inserted into the tissue and constantly perfused, acting as a capillary vessel and enabling passage of the target metabolite in the dialysate by simple diffusion.
**Primordial follicles (PMFs):** after being formed during fetal life, these follicles remain quiescent until their activation to either growing stages or atresia. They consist of immature oocytes surrounded by a single layer of flattened granulosa cells.

alkylating and alkylating-like agents, CPM is known to be most toxic to the ovaries [23]. Indeed, there is strong evidence that CPM exposure to growing follicles is linked to **follicle atresia** and granulosa cell apoptosis [24–27]. Apoptosis is a major mechanism of action of many chemotherapeutic drugs targeting proliferating cells and granulosa cells of growing follicles are certainly impacted by chemotherapy, causing their apoptotic death.

Regarding loss of PMFs, a number of mechanisms have been suggested, including accelerated activation [28–30], atresia, apoptosis, inflammation, and vasculature damage (Figure 2). All these elements make the local environment inhospitable to follicles [22,29,30]. Among all causes of ovarian reserve injury, large-scale follicle activation triggered indirectly by chemotherapy drugs, known as the **burn-out hypothesis**, has been widely investigated as a possible cause of PMF loss [28–30]. Abnormal follicle activation may essentially be due to dysregulation of pathways that control follicle quiescence [28–31]. Indeed, the phosphoinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (Box 1) was shown to be upregulated in a murine model after CPM administration (Figure 3). A possible explanation for this may either be direct activation of the pathway as protection from inflammation and oxidative stress, with potential DNA damage to the oocyte, or indirect activation of PMFs due to a fall in secretion of suppressive factors like anti-Müllerian hormone (AMH) because of death of growing follicles [29,30]. If activation is the cause of PMF loss, then inhibitors of the PI3K/PTEN/Akt pathway should protect the ovarian reserve from CPM toxicity [22]. Other investigations into different origins of massive chemotherapy-induced PMF loss identified induction of apoptosis as a prevailing cause of pathway activation [22]. Indeed, CPM-induced DNA damage to PMFs was shown to trigger apoptosis in mice [32,33]. These investigations suggested a possible role for apoptosis inhibitors as gonad protectors against the toxic effects of CPM. However, no conclusive evidence was gathered by any of the research teams in favor of one hypothesis over another in human ovarian tissue, nor was there proof of effectiveness of any of the gonadoprotective drugs acting on these pathways. Indeed, whether these pathways function synergistically, or have different impacts depending on the chemotherapeutic and biological characteristics of patients, remains to be investigated.

### Is Prevention of Follicle Loss Possible?

Agents used to protect the ovaries from CPM-induced damage should, in theory, protect against direct loss of PMFs, accelerated activation of PMFs, PMF apoptosis, increased atresia of growing follicles, and damage to the ovarian vasculature. Of all potential protectants, only sphingosine-1-phosphate (S1P) and tamoxifen have been studied in the human ovary [22]. S1P prevents CPM-induced apoptotic follicle death in human ovarian xenografts [34,35], while tamoxifen was investigated in a randomized controlled trial and given to women treated with CPM, methotrexate, and 5-fluorouracil. No impact was observed on ovarian function [36,37]. Among protectants against specific damage to pathways, AMH was investigated in mice as a possible candidate [38,39]. Administration of AMH was found to reduce loss of follicles after treatment with CPM and mitigate abnormal PMF recruitment through PI3K/Akt pathway activation [38]. The crucial role of AMH in PMF maintenance has been established in rodents, evidencing fast PMF depletion in knockout mice ($AMH^{-/-}$) [40]. However, there is no evidence as yet that this strategy can prevent chemotherapy-induced PMF loss in humans, where folliculogenesis appears to be differently regulated.

### Gonadotropin-Releasing Hormone (GnRH) Agonist and Ovarian Protection

Numerous investigations have been conducted to assess whether administration of GnRH agonist (GnRHa) during oncological treatment may protect against chemotherapy-induced damage [41], pursuant to the hypothesis that a 'suppressed' quiescent ovary is less susceptible to chemotherapeutic injury [42]. Temporary ovarian suppression with GnRHa during chemotherapy should be considered an option to prevent the negative effects of chemotherapy in premenopausal

**Reactive oxygen species (ROS):** these originate from oxygen metabolism in cells, mainly from mitochondrial activity, but also as byproducts of cytosolic enzymatic activity for detoxification. Hydrogen peroxide ($H_2O_2$) is the most stable of them and can easily diffuse through cell membranes and act as a signaling mediator for cell cycle maintenance, detoxification, and apoptosis regulation. Other ROS, like the hydroxyl radical ($OH^-$) and superoxide ($O_2^-$), have unpaired electrons, which confer higher oxidant activity, with subsequent macromolecular damage in all cell compartments.

**Vitrification:** a cryopreservation technique developed for single cells and tissues, involving rapid cooling to avoid ice formation in subcellular compartments.

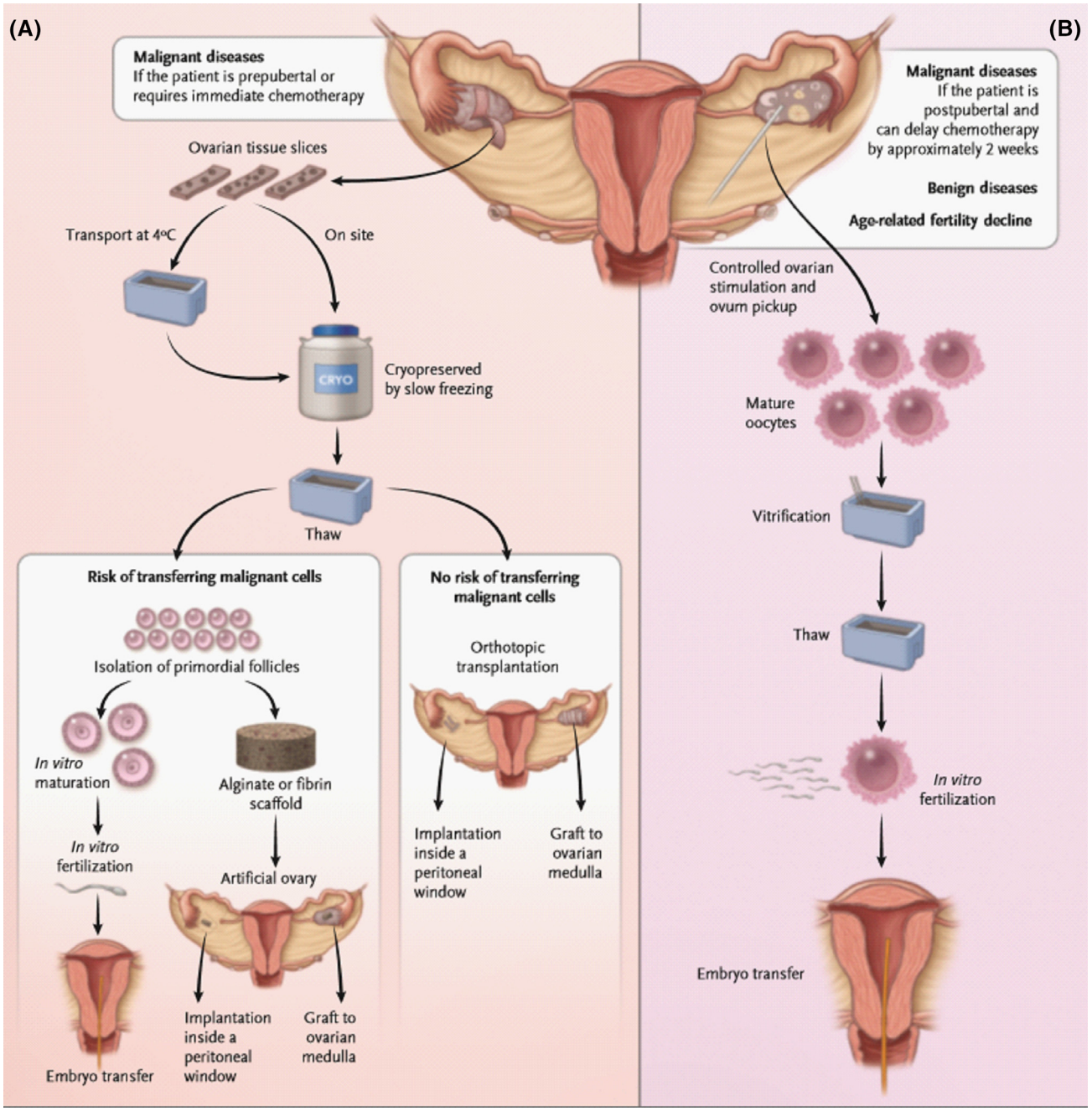


Figure 1. Fertility Preservation Options. (A) If the patient is prepubertal or requires immediate chemotherapy, ovarian tissue is removed in the form of multiple biopsies (or an entire organ) and cut into cortical strips. The tissue is then cryopreserved by slow-freezing on site or transported to a processing site at a temperature of 4°C. After thawing, if there is no risk of transmitting malignant cells (right column), the ovarian tissue can be grafted to the ovarian medulla (if at least one ovary is still present) or reimplanted inside a specially created peritoneal window. If there is a risk of transmitting malignant cells (left column), ovarian follicles can be isolated and *in vitro*-grown to obtain mature eggs, which can be fertilized and transferred to the uterine cavity. Isolated follicles may also be placed inside a scaffold (alginate or fibrin), creating an 'artificial ovary' that can be grafted to the ovarian medulla or peritoneal window. (B) If the patient is postpubertal and can delay chemotherapy by approximately 2 weeks, mature oocytes are removed after ovarian stimulation and vitrified on site. After thawing, they are inseminated and, in the form of embryos, transferred to the uterine cavity. The combined technique can also be applied, involving ovarian tissue cryopreservation followed by controlled ovarian stimulation and vitrification of oocytes. This combined technique theoretically yields a 50–60% chance of obtaining a live birth. Reproduced, with permission, from [1].

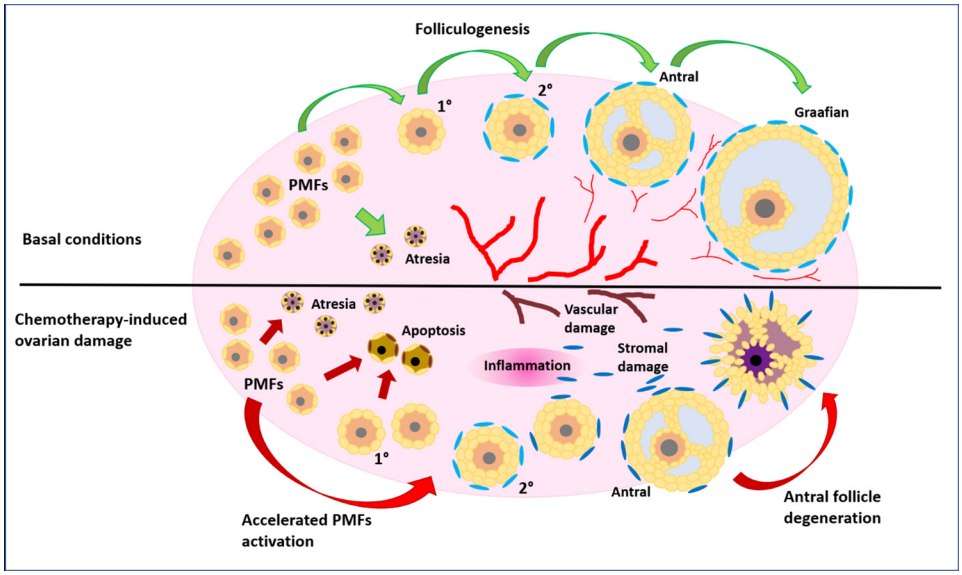


Figure 2. Ovarian Damage Mechanisms of Chemotherapy. Top: ovarian folliculogenesis in basal conditions. From left to right, primordial follicles (PMFs) go either through continuously occurring atresia, or follicle development and growth up to the antral stage. From this latter stage, gonadotropin-dependent selection of a dominant follicle occurs every month for ovulation of a mature oocyte. Bottom: damaging effects of chemotherapy drugs on the ovary. From left to right, loss of PMFs occurs due to apoptosis (increasing the number of dead follicles beyond simply atretic follicles) or accelerated activation of growing stages. Inflammation, together with stromal and vascular damage, then results in a toxic environment for growing follicles, which are highly dependent on the quality of the blood supply for their survival and further growth, leading to their degeneration. Reproduced, with permission, from [48].

women with breast cancer [43,44]. However, as data on post-treatment pregnancies remain limited [45,46], administration of GnRHa during chemotherapy should not be considered a stand-alone fertility-preserving alternative to cryopreservation options (oocytes, ovarian tissue) [47].

Indeed, it is difficult to understand how GnRHa could prevent the massive double-stranded DNA breaks induced by chemotherapy [47,48]. A biological basis and rationale for fertility preservation by GnRHa are still lacking [47,48]. Intensive research is required to develop different potential protectants, acting on a wide range of ovarian pathways damaged by chemotherapy.

### Box 1. Signaling Pathways Involved in Abnormal Follicle Activation

The PI3K/Akt pathway is known to be one of the major elements responsible for primordial follicle activation. This signaling pathway works through a phosphorylation cascade, after binding of numerous cytokines and growth factors to tyrosine-kinase receptors on cell membranes. Upon Akt phosphorylation by various molecules and growth factors or inhibition of phosphatase and tensin homolog (PTEN), a number of effectors are activated, including forkhead box 01-3 (FOXO1-3) and the mechanistic target of rapamycin complex (mTORC1), in order to stimulate granulosa cell growth from flattened to cuboidal and early follicle growth [49,91,96].

The Hippo pathway is one of the major regulators of tissue growth by mechanical force modulation. This signaling pathway can be disrupted by conversion of globular-actin (G-actin) into filamentous-actin (F-actin). It functions through its action on the Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), which, once unphosphorylated, shifts to the nucleus to activate transcription factors like the TEA domain family 1–4 (TEAD1–4). Its disruption has been related to follicle activation and growth after ovarian tissue manipulation and fragmentation [50,51]. However, recent findings in the field cast doubt on its role in accelerated follicle activation occurring soon after ovarian tissue transplantation [52,53].

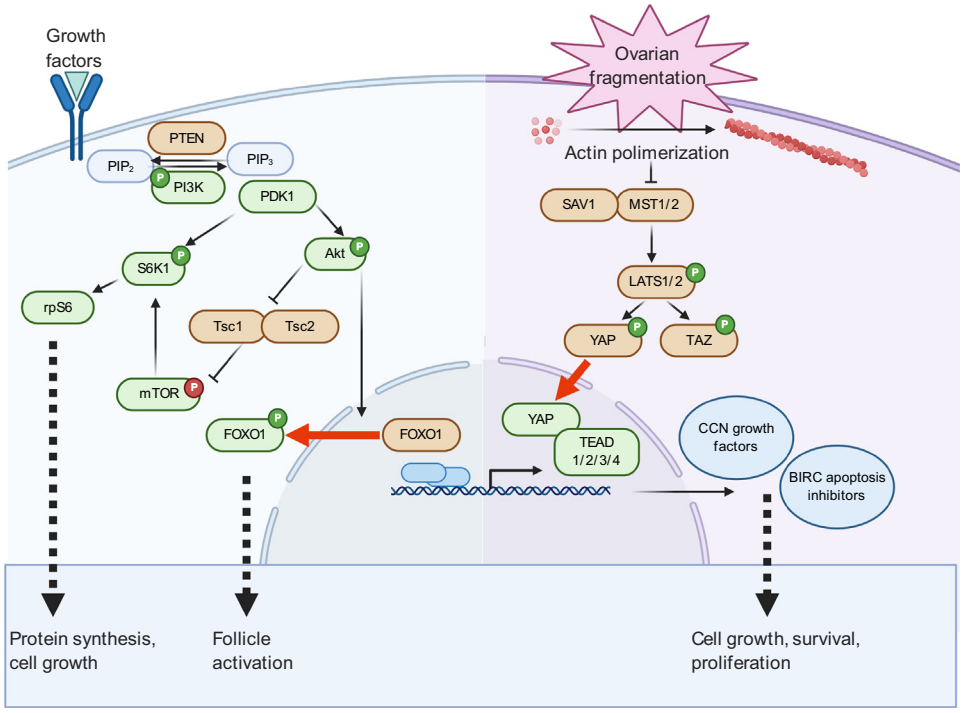


Figure 3. Representation of the PI3K and Hippo Pathways Involved in Follicle Activation. The PI3K/Akt pathway (left) works through a phosphorylation cascade, after binding of numerous cytokines and growth factors to tyrosine-kinase receptors on cell membranes. Upon Akt phosphorylation or inhibition of PTEN, a number of effectors are activated, including FOXO1 and mTOR, in order to stimulate granulosa cell growth from flattened to cuboidal and early follicle growth. The Hippo pat hway (right) can be disrupted by conversion of globular-actin into filamentous-actin. It functions through its action on YAP/TAZ, which, once unphosphorylated, shifts to the nucleus to activate transcription factors like TEAD1-4, related to follicle activation and growth. Activators are represented in green; inhibitors are represented in orange. Abbreviations: Akt, protein kinase B; BIRC, baculoviral inhibitors of apoptosis; FOXO1, forkhead box O1; LATS1/2, large tumor suppressor kinase 1/2; mTOR, mechanistic target of rapamycin; MST1/2, mammalian Ste20-like serine/threonine kinases 1/2; PDK1, pyruvate dehydrogenase lipoamide kinase isozyme 1; PI3K, phosphoinositol-3- kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RPS6, ribosomal protein S6; SAV1, protein Salvador homolog 1; S6K1, ribosomal protein S6 kinase beta-1; TAZ, transcriptional coactivator with PDZ-binding motif; TEAD 1/2/3/4, TEA domain family members 1/2/3/4; TSC1 and TSC2, tuberous sclerosis proteins 1 and 2; YAP, yes-associated protein.

## Stem Cell Therapy for Fertility Rescue

Some research groups are now focused on developing strategies to enhance fertility potential in patients with a diminished ovarian reserve. *In vitro* activation (IVA) of remaining PMFs in the context of a poor ovarian reserve through upregulation of the PI3K/Akt pathway by *in vitro* administration of positive modulators, along with disruption of the Hippo pathway by ovarian cortex fragmentation (Box 1), was first proposed by Kawamura *et al.* [49,50], but other studies failed to confirm the effectiveness of this approach. This combined IVA method was used to enhance follicle growth up to the antral stage in patients with premature ovarian failure, leading to two live births [51]. However, investigations carried out by other groups could not substantiate the role of the Hippo pathway in PMF activation or the positive impact of ovarian tissue fragmentation on its disruption [52,53], casting doubt on its role in the modulation of the ovarian reserve [54] (Figure 3).

Another strategy that has been investigated to boost ovarian function in the presence of a diminished ovarian reserve is use of numerous adult stem cell lines. Improved follicle outcomes in murine models

with a decreased ovarian reserve were observed after infusion of: (i) mesenchymal stem cells (MSCs) from the umbilical cord or from bone marrow [55–61], (ii) amniotic endothelial cells [62,63], (iii) menstrual blood-derived endometrial cells [64,65], and (iv) adipose tissue-derived stem cells (ASCs) [66,67]. Elevated hormone levels and higher numbers of developing follicles add weight to the hypothesis of a beneficial impact of adult stem cells on a limited follicle pool. A possible explanation may be found in the effect of the adult stem cell secretome, rich in growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), fibroblast growth factor (FGF), and insulin growth factor 1 (IGF-1), which can promote follicle development [68–70].

Patients affected by premature ovarian insufficiency have already been treated using infusions of bone marrow-derived MSCs (BM-MSCs), with six pregnancies reported so far [71–73]. However, variability in patient selection and small sample size in published data do not yet allow conclusions to be drawn on the effectiveness of this strategy. While use of stem cells to rescue the remaining follicle pool may arouse increasing interest, the mechanisms by which they have a beneficial impact on the follicle pool still need to be clarified. Moreover, regarding fertility preservation for malignant diseases, additional information is required about the safe implementation of this regenerative medicine approach during oncological treatment.

## Revascularization of Transplanted Human Ovarian Tissue: From Hypoxia to Restoration of Blood Supply

### Hypoxia and Oxidative Stress in Ovarian Grafts

As ovarian cortical strips are transplanted without vascular anastomosis, they experience a period of hypoxia, the duration of which is contingent on the progressive revascularization process. Partial pressure of $O_2$ ($pO_2$) was previously measured in grafted human tissue using **electronic paramagnetic resonance** [74]. Hypoxic values were found to persist for 5 days after transplantation and subsequently improve until they reached a plateau from day 10, suggesting active revascularization through angiogenesis driven by a limited oxygen supply [75]. Ovarian angiogenesis proceeds from both neoangiogenesis, sprouting from existing vessels [76] and vasculogenesis, namely blood vessel formation from vascular progenitors [77]. Invasion by host vascular components starts 3–5 days after grafting and, together with host endothelial compartments, leads to formation of a functional vascular network 10 days after transplantation [78]. Regulation of ovarian angiogenesis is a complex process involving multiple vasoactive and angiogenic factors. The master regulator of response to hypoxia is **hypoxia-inducible factor 1 (HIF1)** [79]. Hypoxia-related responses enhance angiogenesis by upregulating several growth factors. Among them, VEGF is a powerful promoter of neovascularization, widely expressed in the ovary [80,81]. Indeed, its values were observed to increase 40–60-fold in ovarian tissue soon after transplantation [82].

Around 10 days after ovarian tissue grafting, the vasculature appears to be reestablished with functional vessels and sufficient permeability [78]. At this point, namely once oxygen is available after ischemic damage, other harmful components triggered by an increase in **reactive oxygen species (ROS)** may contribute to tissue injury [83]. Their levels are maintained below a certain threshold by a number of scavengers, but when ROS are generated en masse, like after ischemia, they exceed cell antioxidant capacity, leading to oxidative stress [84]. Excessive ROS production can induce protein, lipid, and DNA modifications, with consequent cell damage [85]. ROS generation was directly quantified in human ovarian tissue xenografted to mice by use of **microdialysis** [86]. ROS appear to slowly increase in ovarian grafts and peak at 10 days after transplantation, consistent with the timing of tissue reperfusion itself. A second peak then occurs around 18 days after grafting, indicative of complete reestablishment of the vasculature and aerobic metabolism [86]. Indirect effects of oxidative stress in transplanted ovarian tissue have also been widely

investigated by evaluation of scavenger expression and signs of oxidative damage, quantified by lipid peroxidation [87].

### PMF Pool Behavior After Transplantation

Survival of ovarian follicles in grafted tissue depends on the timing and levels of proangiogenic growth factors generated and their orchestration of the revascularization process soon after transplantation. Indeed, major follicle loss occurs before complete revascularization of the graft [75,86] and affects around 50–90% of the follicle reserve [88].

Of all follicle stages, growing follicles appear to be most sensitive to hypoxia because of their higher metabolic demands, while PMFs are less vulnerable thanks to their quiescent state [89]. However, a significant decrease in PMFs has been documented after transplantation as a consequence of the ischemic/hypoxic period [89,90]. Interestingly, PMF loss is an early event, associated with increased numbers of growing follicles [91–93]. This phenomenon of large-scale PMF activation, known as the burn-out effect and already described as a response to chemotherapy-induced injury in ovarian tissue, acts through activation of a number of signaling pathways involved in granulosa cell survival and growth [91–94]. The PI3K/Akt pathway has been shown to maintain PMF quiescence and is linked to hypoxia-related signaling, as VEGF is able to trigger its activation for granulosa cell survival and proliferation [95]. Transplanted human ovarian tissue was also found to activate this signaling pathway soon after transplantation [91,96]. The hypoxic period is therefore associated not only with direct follicle loss, but also with massive PMF activation, contributing to faster depletion of the ovarian reserve in grafted ovarian cortex. Both these mechanisms were demonstrated in extensive investigations of the follicle pool after transplantation. Indeed, while absolute numbers, namely the direct count of follicles remaining after grafting, suggest direct death, changes in follicle proportions in favor of the growing stage point to abnormal activation. Both processes probably contribute to ovarian reserve depletion, but to what extent is still unknown.

### How to Improve Follicle Outcomes After Ovarian Tissue Transplantation

Numerous investigations have been conducted to address large-scale follicle loss, focusing on boosting revascularization to minimize grafted tissue injury. Administration of growth factors was carried out in animal models, with encouraging results. The short half-life of growth factors is one of the main limitations to their use, but development of biocompatible scaffolds transplanted with tissue may ensure extended release throughout the post-transplantation period, with improved follicle outcomes in both murine [97] and human [98] tissue. Another promising approach is use of erythropoietin, a proangiogenic hormone with a pleiotropic effect as an antioxidant, antiapoptotic, and anti-inflammatory cytokine [99]. However, while its use provided evidence of enhanced revascularization and decreased lipid peroxidation, it failed to exert any beneficial effects on the follicle pool [99,100]. Numerous studies have been performed with other antioxidants, such as superoxide dismutase [101], *N*-acetylcysteine [102], and melatonin [103], or modulators of vessel permeability like verapamil [104], but the transplanted ovarian tissue was of non-human origin and hence could not yield robust data on their effectiveness. In human xenografts, S1P, an angiogenesis enhancer and antiapoptotic drug, was found to boost vascularization after short-term transplantation, but failed to have any impact on PMFs [105].

Other research groups have focused more on holistic approaches to promote revascularization, taking advantage of endogenous activation of angiogenesis in specific conditions, either transplanting ovarian tissue into wound-healing granulation tissue in a murine autografting model [106], or to a freshly decorticated ovarian site in a non-human primate model [107]. Ovarian tissue revascularization was faster in both instances, thanks to the already activated tissue response to hypoxia and inflammation. MSCs have also been investigated in the context of ovarian tissue transplantation for their

potential impact on revascularization and follicle survival and further development. Among them, ASCs have proved their proangiogeneic credentials by secreting VEGF and other growth factors and differentiating into endothelial-like lineages [108,109]. Indeed, ASCs had a beneficial effect on the vasculature and follicle survival and development in rodents [110]. ASCs have also been used for preparation of the transplantation site with a view to maximizing their proangiogenic impact on grafted tissue. They were encapsulated inside a fibrin scaffold and grafted 2 weeks prior to ovarian tissue fragments [111]. A shorter hypoxic period and greater vascularization were observed in human ovarian tissue xenografted with this technique [112]. Moreover, boosting the revascularization process also had a significantly favorable influence on follicle survival and growth [112]. In conclusion, the most promising results so far have been achieved using stem cells, which play a proangiogenic role in numerous ways, acting on different levels in the hypoxia-response cascade of events. They also have antiapoptotic, anti-inflammatory, and immunomodulatory effects, which, combined with enhanced vascularization, may be critical to survival and quiescence of the follicle pool [109–112].

## The Why and How of the Artificial Ovary

### Evaluating the Risk of Reimplantation of Malignant Cells

Indications for ovarian tissue cryopreservation and subsequent transplantation in adults are most commonly hematological malignancies (Hodgkin's and non-Hodgkin's lymphoma and leukemia) and breast cancer [1]. In children, leukemia and myeloproliferative or myelodysplastic diseases tend to dominate, followed by sarcoma [113]. It is therefore vital that we address the threat of reimplanting malignant cells along with grafted tissue (Table 2).

Since malignant leukemic cells are found in the bloodstream, the risk is highest in patients with acute leukemia [114–116]. Even if biopsies destined for cryopreservation are retrieved from patients in complete remission, the risk cannot be ruled out [117–119]. Indeed, while ovaries from leukemia patients in complete remission do not appear to contain enough viable malignant cells to transmit the disease to mice upon xenotransplantation, molecular markers in ovarian tissue may still be positive [118].

Burkitt lymphoma is a rare B cell form of non-Hodgkin's lymphoma, but highly aggressive, with the ovaries most frequently affected in the female genital tract. It is thought to account for approximately 19% of adnexal lymphomas [120,121], so it is essential that ovarian tissue be screened for malignant cells before considering its reimplantation in these patients. Current screening methods include histology, immunohistochemical testing for disease-specific markers, fluorescence *in situ* hybridization, and PCR, as well as 6-month follow-up of immunodeficient mice transplanted with fragments of thawed ovarian tissue.

In view of this risk, various research teams worldwide have been investigating alternative approaches, including: (i) *in vitro* (*ex vivo*) culture of follicles [122–124], and (ii) *in vivo* (post-transplantation) growth of isolated preantral follicles inside a transplantable artificial ovary [92,125–140].

Table 2. Risk of Ovarian Metastasis According to Cancer Type

| High risk (>10%) | Moderate risk (2–10%) | Low risk (0–2%) |
|---|---|---|
| Leukemia<br>Neuroblastoma<br>Burkitt lymphoma | Breast cancer (advanced stage)<br>Colorectal cancer<br>Adenocarcinoma of the cervix<br>Non-Hodgkin's lymphoma<br>Ewing sarcoma<br>Ovarian cancer<br>Borderline ovarian tumor | Breast cancer (early stage)<br>Squamous cell carcinoma of the cervix<br>Hodgkin's lymphoma<br>Rhabdomyosarcoma<br>Soft tissue sarcoma |

## How to Avoid Reimplanting Malignant Cells When the Risk Is Real: Advent of the Bioengineered Transplantable Artificial Ovary

### *Bioengineering a Transplantable Artificial Ovary: Objectives and Required Conditions*

The primary objective of an artificial ovary is to safely transplant ovarian follicles, while ensuring secretion of sex hormones and development of fertilizable mature oocytes. In order to bioengineer an artificial ovary, several key conditions must be met: (i) ensuring scrupulous isolation of follicles; (ii) guaranteeing that no malignant cells find their way back to patients; and (iii) establishing follicle survival and growth, which depends on crosstalk between follicles and surrounding ovarian cells and the ovarian extracellular matrix [126].

### *Follicle Isolation: Quality, Quantity, and Safety*

The aim of the procedure is to dissociate as many intact follicles as possible from the surrounding ovarian stroma. Human ovarian cortex accommodating the majority of primordial and primary follicles is fibrous, so the most effective way of obtaining a high follicle yield and quality usually depends on a combination of mechanical and enzymatic tissue digestion [127]. Importantly, in order to allow its application in patients, reagents like Liberase and DNase must fully comply with good manufacturing practice guidelines [127,128]. The isolation process should also remain alert to the integrity of the follicle basal membrane, which can be monitored by electron microscopy [129].

It is also crucial to prevent any possible contamination of the follicle suspension by malignant cells. To this end, Soares *et al*. [130] devised a specific protocol using a repeated washing process to eliminate malignant cells.

### *Addition of Isolated Ovarian Stromal Cells*

Early follicle development is governed by ovarian autocrine/paracrine regulators, and stromal cells are known to release various factors that positively regulate primordial-to-primary follicle transition [31,131,132]. Upon activation of PMFs, primary follicles secrete other factors to recruit stromal cells to differentiate into functional theca cells [132–134], which participate in follicle growth and supply steroidogenic precursors for estrogen biosynthesis by granulosa cells. Hence, integrating ovarian stromal cells into the artificial ovary could reestablish natural communication between follicles and surrounding stromal cells, potentially enhancing follicle development [126].

Vascularization is also critical to follicle survival and growth, so vascular development is requisite after grafting. Endothelial cells are crucial to this, facilitating survival of different types of cells by boosting the capacity for revascularization after grafting [135]. Cotransplanting isolated endothelial cells along with stromal cells can promote vascularization in grafted tissue [136].

### *3D Matrix to Encapsulate Isolated Preantral Follicles and Ovarian Cells*

Which 3D matrix is appropriate for grafting of isolated human follicles and cells is one of the most challenging and critical questions in the development of a bioengineered artificial ovary. Since its conception, both synthetic [137–139] and natural [92,140–145] polymers have been tested in the search for a suitable artificial ovary prototype. Synthetic polymers allow the mechanical properties to be tailored to specific clinical applications, but they do not contain molecules needed for cell adhesion, although bioactive factors can be incorporated to stimulate cell adhesion [137,146]. Conversely, natural polymers display superior interaction with cells and are useful in cell adhesion, migration, proliferation, and differentiation processes, as they contain biofunctional molecules.

The first natural matrices used to graft isolated mouse preantral follicles were collagen [147] and plasma clots [148]. Subsequent studies followed using different matrices, with a few even reporting the birth of mouse pups [148,149]. Since data on mouse follicles cannot unfortunately

### Clinician's Corner

Ovarian tissue cryopreservation and subsequent transplantation is an effective strategy for both endocrine function and fertility restoration, with more than 200 live births obtained so far. Moreover, it is the only available fertility preservation option for prepubertal girls and patients whose cancer treatment cannot be postponed.

Mounting evidence suggests application of the following patient selection criteria: age less than 35 years, a realistic chance of surviving for 5 years, and at least a 50% risk of premature ovarian insufficiency.

Temporary ovarian suppression with GnRH agonists should not be used as an alternative, but possibly in addition to other cryopreservation options (oocytes and ovarian tissue), since its effectiveness in preserving the follicle pool has not been proved.

Ovarian tissue can be transplanted either orthotopically to the pelvic cavity or heterotopically to other sites like the forearm or abdominal wall, but the orthotopic approach by minimally invasive surgery is the most widely used today. Orthotopic transplantation involves grafting ovarian cortical fragments to the exposed medulla of the decorticated ovary or a newly created peritoneal window, depending on whether or not the patient previously underwent unilateral or bilateral oophorectomy.

Clinical application is limited in case of leukemia and Burkitt lymphoma, as there is a risk of reimplanting malignant cells together with the ovarian tissue. Screening ovarian tissue for malignant cells is vital before contemplating its reimplantation in these groups of patients. Currently accepted methods of screening ovarian tissue include histology, testing for disease-specific markers by immunohistochemistry or fluorescence *in situ* hybridization and PCR, and 6-month follow-up of immunodeficient mice transplanted with fragments of thawed ovarian tissue.

be extrapolated to human follicles, we will concentrate on isolated human follicles. Based on the successful results obtained with mouse follicles in Gosden's study, Dolmans *et al*. [92,140] encapsulated isolated human ovarian preantral follicles in autologous plasma clots and xenografted them to immunodeficient mice for 5 months. After this period, antral follicles were indeed detected in the grafts [92,140]. Despite these favorable findings, plasma clots have an inconsistent composition, degrading rapidly after grafting, which can result in follicle loss and irregular outcomes.

A number of studies have shown that fibrin is able to support survival and growth of human follicles. However, fibrin formulations need a much stiffer consistency than those tested for mouse follicles [127,143]. With this matrix, follicle recovery rates were 22–35% after 1 week of xenografting to immunodeficient mice.

Other natural matrices, like alginate, gelatin, and decellularized ovarian tissue, have also yielded promising data in isolated mouse follicle transplantation [142,150–154]. More recently, Pors *et al*. [155] reported encouraging results with human preantral follicles embedded inside decellularized human ovarian tissue. Isolated follicles were shown to survive for 3 weeks after xenografting to mice.

### *Functionality of the Transplantable Artificial Ovary*

Future research into a transplantable artificial ovary prototype for human follicles requires particular focus on follicle morphometry, growth rates, hormone secretion, molecular markers, and the epigenetic status of oocytes. Only then can we begin to understand how all these factors impact follicle survival and development.

## Concluding Remarks

There is now enough evidence to support the feasibility and efficacy of ovarian tissue cryopreservation and further transplantation for fertility restoration purposes in clinical practice. However, some concerns still preclude application of ovarian tissue transplantation in all patient categories. Indeed, massive follicle loss occurring soon after transplantation limits its effectiveness, especially in patients with a diminished ovarian reserve from the start. There is also a risk of reimplanting malignant cells together with the ovarian tissue in some cancers.

Numerous strategies have been formulated to address these issues over recent years. The most promising place particular emphasis on evaluating the complexity of biological mechanisms implicated in ovarian damage in order to successfully improve outcomes. The first challenge is to develop tools to accurately analyze oocyte quality and metabolic activity in ovarian tissue after grafting (see Outstanding Questions). Early follicle depletion occurring after avascular transplantation of frozen-thawed cortical pieces should then be investigated. Regenerative medicine using MSCs is already yielding promising results in terms of revascularization enhancement and follicle protection. The success of the transplantable artificial ovary is highly dependent on understanding the biological mechanisms involved in folliculogenesis and interaction of follicles with the extracellular matrix for appropriate follicle maintenance and development. Hence, finding the optimal scaffold to accommodate isolated follicles is paramount. Another crucial issue is establishing whether there is any epigenetic impact of isolation and grafting on human ovarian follicles before clinical application.

However, *in vitro* culture is another way of circumventing the potential risk of implanting malignant cells along with ovarian tissue, but progress in this field is cautious. Indeed, finding the best medium and conditions to promote efficient *in vitro* culture of human PMFs is a tough task.

### Outstanding Questions

How can we develop different potential protectants acting on chemotherapy-induced damage to ovarian pathways?

How can we mitigate early follicle depletion after transplantation?

How can we analyze oocyte quality and metabolic activity of ovarian tissue after grafting?

What is the best scaffold for the artificial ovary?

What would be the best medium and conditions to promote *in vitro* culture of human primordial follicles?

What is the epigenetic impact of isolation and grafting on human ovarian follicles?

Last but not least, identification of suitable protectants to mitigate chemotherapy-induced damage to the ovaries would potentially offer another means of preserving and protecting fertility.

While significant progress has been and continues to be made, there are still numerous challenges to be surmounted. Science is slowly but surely delivering the answers we need.

## Acknowledgments

The authors thank Mira Hryniuk, BA, for reviewing the English language of the article and Deborah Godefroidt for her administrative assistance. Grants from the Fonds National de la Recherche Scientifique de Belgique (grant 5/4/150/5, FNRS-PDR Convention T.0077.14, and the Excellence of Science FNRS–EOS number 30443682) were awarded to Marie-Madeleine Dolmans and (F.R.S.-FNRS/FRIA FC29657) to Luciana Cacciottola.

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