Female fertility restoration: Modulation of follicle activation linked to ovarian cryopreservation and transplantation

Jules Bindels

Thesis submitted to obtain the degree of

Doctor of Philosophy in Biomedical and Pharmaceutical Sciences

Academic year 2025 - 2026

University of Liège
Faculty of Medicine
GIGA - Cancer
Laboratory of Biology of Tumor and Development

Supervisor: Dr. Carine Munaut

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Restauration de la fertilité féminine: Modulation de l'activation folliculaire liée à la cryopréservation et à la transplantation ovarienne

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List of abbreviations

4-HC: 4-hydroperoxycyclophosphamide
ACS: Adipose tissue-derived stem cell
Akt: Protein kinase B
AMH: Anti-Müllerian hormone
AP-1: Activator protein 1
ATP: Adenosine triphosphate
BIRC: Baculoviral inhibitors of apoptosis repeats-containing proteins
BMP: Bone morphogenic protein
CPA: Cryoprotectant agents
CRsf: Cooling rate slow-freezing
CRv: Cooling rate vitrification
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
ECM: Extracellular matrix
EPO: Erythropoietin
F-actin: Filamentous actin
FOXL2: Forkhead box L2
FOXO3: Forkhead box O3
FSH: Follicle-stimulating hormone
G-actin: Globular actin
G-CSF: Granulocyte colony-stimulating factor
GnRH: Gonadotropin-releasing hormone
HIF-1: Hypoxia-inducible factor-1
HPO: Hypothalamus-pituitary-ovarian
IA: Intra-auricular
ICR: Institute of Cancer Research
IP: Intraperitoneal
IVF: In vitro fertilization
IVG: In vitro growth
IVM: In vitro maturation
JAK: Janus kinase
JNK: c-Jun N-terminal kinase
LH: Luteinizing hormone
Lhx8: LIM homeobox 8
MAPK: Mitogen-activated protein kinase
MEK: MAPK kinase
MGH: Medical-grade honey
MII: Metaphase II

MSC:	Mesenchymal stem cell
mTOR:	Mammalian target of rapamycin
mTORC1:	mTOR complex 1
mTORC2:	mTOR complex 2
NAC:	N-acetylcysteine
NMRI:	Naval Medical Research Institute
OTCTP:	Ovarian tissue cryopreservation followed by autotransplantation
PCR:	Polymerase chain reaction
PDK1:	Phosphatidylinositol-dependent kinase 1
PGC:	Primordial germ cells
PI3K:	Phosphatidylinositol-3-kinase
PIP2:	Phosphatidylinositol-4,5-biphosphate
PIP3:	Phosphatidylinositol-3,4,5-triphosphate
PMF:	Primordial follicle
POF:	Premature ovarian failure
PTEN:	Phosphatase and tensin homolog
ROS:	Reactive oxygen species
Rps6:	Ribosomal protein s6
RT:	Room temperature
S1P:	sphingosine-1-phosphate
S6K1:	Ribosomal protein S6 kinase beta-1
SCID:	Severe combined immunodeficiency mice
SF:	Slow-freezing
STAT:	Signal transducer and activator of transcription
TAZ:	Transcriptional co-activator with PDZ-binding motif
TEA:	Transcriptional enhancer activator
TEAD:	TEA DNA binding domain
TGF-β:	Transforming growth factor beta
TSC1/TSC2:	Tuberous sclerosis 1 and 2
VEGF:	Vascular endothelial growth factor
YAP:	Yes-associated protein
Z-VAD-FMK:	Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Summary - Résumé

Summary

Cancer therapies such as chemotherapy, while increasingly effective, can severely impair ovarian function, leading to premature ovarian failure and infertility. Ovarian tissue cryopreservation and transplantation (OTCTP) is currently the only viable fertility preservation strategy for prepubertal girls and women requiring immediate treatment. Despite its clinical success, OTCTP is limited by excessive follicle loss after grafting, largely driven by ischemia, apoptosis, and primordial follicle (PMF) hyperactivation.

To address this, we first developed a novel, minimally invasive heterotopic transplantation model, between the skin and cartilage of the ear, which allows for localized post-grafting pharmacological treatment. Comparison with the conventional but invasive kidney capsule model showed that PI3K inhibition with LY294002 produced similar effects on follicle activation at both sites, validating the new model's utility for testing pharmacological interventions.

Building on prior *ex vivo* findings with the mTOR inhibitor rapamycin, we aimed to improve follicle preservation during the transplantation process. Rapamycin addition during the cryopreservation process maintained follicle quiescence and improved fertility restoration *in vivo*, resulting in more offspring with higher live birth rates compared to controls. However, rapamycin treatment triggered feedback activation of the Akt pathway.

We therefore investigated BEZ235, a dual PI3K/mTOR inhibitor. Its addition during cryopreservation significantly preserved the PMF pool and suppressed follicle activation more effectively than single-pathway inhibitors, both *in vitro* and *in vivo*. Post-grafting VEGF/G-CSF injections, intended to enhance vascularization, did not further enhance PMF preservation when combined with BEZ235.

In conclusion, while rapamycin supports follicle dormancy and fertility restoration, dual inhibition of PI3K/mTOR with BEZ235 more effectively reduces post-grafting follicle loss. As fertility preservation is becoming increasingly important for young cancer patients, such strategies may help extend their reproductive lifespan and quality of life post-treatment.

Résumé

Les traitements anticancéreux, tels que la chimiothérapie, bien qu'efficaces, peuvent gravement altérer la fonction ovarienne, entraînant une insuffisance ovarienne prématurée et une infertilité. La cryopréservation et la transplantation de tissu ovarien (CTPTO) constituent actuellement la seule option de préservation de la fertilité pour les filles prépubères et les femmes nécessitant un traitement immédiat. Malgré son succès clinique, la CTPTO est limitée par une perte folliculaire post-greffe importante, induite par l'ischémie, l'apoptose et l'hyperactivation des follicules primordiaux (PMF).

Pour surmonter ces limitations, nous avons développé un nouveau modèle murin de transplantation hétérotopique, peu invasif, entre la peau et le cartilage de l'oreille, qui permet l'administration de traitements pharmacologiques post-greffe localisée. Une Comparaison avec le modèle conventionnel, mais invasif, de la capsule rénale a montré que l'inhibition de PI3K par LY294002 produisait des effets similaires sur l'activation folliculaire dans les deux sites. Ce résultat valide l'utilité du nouveau modèle pour tester de nouveaux agents pharmacologiques.

Sur la base de résultats antérieurs obtenus *ex vivo* avec l'inhibiteur de mTOR, la rapamycine, nous avons cherché à améliorer la préservation folliculaire au cours de la transplantation ovarienne. Nous avons montré que l'ajout de rapamycine pendant le process de cryopréservation permettait de maintenir les follicules dans un état quiescent transitoire et d'améliorer la restauration de la fertilité *in vivo*, avec un nombre de descendants et un taux de naissances vivantes plus élevés que chez les témoins. Cependant, ce traitement induisait une activation compensatoire de la voie Akt.

Pour y remédier, nous avons testé le BEZ235, un inhibiteur dual PI3K/mTOR. Son ajout pendant la cryopréservation a permis de préserver efficacement le pool de PMF et de limiter l'activation folliculaire, en surpassant les inhibiteurs ciblant une seule voie, tant *in vitro* qu'*in vivo*. L'administration post-greffe de VEGF/G-CSF n'a pas renforcé cet effet.

En conclusion, l'inhibition duale des voies PI3K/mTOR par le BEZ235 représente une stratégie prometteuse pour prolonger la longévité des greffes ovariennes. De telles approches pourraient contribuer à étendre la fenêtre de fertilité et à améliorer la qualité de vie des jeunes patientes atteintes de cancer.

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Chapter 1

Introduction

1.1 Cancer therapies and their implication on female fertility

1.1.1 Improvements in cancer therapies and the importance of quality of life

In the United States, approximately 60,000 new cases of cancer are reported annually in women under 40 years of age, with 6,617 prepubertal girls diagnosed in 2020 (1, 2). In Belgium, more recent national estimates reported 72,680 new cancer cases in 2022, with an age-standardised incidence rate of 544 per 100,000 women, approximately 11% above the European average (3). Over the past decades, advancements in cancer diagnosis and therapies have significantly improved the cure rates for many childhood and young adult cancers, allowing 80% of children to survive at least 5 years (4, 5). With the enhanced efficacy of oncologic treatments, clinical focus has increasingly shifted toward improving the quality of life and addressing the adverse effects of therapy. Many side effects and impairments of active cancer treatment are acute and short-term, and a well-established rehabilitation plan can improve common side effects, such as pain, fatigue, and physiological problems (6-8). However, other side effects can have a long-lasting impact, reducing the long-term quality of life (9). One of these possible long-term effects is infertility, and many young women with cancer express concerns about their fertility following treatment (10). Indeed, a critical issue amongst young cancer survivors wishing to start families is to conceive biological children. Therefore, for young patients undergoing gonadotoxic treatments with a potential for complete remission, implementing fertility preservation techniques has become increasingly important (11). To better understand the effects of cancer treatment on the ovaries and to understand the fertility preservation options, it is important to introduce the ovarian physiology.

1.1.2 Physiology of ovaries

The human ovaries are oval-shaped intraperitoneal (IP) organs that play an important role in the female reproductive system. During the reproductive age, each ovary measures 2.5 to 5.0 cm in length, 1.5 to 3 cm in width, and 0.6 to 1.5 cm in thickness. Located inside the ovarian fossa alongside the lateral pelvic wall, they are positioned on each side of the uterus next to the fallopian tubes. Each ovary is held in place via attachment to the broad ligament, extending from the uterus to the wall of the pelvic cavity, and their outer surface is covered by a germinal layer of cuboidal epithelial cells, with underneath a dense connective tissue capsule named the tunica albuginea. The ovary is divided into two principal compartments: the inner medulla, which is surrounded by the outer cortex. This cortex is characterized by connective tissue, in which the majority of follicles, cellular aggregations containing the female gametes, are embedded. In the center of the ovary lies the medulla, a mass of loose connective tissue containing blood and lymphatic vessels, and associated nerves (**Figure 1**). Blood is supplied to the ovary via two sources: the ovarian artery originating from the abdominal aorta, and branches deriving from the uterine artery (12, 13). The ovary has two main functions: the development of an oocyte for reproduction, and the production and secretion of several steroid hormones (14).

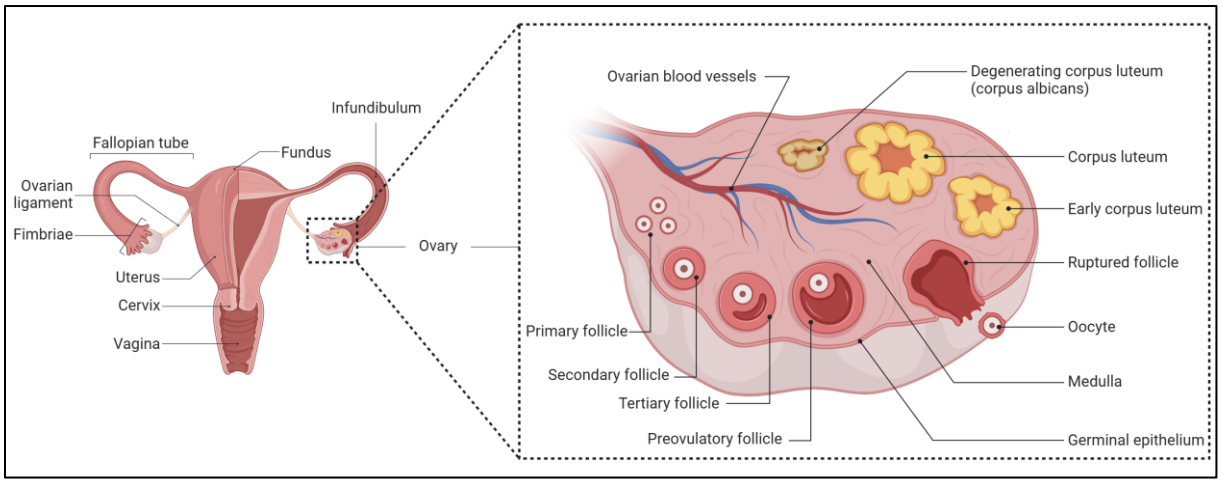

Figure 1. ***Anatomy of the female reproductive system and human ovary.*** *Created with BioRender.com.*

1.1.3 Pre-pubertal ovarian development

The complete follicle pool originates from primordial germ cells (PGC) derived from the inner cell mass of the blastocyst during the embryonic stage. When formed, these cells migrate out of the yolk sac epithelium towards the gonadal ridges, where the early bipotential gonads will develop. In women, due to the absence of the Y chromosome, the gonads will further develop into ovaries and the PGCs into oogonia (15, 16). Via mitotic divisions, the oogonia can reach up to a number of 7 million by the fifth month of gestation (17). At this point, oogonia will stop mitosis and initiate meiosis, transforming into oocytes, where they will be arrested at the first meiotic prophase (18). Only about one-third of the initial oocytes will become surrounded by pregranulosa cells and constitute the final prenatal primordial follicle (PMF) pool, with the remaining oocytes being lost via apoptosis (19, 20). Amongst the reasons for this massive loss of oocytes are failure of meiosis and unrepairable deoxyribonucleic acid (DNA) damage, as well as an insufficiency of pregranulosa cells (20). Therefore, females are born with 1 to 2 million PMFs. After birth, this number will steadily decline throughout childhood until approximately 400,000 PMFs remain at the onset of menses (**Figure 2**) (21).

1.1.4 The onset of menses and folliculogenesis

Between the ages of 10 and 16, menarche will occur, which is the initial menstrual cycle of the female and indicates the onset of fertility (22). This cycle prepares the body for ovulation and potential pregnancy, and is regulated via the complex interactions of the hypothalamus-pituitary-ovarian (HPO) axis via hormonal secretion. This regulation originates from the hypothalamus, where gonadotropin-releasing hormone (GnRH) is secreted and subsequently transported to the anterior pituitary, resulting in the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are transported to the ovaries, stimulating the production of the sex hormones. The menstrual cycle consists of two phases: The follicular/proliferative phase, and the luteal/secretory phase, with ovulation happening in between these two (**Figure 3**) (23).

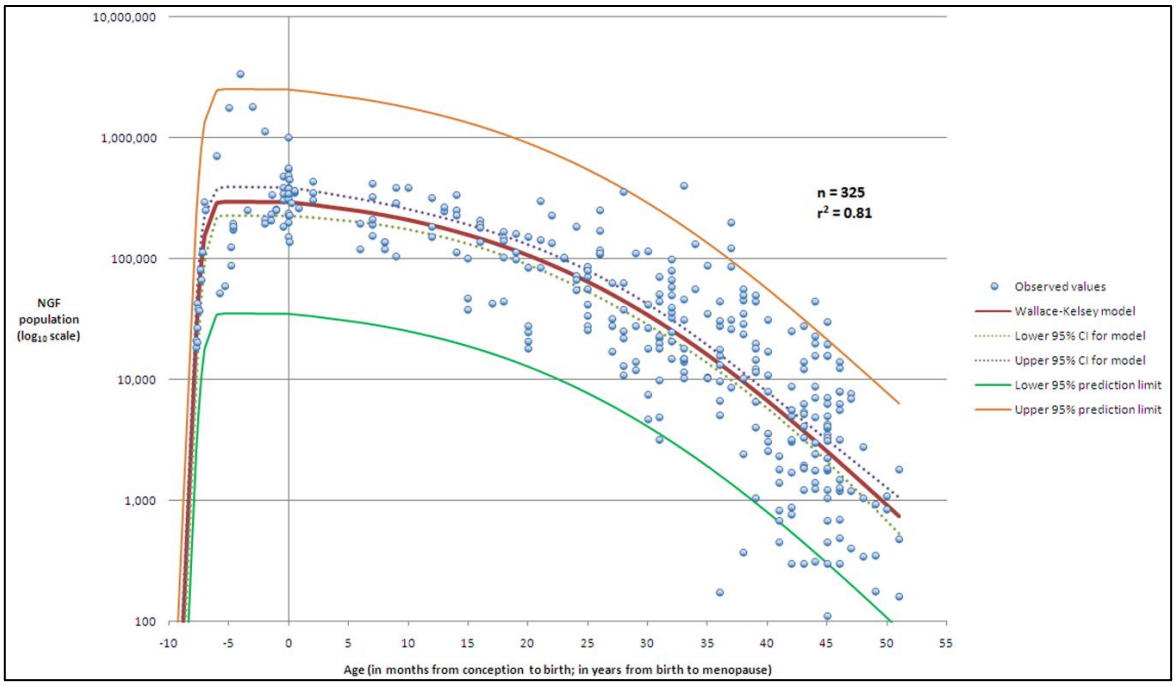

Figure 2. ***Mathematical modeling of the human ovarian reserve from conception until menopause.*** *Graph showing the predicted and observed number of non-growing follicles (NGFs) in the human ovary (logarithmic scale) from mid-gestation through to menopause. Observed data (n = 325) are shown as blue dots. The red curve represents the Wallace-Kelsey model of follicle depletion, with 95% confidence intervals (dotted lines) and 95% prediction limits (solid green and orange curves). The model predicts a peak at approximately 5-6 months of gestation, followed by an exponential decline until menopause. From Wallace et al. 2010 (24).*

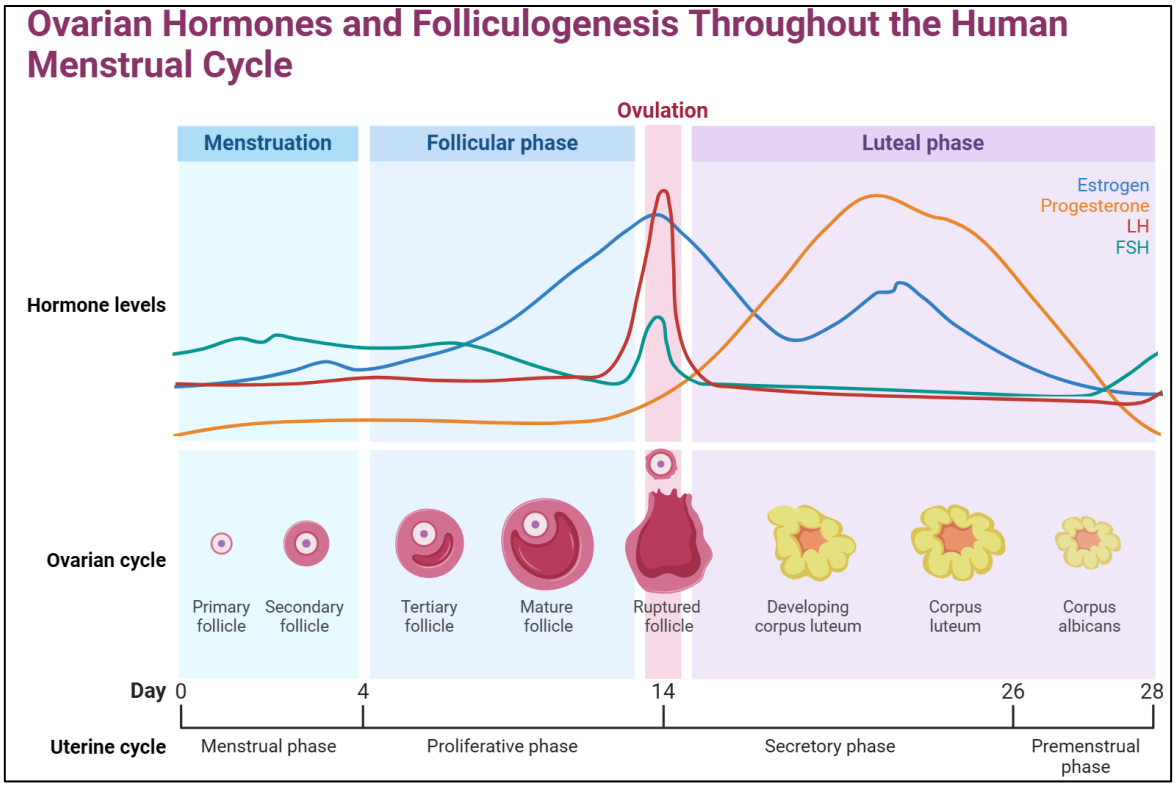

Figure 3. ***The human menstrual cycle.*** *During the proliferative phase, follicles develop under the influence of FSH and LH. Ovulation is induced on the 14th day by a spike in LH levels, releasing the oocyte for fertilization. During the second phase of the cycle, the follicle will develop into the corpus luteum, starting the production of progesterone, allowing the differentiation of the epithelial cells of the endometrium, preparing for implantation. In the absence of pregnancy, the corpus luteum regresses, the endometrium is shed, and FSH levels rise, initiating a new cycle (23). Created with BioRender.com.*

Until the onset of menses, the PMFs have lain dormant, residing in the ovarian cortex. In humans, these follicles measure approximately 35 µm in diameter and constitute the majority of ovarian follicles. Each consists of an oocyte encased by a single layer of squamous granulosa cells. The PMFs have three possible fates: (I) remain dormant, (II) directly die from their dormant state, (III) become activated and join the growing follicles, either going through atresia or becoming a dominant follicle ready for ovulation (25). Starting from puberty, the follicular phase, or "folliculogenesis", takes place, including the maturation of ovarian follicles and proliferation of the endometrium (23, 26). The first phase of folliculogenesis is the initial recruitment, with a variable length of about 120 days. During this phase, follicles grow independently of gonadotropins and develop into primary follicles. This transition is characterized by a change in granulosa cell morphology, from squamous to cuboidal. After the initial recruitment, a phase with slow follicle growth of approximately 70 days takes place. The granulosa cells proliferate and will first form secondary follicles surrounded by two or more layers of granulosa cells. Then, a membrane will form around the outer granulosa cells, with another layer called theca cells encapsulating the preantral follicle. While these preantral follicles grow independently of FSH, the bidirectional communication between the oocyte and surrounding granulosa and theca cells is essential for adequate follicle development. As folliculogenesis continues, an antrum forms, dividing the granulosa cell population into two distinct functional types: mural granulosa cells, which regulate steroidogenesis, and cumulus cells, which surround and support the oocyte. This is followed by the third and final phase, the terminal follicle growth. Depending on the cyclical rise of FSH, a single dominant follicle is selected per cycle that will develop for 15 days until ovulation. At the beginning of this phase, the rise in FSH will result in a massive increase in granulosa cells, while the late-stage survival of the dominant follicle is mainly LH-dependent (23, 26, 27). The dominant follicle has now developed into a Graafian follicle, ready for ovulation. Under the control of estradiol, a rise in LH triggers ovulation. The follicle wall breaks, and the cumulus-oocyte complex is released, containing the fertilizable oocyte. This marks the start of the luteal phase. The granulosa and theca cells in the remaining follicle will differentiate into luteinizing cells and form the corpus luteum. The central role of the corpus luteum is the secretion of progesterone, essential for establishing and maintaining pregnancy. When fertilization has not occurred, the corpus luteum degenerates, and the cycle will start again until menopause (**Figure 4**) (23, 26, 28).

After puberty, the PMF pool continues to decline with an acceleration around the age of 37, when approximately 25,000 follicles remain, until menopause, when less than 1,000 follicles remain around the age of 51. With one mature oocyte being ovulated every 28 days, only approximately 450 follicles reach ovulation, while the large majority will undergo follicle death by atresia (**Figure 2**) (24).

1.1.5 Primordial follicle activation pathways

The PMF pool is non-renewable, and once PMFs are activated, they start an irreversible developmental process. Therefore, the controlled activation and quiescence of PMFs play an important role in determining the female reproductive lifespan. As mentioned before, the initial recruitment of PMFs is not regulated by gonadotropins, but via complex bidirectional signals between the oocyte and granulosa cells, as well as stress or growth factors from the follicle microenvironment

(29, 30). As the mechanisms behind follicle activation are so complex, the understanding of how these signals interact is still limited. However, it is known that under normal physiological conditions, delicately timed activation of PMFs is regulated by an equilibrium between activation and inhibitory signals. In the process of follicle activation, the phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Protein kinase B (Akt) pathway plays a key role and is the clearest and most studied pathway (**Figure 5**). It can be activated via several growth factors, such as the vascular endothelial growth factor (VEGF), as well as kit ligand, a cytokine originating from granulosa cells, highlighting again the importance of signals between the granulosa cells and oocyte (31, 32). Upon binding of these growth factors to their respective tyrosine kinase receptor on the oocyte surface, these receptors stimulate the activity of PI3K, resulting in the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3). This step is negatively regulated by PTEN via converting PIP3 back to PIP2 (33). Via the co-binding to PIP3, phosphatidylinositol-dependent kinase 1 (PDK1) activates AKT. After the translocation of AKT to the nucleus, it inhibits the transcriptional activity of forkhead box O3 (FOXO3). FOXO3 is then translocated into the cytoplasm, where it cannot keep PMFs in a dormant state (**Figure 5**) (31).

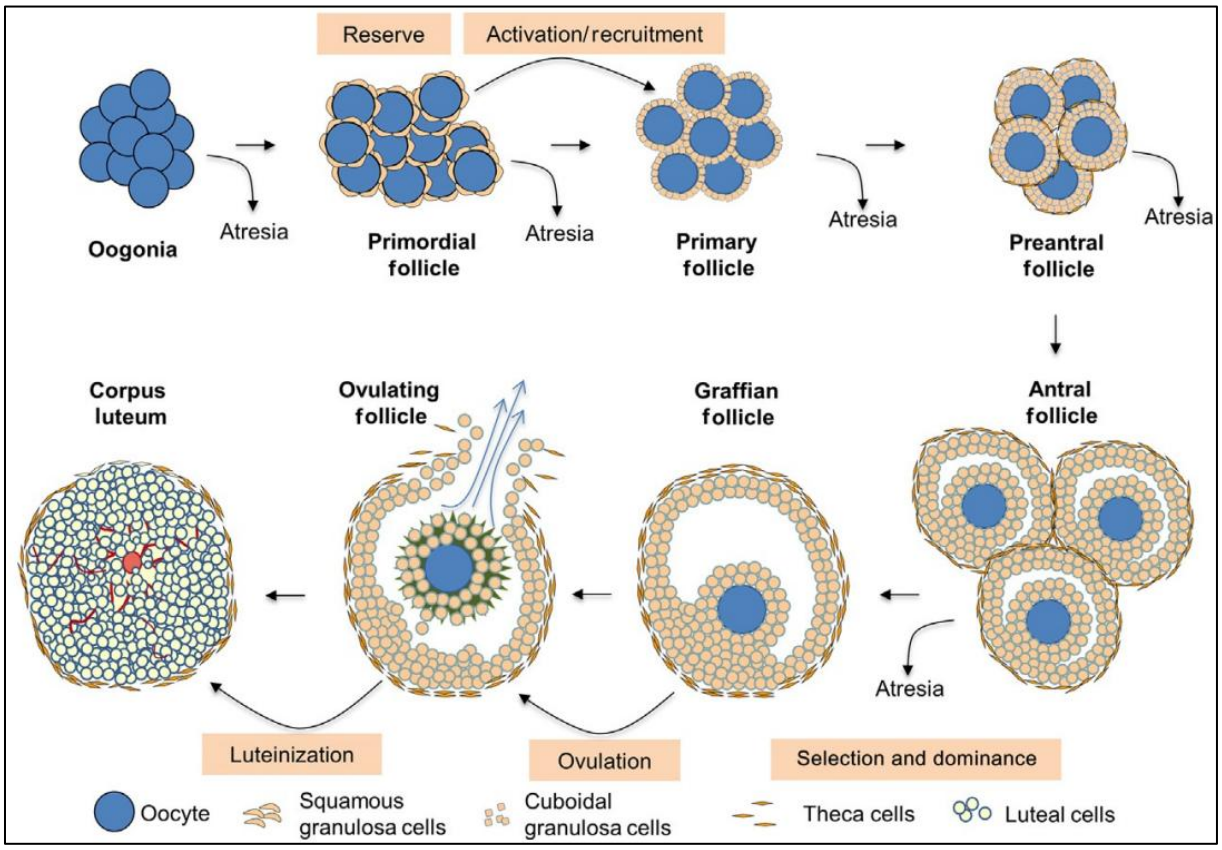

Figure 4. ***Overview of folliculogenesis and human ovarian cycle.*** *Schematic representation of the different stages of follicular development, from the initial pool of primordial follicles to ovulation and corpus luteum formation. The figure illustrates both the gonadotropin-independent (primordial to preantral) and gonadotropin-dependent (antral to ovulatory) phases, as well as key points of follicular selection, atresia, and luteinization. From Puttabyatappa et al. 2018 (28).*

Another important pathway involved in PMF activation and downstream of Akt is the mammalian target of rapamycin (mTOR). It is a key regulator of cell growth and metabolic state, and exists in two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). When PMFs are in a

dormant state, mTORC1 is inhibited by the tuberous sclerosis 1 and 2 (TSC1/TSC2) complex. However, activated Akt can inactivate the TSC1/TSC2 complex, which stops its inhibitory effects on mTORC1. Upon activation, mTORC1 can phosphorylate ribosomal S6 kinases to promote protein synthesis and thus induce cell survival and proliferation (33-35). On the other hand, mTORC2 plays an important role in the complete activation of Akt. Indeed, depletion of mTORC2 inhibited Akt and reduced the phosphorylation of FOXO3, reducing the activation of PMFs. In contrast, the activity of the TSC1/TSC2 complex was not affected by mTORC2 depletion (**Figure 5**) (34).

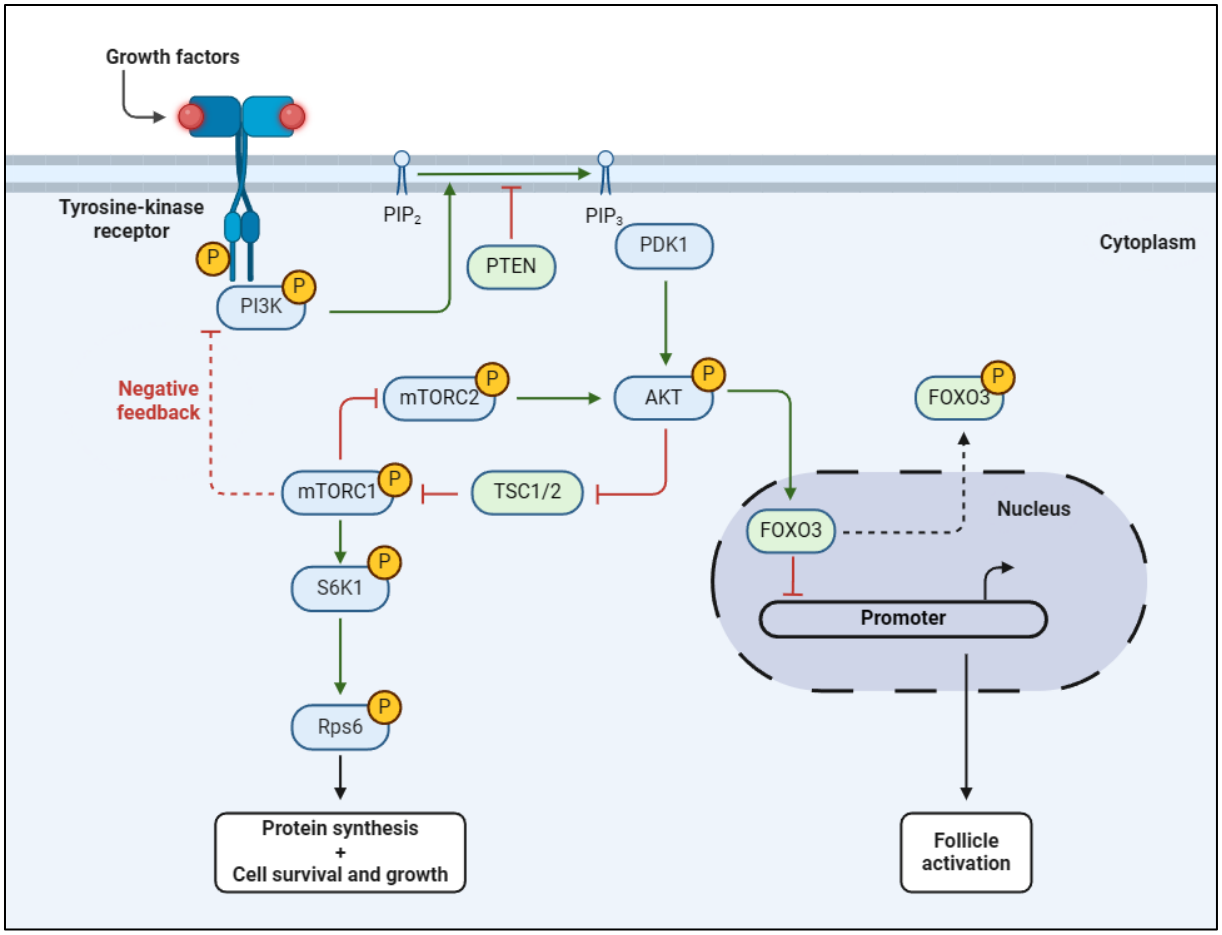

Figure 5. ***PI3K/PTEN/Akt and mTOR pathway activation.*** *Activation signals are shown in green and inhibition signals in red. PI3K: Phosphatidylinositol-3-kinase; PIP2: Phosphatidylinositol-4,5-bisphosphate; PIP3: Phosphatidylinositol-3,4,5-triphosphate; PDK1: Phosphatidylinositol-dependent kinase 1; PTEN: Phosphatase and tensin homologue; Akt: Protein kinase B; FOXO3: Forkhead box O3; mTORC1: Mammalian target of rapamycin complex 1; mTORC2: Mammalian target of rapamycin complex 2; TSC1/2: Tuberous sclerosis 1 and 2 complex; S6K1: Ribosomal protein S6 kinase beta-1; Rps6: Ribosomal protein s6; P: phosphorylation. Created with BioRender.com.*

An important pathway involved in follicle dormancy and activation is the Hippo signaling pathway. It is a pathway involved in organ size control and consists of multiple negative growth regulators (36, 37). When active, the pathway ultimately phosphorylates and inactivates key transcriptional coactivators, yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). Disturbance of this pathway by mechanical stress of the ovary induces a transient increase in polymerization of globular actin (G-actin) to filamentous actin (F-actin), leading to a decrease in YAP phosphorylation. Therefore, YAP/TAZ is translocated to the nucleus where it interacts with transcription factors containing the transcriptional enhancer activator (TEA) DNA binding domain

(TEAD) to increase the expression of connective tissue growth factors and baculoviral inhibitors of apoptosis repeats-containing proteins (BIRC) apoptosis inhibitors (31, 36). Increased expression of these proteins leads to granulosa cell growth and proliferation (38).

One of the key inhibitory signals during the PMF activation process is the paracrine anti-Müllerian hormone (AMH). It is part of the transforming growth factor beta (TGF-β) family of extracellular ligands and is expressed from the granulosa cells of secondary to early antral follicles. It functions to inhibit the transition of primordial to primary follicles, maintaining PMFs in a dormant state (39, 40). While recent advancements have provided valuable insight into the signaling mechanism of AMH, many areas are still not completely understood (41).

Besides these pathways involved in the dormancy and activation of PMFs, the c-Jun N-terminal kinase (JNK) pathway has been shown to be an important factor in the growth of preantral follicles by controlling mitosis (42). As part of the Mitogen-activated protein kinase (MAPK) family, it can be activated by various stimuli, including cytokines and stress factors. These stimuli initiate MAP3Ks activation, which subsequently phosphorylate and activate the MAP2K isoforms MKK4 and MKK7, leading to the activation of JNK. A key downstream target of JNK signaling is the activator protein 1 (AP-1) transcription factor, which is partially regulated through the phosphorylation of c-Jun and related molecules. These factors have been shown to be essential for cell cycle progression and proliferation (42, 43).

As the activation of PMFs and subsequent maturation is such a complex mechanism, many other signals and pathways are involved besides the ones already mentioned. These include bone morphogenic proteins (BMPs), Forkhead box L2 (FOXL2), the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway, SMAD3, and LIM homeobox 8 (Lhx8). In-depth explanation of these signals and pathways would be too extensive for this introduction. However, these are reviewed in Zhang *et al.* 2023 (30). It is important to note that most mechanistic insights into the molecular regulation of follicle activation, including the aforementioned pathways, have been derived from animal studies. Nevertheless, these signaling pathways appear to be well conserved across species, supporting their relevance in understanding human ovarian physiology (30).

1.1.6 Effects of chemotherapy and radiotherapy on fertility - premature ovarian failure

As mentioned in the beginning, advancements in cancer therapies have significantly improved the cure rates for many childhood and young adult cancers. Indeed, aggressive chemotherapy and radiotherapy can cure more than 90% of girls and young women affected by disorders requiring such treatment (44). Unfortunately, these treatments are highly gonadotoxic, which can lead to ovarian failure and the loss of follicles and, consequently, infertility (45-47). Ionizing radiation from pelvic irradiation to treat several types of cancer, *e.g.,* cervical and rectal cancer, can be highly gonadotoxic as the ovaries are located in the radiation field. Indeed, these radiotherapies destroy PMFs in a dose-dependent manner (48). The gonadotoxicity of chemotherapy is characterized by temporary and permanent effects, which can be both directly on the ovaries or the follicles. Among the chemotherapeutic compounds, alkylating agents, *e.g.,* cyclophosphamide, are considered to be the most toxic. Growing follicles are the most susceptible to damage, and several hypotheses have been

proposed to explain gonadotoxicity. Chemotherapy has shown to induce DNA damage via double-stranded DNA breaks in both granulosa cells and oocytes of growing follicles, leading to apoptosis. Furthermore, these drugs could trigger indirect PMF depletion via over recruitment. Besides the effects on follicles itself, chemotherapy can lead to fibrosis and blood vessel damage inside the ovaries (49). All these factors can lead to the depletion of the ovarian reserve and eventually a phenomenon called premature ovarian failure (POF), which is characterized by the absence of menarche or premature depletion of the PMF pool before the age of 40 years (50). The risk of developing POF depends on several factors, including the type, dose, and duration of therapy, but also the age of the patient at the onset of therapy. Indeed, younger girls have a lower risk than older patients of developing POF at the same therapeutic dose (46). A summary of the risk of POF can be found in **Table 1** (51).

Table 1. ***Risk of premature ovarian failure in women****. Adapted from Dolmans et al. 2018 (51).*

High risk	Stem cell transplantation, external beam irradiation to fields including the ovaries, breast cancer adjuvant combination chemotherapy regimens containing cyclophosphamide, methotrexate, fluorouracil, doxorubicin and epirubicin in women aged > 40 years
Intermediate risk	Breast cancer adjuvant chemotherapy regimens containing cyclophosphamide in women aged 30–39 years, or doxorubicin/cyclophosphamide in women aged > 40 years, bevacizumab
Low risk (< 20%)	Combination chemotherapy regimens for non-Hodgkin's lymphoma, acute lymphoblastic or myeloid leukemia, breast cancer adjuvant chemotherapy regimens containing cyclophosphamide in women aged < 30 years, or doxorubicin/Cy in women aged < 40 years
Very low risk or no risk	Vincristine, methotrexate, fluorouracil
Unknown risk	Paclitaxel, taxotere, oxaliplatin, irinotecan, trastuzumab, cetuximab, erlotinib, imatinib

As the cure rates for childhood and young adult cancers keep rising, it is becoming more and more important to consider the quality of life after remission. For many young cancer survivors who aspire to start families, a critical concern is their ability to conceive biological children. Therefore, implementing fertility preservation strategies for prepubertal girls and young women with a child wish undergoing gonadotoxic treatment has increased in interest (52).

1.2 Female fertility preservation techniques

Many young women with cancer express concerns about their fertility following treatment and have a strong desire to conceive biological children in the future. It is therefore important to inform patients about the potential risks of these therapies and discuss fertility preservation strategies before the onset of gonadotoxic therapies. At present, several of these strategies exist, with each one having its benefits and limitations. Selecting the most suitable technique depends on multiple factors, including age, cancer type, urgency of the onset of treatment, and whether the patient is in a relationship (53). A comparison of the benefits and drawbacks of the different female fertility preservation techniques can be found in **Table 2**.

Table 2. ***Benefits and drawbacks of female fertility preservation methods*** *(11, 53-56). PMF: primordial follicles, POF: premature ovarian failure, N.A. = Not applicable.*

Established fertility preservation methods				
Intervention	**Ideal patient**	**Pregnancy rate**	**Benefits**	**Drawbacks**
Ovarian tissue cryopreservation	Prepubertal girls or women without time for ovarian stimulation	20-40% per transplantation (54)	No ovarian stimulation or partner needed	Multiple surgeries required
				Risk of cancer recurrence
			Restores both natural conception and endocrine function	Complications with PMF activation
Ovarian transposition	Patient undergoing pelvic radiotherapy	N.A.	There are almost no prerequisites	Requires surgery, and success rates vary
				Not feasible in combination with chemotherapy
Mature oocyte cryopreservation	Adult women without a male partner	4.5-12% per oocyte (54)	Well-established method	Expensive
			Does not require a male partner/sperm donor	Requires time, as hormonal stimulation is required
			More preferable for patients with ethical concerns	Less feasible for women with hormone-sensitive cancers
Embryo cryopreservation	Adult women with a male partner	30-35% per embryo (54)	Well-established method	Expensive
				Requires time, as hormonal stimulation is required
			Offers the possibility of preimplantation genetic diagnosis	Less feasible for women with hormone-sensitive cancers
				Requires a male partner or sperm donor
***In vitro* maturation**	Women without time for ovarian stimulation and risk re-implantation of cancer	N.A.	No treatment delay	Limited chance of success before puberty
			No risk of malignant contamination	
Experimental fertility preservation methods				
Intervention	**Ideal patient**	**Pregnancy rate**	**Benefits**	**Drawbacks**
Artificial ovary	Prepubertal girls or women without time for ovarian stimulation and risk re-implantation of cancer	N.A.	No delay in cancer therapy	Limited evidence in clinical trials
			Suitable for patients with POF	
Hormone treatment	Adult women undergoing chemotherapy	N.A.	No delay in cancer therapy	Unproven efficacy
			No surgery needed	Not feasible in combination with radiotherapy
			Cheap and easy	Risk for osteoporosis
***Ex vivo* ovarian perfusion**	Prepubertal girls or women without time for ovarian stimulation and risk re-implantation of cancer	N.A.	No delay in cancer therapy	Technique is still in its early days of development
			No risk of malignant contamination	

Besides cancer patients undergoing gonadotoxic therapies, fertility preservation can be proposed for several other reasons, including patients with endometriosis, ovarian torsion, and recurring ovarian cysts. Other indications might be genetic disorders with a risk of POF, like Turner's syndrome, a disorder known to deplete the follicle pool at a young age (57). Furthermore, as many women are nowadays attempting to conceive at a later stage in their lives, due to career choices, for instance, fertility preservation has become an option for personal reasons during the last few years (58) (**Table 3**).

Table 3. ***Indications for fertility preservation****. From Dolmans et al. 2021 (58).*

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

1.2.1 Ovarian transposition

Ovarian transposition, or "oophoropexy", is an applicable method offered to patients undergoing pelvic irradiation. In this procedure, ovaries are transposed either under the uterus or out of the radiation field, and it can be performed immediately before the scheduled therapy through laparoscopic section, to minimize the harmful effects of irradiation on ovarian function. Fertility preservation outcomes using this method are directly influenced by factors such as patient age, radiation dose, ovarian shielding, and the use of concomitant chemotherapy. Consequently, the overall efficiency is often unsatisfactory, and there is an increased risk of additional complications (53).

1.2.2 Mature oocyte and embryo cryopreservation

Mature oocyte or embryo cryopreservation is currently the golden standard fertility preservation method for cancer patients. For single women who do not want sperm donation or embryo freezing, mature oocyte cryopreservation is the best option. This approach is also suitable for women with a partner who prefer not to create embryos at the time of fertility preservation. Conversely, embryo freezing is a suitable option for women wanting to conceive and have a male partner, or who want sperm donation. These techniques require ovarian stimulation, making them unsuitable for prepubertal girls and patients with aggressive cancers requiring immediate treatment, as it will lead to a delay in the onset of treatment. In patients with oestrogen-dependent cancers, conventional ovarian stimulation protocols are not suitable as they can increase blood oestrogen levels. However, the use of aromatase inhibitors (a key enzyme in oestrogen synthesis) during stimulation has been shown to effectively mitigate this risk, making the approach safe in these cases (59). The pregnancy rates are approximately 30-35% per embryo and 4.5-12% per frozen/thawed oocyte (54).

1.2.3 *In vitro* growth and maturation

A fertility preservation technique that was declared non-experimental a few years ago is *in vitro* maturation (IVM) of immature oocytes from small antral follicles (60, 61). It is an excellent technique for cancer patients whose treatment needs to commence immediately or who are suffering from oestrogen-dependent cancers. However, it remains a complex technique with a lower success rate

compared to conventional *in vitro* fertilization (IVF). Furthermore, the mature oocyte yield and overall quality are much lower in IVM compared to conventional ovarian stimulation in cancer patients, showing the need for improvements in the IVM program (61). Besides the collection of oocytes from small antral follicles, it is also possible to collect PMFs and perform complete *in vitro* growth (IVG) and IVM to produce fertile metaphase II (MII) oocytes. Although IVM is already a complex technique, IVG requires even more extensive culture systems, as isolated PMFs remain quiescent and thus need to be maintained in small pieces of ovarian cortex containing stromal cells. While IVM has been declared non-experimental, IVG is still deemed experimental since most PMFs will never reach the secondary stage, yielding too few mature oocytes (62).

1.2.4 Experimental techniques

1.2.4.1 Artificial ovary

In recent years, a new experimental fertility preservation technique has been investigated for girls and women who cannot undergo oocyte/embryo cryopreservation and ovarian tissue transplantation: the artificial ovary. Indeed, for several cancer types, *e.g.,* leukaemia, there is a risk of re-implanting malignant cells together with the grafted tissue during ovarian tissue transplantation (63). The two main goals of this technique are to safely encapsulate and transport isolated primordial and primary follicles in a biomimetic scaffold, and to support the survival and growth after the transplantation, ensuring the production of hormones and eventually fertilizable mature oocytes. As the crosstalk between follicles, surrounding cells, and the extracellular matrix (ECM) is crucial for follicle survival and health, the production of an environment that best mimics the natural ovary is essential (64). Many different scaffolds have been tested, including ECM-based decellularized ovaries, alginate and fibrin matrices, 3D printed microporous hydrogel scaffolds, and synthetic hydrogels (65-68). While many different *in vivo* animal studies showed promising results on the restoration of the endocrine function of the gonads with successful follicle development, more research is needed before it can be implemented in the clinic (65).

1.2.4.2 Ex vivo ovarian perfusion

Another newly investigated technique for patients at risk of re-implanting malignant cells during ovarian tissue transplantation is *ex vivo* ovarian perfusion to mature oocytes. In this technique, ovaries from cancer patients are explanted and perfused with gonadotropins in a bioreactor to support follicular development and oocyte maturation. After maturation, oocytes can be retrieved and cryopreserved for future fertilization. The main benefit of this novel technique is that it overcomes several limitations of IVM, including diffusion limitations and hypoxic stress. Although preclinical trials with sheep ovaries have shown promising results, this technique is still in its early stages of development (69).

1.2.4.3 Hormone treatments, apoptosis, and follicle activation pathway inhibition

There is a growing interest in using GnRH analogues (agonists and antagonists) as prophylaxis to chemotherapeutic gonadotoxicity due to their ovarian suppressive effects, making them a potential option to prevent infertility caused by such chemotherapy and/or radiotherapy. As chemotherapy

mainly affects follicles with rapid cellular turnover, GnRH analogues could protect growing follicles by inhibiting ovarian cellular turnover, potentially decreasing the chance of cellular destruction during gonadotoxic cancer treatment. While some studies show the beneficial effects of using GnRH analogues in combination with other fertility preservation techniques, future clinical studies are needed to confirm or deny their role in protecting the ovaries during treatment (70, 71).

Other future alternatives to current fertility preservation methods include kinase and PI3K/Akt/mTOR pathway inhibitors. These treatments function by preventing apoptosis or excessive PMF activation induced by alkylating chemotherapeutic agents, respectively. Vascular damage is another key factor contributing to the gonadotoxicity effect of chemotherapy, indirectly compromising the ovarian reserve. Granulocyte colony-stimulating factor (G-CSF), a glycoprotein that stimulates the bone marrow to produce granulocytes and stem cells, has been explored for its potential to protect ovarian tissue (72). Studies have identified G-CSF as a crucial factor in preserving the PMF pool, showing that the combined administration of VEGF and G-CSF increased follicle numbers in mice (73). While animal studies have been promising, further studies are necessary to decrease the limitations of these techniques. These limitations include possible interference with cancer treatments and the non-specificity of these agents to ovaries (72).

1.3 Ovarian tissue cryopreservation and transplantation

Ovarian tissue cryopreservation followed by autotransplantation (OTCTP) is a fertility preservation technique requiring the surgical removal of ovarian tissue before the onset of cancer treatment. After cutting the tissue into cortical strips containing the PMFs, cryopreservation is performed. When patients are in remission and desire to conceive, these strips can be thawed and autotransplanted. As this technique does not require ovarian stimulation and thus does not result in a delay in cancer treatment, it is a promising technique for both prepubertal girls and young women requiring urgent treatment (51, 74). One of the advantages of OTCTP is that, unlike freezing individual oocytes or embryos, ovarian tissue cryopreservation can preserve hundreds of PMFs more effectively at once (74). Additionally, it can restore both natural fertility as well as the endocrine function of the gonads (75). Before considering OTCTP, a risk assessment should be made, and only patients meeting the Edinburgh selection criteria are offered cryopreservation of ovarian tissue (**Table 4**) (76). An adequate ovarian reserve is also a critical prerequisite for successful OTCTP, as the number and quality of PMFs within the extracted ovarian tissue directly influence post-grafting function and reproductive outcomes (77).

While most of these criteria are still valid today, the results from several clinical centers showed that chemotherapeutic therapy before ovarian tissue cryopreservation does not affect pregnancy and live birth rates. Therefore, patients who have already received certain types of chemotherapy can still be recommended for OTCTP at present (78).

Table 4. ***A summary of the Edinburgh selection criteria for ovarian tissue cryopreservation.*** *From Wallace et al. 2014 (76).*

The Edinburgh selection criteria
Age younger than 35 years
No previous chemotherapy or radiotherapy if aged 15 years or older at diagnosis, but mild, non-gonadotoxic chemotherapy is acceptable if younger than 15 years
A realistic chance of surviving for 5 years
A high risk of premature ovarian insufficiency (>50%)
Informed consent (from parents and, where possible, the patient)
Negative serology results for HIV, syphilis, and hepatitis B
Not pregnant and no existing children

In 2004, Donnez *et al*. reported the first successful live birth after orthotopic ovarian tissue transplantation in a patient who suffered from POF after her treatment (79). Since then, there have been many human studies reporting successful pregnancies after OTCTP, with already 300 live births reported worldwide, and approximately 40% of the women having at least one live birth (58, 78, 80, 81). As this technique currently represents the only fertility preservation option for prepubertal girls, it is noteworthy that in 2015, the first live birth resulting from the transplantation of ovarian cortical tissue cryopreserved before menarche was reported. The patient had undergone ovarian tissue cryopreservation at the age of 14, with autotransplantation performed ten years later following the onset of POF (82, 83). One year later, in 2016, a healthy baby was born to another woman after undergoing IVF treatment using an oocyte derived from cryopreserved and transplanted tissue, whose ovarian tissue had been cryopreserved pre-pubertally at the age of 9 (84). With the increasing frequency of studies supporting the effectiveness of ovarian tissue cryopreservation, the American Society for Reproductive Medicine has recognized this method as a safe and clinically accepted fertility preservation technique (85).

1.3.1 Procedural details of ovarian tissue cryopreservation and transplantation

1.3.1.1 Extraction and preparation of ovarian tissue

The amount of ovarian tissue to be extracted varies according to the patient's age, risk of POF, and ovarian volume. Entire ovary extraction (oophorectomy) via surgery is often performed in the case of pelvic or total body irradiation, as well as in children due to the small size of the ovaries. Otherwise, several ovarian tissue fragments (10 x 0.5 x 1.5 mm) are recovered by laparoscopy (51, 86). Concerning tissue fragments, it is important to take pieces of ovarian cortex with a thickness of at least 1 mm, since superficial and thin fragments may not contain follicles (44). After extraction, the tissue is placed in a transport solution and kept on ice between 2 and 8 °C for transportation. While the transport time should be kept to a minimum, many studies have shown that ovarian tissue is mostly unaffected, with no negative influence on follicle quality during storage at 4 °C up to 24 h. However, using the right transport medium, such as Custodial®, is crucial to prolong ischaemia tolerance (87). After transportation, most of the medulla is removed from the ovarian tissue, leaving only a thin layer of the medulla on the cortex to facilitate revascularization after grafting, and cortex fragments are prepared (approximately 8 x 4 x 1 mm). Before freezing, part of the tissue is used for quality analysis of PMF

density and viability. Furthermore, histological analysis is carried out to detect and exclude malignant cells (88, 89).

1.3.1.2 Cryopreservation of ovarian cortex fragments

The main principle of cryopreservation is to reduce the temperature of cells or tissues to extreme sub-zero values in order to stop the metabolism of cells, allowing the banking of biological material over a long period of time (90). An important thing to understand is that exposing cells to temperatures below 0 °C is generally lethal. Given that water comprises approximately 80% of organ and tissue mass, the freezing of both intra- and extracellular water plays a major role in triggering harmful biochemical and structural changes, which are believed to be the primary cause of freezing injury. The harmful effects of freezing can be explained by two mechanisms: ice crystal formation disrupting cell membranes, preventing the recovery of structurally intact cells after thawing; and the formation of intracellular ice crystals during cooling, leading to lethal rises in solute concentration within the remaining liquid phase. To mitigate these effects, using cryoprotectant agents (CPAs) and selecting the optimal cooling procedure is critical (90, 91).

CPAs are chemicals added to the freezing medium, improving the survival rate by protecting cells and tissue during cryopreservation against the toxic effects of sub-zero temperatures. Based on the principle of molar freezing point depression in solute mixtures, it is proposed that during cooling, the rise in salt concentration, particularly sodium chloride, the primary component of most cell media, would be mitigated by the presence of CPAs. This would prevent salt levels from reaching a critical, damaging concentration while allowing the system to cool sufficiently. Additionally, the increasing viscosity of the medium at lower temperatures may further inhibit or slow ice crystal growth through kinetic effects (92). CPAs can be categorized into two groups: permeating and non-permeating agents. Permeating agents are relatively small, highly water-soluble at low temperatures, and can easily cross cell membranes. Because permeating agents interact strongly with water through hydrogen bonding, the freezing point of water is lowered, and fewer water molecules are available to cross-react to form critical nucleation sites for ice crystal formation inside the cells. Examples of permeating agents are glycerol, the first CPA discovered, and dimethyl sulfoxide (DMSO) (90). Non-permeating agents, as their name suggests, exert their protective effects extracellularly. They are typically large, covalently linked polymers, and they act by increasing the osmolarity of the extracellular medium, resulting in controlled cell dehydration. This decrease in intracellular water leads to a higher solute concentration inside the cell, inhibiting crystallization. Non-permeating CPAs include polyethylene glycol and sucrose (90, 93). While these CPAs are highly important for successful cryopreservation, their use is limited by their toxicity at high concentrations. The mechanism and severity of damage depend on the type of CPAs used, as well as the time of exposure and temperature. Examples of toxicity include osmotic stress and cell membrane damage. To minimize toxicity while ensuring adequate cryoprotection, a combination of permeating and non-permeating agents at reduced concentrations is commonly employed (90, 94).

Besides the use of CPAs, the selection of the optimal cooling method is important for successful cryopreservation. Cooling at a too-slow rate will lead to extracellular ice crystal formation, resulting in high electrolyte concentrations and thus dehydration of cells, causing irreversible cell damage. At rapid cooling rates, intracellular water is supercooled, eventually leading to crystallization, which is almost

universally lethal. These findings indicate the existence of an optimal intermediate cooling rate that maximizes cell viability. This rate falls within a critical range between excessively slow and overly rapid cooling, where a balance is maintained between cell dehydration and crystallization. This optimal cooling method is called slow-freezing (SF) and is represented by the classical inverted 'U' curve as shown in **Figure 6** (93). Another method for cryopreservation is vitrification. This technique prevents ice crystal formation by ultra-rapidly cooling biological samples in the presence of low concentrations of cryoprotectants. Instead of freezing, the sample transitions into a glass-like, solid state without crystallization, which helps preserve cellular structure and function (**Figure 6**) (93).

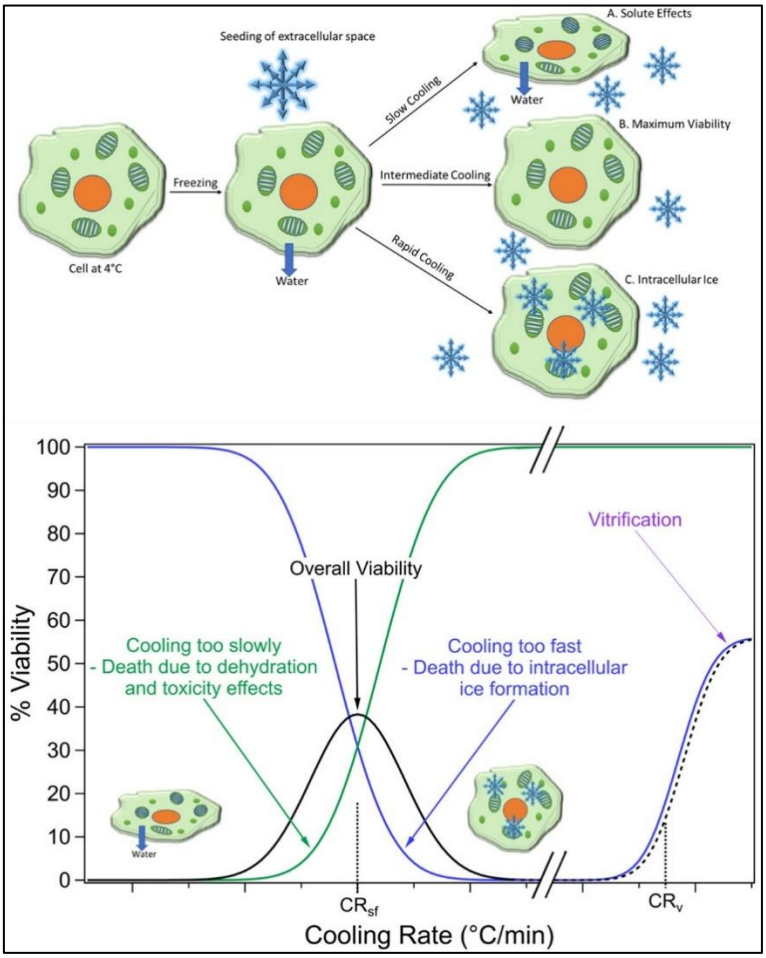

Figure 6. ***Cell survival at different cooling rates.*** *Top: Illustration of the effects of different cooling rates on cell survival during freezing; with slow cooling (A), there is shrinkage due to water loss and dehydration; solute concentration effects may cause cell death. With intermediate cooling (B), there is a balance between solute effects and intracellular ice formation, leading to maximum viability. With rapid cooling (C), there is supercooling of the intracellular space, which leads to intracellular ice formation and cell death. Bottom: Graphical representation of cell viability, highlighting damage due to cooling too slowly or too quickly, with an optimal cooling rate (CRsf) that maximises survival. When the cooling rate is higher than a critical cooling rate (CRv), cells are vitrified without freezing (or ice formation), and high cell survival ensues. From Raju et al. 2021 (93).*

In summary, cryopreservation can be achieved through either SF or vitrification, each with its advantages and limitations. Slow freezing is a straightforward technique that minimizes severe tissue damage; however, it carries a risk of ice crystal formation, requires specialized and costly laboratory equipment, and is a time-intensive process that takes several hours to complete. Vitrification, on the other hand, significantly reduces the risk of ice crystal formation, requires less handling time, and can be performed with less expensive equipment, making it a more efficient alternative in certain

applications. (95). At present, SF is the most commonly used technique for cryopreservation of ovarian tissue. Indeed, more than 95% of live births took place after transplantation of frozen/thawed ovarian fragments, while vitrification has only resulted in five live births (96-98). Therefore, we will focus further on the SF technique.

Prior to freezing, the cortical strips are placed in a freezing medium and equilibrated for 15-30 minutes at 4 °C to allow the CPAs to adequately enter the tissue. Ovarian cortical strips are then transferred to cryogenic vials containing 1 mL of freezing medium, which differs according to protocol. Cryovials are placed in a programmable freezer and subjected to a controlled cooling process at a rate of 2 °C/min. Cooling is then temporarily halted to allow manual seeding for ice nucleation at an optimal temperature, typically between -7 °C and -9 °C, depending on the cryoprotectant properties in the freezing solution. Manual seeding is performed by briefly touching each cryovial with a cryogenically cooled spatula to initiate ice formation. The cooling process then resumes at a slow rate of 0.3 °C/min until reaching -40 °C, followed by a more rapid cooling phase at rates ranging from 1 to 10 °C/min. Once freezing is complete, the cryovials are plunged into liquid nitrogen and stored at -196 °C to maintain long-term preservation (99).

1.3.1.3 Thawing procedure

The use of an optimal thawing procedure is crucial to ensure maximum viability. Similar to SF, ice crystal formation can occur during thawing. Many thawing protocols exist, but samples are often rewarmed in the air for 2 minutes before being transferred to a water bath at room temperature (RT) or 37 °C to complete thawing. As the CPAs can be toxic at RT, it is important to quickly remove them after thawing by performing three washes in successive changes of fresh medium with or without decreasing CPA concentrations (99-101).

1.3.1.4 Autotransplantation of ovarian cortex fragments

When patients in remission with cryopreserved ovarian tissue show signs of POF and have the desire to conceive, autotransplantation of ovarian cortex fragments can be performed. This can be achieved through orthotopic or heterotopic transplantation, each with distinct advantages and limitations.

First described by Donnez *et al*., orthotopic transplantation involves either the grafting of ovarian tissue to the medulla of the remaining ovary or a crafted peritoneal pocket (79, 102, 103). If at least one ovary is remaining, a large piece of ovarian cortex is removed to gain access to the medulla via laparoscopy, and frozen/thawed ovarian cortical fragments are placed and fixed onto the medulla. If both ovaries are absent, a peritoneal window can be created wherein the cortical strips can be placed and fixed. The main advantage of orthotopic sites is that natural pregnancies can take place (58). However, its limitations include the restricted space available for grafting due to ovary size, as well as the invasiveness of the procedure, which may lead to severe pelvic adhesions (104).

To address these challenges, heterotopic transplantation has been explored as an alternative. Proposed sites include the rectus muscle, breast tissue, and forearm, each offering benefits such as minimizing the need for invasive surgery**,** providing easy graft accessibility, and accommodating a greater number of cortical fragments (105). However, heterotopic sites may not provide an optimal

environment for follicular development due to differences in temperature, paracrine factors, and blood supply compared with the IP environment. Furthermore, unlike orthotopic grafting, natural pregnancies cannot occur after heterotopic transplantation, and, therefore, oocyte retrieval followed by IVF is required to achieve conception (104, 106).

Long-term graft function and success rates vary widely across patients. A review analysing 285 women undergoing OTCTP found that approximately 90% experienced a resumption of ovarian endocrine function, typically occurring 4 to 5 months post-transplantation, which aligns with the timeframe required for folliculogenesis. However, around 20% of patients required a second transplantation due to insufficient recovery of ovarian function following the initial grafting (107). The function and lifespan of grafts depend on several factors, including age at the time of cryopreservation, PMF reserve, and exposure to gonadotoxic treatment prior to tissue freezing (44). On average, ovarian function lasts between 4 to 5 years, though its duration varies greatly, ranging from a few months to over 10 years (86, 108, 109).

1.3.2 Risks and limitations of ovarian tissue cryopreservation followed by transplantation

While OTCTP is a promising method to preserve the fertility of prepubertal girls and young cancer patients with aggressive malignancies, multiple risks and limitations are associated with the technique.

When OTCTP was first introduced, a limitation was the risk of reintroducing malignant cells via autotransplantation of cryopreserved ovarian fragments. However, advances in tissue screening and selection have substantially reduced concerns regarding the reintroduction of malignant cells, and the risk of disease recurrence through transplantation of cryopreserved ovarian tissue is now regarded as very low. Nevertheless, this technique is not recommended for patients with hematologic malignancies, as there is a high risk of malignant cells being present in ovarian tissue. Since malignant leukemic cells are found in the bloodstream, the risk is highest in patients with acute leukaemia, one of the most common cancers in children (110, 111). Experimental studies have detected leukemic cells in the cryopreserved ovarian tissue of leukaemia patients and have demonstrated the potential for disease transmission through grafting in xenotransplantation models (63, 112). It is therefore essential that ovarian tissue is screened for malignant cells prior to reimplantation. Current screening methods comprise histological analysis, immunohistochemical detection of disease-specific markers, and polymerase chain reaction (PCR). Additionally, a 6-month follow-up in immunodeficient mice transplanted with thawed ovarian tissue fragments is used to assess potential disease transmission (110). However, no case of cancer recurrence after ovarian tissue transplantation has ever been reported, and the overall risk of reintroducing malignant cells is very low (95).

Although surgical complications during ovarian tissue retrieval or transplantation are rare, a comprehensive preoperative evaluation is crucial to identify women at high surgical risk. Additionally, when considering ovarian tissue removal, it is important to account for the patient's potentially weakened condition due to their underlying oncological disease (74, 113). Reports from multiple centers indicate that complication rates are low, ranging from 0.2% to 1.4%, with severe complications occurring in less than 1% of cases (114, 115).

Beyond the risks associated with surgery and cancer recurrence, the high variability in graft longevity remains a significant limitation of OTCTP. A recent review showed the lifespan of ovarian tissue transplanted to heterotopic sites varies between 9 months and 3 years, with the median graft survival of orthotopically transplanted tissue being around 5 years (116). This variability can be attributed to multiple factors, including patient age at cryopreservation, baseline PMF reserve, cryopreservation and transplantation methods, and the number of cortical fragments grafted (117). Additionally, ovarian damage during cryopreservation may further contribute to shortened graft function. Studies have shown that the freezing process negatively impacts ovarian stromal tissue (118), theca cell formation (119), and the function of granulosa cells (101, 120). Furthermore, freezing and thawing can lead to follicular apoptosis (121).

The primary factor limiting graft lifespan is the substantial follicular loss during both cryopreservation and transplantation, with an estimated 80% of follicles lost throughout the OTCTP process (122, 123). The vast majority of these follicles are lost in the final stage of the procedure after grafting (123). To assess follicle loss or survival in grafts, it is essential to distinguish between two distinct follicle populations: dormant PMFs, which represent the ovarian reserve and fertility potential, and developing follicles, which are actively growing. Antral follicles, the largest growing follicles, are typically lost due to mechanical injury during tissue preparation. Smaller developing follicles are highly susceptible to freezing and thawing and are often lost during the cryopreservation process (123, 124). In contrast, PMFs are more resistant to cryopreservation-related damage compared to growing follicles (118, 123, 125).

The rapid depletion of follicles directly after grafting is driven by three key mechanisms:

1. Ischemic injury in the first few days post-grafting, as ovarian cortical pieces are transplanted without vascular anastomosis, leading to oxygen deprivation and oxidative stress (126, 127).
2. Apoptosis of follicles shortly after transplantation further reducing the available follicle pool (95, 128, 129).
3. Excessive recruitment of PMFs, also known as “follicle burn-out,” leading to accelerated depletion of the ovarian reserve (123, 130, 131).

1.3.3 Initiators of massive follicle loss directly after grafting

In this section of the introduction, the three aforementioned key mechanisms of follicle loss after grafting will be explained in further detail, together with possible solutions to preserve the follicle pool. As our main focus was on preventing the excessive recruitment of PMFs, this part will be the most extensive.

1.3.3.1 Slow neovascularization resulting in ischemic injury

Ovarian cortical fragments are grafted without vascular anastomosis, and several studies have shown transient hypoxia during the first five days post-ovarian tissue grafting, with oxygenation and neovascularization being improved over the next five days. During this initial ischemic phase, follicles and the ovarian microenvironment are exposed to hypoxic conditions until revascularization takes place. These hypoxic conditions can lead to necrosis and apoptotic pathway activation, resulting in the

massive decrease of the follicle pool (132-134). Additionally, granulosa cell proliferation can be induced through hypoxia-inducible factor-1 (HIF-1) pathways, further contributing to the premature depletion of the follicle pool (132). An additional issue is that hypoxia triggers a rapid metabolic shift. During this early ischemic phase, oxidative phosphorylation is inhibited, adenosine triphosphate (ATP) production falls rapidly, and cells revert to anaerobic glycolysis, resulting in depleted ATP levels and increased lactate accumulation. The hypoxic tissue is not able to meet the energy demands required to keep the tissue healthy, although PMFs are more resistant and can remain intact, even surrounded by damaged stromal cells (135). During revascularization, the tissue may switch back toward aerobic metabolism, but the prior energy deficit may have already induced mitochondrial damage, membrane potential loss, and activation of cell death or follicle activation pathways, with studies showing a high glucose/lactate ratio until 10 days post grafting (136). This energetic stress likely contributes to the "burn-out" of PMFs. ATP depletion, causing autophagy to recycle cellular components and maintain the energy supply, can lead to follicular atresia. Therefore, limiting hypoxia, oxidative stress, and improving neovascularization post-grafting is an important aspect of protecting the follicle pool, with many different protective methods having been tested before.

Several studies focused on the use of antioxidants, with melatonin being an extensively researched compound to address oxidative stress-induced damage. Multiple animal models showed that melatonin improved graft survival and function, possibly through decreasing inflammatory factors (137-139). Furthermore, N-acetylcysteine (NAC) administration was associated with reduced ischemic injury through increased expression of antioxidant defence biomarkers and a decrease in inflammation markers (140). Indeed, NAC can directly scavenge reactive oxygen species (ROS) and indirectly increase levels of other antioxidant components, such as glutathione (141). Reduced ischemic injury with increased follicle survival has also been reported in animals treated with erythropoietin (EPO) (139, 142). While these studies show promising results, more research on human tissue should be performed before it can be implemented in the clinic.

Besides antioxidants, the use of mesenchymal stem cells (MSCs) prior to transplantation has been evaluated. Indeed, hypoxia-preconditioned human umbilical cord MSCs decreased the ischaemic period and boosted vascularization, with a significantly higher resting follicle density (143). Additionally, ovarian tissue transplantation with adipose tissue-derived stem cells (ACSs) enhanced neovascularization with improved follicle survival through the shortening of the hypoxic period, resulting in similar PMF densities between grafted and control ovaries. Indeed, ACSs increased levels of VEGF and maintained adequate vessel differentiation (144).

The main angiogenic compound researched to improve neovascularization post-grafting is VEGF, especially VEGF-A (further referred to as VEGF), a potent regulator of angiogenesis. Besides stimulating the survival, migration, and proliferation of vascular endothelial cells, VEGF is involved in folliculogenesis via the presence of VEGF receptors in granulosa cells (145, 146). Indeed, several studies have revealed that VEGF is essential in all follicle development steps, mainly from secondary follicles to corpus luteum (145, 147). Since neovascularization typically occurs around five days after ovarian tissue grafting in humans, enhancing angiogenesis-related signaling pathways through VEGF appears to be a promising strategy for mitigating ischemia-induced ovarian damage. It is important to recognize

that many angiogenic factors, including VEGF, have a relatively short half-life ranging from 30 minutes to around 18 hours, meaning that direct application is unlikely to produce long-term benefits (145, 148, 149). To address this limitation, various techniques have been developed that involve transplanting ovarian tissue within hydrogel or collagen matrices embedded with angiogenic compounds. These matrices enable a controlled release of the factors, thereby enhancing their long-term efficacy (145, 146, 150, 151). Our team previously investigated the role of VEGF in improving angiogenesis using collagen matrices. In one of these studies, sheep ovarian tissue was encapsulated with $VEGF_{111}$ in a collagen matrix before xenotransplantation into severe combined immunodeficiency (SCID) mice, leading to improved revascularization and reduced loss of primary follicles three weeks after grafting (151). A follow-up study with $VEGF_{165}$ demonstrated its ability to enhance vascularization by significantly increasing the number of functional vessels as early as three days post-grafting. However, no significant difference in the PMF pool was observed (146). Conversely, *Skaznik-Wikiel et al.* previously reported that injection of VEGF in combination with G-CSF after orthotopic ovarian transplantation significantly improved the preservation of the PMF pool compared to saline controls in mice (73). These findings underscore the importance of improving revascularization but also highlight the need for additional strategies to optimize ovarian tissue viability due to the variable results on PMF preservation obtained when using VEGF.

More recently, medical-grade honey (MGH) has been investigated for its potential to enhance angiogenesis following ovarian tissue grafting. Studies have shown that MGH can stimulate angiogenic responses comparable to those of VEGF, including increased endothelial cell density in transplanted tissues. In one study, MGH was applied to frozen-thawed bovine ovarian tissue, which was then xenografted into immunodeficient mice. The MGH-treated grafts demonstrated a significantly higher number of PMFs and greater endothelial cell density compared to control tissues at both 7- and 28-days post-transplantation (152, 153).

1.3.3.2 Follicle apoptosis

The second major factor contributing to the rapid depletion of follicles is follicular apoptosis during the OTCTP process. As mentioned before, the cryopreservation procedure itself can trigger apoptosis, with multiple studies reporting an increase in apoptotic cells after thawing. Specifically, freezing and thawing have been shown to increase TUNEL-positive nuclei and elevate levels of the apoptotic marker activated caspase 3 (128, 154). As described in the previous section, ischaemia post-grafting, resulting from slow neovascularization, can further contribute to follicular apoptosis, with a notable rise in apoptotic follicles shortly after transplantation (128, 129). Given these challenges, strategies to limit apoptosis post-grafting, alongside improvements in angiogenesis, are essential for preserving the follicle pool. The main anti-apoptotic agents studied are sphingosine-1-phosphate (S1P) and Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK).

S1P functions as the natural inhibitor of ceramide, a second messenger in activating the apoptotic cascade (155, 156). Studies have demonstrated that cryopreservation of ovarian cortical fragments with S1P enhances PMF survival and mitigates follicular atresia during freezing (157). Additionally, a xenograft model revealed that S1P significantly reduced the proportion of apoptotic follicles (158). However, conflicting results have been found. Indeed, one study reported that S1P-treated

cryopreserved tissue exhibited lower follicle density and higher levels of double-strand DNA damage compared to controls, highlighting the need for further investigation into its safety and efficacy (128, 159).

Another promising anti-apoptotic agent is Z-VAD-FMK, a caspase inhibitor acting through the irreversible binding to the catalytic site of caspases, thereby preventing apoptosis (160). Several studies have shown that Z-VAD-FMK treatment improves follicle preservation during the cryopreservation process, as well as preserving follicle densities, reducing double-strand DNA breaks, and promoting angiogenesis in murine xenotransplantation models (128, 134, 161). While these studies are promising, the safety of Z-VAD-FMK and its potential effects on oocytes and future offspring should be considered before clinical application.

1.3.3.3 Excessive follicle recruitment - The problem

The third main factor contributing to the rapid depletion of the follicle pool is the massive recruitment of PMFs following the transplantation of ovarian cortical fragments. Numerous animal studies found that the loss of PMFs is accompanied by an increase in growing follicles exhibiting elevated proliferation post-grafting, indicating an increase in follicle activation (130, 131, 162). Similarly, clinical studies have demonstrated excessive follicle recruitment after ovarian cortex transplantation. Indeed, AMH levels initially rise to high levels approximately four months after grafting, only to decline to very low levels another 4 four months later. This pattern indicates that the over-recruitment of PMFs leads to accelerated depletion of the follicle pool (163).

Under normal physiological conditions, a tightly regulated equilibrium between activation and inhibitory signals preserves the long-term reproductive capacity of the ovary by keeping the majority of PMFs in a quiescent state. However, during the OTCTP process, this balance is disrupted. Indeed, there is an upregulation of follicle activation pathways, such as the PI3K/PTEN/Akt and mTOR pathways, and a reduction in inhibitory signals like AMH, which is normally secreted by growing follicles. Consequently, this imbalance accelerates follicle recruitment, ultimately depleting the follicle reserve and reducing graft longevity (**Figure 7**). It is important to note that most mechanistic insights into AMH's inhibitory role in PMF activation originate from animal models, as *in vivo* human data is limited due to ethical and practical constraints. (131, 164).

Several possible explanations for this disruption have been proposed. One possibility is that dormant follicles are less susceptible to the damaging effects of cryopreservation and ischaemia compared to the metabolically active growing follicles (130). Indeed, the majority of growing follicles do not survive the cryopreservation process (164). Moreover, the separation of the ovarian cortex from the medulla during the preparation of extracted ovarian tissue results in the removal of most growing follicles (87). Since these growing follicles play a crucial role in maintaining PMFs in a dormant state through the production and secretion of AMH, their removal further shifts the balance toward excessive follicle activation following grafting (130). In addition, ovarian fragmentation during tissue preparation may trigger follicle activation through the Hippo pathway, as studies in murine models have shown that fragmented ovarian tissue contains fewer PMFs and a higher density of mature follicles compared to intact ovaries (38, 159).

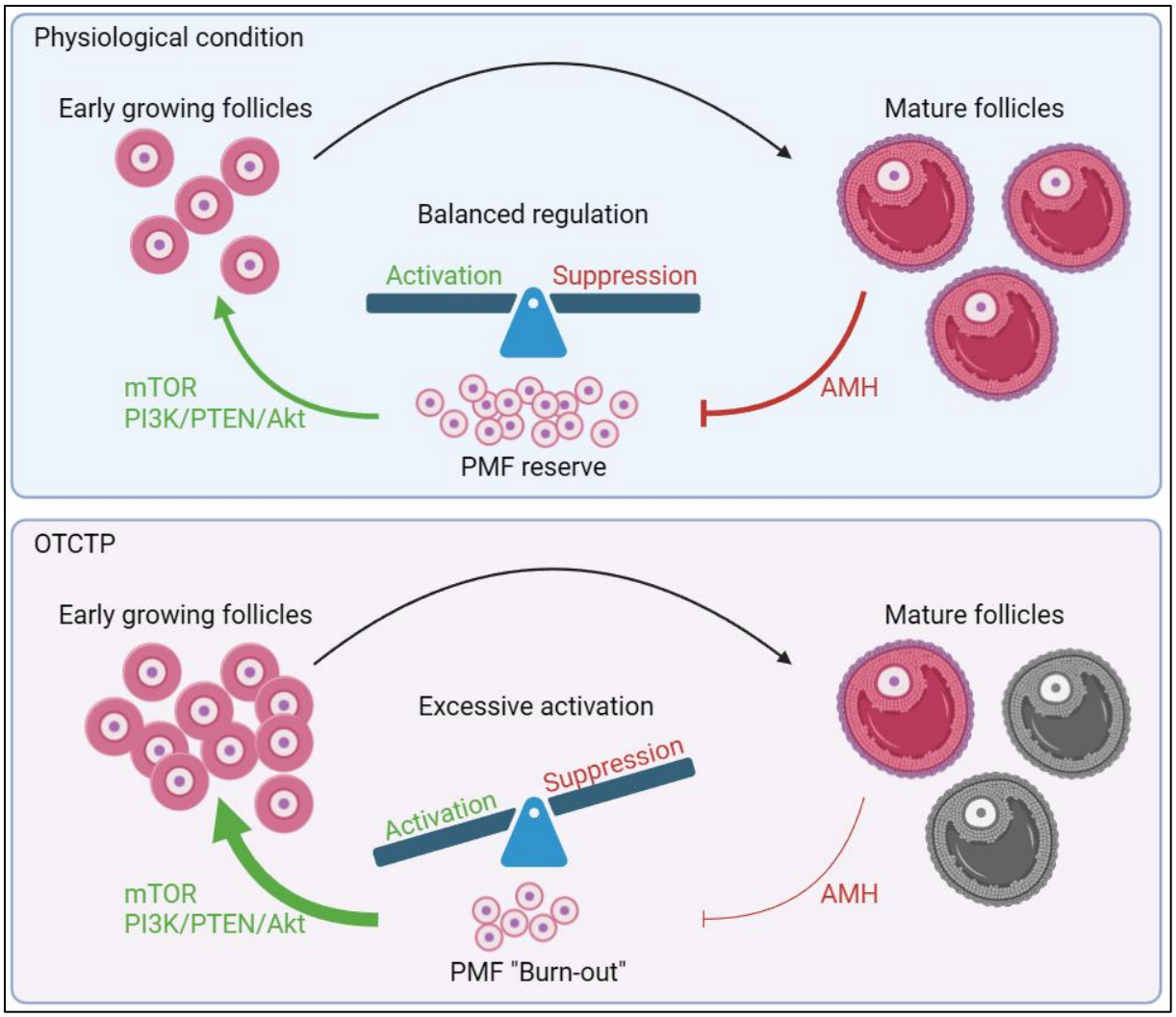

Figure 7. ***Follicle activation and suppression under normal physiological conditions and during OTCTP.*** *Top: Under normal physiological conditions, a tightly regulated equilibrium between activation signals (PI3K/PTEN/Akt and mTOR) and inhibitory signals (anti-Müllerian hormone; AMH) preserves the long-term reproductive capacity of the ovary by keeping the majority of primordial follicles (PMFs) in a dormant state. Bottom: During ovarian tissue cryopreservation and transplantation (OTCTP), this balance is disrupted. Many growing and mature follicles are removed during the tissue preparation process, or do not survive the cryopreservation and thawing. Therefore, there is a reduction of inhibitory signals like AMH, which is normally secreted by growing follicles. Consequently, this imbalance accelerates follicle recruitment, causing follicle "burn-out", ultimately depleting the follicle reserve and reducing graft longevity. Created with BioRender.com.*

Many studies have investigated whether the majority of this activation occurs during cryopreservation or after transplantation. Xenograft models have shown a substantial decline in PMF density following grafting, accompanied by a significant increase in the number of growing follicles compared to frozen/thawed non-grafted controls. Additionally, follicle proliferation was markedly elevated post-transplantation (130, 165). Notably, one study found a significant increase in Akt activation in PMFs within the first few days after xenotransplantation, further supporting that grafting induces follicle activation (166). Other xenograft studies reported similar follicle development in both fresh and cryopreserved ovarian tissue after transplantation, with a significantly higher proportion of growing follicles and a lower proportion of PMFs compared to non-grafted controls (119, 131). These results suggest that the massive follicle activation occurs in the first few days after grafting, rather than as a direct consequence of cryopreservation. However, our team previously demonstrated that the

freezing process itself induced follicle activation via the PI3K/PTEN/Akt and mTOR pathways, indicating follicle activation occurs both during cryopreservation and transplantation (167).

1.3.3.4 Excessive follicle recruitment - Use of pharmacological inhibitors, agonists, and AMH

Since the massive recruitment of follicles after grafting depletes the PMF pool and shortens graft longevity, many studies have explored ways to limit follicle activation using pharmacological inhibitors, agonists, and the natural inhibitor AMH.

One recent study investigated the effects of p300 inhibitors or agonists on follicle activation. p300 is a histone acetylase, acting as a key transcriptional regulator of cell growth and development. The study found that culturing 3-day-old mouse ovaries with a p300 agonist led to reduced follicle activation after four days of culture. However, p300 also suppressed VEGF transcription, which could potentially impair revascularization after ovarian transplantation and exacerbate ischemic damage (168).

Another approach involved U0126, a MAPK kinase (MEK) inhibitor, which was tested during mouse ovarian culture. After five days, treated ovaries exhibited a higher PMF density with fewer developing follicles (169). These findings align with those of Zhao *et al*., who added U0126 to neonatal mouse ovarian cultures and observed reduced follicle activation (170).

Other strategies involved inhibition of the JNK pathway. The culture of ovine cortical fragments with inhibitors of c-Jun phosphorylation inhibited PMF activation, possibly through the suppression of FOXO3 translocation from the nucleus (171).

Given the involvement of the PI3K/PTEN/Akt and mTOR pathways in PMF activation, various inhibitors targeting these pathways have also been studied (**Figure 8**). When 3-day-old mouse ovaries were cultured with the Akt inhibitor MK2206, researchers observed a higher PMF density and a decreased number of primary follicles after four days. Furthermore, MK2206 inhibited follicle activation after 8 days of culture (172). In addition to its effects on the PI3K/PTEN/Akt pathway, MK2206 was found to enhance Hippo pathway activity, which may further contribute to the preservation of the PMF pool (172, 173).

LY294002, a reversible inhibitor of PI3K, has been studied in various models for its ability to suppress follicle activation. When added to the culture of foetal (E17.5) murine ovaries, LY294002 reduced follicle development after five days of culture (169). These findings align with a study by Bezerra *et al*., who cultured ovine ovarian cortex fragments for 7 days with LY294002 (174). Additionally, LY294002 treatment led to decreased phosphorylated Akt staining in oocytes. However, an increased proportion of apoptotic follicles was also reported, suggesting potential cytotoxicity associated with this pharmacological agent (175).

Our team previously investigated using LY294002 during cryopreservation and culture of ovaries, together with rapamycin, a naturally occurring specific mTORC1 inhibitor. LY294002 effectively suppressed PI3K/PTEN/Akt signaling in cultured mouse ovaries, while rapamycin inhibited the mTOR pathway during cryopreservation. When ovaries were cryopreserved with rapamycin and subsequently cultured for 24 hours with LY294002, dual inhibition of both pathways was achieved. Furthermore, a higher PMF density was observed in ovaries cultured with rapamycin compared to an

ovary cultured without an inhibitor, indicating rapamycin's potential to preserve the PMF pool *in vitro* and potentially *in vivo* (167).

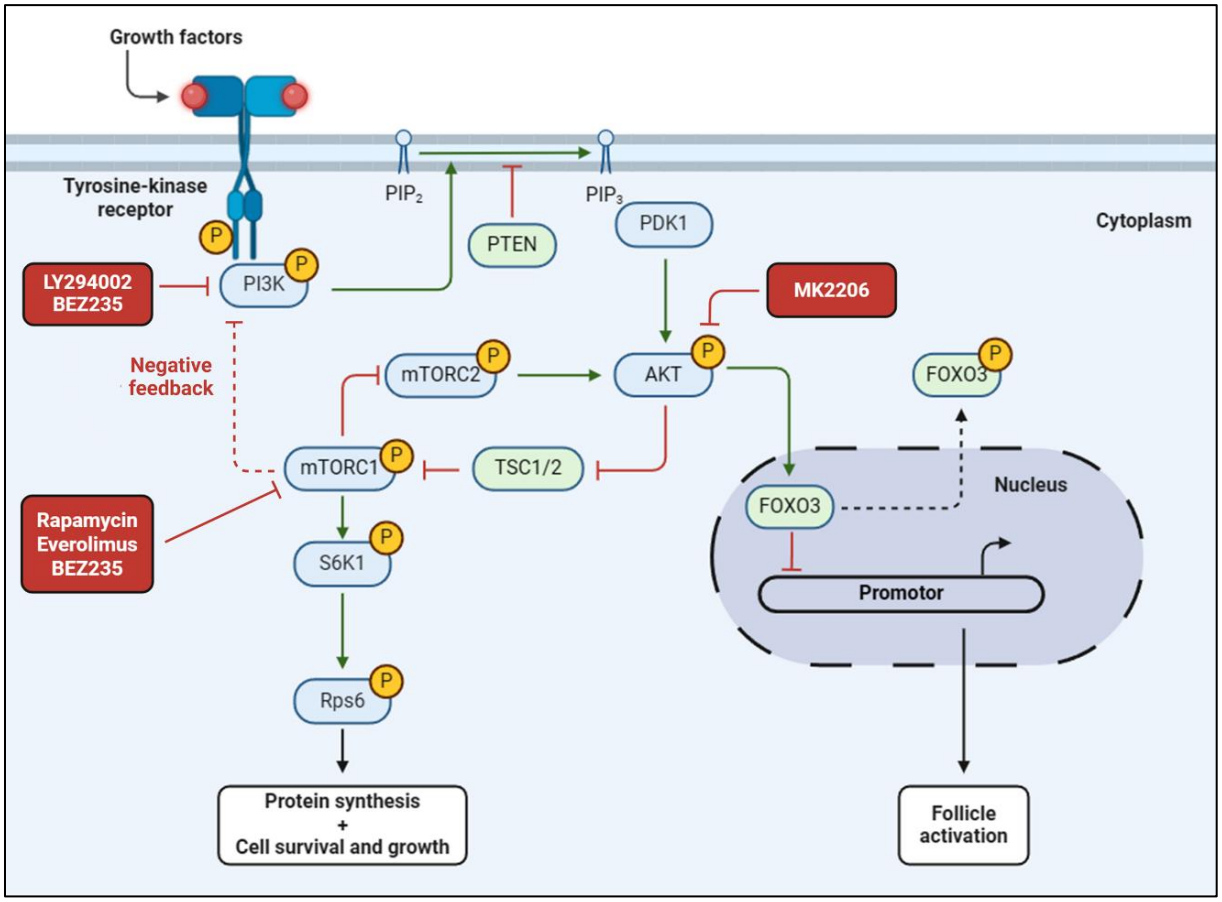

Figure 8. ***Pharmacological inhibitors of the PI3K/PTEN/Akt and mTOR pathways****. Created with BioRender.com.*

In addition to our work, numerous other studies have examined the potential protective effects of rapamycin to keep follicles in a dormant state in multiple experimental settings (176-180). *In vitro*, rapamycin reduced the ratio of growing to PMFs in rat ovaries cultured for three days (176). *In vivo*, vitrified murine ovaries pre-treated with rapamycin and heterotopically transplanted showed reduced mTOR pathway activation, although no significant difference in PMF density was observed two weeks post-transplantation (179). Conversely, IP injection of rapamycin in rats post-grafting of frozen/thawed ovaries protected the PMF pool through decreased activation of the mTOR pathway (180). The results are in line with another study in which rats were injected with rapamycin, preserving the follicle pool (177).

These promising results support the potential of rapamycin to preserve the PMF pool after ovarian grafting and potentially increase graft longevity. As rapamycin is already used in several clinical settings, including as an immunosuppressant and to prevent acute organ rejection, its application during OTCTP in humans appears feasible (181). However, as the ultimate goal is to improve the restoration of fertility, more studies are needed to evaluate the long-term effects of rapamycin and whether it can enhance fertility restoration in mice through orthotopic transplantation.

Everolimus, a more water-soluble analogue of rapamycin, also reduced follicle activation by inhibiting mTOR in murine ovaries cultured for two days (182). In another study, everolimus injection

in rats led to decreased activation of Akt and downstream mTOR targets after eight weeks. This finding is particularly interesting, as it suggests that everolimus, primarily an mTORC1 inhibitor, may also impact mTORC2, which regulates Akt signaling. However, despite these effects, everolimus did not improve PMF preservation in that specific experimental context (183).

While PI3K and mTOR inhibitors have shown promising results in maintaining PMF in a dormant state across multiple experimental models, targeting both pathways simultaneously may offer enhanced protection. Dual inhibition can help prevent compensatory feedback loops that may arise when only one pathway is blocked. BEZ235, a dual PI3K/mTOR inhibitor, could be a candidate for preserving the follicle pool during OTCTP. Although its effect on follicle activation has not yet been directly studied, BEZ235 has been shown to suppress growth and proliferation in ovarian cancer cell lines by effectively inhibiting the PI3K/PTEN/Akt and mTOR signaling pathways (184).

In addition to pharmacological inhibitors, the natural inhibitor AMH has been evaluated in several experimental models, though its role in follicle preservation remains inconclusive (185-187). A key advantage of AMH is its ovary-specific mechanism of action, which may reduce the risk of systemic side effects often associated with pharmacological agents (188, 189). In one study, AMH injection before and/or after OTCTP in mice did not significantly impact the PMF pool compared to controls (185). Conversely, co-transplantation of ovarian tissue with endothelial cells constitutively secreting AMH led to a higher PMF density compared to the control group (187). These mixed results have raised questions about the consistency of AMH's protective effects. However, more recent studies are more promising (188, 189). One study reported that mice injected with AMH following ovarian transplantation retained a larger PMF pool than controls (188). In another study, human ovarian cortical biopsies were cultured with the active cyclophosphamide metabolite 4-hydroperoxycyclophosphamide (4-HC) alongside either rapamycin or AMH. While rapamycin only mitigated follicle activation, AMH significantly reduced both follicle damage and activation induced by 4-HC exposure (189). Taken together, these findings suggest that AMH holds promise as a targeted, ovary-specific strategy for preserving the PMF pool during OTCTP. Nevertheless, further studies are needed to better define its long-term efficacy and potential clinical applications in fertility preservation.

While these findings highlight the promise of several agents in limiting follicle hyperactivation post-grafting, a major concern is their possible toxicity to oocytes, which could compromise the health of future offspring. To address this, the offspring of female animals undergoing OTCTP with therapeutic interventions should be carefully examined for any adverse health effects.

1.4 The use of animal models

Using animal models offers several advantages over human tissue in OTCTP research. Animal models provide a controlled environment for studying the biological mechanisms underlying follicle development, ischemic injury, and therapeutic interventions. Their shorter reproductive cycles and lifespan allow for an easier evaluation of long-term outcomes, such as fertility restoration and offspring health, in a relatively short timeframe. Ethical and legal limitations make access to human ovarian tissue challenging, particularly from healthy donors, whereas animal tissue is more readily available and less restricted. Additionally, genetic homogeneity in animal models reduces variability, improving

reproducibility and statistical power. Several animal species have been used for OTCTP studies, including sheep, monkeys, and rodents.

Sheep are frequently used due to the structural and functional similarities of their ovaries to those of humans. The sheep ovary has a dense fibrous stroma and a high density of PMFs within the cortex, closely resembling the human ovary. Furthermore, ovine ovaries are approximately 80% the size of premenopausal human ovaries. Successful pregnancies and restoration of hormone production have been reported following transplantation of cryopreserved ovarian cortex fragments in sheep models (190-192).

Baboons, large non-human primates, have also been used in reproductive research due to their similarities to humans in pre-antral follicle morphology, menstrual cycles, and hormonal patterns (193, 194). However, due to logical ethical reasons, the use of non-human primates has declined over time (195).

Rodents, particularly mice, are the most commonly used models in OTCTP research. Mice serve as an ideal preclinical model due to their genetic and physiological similarities to humans: 99% of human genes are represented in mice, and 93% of mouse genomic regions correspond to those in the human genome. Moreover, mouse ovarian function and follicle activation closely parallel those of humans (as discussed in the following section). Various strains are utilized, including C57BL/6, Institute of Cancer Research (ICR), Naval Medical Research Institute (NMRI), and SCID mice. C57BL/6 mice, the first inbred strain to have a fully sequenced genome, offer high genetic homogeneity, enhancing reproducibility across studies. In contrast, ICR and NMRI mice are outbred populations that retain greater genetic variability, making them more reflective of human population diversity. SCID mice, which lack functional immune responses, are particularly valuable in xenotransplantation studies involving human ovarian tissue, as they minimize graft rejection. Xenotransplantation offers a direct representation of the physiological and pathological changes that occur in human ovarian tissue following transplantation, making it a valuable model for informing clinical practice. Key advantages of this approach include: The ability to directly assess how human ovarian tissue responds to transplantation and analyze follicle development and activation, as well as evaluate potential therapeutic interventions (196).

1.4.1 The murine reproductive system and estrus cycle

One of the major anatomical differences between the murine and human reproductive systems lies in the structure of the uterus. Mice possess a bicornuate uterus, consisting of two lateral uterine horns and a single uterine body, while humans have a simplex uterus with a single, unified cavity. In mice, the oviducts are slender, tightly coiled tubes located at the distal end of each horn, connecting to the ovaries. Each mouse ovary is enclosed within a membranous bursa, a structure absent in humans. Morphologically, the mouse ovary is spherical, whereas the human ovary is ovoid (**Figure 9**) (197). Furthermore, there are some anatomical differences in the ovary itself between human and mouse. In the human ovary, PMFs are strictly located in the outer ovarian cortex. This cortex is collagen-rich, having a well-defined boundary with the inner medulla, which typically does not contain PMFs. In mice, this boundary between cortex and medulla is less pronounced. PMFs are therefore not strictly confined

to the cortex, extending closer toward the medullary region. In addition, the human cortex is dense with higher ECM stiffness due to an abundance of collagen. However, the murine ovary is less collagen-rich, resulting in softer tissue (198, 199). The comparison of the reproductive tract is summarized in **Table 5**.

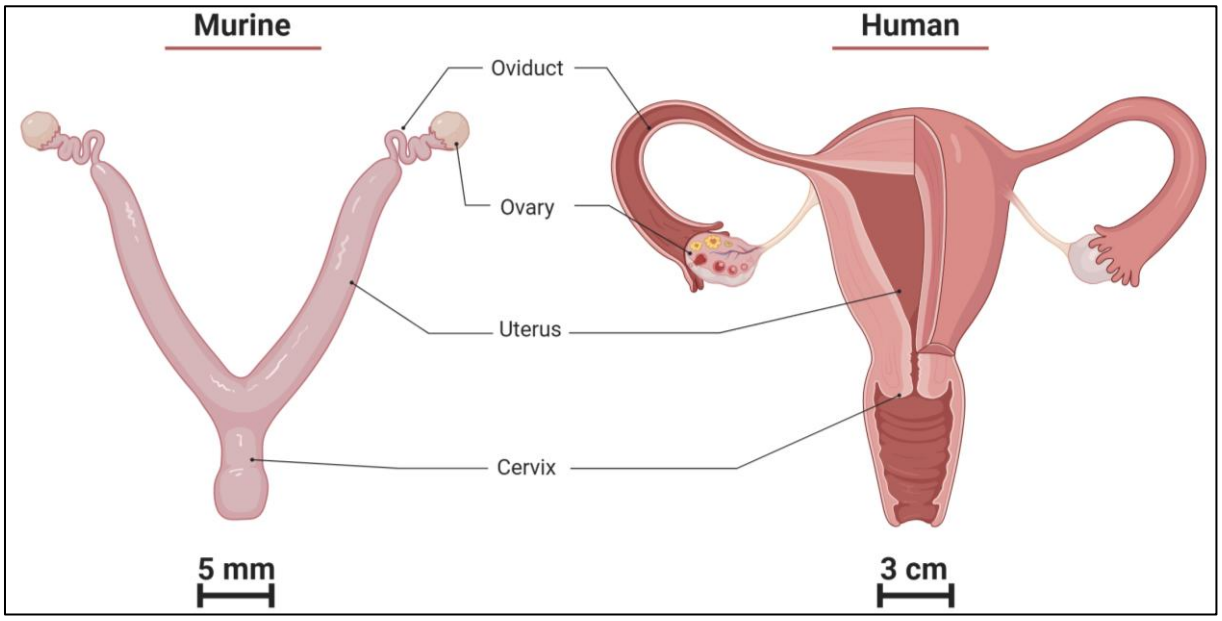

Figure 9. ***Comparison of the murine and human female reproductive system****. Created with BioRender.com.*

Table 5. ***Comparison of murine and human reproductive tract*** *(197-199). PMF = Primordial follicle.*

	Mouse	**Human**
Uterus type	Bicornuate (two lateral horns and one uterine body)	Simplex (single uterine cavity)
Oviduct structure	Thin, tightly coiled tubes at the end of each uterine horn	Less coiled fallopian tubes
Ovary position	Encapsulated by a membranous bursa	No bursa; the ovaries are free in the pelvic cavity
Ovary shape	Spherical	Ovoid
Ovarian density	Low	High
Cortical stiffness	Soft, less fibrous	Dense, collagen-rich
PMF localization	Primarily the cortex, but extends closer to the medullary region	Strictly in the cortex

In addition to structural differences, mice and humans also differ in key aspects of folliculogenesis and reproductive cycles, as summarized in **Table 6**. At birth, C57BL/6 mouse ovaries contain approximately 8,000 healthy PMFs (200), whereas the human ovarian reserve ranges between 1 and 2 million PMFs (21). Mice reach sexual maturity around 35 days of age (201), while in humans, menarche typically occurs between the ages of 10 and 16 years (22). Follicular growth in mice takes approximately three weeks (202), in contrast to the six-month maturation period observed in humans

(23). Mice undergo estrous cycles lasting 4 to 6 days, whereas humans experience menstrual cycles averaging 28 days. Although both species exhibit the simultaneous maturation of multiple follicles, humans typically ovulate a single dominant follicle per cycle, while mice release multiple oocytes. The gestation period in mice ranges from 18 to 21 days, compared to approximately 39-40 weeks in humans (197). C57BL/6 mice begin to show signs of reproductive decline between 6 and 8 months of age (203), whereas the average reproductive lifespan in women born after 1970 is approximately 38 years (204).

Table 6. ***Overview of reproductive parameters in mice and humans.***

	Mice	**Humans**
PMF reserve at birth	~8000	1-2 million
Follicular growth duration	~3 weeks	~6 months
Cycle type	Estrus cycle	Menstrual cycle
Cycle length	4-6 days	28 days
Follicle maturation per cycle	Multiple follicles; several oocytes released	Multiple follicles mature; typically, one ovulates
Gestation length	18-21 days	39-40 weeks
Reproductive lifespan	6-8 months	~38 years

The reproductive cycle in mice is referred to as the estrus cycle and contains four phases, namely proestrus, estrus, metestrus, and diestrus. The proestrus phase is characterized by increased estrogen, follicular growth, and uterine preparation for potential fertilization. It precedes the estrus phase, during which ovulation takes place and the mice are susceptible to males. Metestrus is a brief transition in the cycle, marked by an increase in progesterone and a transition to luteal dominance. Diestrus is a resting phase, characterized by high progesterone and low estrogen, when females are no longer susceptible to males (**Figure 10**) (205, 206). The cytological characteristics of the four phases of the estrus cycle can be distinguished through vaginal smear analysis. In the proestrus phase, smears are dominated by nucleated epithelial cells, which appear round with large nuclei. During the estrus phase, the smear shows mostly cornified epithelial cells, which are irregular and anucleate. The metestrus phase is characterized by a mixed population of cells, including remaining cornified epithelial cells, some nucleated epithelial cells, and an increasing number of leukocytes. Finally, the diestrus phase is dominated by leukocytes, with few or no epithelial cells (**Figure 10**) (207).

1.4.2 The different murine transplantation models

As previously described, mice serve as an ideal preclinical model for investigating follicle activation and pharmaceutical interventions. Two primary transplantation approaches are used: orthotopic and heterotopic transplantation. Orthotopic transplantation refers to the grafting of tissue in its natural anatomical location, which, in the case of murine ovarian tissue, is the ovarian bursa. This site is

particularly advantageous due to its rich vascularization and a microenvironment that supports follicular growth and development. Moreover, it is the only site that enables natural conception following grafting, making it the preferred model for evaluating fertility restoration. However, orthotopic transplantation also has limitations, including the need for invasive surgical procedures and the lack of fixation of the grafts, which may impact graft stability (208, 209).

In contrast, several heterotopic transplantation sites have been evaluated (196, 210). The establishment of a new vascular network, or neovascularization, from the surrounding tissue is essential for long-term graft survival. Therefore, factors such as local blood supply, available space for growth, and technical accessibility of the transplantation site significantly influence graft outcomes. Common heterotopic transplantation sites include subcutaneous tissue (208, 211), under the kidney capsule (143, 212), the back muscle (213), and IP (132, 214).

The kidney capsule provides a highly vascularized environment and is rich in angiogenic factors, making it an excellent transplantation site. However, this site requires invasive surgery, and the limited space may impair the growth of larger follicles (196). Subcutaneous transplantation is less invasive, easier to perform, and allows easy external monitoring. Nonetheless, this site is characterized by low vascular density, and grafts are more susceptible to external factors such as temperature fluctuations, pressure, and mouse activity (196, 215). Transplantation into the dorsal muscle has been associated with high graft survival and reduced follicular apoptosis. However, in the early post-transplantation period, before the graft integrates with surrounding tissues, there is a risk of graft displacement due to mouse activity, leading to potential model failure (196). Lastly, IP transplantation has demonstrated a supportive environment for follicular development and offers strong translational relevance, as it mimics the orthotopic graft site used in clinical OTCTP procedures (215).

Concisely, transplantation site selection in mouse models has to be determined by the specific objectives of the study. Although orthotopic transplantation is crucial for the evaluation of fertility restoration through natural conception, heterotopic sites are suitable for examining graft survival, vascularization, and follicle growth. All sites have specific benefits and limitations in terms of invasiveness, vascular support, monitoring feasibility, and translational applicability. Therefore, the selection of the most appropriate model depends on whether the focus is on mechanistic insights, therapeutic intervention testing, or functional fertility outcomes.

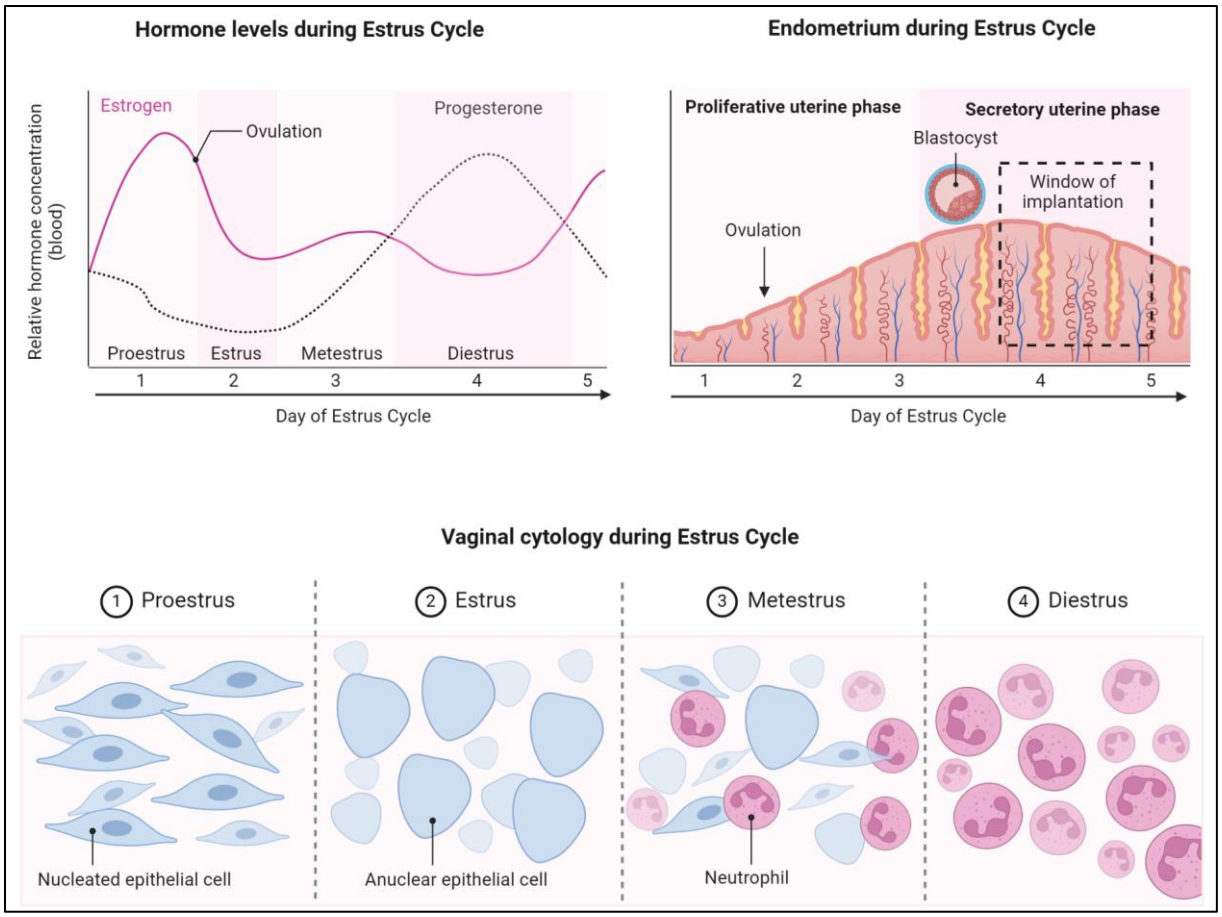

Figure 10. ***The estrus cycle in mice.*** *The estrus cycle in mice consists of four stages: proestrus, estrus, metestrus, and diestrus. Throughout this cycle, the levels of the sex hormones estrogen and progesterone fluctuate, and the vaginal cytology changes, showing a mix of nucleated endothelial cells, folded endothelial cells, and neutrophils. Ovulation triggers the proliferation of the endometrium, and the implantation window occurs when progesterone levels and neutrophil counts are elevated. Created with BioRender.com.*

Chapter 2

Objectives and outlines of the thesis

2.1 Objectives

For prepubertal patients and women needing immediate treatment for aggressive malignancies, ovarian tissue cryopreservation followed by autotransplantation is currently the only fertility preservation method. While this attractive approach has already led to over 300 live births, it still requires improvements due to several limitations. Amongst these limitations is massive follicular loss directly after grafting of cryopreserved tissue, partly due to increased activation of PMFs, which could potentially reduce the longevity of the graft. This thesis aimed to explore strategies for improving the OTCTP procedure, focusing on reducing PMF loss and enhancing fertility restoration. Our five main objectives were:

1. To develop a minimally invasive heterotopic ovarian transplantation model between the skin and cartilage of the ear, allowing for localized post-grafting pharmacological intervention and enabling dynamic graft monitoring.
2. To validate the new ear transplantation model by comparing it to the conventional but invasive kidney capsule model, particularly in the context of pharmacological modulation using the PI3K inhibitor LY294002.
3. To investigate whether adding the mTOR inhibitor rapamycin to the cryopreservation medium could maintain follicular dormancy and reduce PMF activation *in vivo*.
4. To evaluate whether adding rapamycin to the slow-freezing medium can enhance fertility restoration in mice through orthotopic autotransplantation after chemically-induced ovarian failure.
5. To determine if dual inhibition of PI3K and mTOR with BEZ235 offers superior protection of the PMF pool compared to single inhibitors or the natural inhibitor AMH, and to assess whether combining BEZ235 with angiogenic support further improves graft survival.

2.2 Experimental approach

To experimentally address these objectives, we designed a series of *in vitro* and *in vivo* studies, each contributing to the refinement of OTCTP protocols. The results of these studies are summarized below and are detailed in four articles.

Objective 1 was addressed in **Article 1**: *Evaluation of an alternative heterotopic transplantation model for ovarian tissue to test pharmaceutical improvements for fertility restoration*.

To support localized post-grafting treatments, enable graft monitoring, and reduce surgical invasiveness, we developed a heterotopic ovarian transplantation model between the skin and cartilage of the ear. Cryopreserved murine ovaries were grafted into this site and analyzed at 3 days and 3 weeks post-transplantation. Histological assessment revealed ovarian revascularization, minimal fibrosis, and favorable conditions for follicle development, without significant apoptosis. These findings validated the ear site as a promising, minimally invasive model to evaluate graft outcomes and pharmacological interventions.

Objectives 2 and 3 were jointly addressed in **Article 2**: *The mTOR Inhibitor Rapamycin Counteracts Follicle Activation Induced by Ovarian Cryopreservation in Murine Transplantation Models*.

To further validate the novel ear model (objective 2), we compared it with the more invasive but conventional transplantation under the kidney capsule. Fresh four-week-old C57BL/6 mouse ovaries were autotransplanted to either site. To test pharmacological treatment administration, the PI3K inhibitor LY294002, vehicle control, or neither, was injected locally for ovaries transplanted into the ear, or systemically for ovaries transplanted under the kidney capsule. Three weeks after transplantation, both sites showed similar PMF densities, vascularization, follicle proliferation, and mTOR pathway activation, without signs of apoptosis. No differences in weight gain were observed between LY294002-treated and untreated mice in both models. However, behavioral analysis revealed that mice receiving IP injections of LY294002 were significantly less active than those receiving local injections or controls. Furthermore, both administration routes produced comparable effects on follicle density, without any observation of apoptosis or DNA damage.

For objective 3, we aimed to preserve the PMF pool by keeping them in a dormant state via the inhibition of follicle activation pathways using the pharmacological mTOR inhibitor rapamycin during the ovarian preparation and slow-freezing process. Therefore, four-week-old C57BL/6 mouse ovaries, either fresh, slow-frozen, or slow-frozen with rapamycin, were autotransplanted under the kidney capsule of mice and recovered three weeks later for analysis. We observed that rapamycin significantly reduced both follicle proliferation and activation of the Akt and mTOR pathways, thus keeping PMFs in a dormant state. However, rapamycin did not prevent cryopreservation-induced depletion of the PMF pool.

Objective 4 was explored in **Article 3**: *Ovarian cryopreservation with rapamycin improves fertility restoration in a murine orthotopic transplantation model.*

While the previous heterotopic transplantation models are suitable for testing the effects of inhibitors on follicle quantities and activation, the ultimate goal is to improve fertility restoration. Therefore, we investigated whether adding rapamycin during cryopreservation can enhance fertility restoration in mice through orthotopic autotransplantation after chemically-induced ovarian failure. Four-week-old C57BL/6 mice underwent unilateral oophorectomy with subsequent slow-freezing of ovaries with or without rapamycin. After chemically disabling the remaining ovary, orthotopic autotransplantation was performed, and the mice were mated for four months to investigate pregnancy outcomes. Mice in the rapamycin group exhibited improved fertility restoration, with a significantly higher live birth rate and total pup counts compared to controls. Additionally, rapamycin enhanced graft survival, as indicated by the presence of PMFs approximately six months after grafting.

Objective 5 was the focus of **Article 4**: *BEZ235-Mediated PI3K/mTOR Dual Inhibition Improves Ovarian Follicle Survival in a Preclinical Model.*

Although mTOR inhibitors, such as rapamycin, can be promising agents to protect the PMF pool during cryopreservation, many studies have shown increased activation of the Akt pathway following mTOR inhibition due to feedback mechanisms. For this reason, we tested whether BEZ235 could more profoundly protect the PMF pool compared to single inhibitors or the natural inhibitor AMH. Therefore, four-week-old C57BL/6 mouse ovaries were subjected to whole organotypic culture under chemotherapeutic conditions, in media supplemented with the different inhibitors. BEZ235 significantly counteracted chemotherapy-induced activation of both Akt and mTOR pathways, whereas

rapamycin and LY29400 inhibited only mTOR or Akt, respectively. Furthermore, combining BEZ235 and AMH did not provide any additional or synergistic benefit beyond that achieved with BEZ235 alone. Additionally, murine ovaries were cryopreserved with the different pharmacological inhibitors to compare their effects on follicle activation during the slow-freezing and thawing process. BEZ235 significantly counteracted Akt and mTOR pathway activation, while rapamycin and LY294002 both only significantly decreased Rps6 activation. Furthermore, the effects of adding BEZ235 or rapamycin during slow-freezing were compared in a heterotopic ovarian transplantation model. Ovaries slow-frozen with BEZ235 retained a higher percentage of primordial follicles and showed reduced follicle proliferation and activation compared to both control and rapamycin three weeks after transplantation. Finally, to assess whether vascular support could enhance BEZ235's protective effects, we combined it with post-graft injection of VEGF/G-CSF. This angiogenic treatment, however, did not improve outcomes, likely reflecting a ceiling effect of BEZ235-mediated protection.

Chapter 3

Results

Article 1

Evaluation of an alternative heterotopic transplantation model for ovarian tissue to test pharmaceuticals improvements for fertility restoration

Carmen Terren, **Jules Bindels**, Michelle Nisolle, Agnès Noël and Carine Munaut

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SHORT COMMUNICATION Open Access

Evaluation of an alternative heterotopic transplantation model for ovarian tissue to test pharmaceuticals improvements for fertility restoration

Carmen Terren[1], Jules Bindels[1], Michelle Nisolle[1,2], Agnès Noël[1] and Carine Munaut[1*]

Abstract

Background: Ovarian tissue cryopreservation and transplantation (OTCTP) is currently the main option available to preserve fertility in prepubertal patients undergoing aggressive cancer therapy treatments. However, a major limitation of OTCTP is follicle loss after transplantation. The mouse is a model of choice for studying ovarian function and follicle development after ovarian tissue grafting in vivo. In these mouse models, ovarian tissue or ovaries can be transplanted to different sites. Our aim was to evaluate a new alternative to heterotopic transplantation models that could be useful to test pharmaceutical improvement for ovarian grafts after OTCTP.

Methods: Slow frozen murine whole ovaries were transplanted into the mouse ears (between the external ear skin layer and the cartilage). Ovarian transplants were recovered after 3, 14 or 21 days. Grafts were analyzed by immunohistochemistry and follicle density analyses were performed.

Results: An increase of ovarian vascularization (CD31 and Dextran-FITC positive staining), as well as cellular proliferation (Ki67 staining) were observed 3 weeks after transplantation in comparison to 3 days. Fibrosis density, evaluated after Van Gieson staining, decreased 3 weeks after transplantation. Furthermore, transplantation of cryopreserved ovaries into ovariectomized mice favored follicle activation compared to transplantation into non-ovariectomized mice.

Conclusion: The present study indicates that surgical tissue insertion in the highly vascularized murine ear is an effective model for ovarian grafting. This model could be helpful in research to test pharmaceutical strategies to improve the function and survival of cryopreserved and transplanted ovarian tissue.

Keywords: Ovarian tissue transplantation, Heterotopic transplantation, Animal model, Fertility restoration

Background

Cryopreservation of ovarian tissue followed by its autotransplantation is currently the main option to preserve the fertility of prepubertal patients or when oncological care is urgent [1, 2]. However, this technique has certain limitations, including follicular loss immediately after grafting, possibly due to slow neovascularization, apoptosis [3, 4] and/or massive follicular recruitment, which is also known as follicular burnout [5]. Primordial follicle overactivation leads to a depletion of the ovarian reserve and thus to a reduced lifetime of the transplant [5]. Experimental *in vivo* models are of high value in this research field in order to test pharmacological strategies to limit transplantation-induced follicle loss and therefore to increase graft lifetime as well as the chances of

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pregnancies. In the literature, a number of transplantation sites have been investigated, including grafting intraperitoneally [6, 7], into the ovarian bursa [8, 9], under the kidney capsule [10, 11], intramuscularly [12] or subcutaneously [13, 14]. A good transplantation site promotes fast revascularization of ovarian tissue, limiting the duration of ischemia and hence ovarian damage. The ovarian bursa is an interesting transplantation site as it is highly vascularized and natural pregnancies after murine ovarian transplantation are possible [8, 14, 15]. However, grafting beneath the murine ovarian bursa has several disadvantages compared to other sites. Indeed, only small tissue pieces can be inserted into the bursa via a small slit under microscopic control. In a recent study, the ovarian bursa site showed however the best ovarian tissue quality compared to subcutaneous graft sites [14]. This could be explained by the fact that in the subcutaneous site, the environment, pressure changes and temperature could influence ovarian quality. However, the subcutaneous graft site involves a simple surgical procedure and allows for external follow-up of follicular growth [16].

The intraperitoneal site may be an equivalent to the orthotopic graft site used in clinic [17]. Indeed, it provides a favorable environment for follicular development [16]. However, invasive surgery is necessary.

Our aim was to evaluate an alternative to heterotopic and/or other classical transplantation models for ovarian grafts through an adaptation of the "ear sponge assay" which was previously set up to study angiogenesis and lymphangiogenesis [20, 21]. This model involves ovarian tissue transplantation into the mice ear (between the skin layer and the cartilage). The first important advantage is to be less invasive than conventional models. Secondly, the transplantation site is highly vascularized. This feature is of high value in the case of ovarian grafts since a major follicle loss has been documented during the avascular ovarian transplantation [8, 18, 19, 22–24]. Finally, transplanted tissues remain easily accessible for subsequent therapeutic local treatments to limit follicle loss observed after transplantation of cryostored ovarian tissue.

We therefore evaluated this model by studying graft revascularization and survival after cryopreservation and transplantation.

Materials and methods

Experimental design

Mice were bred and maintained within the accredited Mouse Facility and Transgenics GIGA platform of the University of Liège (Belgium). The first experiment consists of the model validation. Therefore, slow frozen (SF) ovaries from SCID mice (6–10 weeks old) were transplanted ectopically into the ear of SCID mice (12–14 weeks old, not ovariectomized) for either 3 days or 3 weeks ($n=4$–5 ovaries per group with one graft per mice). We also performed a comparison of follicle development of SF ovaries from BALB/c mice (7 weeks old) transplanted into ovariectomized or non-ovariectomized SCID mice (7-11 weeks old) with transplants recovery after 2 weeks ($n=11$–12 ovaries per group with one graft per mice). Transplanted ovaries from all experiments were fixed in 4% formaldehyde for histological assessment. The Animal Ethics Committee of the University of Liège approved this study (#1934) and all experiments were performed in accordance with relevant guidelines and regulations.

Ovariectomy, slow freezing (SF) and thawing procedure

This procedure was previously described [25].

Transplantation procedure

Mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and xylazine (10 mg/kg body weight) by intra-peritoneal injection and a small horizontal incision was performed in the basal, external, and central parts of the ear and the external mouse ear skin layer was smoothly detached from the cartilage with thin forceps. The cryopreserved ovary (whole ovary) was introduced inside the hole, between the external mouse skin layer and the cartilage (Fig. 1). A suture point was made to close the skin incision.

Histological assessment

Intravenous injection of 200 µl dextran-fluorescein isothio-cyanate (FITC, 2.5 mg/ml in PBS) was given to mice before sacrifice and then the skin of the ear was cut around the transplanted ovary. Ovaries fixed in 4% formaldehyde were paraffin-embedded and serially sectioned (5 µm sections). The immunohistochemical detection of vascular endothelial cells (CD31) and functional blood vessels (dextran-FITC) was performed using specific primary antibodies (rabbit anti CD31 1/200 during 1 h (Abcam, Cambridge, UK) and anti-fluorescein-POD/HRP during 30 min (Roche, Basel, Switzerland). Fibrosis and nucleus density were analyzed thanks to Van Gieson staining. Apoptosis and cell proliferation in the ovaries were evidenced by immunostaining of caspase-3 (1/300 overnight at 4 °C, Cell Signaling, Danvers, USA) and Ki67 (1/100 during 1 h, Abcam, Cambridge, UK), respectively.

Density of immunostaining was determined by computer-assisted image analysis as previously described [25]. For follicle quantification, hematoxylin and eosin sections were analyzed by light microscopy for the presence of primordial, primary and secondary or more mature follicles based on morphological classification of mouse follicles [26]. The follicular densities (number/

Fig. 1 Photograph of the place where ovaries were grafted. A horizontal incision was performed in the basal, external and central part of the mouse ear and the external mouse ear skin layer was smoothly detached from the cartilage with thin forceps. The ovary was introduced inside the hole, between the external mouse skin layer and the cartilage. A suture point was made to close the skin incision

mm^2) were calculated after manually outlining the ovarian surface (NDP view software–NDP.view2 Viewing software U12388-01, Hamamatsu Photonics K.K., Japan).

Statistics

GraphPad Prism (GraphPad, San Diego, CA, USA) was used for statistical analyses. All data are presented as means ± SEM. The Mann Whitney test was applied and p-value < 0.05 was considered statistically significant.

Results

Grafts are revascularized 3 weeks after transplantation with an increased cell proliferation

A significant increase of vascular endothelial cells (CD31 staining) and functional blood vessels (FITC staining) was observed after 3 weeks of transplantation compared to 3 days (Fig. 2A-B). No modulation in apoptosis (Fig. 2C) was observed. Cell proliferation, as detected by the Ki67 staining quantification was increased after 3 weeks (Fig. 2D). Furthermore, a decrease in fibrosis density was observed after 3 weeks of transplantation compared to 3 days (Fig. 2E) but no modulation in nuclear density was observed (Fig. 2E).

Follicle activation is more pronounced after ovarian transplantation into ovariectomized compared to non-ovariectomized mice

A significant increase of secondary or more mature follicles was observed in ovaries transplanted into ovariectomized mice accompanied with a decrease in primary follicle density compared to non-ovariectomized mice. However, primordial follicle density was similar between the two experimental groups (Fig. 3 A-C).

Discussion

A number of different ovarian transplantation models exists (e.g., intraperitoneal, under the kidney capsule, into the ovarian bursa, subcutaneous, ...). Their main inconvenient is that they are all invasive, with the exception of subcutaneous transplantation sites [8, 9, 11, 16, 18, 19]. Our aim was therefore to develop a simpler and less invasive model, which is in addition easily accessible for local injections of different agents to improve graft health.

First, SF ovaries were transplanted into mice ears for 3 days or 3 weeks. An increase of ovarian vascularization was observed 3 weeks after transplantation compared to 3 days (CD31 and FITC staining). Indeed, hypoxic period was identified during the first 5 days after transplantation followed by gradual oxygenation of the ovarian transplant over the next 5 days [6]. Additionally, nuclear density and apoptosis remained stable through the transplantation time, indicating that cellular death is not increased 3 weeks after transplantation compared to 3 days. A decrease in fibrosis density was observed 3 weeks after transplantation compared to 3 days. However, an increase in fibrosis density was observed when marmoset, bovine or human ovarian tissue was transplanted subcutaneously into immunodeficient mice for 7 days compared to 3 days [19]. This observation determines an advantage of the ear transplantation site compared to the subcutaneous transplantation site.

On the other hand, Ki67 staining increased after 3 weeks of transplantation compared to 3 days. This could indicate follicle activation linked to transplantation. Altogether, these results indicate that this model could be usable as an alternative to invasive models.

In order to determine whether the mice own ovaries have an influence on the transplanted ovaries, a comparison of follicle development of ovaries transplanted into ovariectomized or non-ovariectomized mice has been performed. A significant increase of secondary or more mature follicles was observed in ovaries transplanted into ovariectomized mice accompanied with a decrease in primary follicle density compared to ovaries transplanted into non-ovariectomized mice. One of the likely explanations for the transplantation-induced

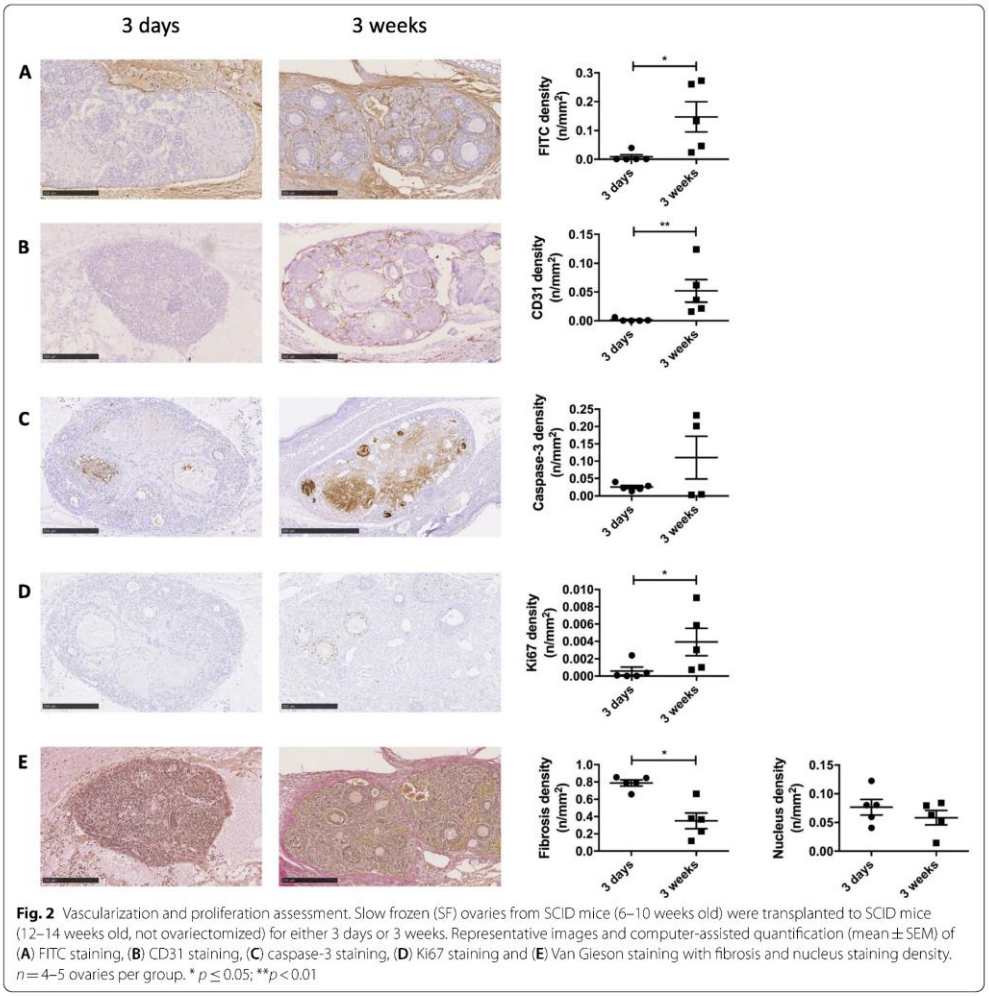

Fig. 2 Vascularization and proliferation assessment. Slow frozen (SF) ovaries from SCID mice (6–10 weeks old) were transplanted to SCID mice (12–14 weeks old, not ovariectomized) for either 3 days or 3 weeks. Representative images and computer-assisted quantification (mean ± SEM) of (**A**) FITC staining, (**B**) CD31 staining, (**C**) caspase-3 staining, (**D**) Ki67 staining and (**E**) Van Gieson staining with fibrosis and nucleus staining density. $n = 4–5$ ovaries per group. * $p \leq 0.05$; ** $p < 0.01$

activation of primordial follicles in ovariectomized mice lies in the absence of the mice own ovaries. In normal physiology, growing follicles maintain the quiescence of primordial follicles via the production of inhibitory factors, such as antiMullerian hormone (AMH) [20]. Furthermore, in a cryopreserved ovary, most of the mature follicles do not resist the cryopreservation process while primordial follicles, which have a low metabolic activity, are more tolerant to cryopreservation [21]. Therefore, in an ovariectomized mice, there is no AMH secretion (neither from the transplanted ovary nor from the removed mice own ovaries). An accelerate follicle activation was therefore observed. Indeed, the absence of growing follicles disrupts the balance between stimulatory and inhibitory factors in the graft, thereby leading to follicle activation [5, 19, 27]. In contrast, when the recipient mice are non-ovariectomized, the mice own ovaries are secreting AMH, acting on the transplanted ovary to limit primordial follicle activation.

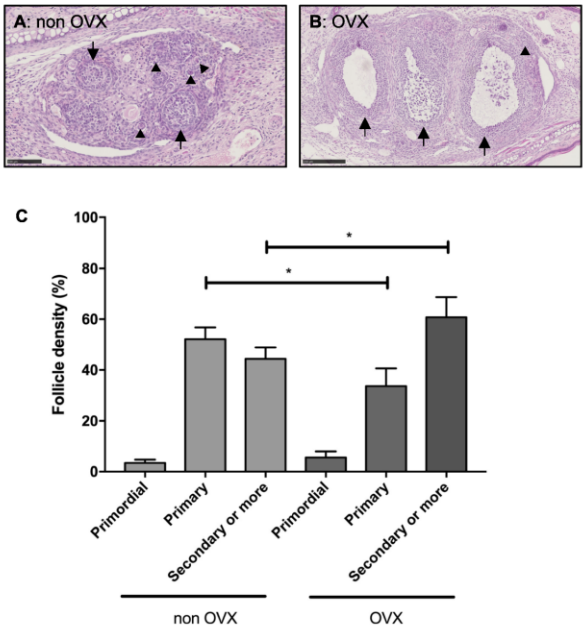

Fig. 3 Follicle development after transplantation into ovariectomized or non-ovariectomized mice. Transplantation of slow frozen (SF) ovaries from BALB/c mice (7 weeks old) into SCID mice (7-11 weeks old) for 2 weeks. Representative images of hematoxylin & eosin staining of ovaries transplanted into a non-ovariectomized (**A**) and an ovariectomized (**B**) mice. Arrowheads show primary follicles and arrows show secondary or more mature follicles. (**C**) Follicle density of primordial, primary or secondary or more mature follicles (means ± SEM). $n = 11–12$ ovaries per group. * $p \leq 0.05$. OVX = ovariectomy

Conclusion

In conclusion, the ear transplant model could be suitable for ovarian tissue transplantation due to the high revascularization rate. Furthermore, transplantation of cryopreserved ovaries into ovariectomized mice favors follicle activation as compared to transplantation into a non-ovariectomized mice. This new model is of particular interest for the testing of various pharmaceuticals strategies to limit follicle loss associated to the avascular ovarian auto-transplantation.

Abbreviations

OTCTP: Ovarian tissue cryopreservation and transplantation; FITC: Fluorescein isothio-cyanate; AMH: AntiMullerian hormone; SF: Slow freezing; OVX: Ovariectomy.

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Authors' contributions

C.T. performed the experimental work, prepared all figures and wrote the draft of the manuscript. A.N. conceived and designed the study. C.M. conceived, designed the study, interpreted data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data underlying this article will be shared on reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate

The Animal Ethics Committee of the University of Liège approved this study (# 1934) and all experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable

Competing interests
The authors declare no competing interests.

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Publisher's Note

Article 2

The mTOR Inhibitor Rapamycin Counteracts Follicle Activation Induced by Ovarian Cryopreservation in Murine Transplantation Models

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Article

The mTOR Inhibitor Rapamycin Counteracts Follicle Activation Induced by Ovarian Cryopreservation in Murine Transplantation Models

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Abstract: *Background and Objectives*: Ovarian tissue cryopreservation followed by autotransplantation (OTCTP) is currently the only fertility preservation option for prepubertal patients. Once in remission, the autotransplantation of frozen/thawed tissue is performed when patients want to conceive. A major issue of the procedure is follicular loss directly after grafting mainly due to follicle activation. To improve follicular survival during the OTCTP procedure, we inhibited the mTOR pathway involved in follicle activation using rapamycin, an mTOR inhibitor. Next, we compared two different in vivo models of transplantation: the recently described non-invasive heterotopic transplantation model between the skin layers of the ears, and the more conventional and invasive transplantation under the kidney capsule. *Materials and Methods*: To study the effects of adding rapamycin during cryopreservation, 4-week-old C57BL/6 mouse ovaries, either fresh, slow-frozen, or slow-frozen with rapamycin, were autotransplanted under the kidney capsule of mice and recovered three weeks later for immunohistochemical (IHC) analysis. To compare the ear with the kidney capsule transplantation model, fresh 4-week-old C57BL/6 mouse ovaries were autotransplanted to either site, followed by an injection of either LY294002, a PI3K inhibitor, vehicle control, or neither, and these were recovered three weeks later for IHC analysis. *Results*: Rapamycin counteracts cryopreservation-induced follicle proliferation, as well as AKT and mTOR pathway activation, in ovaries autotransplanted for three weeks under the kidney capsule of mice. Analyses of follicle proliferation, mTOR activation, and the effects of LY294002 treatment were similar in transplanted ovaries using either the ear or kidney capsule transplantation model. *Conclusions*: By adding rapamycin during the OTCTP procedure, we were able to transiently maintain primordial follicles in a quiescent state. This is a promising method for improving the longevity of the ovarian graft. Furthermore, both the ear and kidney capsule transplantation models were suitable for investigating follicle activation and proliferation and pharmacological strategies.

Keywords: ovarian cryopreservation; follicle activation; fertility preservation; heterotopic transplantation; animal models

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1. Introduction

In recent decades, the chances of surviving cancer have substantially increased thanks to the improvement of cancer therapies. As a result of this, considering the quality of life after remission has become increasingly important. One of the important issues among young cancer survivors hoping to one day become parents is the ability to have biological children. Unfortunately, certain chemo- and radiotherapies (such as alkylating agents) can increase the risk of ovarian failure and infertility due to gonadotoxic effects, removing the possibility of natural pregnancies [1,2].

One of the available fertility preservation options is the cryopreservation of cortical ovarian tissue followed by autotransplantation (OTCTP). At present, for prepubertal girls and young women who require urgent oncological treatment, OTCTP is the only effective fertility preservation technique [3]. Currently, more than 130 live births have taken place after the use of OTCTP, with a live birth rate between 25 and 42% and mean graft longevity of 27 months [4–8]. A major advantage of OTCTP is the possibility to perform the preservation at any moment of the menstrual cycle without delaying the oncological treatment, as compared to, e.g., mature oocyte cryopreservation [9]. Furthermore, it can restore both the endocrine function of the gonads and natural fertility, and it is not limited to a single pregnancy but allows for multiple births [10,11]. However, a major issue of this technique is the loss of follicles directly after grafting. Indeed, multiple studies have shown a decrease in primordial follicles in transplanted ovaries compared to non-transplanted ovaries [12–14]. The depletion of the follicle reserve, reducing the longevity of transplants, is possibly due to transient ischemia, apoptosis, and/or massive follicle recruitment, which is known as follicular "burn-out" [15]. Previous approaches to enhance graft longevity, e.g., enhancing revascularization or limiting apoptosis [16–20], have led to some but still insubstantial improvements in graft survival [21,22]. However, recent studies tested an ovarian tissue transplantation mice model that used adipose-tissue-derived stem cells, and the results showed substantially enhanced graft vascularization and the increased quiescence of primordial follicles, leading to a larger follicle pool both after short- and long-term grafting [23–26]. In order to increase graft longevity, which eventually leads to enhanced pregnancy rates after the use of OTCTP, additional studies are still required to find new approaches that limit follicle "burn-out".

Previous research showed the involvement of multiple molecular pathways, such as phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT and the mammalian target of rapamycin (mTOR) pathways, in follicle activation; the manipulation of these pathways in cultured ovaries resulted in a significant change in primordial follicle levels compared to control ovaries [27,28]. Under normal physiological conditions, the primordial follicle reserve is maintained via the complex balance between activation signals provided by, but not limited to, the PI3K/PTEN/AKT and mTOR pathways and inhibition via the anti-Müllerian hormone (AMH) secreted by growing follicles. However, due to ovarian cryopreservation followed by autotransplantation, this balance is dysregulated; follicle inhibition signals are diminished as many growing follicles do not survive freezing and thawing. This leads to an imbalance towards follicle activation, eventually resulting in massive follicle recruitment and thus a decrease in the follicle reserve [15,29,30].

Currently, different inhibitors of the follicle activation pathways have been tested on ovaries in various experimental settings. The PI3K inhibitor LY294002 (LY) was investigated both in vitro and in vivo, and the results showed that it could inhibit ovarian carcinoma cell growth [31]. In vivo studies with the natural follicle inhibitor, AMH, have shown controversial results on follicle preservation when injected into mice before or after OTCTP [32,33]. However, a recent study showed that mice administrated with AMH after ovarian transplantation had a higher primordial follicle reserve compared to mice injected with the control [34]. Other studies focused on a specific mTOR inhibitor, rapamycin, which is already used in clinics to, e.g., prevent rejection after organ transplantation [35,36]. Rapamycin has been shown to decrease the ratio of growing follicles to primordial follicles in cultured cisplatin-treated fresh rat ovaries [35]. Furthermore, it was observed that the injection of rapamycin in rats could preserve the primordial follicle pool [37], and SCID mice transplanted with vitrified human ovarian tissue followed by rapamycin injection showed a higher percentage of remaining primordial follicles compared to control mice [38]. So far, these promising results with rapamycin were obtained using fresh or vitrified ovaries. To be used in a clinic, a study focusing on slow-frozen (SF) ovaries is required, which is currently the gold-standard ovarian tissue cryopreservation method [39].

Our previous research showed that the slow-freezing of ovaries induced follicle activation via the PI3K/PTEN/AKT and mTOR pathways, which could be counteracted by

adding LY294002 or rapamycin to the freezing medium. Furthermore, the culture-induced activation of follicle activation pathways could be counteracted by cryopreservation with rapamycin and culture with LY294002 [40]. As these in vitro results with rapamycin were very promising, our first aim was to confirm in vivo that rapamycin could counteract cryopreservation- and transplantation-induced follicle activation using the widely used experimental ovarian transplantation model, namely, transplanting ovaries under the kidney capsule of mice [41,42].

Many different ovarian transplantation sites have been investigated to test pharmacological strategies, with every site having its advantages [13,41–48]. Our team previously evaluated an alternative heterotopic transplantation site for ovarian grafts between the skin layer and cartilage of the ears, adapted from the ear sponge assay that was set up to study (lymph) angiogenesis [49,50]. This site was chosen as the transplantation procedure is less invasive compared to more conventional models. Additionally, the ears are highly vascularized, being of great importance as major follicle "burn out" has been reported due to ischemia [12–14]. Our second aim was to compare this new model to the more conventional kidney capsule transplantation site.

2. Materials and Methods

2.1. Experimental Design

The experimental designs can be found in Figure 1. For the first aim, female C57BL/6 mice (n = 27, 4 weeks old) were obtained from Charles River Laboratories (France) and maintained at the accredited Mouse Facility and Transgenics GIGA platform of the University of Liège (Belgium). The mice were kept at ±21 °C in a 12 h light/dark cycle with a maximum of 5 mice per cage.

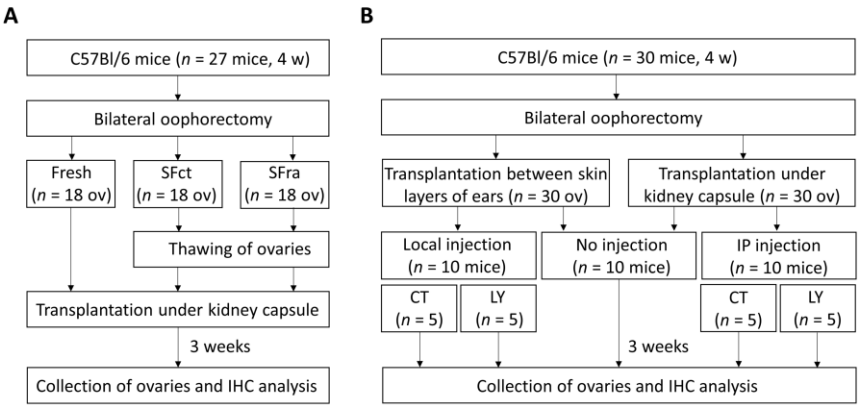

Figure 1. Experimental design for the mice model used to investigate the effects of adding rapamycin to the freezing medium on follicle activation and proliferation (**A**) as well as the comparison between two ovarian transplantation sites, namely, between the skin layers of the ears and under the kidney capsule (**B**). 4 w = 4 weeks old, Fresh = ovaries transplanted directly after oophorectomy, SFct = slow-freezing in control medium, SFra = slow-freezing with rapamycin, IHC = immunohistochemistry, IP = intraperitoneal, CT = control injection, LY = injection with LY294002.

Ovaries collected from these mice, either fresh, SF with control medium (SFct), or SF with rapamycin (SFra), were autotransplanted under the kidney capsule (n = 18 ovaries per group with 2 grafts per mouse).

For the second aim, fresh ovaries from C57BL/6 mice (n = 10, 4 weeks old) were autotransplanted either under the kidney capsule or between the skin layers of the ears (n = 7–10 ovaries per group with 2 grafts per mouse). Furthermore, as a follow-up to test

the treatment administration after transplantation, autotransplantation of fresh C57BL/6 mice ovaries was performed (n = 20, 4 weeks old) to either transplantation site, followed by injection with LY294002 or vehicle control locally for ovaries transplanted into the ear (injected in both ears), or systemically (intraperitoneally; IP) for ovaries transplanted under the kidney capsule (n = 8–10 ovaries per group with 2 grafts per mouse; injections given every other day with a total of 4 injections; 16.67 mg/kg LY294002 per injection). The weight and behavior of the mice were monitored for a total of 3 weeks to assess the side effects of LY294002 treatment. Behavior scoring was adapted from Herrmann et al. and performed as described in Table 1 [51].

Table 1. Mice behavioral score conditions. Adapted from Herrmann et al. [51].

Behavioral Score	Score Condition
0	No activity; breathing issues; death anticipated
1	No activity; no food intake
2	Low active state and sleepy; unreactive to human interaction
3	Low to normal active state; reactive to human interaction
4	Normal active state and fast; tries to escape when scruffed
5	Very active state, strong and fast; agitated when scruffed

Grafted ovaries from all experiments were collected 3 weeks after transplantation and fixed in 4% formaldehyde overnight, after which ovaries were put in 70% ethanol. Fixed ovaries were embedded in paraffin and cut into 5 µm sections using a microtome and mounted on slides for histological assessment.

The Animal Ethics Committee of the University of Liège approved this study (#1934) and all experiments were performed in accordance with relevant guidelines and regulations.

2.2. Oophorectomy and Preparation of Ovaries

For all experiments, bilateral oophorectomy was performed on the mice under gas anesthesia (Isoflurane, Dechra, Northwich, UK), and the ovaries were placed in Leibovitz L-15 medium (Lonza, Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Gibco, Waltham, MA, USA), a transport solution. The oviduct and fat tissue taken during oophorectomy were removed from the ovaries under a binocular microscope using a scalpel. Fresh ovaries were then directly autotransplanted, and ovaries that were going to be slow-frozen were prepared accordingly.

2.3. Ovarian Slow-Freezing and Thawing Method

Designated ovaries were SF and thawed as described before [52]. Briefly, whole ovaries were put in a freezing solution containing Leibovitz L-15 medium supplemented with 10% FBS, 10% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany), and 0.1 M sucrose, and equilibrated for 30 min at 4 °C. After equilibration, ovaries were put in cryovial tubes (Simport, Montreal, QC, Canada) containing freezing solution and thereafter SF in a programmable freezing machine (CL-8800i System; CryoLogic, Mulgrave, Victoria, Australia) as previously described and stored in liquid nitrogen [53]. For ovaries SF with rapamycin (1 µM, InvivoGen, Toulouse, France), the inhibitor was added to the transport and freezing solutions. Thawing was performed by incubating cryovials for 2 min at room temperature (RT) followed by a 2 min incubation at 37 °C in a water bath. Ovaries were then washed in Leibovitz L-15 medium 3 times for 5 min at 37 °C to remove any remaining cryoprotectants and/or rapamycin.

2.4. Autotransplantation Procedures

2.4.1. Transplantation under the Kidney Capsule

The transplantation of ovaries under the kidney capsule was based on an article by Nicholson et al. [54]. Briefly, the kidney was exteriorized under gas anesthesia (isoflurane) and kept hydrated by applying saline solution. Using thin-tip forceps, the kidney capsule

was carefully lifted from the kidney parenchyma and a small incision was made using tiny spring scissors. A small pocket for the ovaries was created by manipulating a rounded closed-end glass Pasteur pipette under the capsule. Both whole ovaries were then inserted into the pocket using the glass pipette and gently pushed away from the hole to decrease the risk of the ovaries slipping out of the pocket. Once the grafting was complete, the peritoneum was closed using a double suture, and the skin was closed using surgical wound clips.

2.4.2. Transplantation between Skin Layers of the Ears

A minor incision was made in the basal, external, and central parts of the mouse ear under gas anesthesia (isoflurane), and the external skin layer was detached from the cartilage using thin-tip forceps. The whole fresh ovary was placed through the incision between the skin layer and the cartilage, and the skin was subsequently sutured to close the hole [55].

2.5. Histological Assessment

In order to perform follicle identification and quantification, ovarian sections were labeled with LIM-homeobox protein 8 (LHX8; Abcam ab41519, Cambridge, UK) transcription factor and/or DEAD-box helicase 4 (DDX4; Abcam ab13840, Cambridge, UK). To analyze follicle proliferation and activation of the AKT and mTOR pathways, sections were labeled for KI67 (Abcam ab16667, Cambridge, UK), phosphor-AKT (pAKT; Abcam ab81283, Cambridge, UK), and phosphor-RPS6 (pRPS6; Cell Signaling #2211, Danvers, MA, USA), respectively. Apoptosis was revealed by immunostaining cleaved caspase-3 (Cell Signaling #9661, Danvers, MA, USA) and TUNEL staining (Roche 11684795910, Mannheim, Germany; following the manufacturer's instructions). Vascular endothelial cells were evidenced by immunostaining CD31 (Abcam ab28364, Cambridge, UK). Fibrosis was identified using Van Gieson staining.

Briefly, for immunostainings, ovarian sections were deparaffinized and rehydrated, followed by antigen retrieval using an autoclave (11 min, 126 °C, 1.4 Bar) in either citrate (for LHX8, DDX4, pRPS6, KI67, and cleaved caspase-3) or target buffer (for pAKT and CD31) (Dako, Glostrup, Denmark). After cooling down for 20 min, endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 20 min at RT. Non-specific binding sites were blocked by incubation with Animal-Free Blocking Solution (Cell Signaling, Danvers, MA, USA) for 20 min at RT. Primary antibodies (diluted in REAL antibody diluent (Dako, Glostrup, Denmark)) were incubated for 1 h at RT except for cleaved caspase-3, which was incubated overnight at 4 °C (LHX8 1/100; DDX4 1/600; KI67 1/100; pAKT 1/250; pRPS6 1/400; cleaved caspase-3 1/300; CD31 1/200). Afterward, sections were incubated with the secondary antibody linked with horseradish peroxidase (HRP; ENVISION/HRP ready to use, Dako, Glostrup, Denmark) for 30 min at RT. For visible staining, the revelation was performed with DAB+ (Dako, Glostrup, Denmark) followed by hematoxylin counterstaining, and sections were mounted using Entellan new mounting medium (Sigma-Aldrich, St. Louis, MO, USA). For fluorescent staining, the fluorescein tyramide kit (PerkinElmer, Waltham, MA, USA) was used for 10 min and sections were mounted with DAPI FluoromountG mounting medium (SouthernBiotech, Birmingham, AL, USA). Stained sections were then scanned using either the NanoZoomer 2.0 HT digital slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan) or the Olympus SLIDEVIEW VS200 high digital slide scanner (Olympus Corporation, Tokyo, Japan).

2.6. Follicle Quantification

Scanned sections labeled for LHX8 were analyzed using the NDP.view2 software (Hamamatsu Photonics K.K., Hamamatsu, Japan). At least four to five 5 μm sections per ovary were analyzed blind, with each section being taken 50 μm further down the ovary compared to the previous section. Follicles were classified into primordial, primary, and secondary or more growing according to morphological mouse follicle classification by

manually looking at each section and counting and classifying all follicles accordingly [56]. Total follicle density was defined as the number of follicles per mm^2 (n/mm^2) after manually outlining the ovarian surface of each section. Results are expressed both as the number of each follicle type per mm^2 and the percentage of each type relative to the total number of follicles per section. Each section was analyzed individually, followed by calculating the mean of the results of the analyzed sections per ovary.

To analyze follicle proliferation and activation, ovarian sections were double-stained for DDX4 in combination with KI67, pAKT, or pRPS6. Primordial and primary follicles were manually counted using DDX4 staining and classified as stained or non-stained by manually looking at the follicles and determining whether they are stained or not for the desired protein (NDP view software). Results are expressed as the percentage of stained relative to the total primordial and primary follicles. At least two to three 5 μm sections per ovary were analyzed blinded, with each section being taken 50 μm further down the ovary compared to the previous section. Each section was analyzed individually, followed by calculating the mean of the results of the sections per ovary.

2.7. Statistical Analysis

GraphPad Prism 8 (GraphPad, San Diego, CA, USA) was used to perform all statistical analyses. The Kruskal–Wallis test with Dunn's multiple comparison post hoc test was applied when comparing three or more experimental groups. For comparisons between two experimental groups, the Mann–Whitney test was performed. For both tests, $p < 0.05$ was considered statistically significant.

3. Results

3.1. In Vivo Effects of Adding Rapamycin to the Freezing Medium in Ovaries Heterotopically Transplanted under the Kidney Capsule of Mice

To investigate in vivo whether the addition of rapamycin to the freezing medium can counteract follicle activation and proliferation induced by cryopreservation and/or transplantation, the heterotopic kidney transplantation mice model was used. Immunohistochemical staining was performed to calculate follicle densities and analyze the activation of follicle activation pathways, as well as apoptosis, angiogenesis, and fibrosis.

3.1.1. The Primordial Follicle Pool Is Decreased by Slow-Freezing with or without Rapamycin Compared to Fresh Ovaries

Ovaries were recovered three weeks after transplantation. The follicular densities were analyzed by manually counting primordial, primary, and secondary or more growing follicles using LHX8 labeling (Figure 2A–C). The percentage of primordial follicles, as well as the total number of primordial follicles per mm^2, was lower when ovaries were SF in the control medium compared to fresh ovaries. The addition of rapamycin to the freezing medium did not reverse this effect. No significant difference in the primary follicle percentage and total number was found in SFct ovaries compared to fresh ovaries. The addition of rapamycin significantly decreased the total number and the percentage of primary follicles compared to fresh ovaries, with no significant difference between SFct and SFra. Furthermore, slow-freezing, both with or without rapamycin, resulted in a significantly higher percentage of secondary or more growing follicles compared to fresh ovaries. However, no difference was observed in the total number of secondary or more growing follicles between the groups (Figure 2D–E).

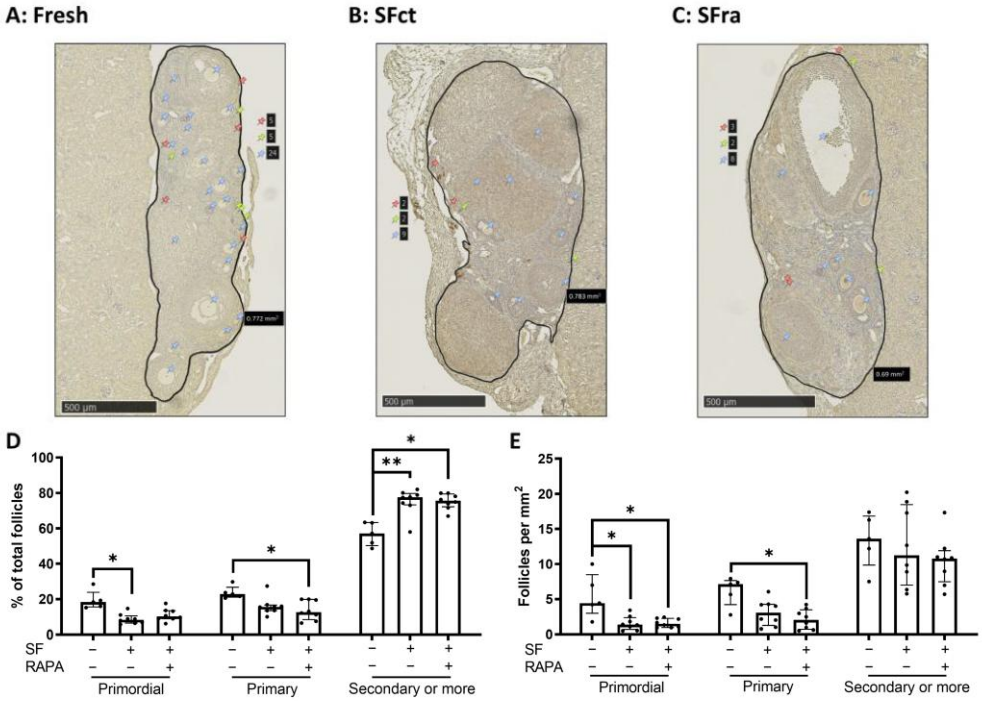

Figure 2. Follicle density assessment in fresh, SFct, or SFra mice ovaries autotransplanted under the kidney capsule of C57BL/6 mice (4 weeks old) for three weeks. Representative images of LHX8 staining of fresh (**A**), SFct (**B**), or SFra (**C**) mice ovaries transplanted under the kidney capsule. Red pins indicate primordial follicles, yellow pins primary follicles, and blue pins secondary or more growing follicles. Follicle density was either expressed in the percentage of primordial, primary, and secondary or more growing follicles relevant to the total amount per section (**D**) or as the total number of each follicle type per mm^2 (**E**) (median with interquartile range). n = 5–8 ovaries per group. * $p \leq 0.05$, ** $p \leq 0.01$. RAPA = rapamycin.

3.1.2. Addition of Rapamycin to the Freezing Medium Counteracts Follicle Proliferation and Activation Induced by Slow-Freezing and/or Transplantation In Vivo

To further analyze whether adding rapamycin to the freezing medium modulates the cryopreservation/transplantation-induced proliferation and activation of the AKT and mTOR pathways, ovarian sections were stained for KI67, pAKT, and pRPS6, respectively. The percentage of primordial and primary follicles labeled for KI67 displayed a trend toward being higher in SF ovaries in the control medium compared to fresh ovaries. The addition of rapamycin to the freezing medium showed a lower amount of KI67-labeled follicles compared to fresh and SFct conditions (Figure 3A). In addition, significantly more primordial and primary follicles were stained for pAKT (Figure 3B) and pRPS6 (Figure 3C) in SF ovaries with the control medium compared to fresh ovaries. We found that the addition of rapamycin to the freezing medium was able to counteract this effect. Indeed, similar amounts of pAKT- and pRPS6-stained primordial and primary follicles were found in SF ovaries with rapamycin and fresh ovaries three weeks after transplantation.

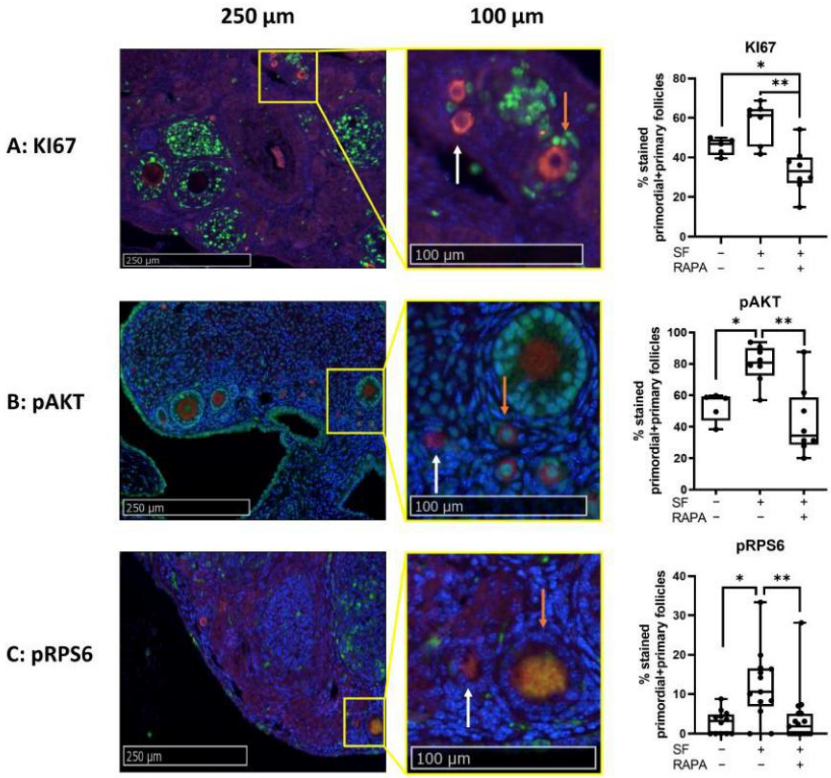

Figure 3. Effects of adding rapamycin to the freezing medium on follicle activation and proliferation in fresh, SFct, or SFra mice ovaries autotransplanted under the kidney capsule of C57BL/6 mice (4 weeks old) for three weeks. Immunohistochemistry (IHC)-assisted quantification (median + min to max) of the percentage of primordial and primary follicles labeled for KI67 (**A**), pAKT (**B**), and pRPS6 (**C**), including representative images of ovaries in the fresh group. Red staining = DDX4, green staining = KI67, pAKT, or pRPS6. White arrow = non-green-stained follicle, orange arrow = green-stained follicle. * $p \leq 0.05$, ** $p \leq 0.01$. SF = slow-frozen/thawed ovaries, RAPA = rapamycin. (**A**,**B**) n = 5–8 ovaries per group, (**C**) n = 12–17 ovaries per group.

3.1.3. No Difference in Apoptosis, Vascular Endothelial Cells, and Fibrosis Was Observed between Fresh, SF, or SF Ovaries, with Rapamycin

No apoptosis was observed three weeks after transplantation in the ovarian sections of all experimental groups by active caspase-3 labeling (Figure S1A) and by analyzing DNA strand breaks using a TUNEL assay (Figure S1B). Furthermore, no difference in the number of vascular endothelial cells (CD31 analysis) was found between the groups (Figure S1C). Additionally, Van Gieson staining showed no differences in fibrosis between the three experimental groups (Figure S1D).

3.2. Comparison of Two Different Ovarian Tissue Transplantation Models

In order to compare the newly described transplantation site between the skin layers of the ears with the more conventional site under the kidney capsule, fresh mice ovaries were autotransplanted to either site with or without LY294002 injection locally in the ears or IP for ovaries transplanted under the kidney capsule.

Mice were monitored for weight gain and their behavior. Transplanted ovaries were recovered after three weeks, and ovarian sections were stained to investigate follicle activation and proliferation and apoptosis.

3.2.1. Graft Vascularization and Follicle Reserve Are Similar between Transplantation Sites, with No Observation of Apoptosis

No significant differences were observed in primordial and primary follicles with respect to the percentage of total follicles as the total number per mm^2 between the two transplantation sites. A significantly higher percentage of secondary or more growing follicles was observed in ovaries transplanted between the skin layers of the ears compared to ovaries transplanted under the kidney capsule. However, this effect was not observed when examining total secondary or more growing follicles per mm^2 (Figure 4). The analysis of active caspase-3 showed no apoptotic activity for both transplantation sites (Figure 5A) and no differences in blood vessel formation (CD31 staining) (Figure 5B).

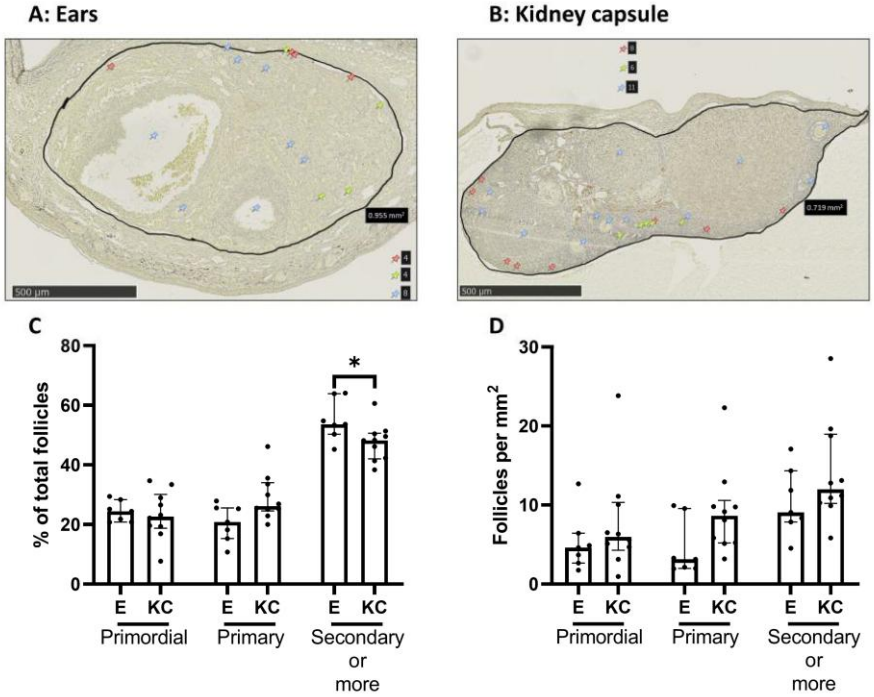

Figure 4. Comparison of follicle densities between fresh mice ovaries autotransplanted either between the skin layers of the ear or under the kidney capsule of C57BL/6 mice (4 weeks old). Representative images of LHX8 staining of fresh mice ovaries transplanted either between the skin layers of the ears (**A**) or under the kidney capsule (**B**). Red pins indicate primordial follicles, yellow pins primary follicles, and blue pins secondary or more growing follicles. Follicle density was either expressed in the percentage of primordial, primary, and secondary or more growing follicles relevant to the total amount per section (**C**) or as the total number of each follicle type per mm^2 (**D**) (median with interquartile range). n = 7–10 ovaries per group. * $p \leq 0.05$. E = transplantation of ovaries between skin layers of ears, KC = transplantation of ovaries under the kidney capsule.

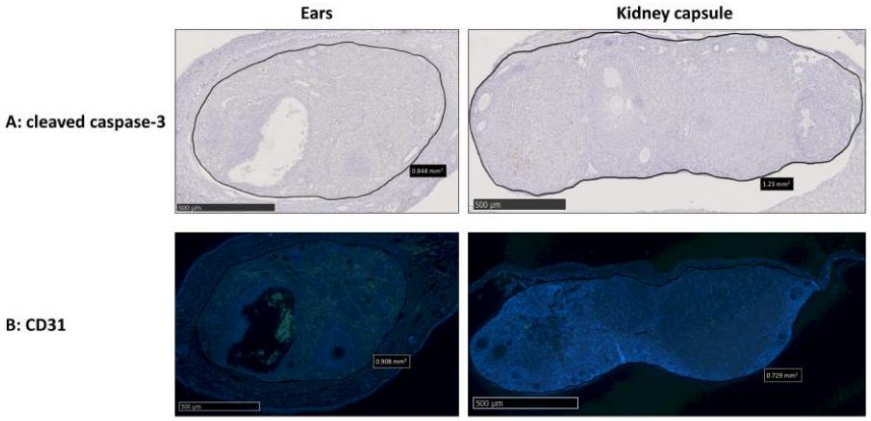

Figure 5. Comparison of apoptosis and vascular endothelial cells between fresh mice ovaries auto-transplanted either between the skin layers of the ear or under the kidney capsule of C57BL/6 mice (4 weeks old). Representative images of cleaved caspase-3 (**A**) and CD31 (**B**) staining of fresh mice ovaries transplanted either between the skin layers of the ears or under the kidney capsule. Green staining = CD31. *n* = 7–10 ovaries per group.

The percentage of primordial and primary follicles labeled for cell proliferation (KI67) (Figure 6A) and the activation of the mTOR pathway (pRPS6) (Figure 6C) was similar between the transplantation sites. However, significantly fewer primordial and primary follicles were labeled with pAKT in ovaries transplanted between the skin layers of the ears compared to ovaries transplanted under the kidney capsule (Figure 6B).

3.2.2. Behavioral Score of Mice after LY294002 Injection in Both Transplantation Models

In order to analyze whether the injection of LY294002 locally in the ear had similar side effects to the IP injection, weight gain and behavioral scores were monitored up to three weeks after injection (Table 1). No differences were observed in weight gain between the four experimental groups after transplantation (Figure 7A). However, the behavioral score was significantly lower for mice systemically (IP) injected with LY294002 compared to mice injected locally in the ears, as well as compared to mice injected with the control (Figure 7B). No differences were observed between the two types of surgery with respect to both weight gain and behavioral score (Figure 7A–B).

3.2.3. Follicle Density, Activation, and Proliferation Are Similar in Both Transplantation Models

Primordial, primary, and secondary or more growing follicle densities, with respect to both the percentage of each type compared to the total amount per section and the total number of each follicle type per mm^2, were similar between ovaries after the local injection of LY294002 in the ears and IP injection for ovaries transplanted under the kidney capsule. However, IP injection with LY294002 resulted in a higher percentage and total number per mm^2 of primordial follicles compared to the control injection in the ears and a higher total number of secondary or more growing follicles (Figure 8).

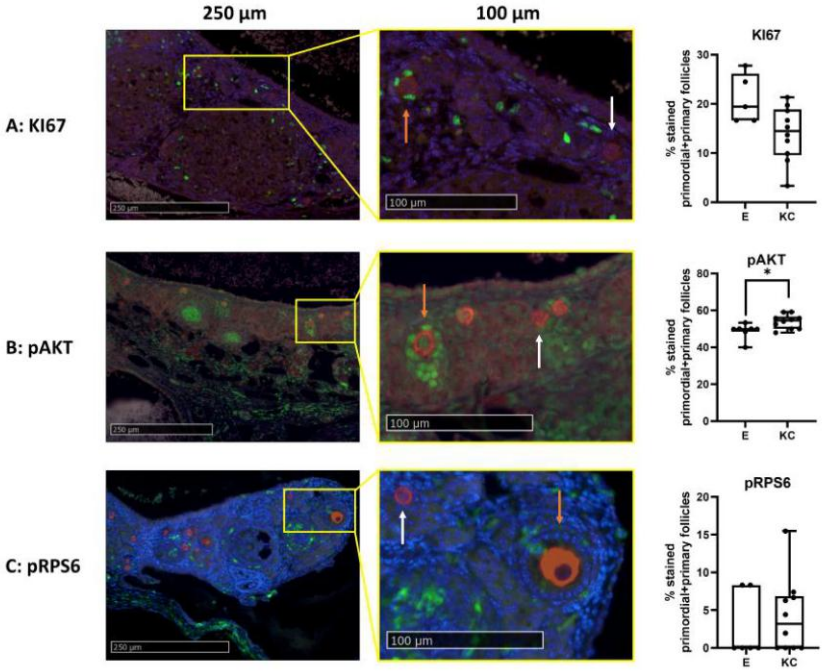

Figure 6. Comparison of follicle activation and proliferation between fresh mice ovaries autotransplanted either between the skin layers of the ear or under the kidney capsule of C57BL/6 mice (4 weeks old). IHC-assisted quantification (median + min to max) of the percentage of primordial and primary follicles labeled for KI67 (**A**), pAKT (**B**), and pRPS6 (**C**), including representative images of ovaries transplanted under the kidney capsule. Red staining = DDX4, green staining = KI67, pAKT or pRPS6. White arrow = non-green-stained follicle, orange arrow = green-stained follicle. * $p \leq 0.05$. n = 5–10 ovaries per group. E = transplantation of ovaries between skin layers of ears, KC = transplantation of ovaries under the kidney capsule.

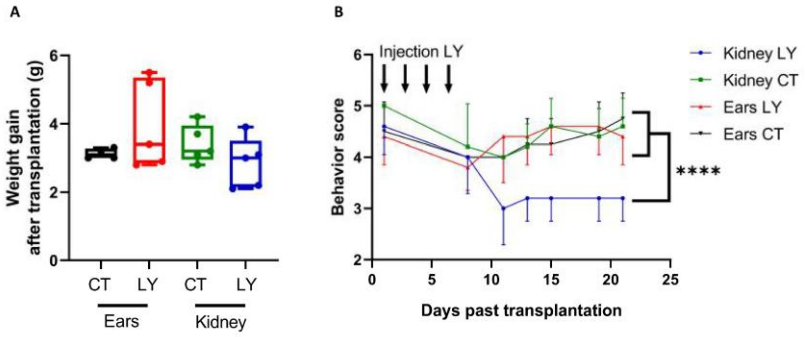

Figure 7. Effect of LY294002 (LY) injection on weight gain and mice behavior after ovarian autotransplantation into C57BL/6 mice (4 weeks old). Ears were injected locally and the kidney capsule was injected intraperitoneally. Weight gain (in grams; median + min to max) (**A**) and behavior score (0 = least active, 5 = most active; mean ± SEM) (**B**) of treated or control mice monitored for three weeks after transplantation. n = 4–5 mice per group. **** $p \leq 0.0001$. CT = vehicle control, LY = LY294002.

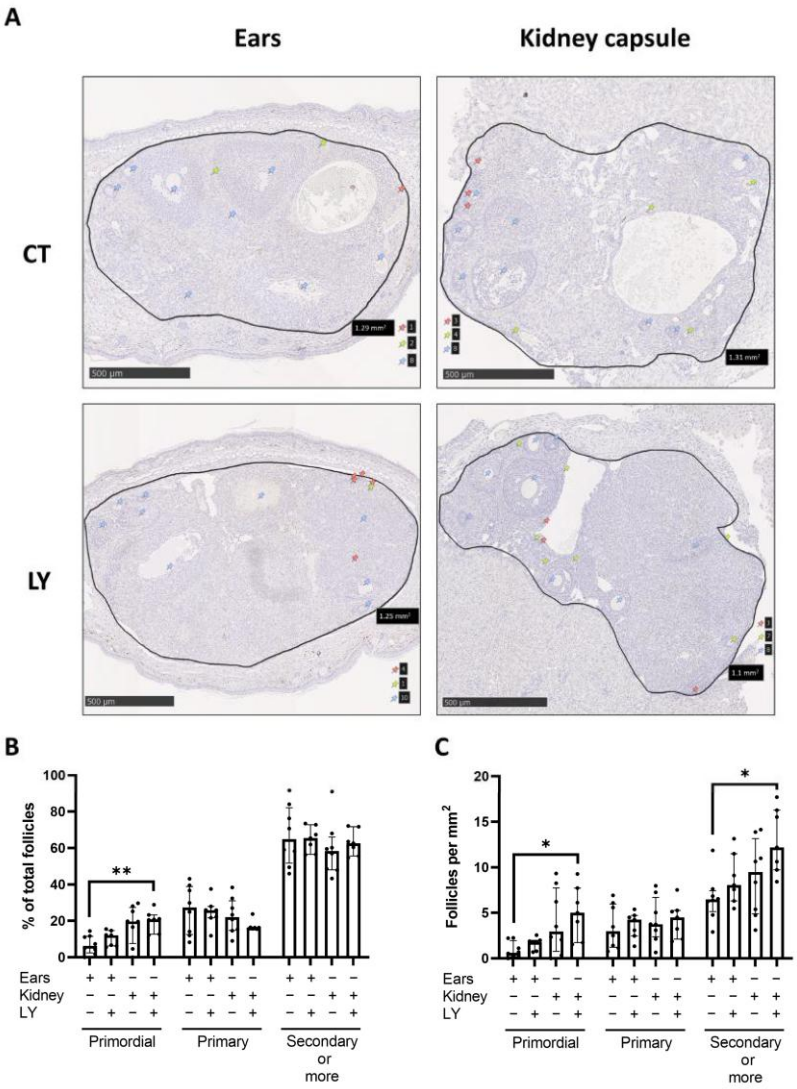

Figure 8. Comparison of follicle densities for LY injection after ovarian autotransplantation into C57BL/6 mice (4 weeks old) either locally in the ears or intraperitoneally (IP) when ovaries were transplanted under the kidney capsule. (**A**) Representative images of LHX8 staining of fresh mice ovaries transplanted either between the skin layers of the ears or under the kidney capsule, followed by local injection with LY or vehicle control for ovaries transplanted between skin layers of the ears, or IP for ovaries transplanted under the kidney capsule. Red pins indicate primordial follicles, yellow pins primary follicles, and blue pins secondary or more growing follicles. Follicle density was either expressed in the percentage of primordial, primary, and secondary or more growing follicles relevant to the total amount per section (**B**) or as the total number of each follicle type per mm^2 (**C**) (median with interquartile range). n = 7–8 ovaries per group. * $p \leq 0.05$, ** $p \leq 0.01$. CT = control injection, LY = injection with LY.

Furthermore, no apoptotic cells were observed in ovarian sections for all four experimental groups (active caspase-3 and TUNEL staining) (Figure S2).

Additionally, local injection in the ears or IP injection did not affect the percentage of primordial and primary follicles stained for KI67 (Figure 9A), pAKT (Figure 9B), or pRPS6 (Figure 9C). However, the percentage of pAKT-stained primordial and primary follicles was significantly lower when mice were injected with LY294002, either locally in the ear or IP, compared to ovaries transplanted under the kidney capsule with the control injection (Figure 9B).

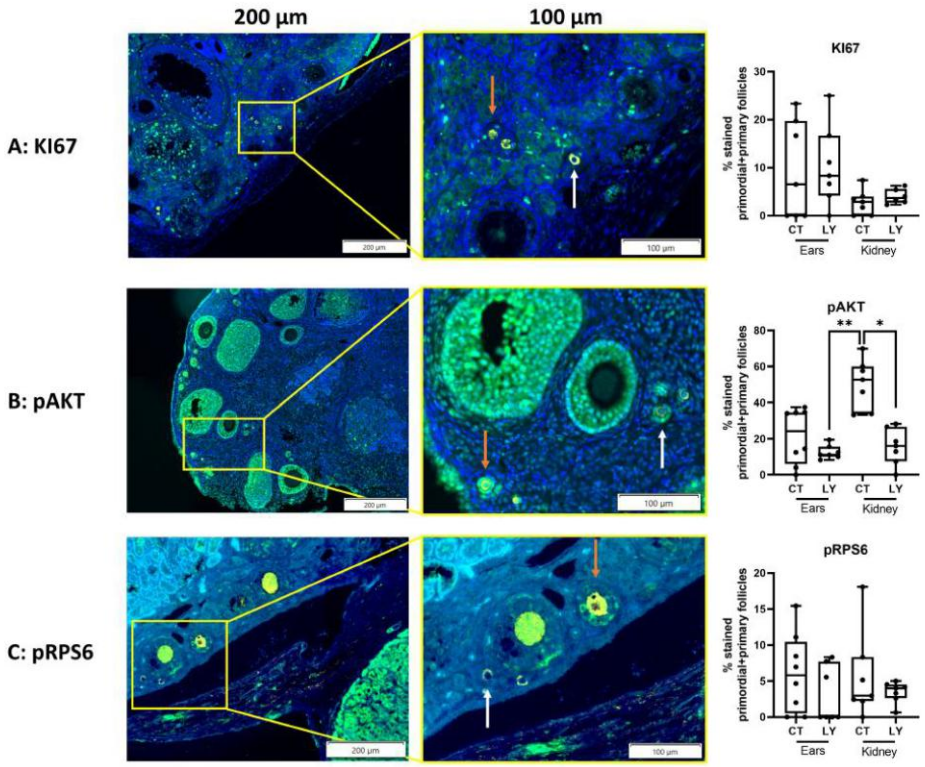

Figure 9. Comparison of follicle activation and proliferation for LY injection after ovarian autotransplantation into C57BL/6 mice (4 weeks old). Ears were injected locally and the kidney capsule was injected intraperitoneally. IHC-assisted quantification (median + min to max) of the percentage of primordial and primary follicles labeled for KI67 (**A**), pAKT (**B**), and pRPS6 (**C**), including representative images of ovaries transplanted under the kidney capsule followed by control IP injection. Yellow staining = DDX4, green staining = KI67, pAKT, or pRPS6. White arrow = non-green-stained follicle, orange arrow = green-stained follicle. * $p \leq 0.05$, ** $p \leq 0.01$. n = 6–8 ovaries per group. CT = vehicle control, LY = LY294002.

4. Discussion

OTCTP is currently the only fertility preservation option for prepubertal patients and young women in need of urgent treatment for severe malignancies [3]. This technique has already led to more than 130 live births, and this number is expected to grow [7,8]. However, the technique still has several limitations. One of the major issues of OTCTP is follicular loss directly after grafting partly due to massive primordial follicle recruitment [15]. Our

team previously showed that the slow-freezing of ovaries induced follicle activation via the PI3K/PTEN/AKT and mTOR pathways. Additionally, this could be counteracted by adding LY294002 or rapamycin to the freezing medium. Furthermore, we found that the best combination of inhibitors to counteract the culture-induced activation of follicle activation pathways was adding rapamycin to the freezing medium and performing the culture with LY294002 [40]. These promising in vitro results encouraged us to test adding rapamycin to the freezing medium in vivo using the widely used ovarian transplantation mice model, transplanting ovaries under the kidney capsule [41,42]. Our results indicated that the slow-freezing and thawing of ovaries before transplantation caused follicle proliferation to be higher and increased follicle activation via the PI3K/PTEN/AKT and mTOR pathways compared to the transplantation of fresh ovaries three weeks after transplantation. Furthermore, we showed that the addition of rapamycin to the freezing medium was able to significantly counteract follicle proliferation and activation, thus keeping primordial follicles in a dormant state. Interestingly, we saw that the addition of rapamycin resulted in fewer pAKT-labeled primordial and primary follicles. Other studies showed an increase in AKT activation following mTOR inhibition, possibly due to a feedback loop [40,57]. However, another study concluded that low concentrations of rapamycin are responsible for increased AKT activation via mTORC1 signaling, while higher concentrations of rapamycin resulted in decreased AKT phosphorylation mainly via the mTORC2 pathway, which could explain the results that we observed [58]. The mTOR pathway is additionally known for its function in cell proliferation [59]. Indeed, we observed that rapamycin was able to counteract cryopreservation-induced follicle proliferation. Furthermore, our results showed that even though the contact time between rapamycin and the ovary is relatively short during the freezing process, its positive effects can still be observed three weeks after transplantation.

To our knowledge, the addition of rapamycin during the cryopreservation process followed by transplantation has not yet been analyzed. Recent studies testing the inhibitor focused on the injection of rapamycin after transplantation [37,38,60]. However, a new vitrification protocol including pre-treating ovaries with rapamycin was tested. This study showed that rapamycin was able to inhibit mTOR pathway activation in ovaries directly after the thawing process and in ovaries five days after grafting in mice [61]. In addition, Chen et al. showed that the injection of rapamycin in mice resulted in lower levels of follicle proliferation [62].

Next, we found no apoptosis, DNA damage, or fibrosis in fresh ovaries and slow-frozen ovaries with or without rapamycin three weeks after transplantation. An explanation for this could be that three weeks is too long for analyzing these factors after transplantation. Indeed, double-stranded DNA breaks can be repaired in a few hours [63]. To more confidently state that there are indeed no effects on apoptosis, DNA damage, and fibrosis, these factors should be analyzed at a shorter time point after ovarian transplantation. However, our results indicate that rapamycin does not cause long-term tissue or DNA damage. Furthermore, CD31 analysis revealed no differences in blood vessel formation after transplantation between the three experimental groups.

Follicle quantification showed that the slow-freezing of ovaries followed by transplantation caused a lower primordial follicle pool. Indeed, a lower percentage and total amount of primordial follicles per mm^2 were observed in slow-frozen ovaries compared to fresh ovaries. Ovaries cryopreserved with rapamycin showed no difference in terms of the percentage or number of primordial follicles per mm^2 compared to cryopreservation without rapamycin. Cryopreservation with rapamycin resulted in a significantly lower percentage and number of primary follicles per mm^2 compared to fresh ovaries, and a similar trend could be observed for the SF ovaries in the control medium. Furthermore, the percentage of secondary or more growing follicles was significantly higher when ovaries were slow-frozen before transplantation either with or without rapamycin in the freezing medium. One of the explanations for this could be that cryopreservation causes more primary follicles to develop into secondary or more growing follicles.

While these results showed that the addition of rapamycin to the freezing medium was not able to counteract a cryopreservation-induced decrease in primordial follicles, the quality of follicles in SF ovaries with rapamycin may be better compared to follicles in SF ovaries with the control medium. To investigate this, follicle health and quality should be examined and compared between SF ovaries with or without rapamycin.

In this study, we promisingly found that the addition of rapamycin to the freezing medium resulted in the lower activation and proliferation of primordial and primary follicles. As other research found promising results when injecting mice post-transplantation with follicle activation inhibitors, e.g., a recent study with AMH, the natural inhibitor, a follow-up study could try to combine the cryopreservation of ovaries, including rapamycin, with the injection of follicle activation inhibitors (e.g., AMH) and/or antiapoptotic/angiogenic agents to increase graft quality even more [34].

For our experiments, mice models were used. While the morphology of human and mouse reproductive systems are distinctive (e.g., the mouse has a bicornuate uterus compared to the human single uterus), their reproductive cycles are very similar [64]. Indeed, similarly to humans, the mouse reproductive cycle oscillates periodically via fluctuations in progesterone and estrogen concentrations, which shows that mice models are suitable for answering certain reproductive research questions [64]. In order to increase translatability, a xenograft study could be performed, transplanting human ovarian tissue into immune-deficient mice. Furthermore, as rapamycin is already used in the clinic, for instance, to prevent rejection after organ transplantation, starting the use of rapamycin during ovarian cryopreservation in humans should not be too difficult [35,36].

Our next aim was to compare the recently described ovarian transplantation model between the skin layers of the ears with the more conventional and invasive model under the kidney capsule. As existing ovarian transplantation models have different inconveniences, e.g., the need for invasive surgery or long periods of ischemia after transplantation, a less invasive model was developed, and in addition, ovaries transplanted between the skin layers of the ears remained easily accessible for local treatment injection and observational studies [12–14,55]. We previously showed that SF/thawed mice ovaries transplanted for either three days or three weeks into the ears of SCID mice were revascularized three weeks after transplantation, with an increased proliferation of cells. Furthermore, we found no modulation in apoptosis and a decrease in fibrosis three weeks after transplantation compared to three days [55]. These results indicated that this new ear transplantation model could be suitable for analyzing follicle activation and proliferation and for testing pharmacological strategies. In order to further complete this study, this new transplantation site was compared with transplantation under the kidney capsule by autotransplanting mouse ovaries to either site, followed by the injection of either LY294002 or vehicle control, or neither, and recovered three weeks later for IHC analysis.

Ovaries transplanted to either side without follow-up injections showed no differences in the primordial follicle pool. Furthermore, no apoptosis was observed for both transplantation sides, indicating no long-term effects of the transplantation progress on cellular death. CD31 staining demonstrated similar levels of vascular endothelial cells between the sites, showing that vascularization is comparable in ovaries transplanted to either site. Additionally, no differences in follicle proliferation and the activation of the mTOR pathway were found in ovaries transplanted for three weeks between the skin layers of the ears or under the kidney capsule. However, the activation of the AKT pathway was significantly lower in ovaries that were transplanted using the ear model compared to the kidney capsule model, indicating that primordial and primary follicles might be in a more dormant state after ovarian transplantation between the skin layers of the ear compared to transplantation under the kidney capsule. Taking these results together, it is established that the new non-invasive ear model is as suitable for ovarian transplantation as the more conventional kidney capsule model.

As one of the main advantages of the new ear model is that it provides easy accessibility for local treatment injections, we wanted to compare the effects of local treatment for ovaries

transplanted between the skin layers of the ears with the effects of IP treatment for ovaries transplanted under the kidney capsule, followed by the recovery of the ovaries three weeks later. For the treatment, the strong PI3K inhibitor LY294002 was chosen [65]. In a previous in vitro organotypic ovarian culture study, our team showed that the culture-induced activation of the PI3K/PTEN/AKT pathway was reversed by the addition of LY294002 to the culture medium [40]. This led us to test this inhibitor in the comparison between the ear and kidney capsule transplantation model.

Body weight and mice behavior were monitored for three weeks after transplantation and injection in order to analyze the toxic side effects of LY294002. No difference in weight gain was observed when mice were injected with or without LY294002, either locally or IP, indicating that the inhibitor has no negative effects on the growth of mice. This is in agreement with another study, in which they injected xenografted BALB/C mice with LY294002 (IP) and found no effects on weight gain [66]. Behavioral analysis showed mice injected IP with LY294002 were significantly less active compared to the local injection of LY294002 in the ears and compared to mice injected with the control. This indicates that mice potentially suffer more from the IP injection of LY294002 compared to local injection. This could be explained by the fact that after local injection, the treatment mainly remains at the injection site and surrounding tissue, while IP injection causes the treatment to be systemically distributed, meaning that it could affect the entire body [67]. Furthermore, no differences in weight gain and behavioral score were observed between the two types of surgery. This suggests that even though the kidney model is more invasive, it does not affect the growth or behavior of mice differently compared to the less invasive ear model.

In order to examine whether the local and IP injections of LY294002 have similar or different effects on the follicle pool in transplanted ovaries, follicle density analysis was performed. This analysis revealed that the injection of LY294002 locally for the ear model and IP injection for the kidney capsule model had the same effect on follicle density both with respect to the percentage of primordial, primary, and secondary or more growing follicles compared to the total amount of follicles per section and the total number of each follicle type per mm^2. Furthermore, no apoptosis and no DNA damage were observed in transplanted ovaries after injections with or without LY294002, for both the ear and kidney capsule models. Therefore, LY294002 showed no toxic long-term side effects on ovaries three weeks after transplantation and injection in both models. The injection of LY294002, both locally or IP, did not affect follicle proliferation and the activation of the mTOR pathway compared to the control injection in ovaries transplanted for three weeks. An explanation for this could be that the effects of LY294002 on proliferation and mTOR pathway activation are only possible in the short term, and three weeks after transplantation and injection is too long for detecting the effects. However, AKT activation was lower in mice treated with LY294002 via IP injections compared to the IP control injection. This effect was not observed for local injections. There is a trend that AKT activation is lower in ovaries with the local control injection compared to the IP control injection. This could explain why LY294002 seems to only have an effect for IP injection, as transplantation under the kidney capsule tends to activate the AKT pathway to a greater extent compared to transplantation between the skin layers of the ears.

5. Conclusions

Our results indicate that the addition of the mTOR inhibitor rapamycin during the OTCTP procedure was able to transiently maintain primordial follicles in a quiescent state. Limiting the massive follicle activation of the primordial follicle pool is a promising method that can improve the longevity of the ovarian graft for fertility restoration, which could prolong the time frame in which recovered patients could become pregnant, and thus increase the overall possibility for pregnancies after the use of OTCTP.

Furthermore, follicle activation and proliferation could be analyzed similarly when ovaries were transplanted between the skin layers of the ears and when transplanted under the kidney capsule. The injection of the PI3K inhibitor LY294002 locally for ovaries

transplanted between the skin layers of the ears or IP for ovaries transplanted under the kidney capsule seemed to have similar effects on follicle proliferation and activation, with a decrease in side effects when injected locally, indicating that both sites could be used to test pharmacological strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/medicina59081474/s1, Figure S1: Effects of adding rapamycin to the freezing medium on apoptosis, vascular endothelial cells, and fibrosis in fresh, slow-frozen (SF) control (SFct), or slow-frozen with rapamycin (SFra) mice ovaries autotransplanted under the kidney capsule of C57BL/6 mice (4 weeks old) for three weeks; Figure S2: Comparison of apoptosis for LY294002 (LY) injection after ovarian autotransplantation into C57BL/6 mice (4 weeks old) either locally in the ears or IP when ovaries were transplanted under the kidney capsule.

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Supplementary

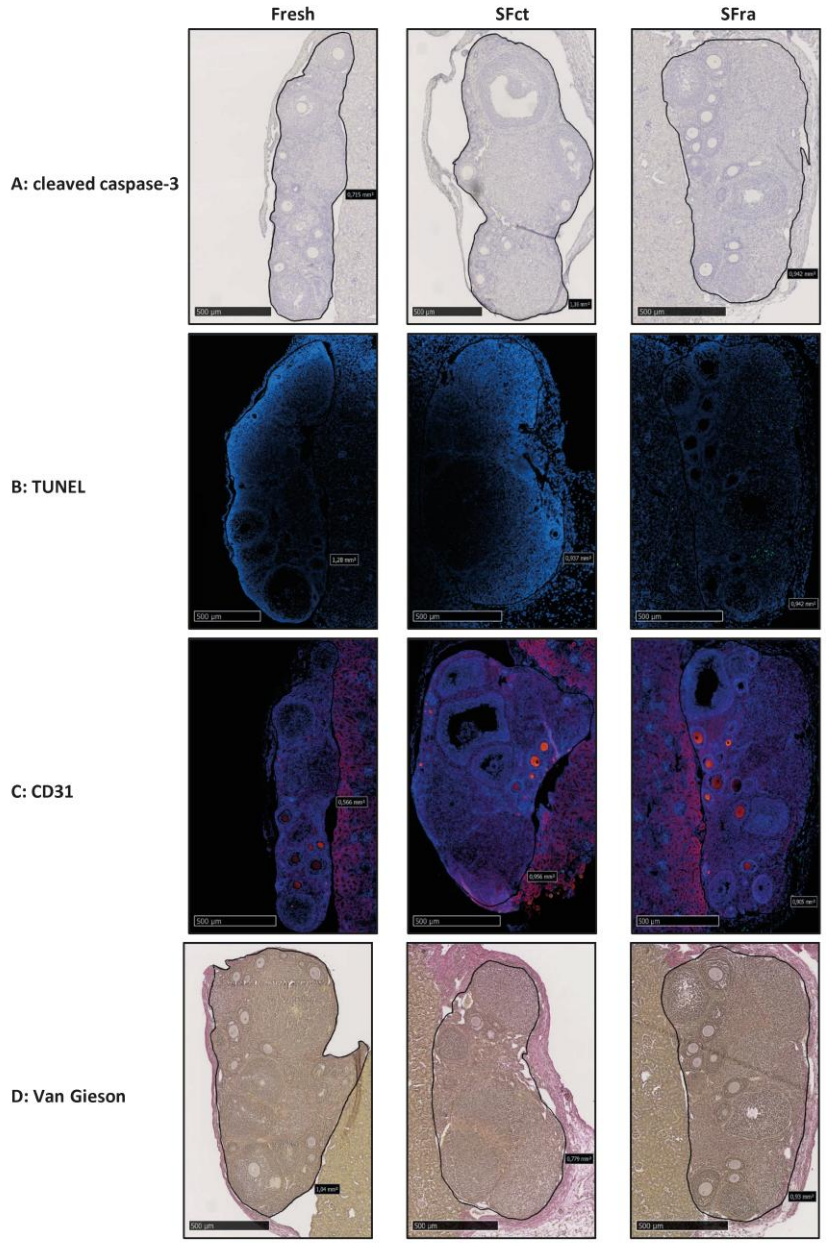

Figure S1. Effects of adding rapamycin to the freezing medium on apoptosis, vascular endothelial cells and fibrosis in mice ovaries fresh, slow-frozen (SF) control (SFct) or slow-frozen with rapamycin (SFra), autotransplanted under the kidney capsule of C57Bl/6 mice (4-weeks-old) for three weeks. Representative images of cleaved caspase-3 (**A**), TUNEL (**B**), CD31 (**C**) and Van Gieson (**D**) staining of fresh, SFct or SFra mice ovaries transplanted under the kidney capsule. Red staining = DDX4, green staining = TUNEL or CD31. Van Gieson: nucleus in black, cytoplasm in yellow, collagen and muscle in red. *n* = 5-8 ovaries per group.

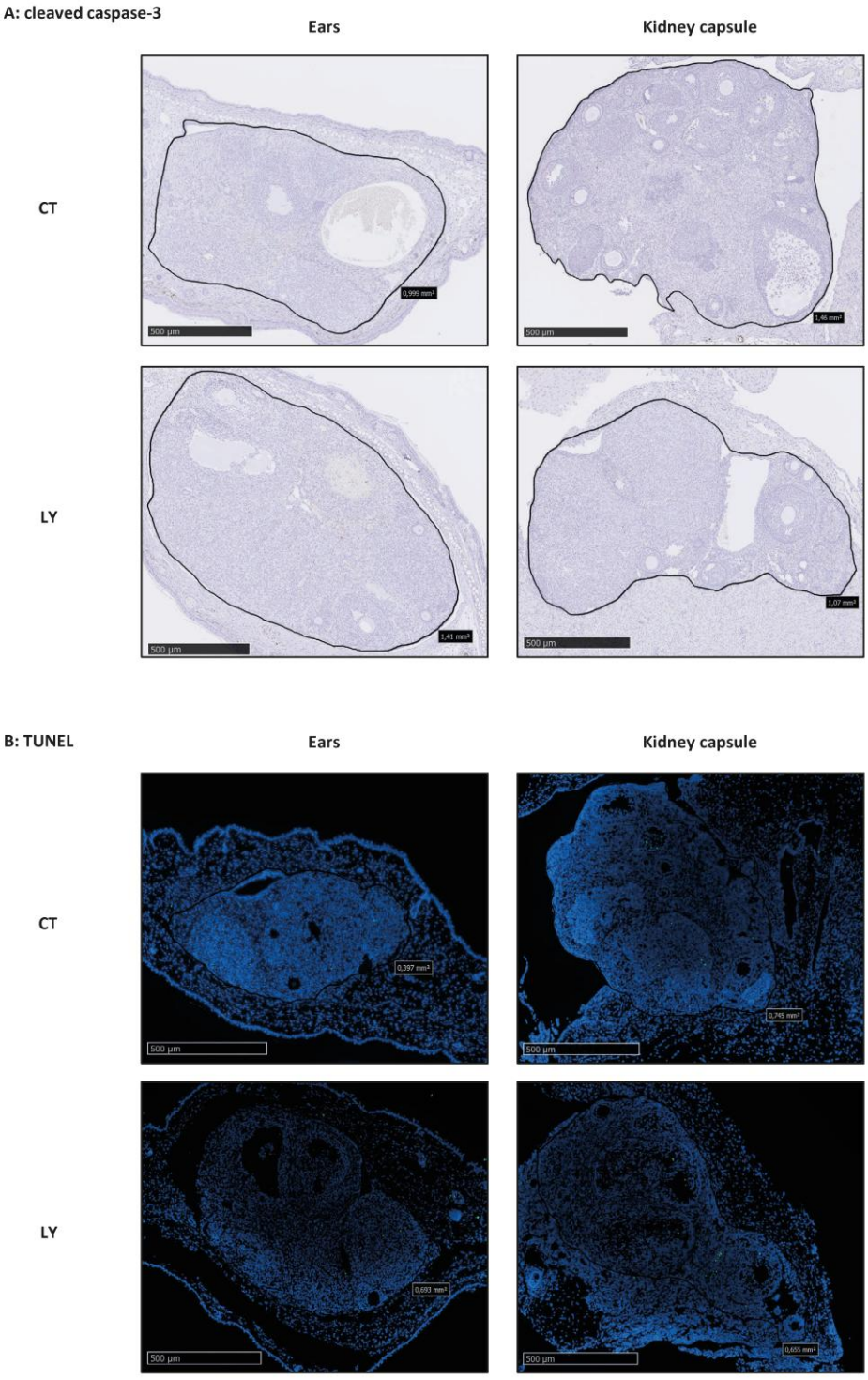

Figure S2. Comparison of apoptosis for LY294002 (LY) injection after ovarian autotransplantation into C57Bl/6 mice (4-weeks-old) either locally in the ears or IP when ovaries were transplanted under the kidney capsule. Representative images of cleaved caspase-3 (**A**) and TUNEL (**B**) staining of fresh mice ovaries transplanted either between the skin layers of the ears or under the kidney capsule, followed by local injection with LY or vehicle control for ovaries transplanted between skin layers of the ears, or intraperitoneal (IP) for ovaries transplanted under the kidney capsule. Green staining = TUNEL. *n* = 6-8 ovaries per group. Ears = transplantation of ovaries between skin layers of ears followed by local LY/control injection, Kidney capsule = transplantation of ovaries under the kidney capsule followed by IP LY/control injection, CT = control injection, LY = injection with LY.

Article 3

Ovarian cryopreservation with rapamycin improves fertility restoration in a murine orthotopic transplantation model

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Ovarian cryopreservation with rapamycin improves fertility restoration in a murine orthotopic transplantation model

Jules Bindels[1], Marlyne Squatrito[1], Laëtitia Bernet[1], Michelle Nisolle[2] & Carine Munaut[1]✉

Currently, the only fertility preservation option of prepubertal patients is ovarian tissue cryopreservation followed by autotransplantation (OTCTP). Once in remission and patients desire to conceive, autotransplantation of frozen/thawed tissue is performed. A major issue of this technique is follicular loss directly after transplantation, mainly due to follicle activation. Our previous research showed that adding rapamycin to the freezing medium counteracted follicle proliferation and activation induced by OTCTP in heterotopic autotransplantation of ovaries in mice. Our current study aimed to test the potential of this approach to improve fertility restoration in mice. Forty 4-week-old female C57BL/6 mice underwent unilateral oophorectomy followed by slow-freezing of ovaries with or without rapamycin. After chemically disabling the remaining ovary, orthotopic autotransplantation was performed. After recovery, estrous cycle analysis was conducted using daily vaginal smears. The mice were mated with males for 4 months, and pregnancy outcomes were recorded. After mating, half the females were super-ovulated for oocyte quantification and ovarian analysis, while the others had their ovaries collected for analysis of remaining primordial follicles using immunohistochemistry. Female mice whose ovaries were cryopreserved with rapamycin prior to chemically disabling the remaining ovary and orthotopic autotransplantation, gave birth to more pups (102 rapamycin, 48 control). The live birth rate was also higher ($P = 0.0025$) when ovaries were cryopreserved in rapamycin compared to control medium. Additionally, more mice in the rapamycin group gave birth (13 rapamycin, 8 control) with a higher average litter size ($P = 0.0837$). More mice had primordial follicles left at the end of the experiment in the rapamycin group ($P = 0.0397$). Superovulation showed a similar number of oocytes collected ($P = 0.4462$). While rapamycin did not influence cyst formation after autotransplantation, mice that developed ovarian cysts gave birth to fewer pups per dam ($P = 0.0119$) with a lower live birth rate compared to mice without ovarian cysts ($P = 0.0032$). The use of rapamycin improved fertility restoration in mice. Using rapamycin during OTCTP in humans could potentially resolve the massive follicular loss directly after grafting, and thus eventually lead to better opportunities for women to become pregnant.

Keywords Ovarian cryopreservation, Follicle activation, Fertility preservation, Rapamycin, Ovarian transplantation, Animal models

Abbreviations

DDX4	DEAD-box helicase 4
DMSO	dimethylsulfoxide
FBS	Fetal bovine serum
hCG	human chorionic gonadotrophin
IHC	Immunohistochemistry
IP	Intraperitoneally
mTOR	Mammalian target of rapamycin
OTCTP	Ovarian tissue cryopreservation followed by autotransplantation
PI3K	Phosphatidylinositol-3-kinase

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PTEN	Phosphatase and tensin homolog
RT	Room temperature
SF	Slow-frozen
SFct	Ovaries slow-frozen in control medium
SFrapa	Ovaries slow-frozen in medium supplemented with rapamycin
VCD	4-vinylcyclohexene diepoxide

Advancements in cancer treatments have significantly raised the likelihood of survival over recent decades. Consequently, the long-term quality of life after remission has gained heightened significance. For many young cancer survivors who aspire to start families, a critical concern is their ability to conceive biological children. Unfortunately, specific chemotherapy and radiotherapy treatments can lead to ovarian failure and, consequently, infertility[1–3]. Currently, the only option available to preserve the fertility of prepubertal girls and young women requiring urgent oncological care is the cryopreservation of cortical ovarian tissue followed by autotransplantation (OTCTP)[4]. One of the major advantages of this technique is the ability to restore natural fertility. To date, over 200 live births have taken place after the use of OTCTP[5,6]. However, this technique has certain limitations, including the loss of follicles directly after transplantation[7,8]. This rapid depletion of the follicle reserve is possibly due to apoptosis, delayed neovascularization, and/or immense follicle recruitment, known as follicular "burn-out", and can drastically decrease graft longevity[9–11]. Under physiological conditions, the primordial follicle reserve is preserved through a balance between activation signals, provided by, e.g., granulosa cells, and inhibition signals provided by growing follicles. Unfortunately, many growing follicles do not survive the OTCTP process, resulting in a reduction of the inhibition signals and an imbalance towards follicle activation. This leads to excessive follicle recruitment and a decrease in the primordial follicle reserve[9,12,13]. Two of the pathways involved in follicle activation are the phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT and the mammalian target of rapamycin (mTOR) pathways. Various inhibitors of these pathways have been investigated to limit follicle "burn out" and protect the follicle reserve[13–19]. One of these inhibitors is rapamycin, a specific mTOR inhibitor already used as an immunosuppressant in clinical settings after organ transplantation[20,21]. Rapamycin has shown promising results in protecting the primordial follicle pool in several experimental settings, e.g., in cultured cisplatin-treated fresh rat ovaries[17]. Furthermore, the injection of rapamycin has been shown to effectively preserve the primordial follicle pool in various models, including rats and SCID mice transplanted with ovarian tissue[18,19]. These findings, obtained with fresh or vitrified ovaries, highlight the potential of rapamycin in protecting the primordial follicle reserve. However, further investigation, particularly focusing on slow-frozen (SF) ovaries, the current gold-standard cryopreservation method, is essential before clinical application[22]. In vitro, we previously found that adding rapamycin to the freezing medium could counteract slow-freezing-induced follicle activation in mouse ovaries[23]. Following these promising in vitro results, we aimed to confirm in vivo that cryopreservation- and/or transplantation-induced primordial follicle activation could be inhibited by freezing ovaries with rapamycin using a heterotopic ovarian transplantation mouse model. Indeed, we found that the addition of rapamycin to the freezing medium can counteract follicle proliferation and activation induced by the slow-freezing and transplantation process three weeks after autotransplanting ovaries under the kidney capsule of mice[24]. While the heterotopic transplantation experiment demonstrated that rapamycin can maintain primordial follicles in a quiescent state, our ultimate goal is to improve the restoration of fertility. Therefore, we aimed to investigate whether adding rapamycin to the slow-freezing medium can enhance fertility restoration in mice through an orthotopic autotransplantation after chemically-induced ovarian failure.

Methods

Experimental design (Fig. 1)

A total of 40 four-week-old female C57BL/6 mice were obtained from Charles River Laboratories (France) and maintained at the accredited Mouse Facility of the University of Liège (Belgium). The mice were housed at

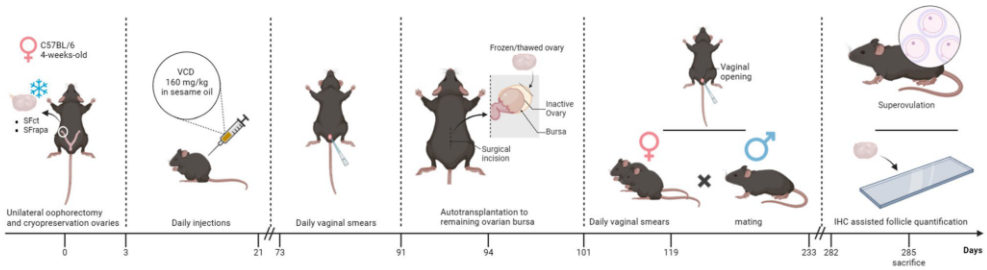

Fig. 1. Schematic of the experimental design used in the murine model to investigate the effects of adding rapamycin to the freezing medium on fertility restoration. VCD = 4-vinylcyclohexene diepoxide, IHC = immunohistochemistry, SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin.

±21 °C in a 12 h light/dark cycle with a maximum of five mice per cage, with food and water provided ad libitum. All mice underwent unilateral oophorectomy and the ovaries were slow-frozen with or without rapamycin in the freezing medium. The mice were randomly assigned to the groups. To induce failure of the remaining ovary, the mice were intraperitoneally (IP) injected daily with 160 mg/kg (100 µl) 4-vinylcyclohexene diepoxide (VCD; Merck, Darmstadt, Germany) diluted in sesame oil (Biofood) for a total of 19 days[25]. Once the failure of the remaining ovaries was verified using vaginal cytology, the cryopreserved ovaries were thawed and orthotopically autotransplanted to the remaining ovarian bursa[26,27]. Afterward, the recovery of estrous cyclicity was monitored using vaginal cytology. Fertility restoration was analyzed by mating the females with males, and recording details on deliveries and pup numbers. After 4 months of mating, 20 females were sacrificed by cervical dislocation and their ovaries were collected and fixed in 4% formaldehyde overnight. The fixed ovaries were embedded in paraffin, sectioned at 5 µm using a microtome, and mounted on slides for immunohistochemistry. Simultaneously, 16 females underwent superovulation, followed by their sacrifice by cervical dislocation, and subsequent collection and counting of oocytes. As manipulation of ovaries can lead to cyst formation, the ovaries from super-ovulated mice were analyzed for the presence of cysts[28,29].

Oophorectomy, slow-freezing, and thawing procedure

Using gas anesthesia (Isoflurane, Dechra, Northwich, UK), unilateral oophorectomy was performed on all mice, and the ovaries were placed in a slow-freezing transport medium consisting of Leibovitz L-15 medium (Lonza, Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Gibco, Waltham, MA, USA). The adjacent fat tissue and oviduct were removed from the ovaries using a scalpel under a binocular microscope, after which the ovaries were cryopreserved as described before[27]. Briefly, whole ovaries were placed in a slow-freezing medium consisting of Leibovitz L-15 medium supplemented with 10% FBS, 10% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany), and 0.1 M sucrose. After a 30-minute equilibration at 4 °C, ovaries were placed in cryovial tubes (Simport, Montreal, QC, Canada) containing the slow-freezing medium and thereafter cooled down in a programmable freezing machine (CL-8800i System; CryoLogic, Mulgrave, Victoria, Australia) as previously described and stored in liquid nitrogen[30]. For the SFrapa group, rapamycin (1 µM, InvivoGen, Toulouse, France) was added to the transport and slow-freezing media. The 1 µM concentration of rapamycin was selected based on our previous in vitro experiments and supportive data from the literature[31]. These prior studies indicated that 1 µM effectively reduced follicle activation without causing detectable toxicity, and therefore no additional dose-response tests were performed for the present study. The thawing procedure involved incubating the cryovials at room temperature (RT) for 2 min followed by a 2-minute incubation in a 37 °C water bath. Cryoprotectants and/or rapamycin were removed by washing the ovaries in Leibovitz L-15 medium 3 times for 5 min at 37 °C.

Vaginal cytology

To validate ovarian failure, the status of the estrous cycle was examined once a day (between 8:30 and 10:30) for a total of 3 times 5 consecutive days by collecting smears via vaginal lavage. The estrous cycle stage was determined by examining the cell population in the smears using a hematoxylin-eosin staining[26]. Briefly, the murine estrous cycle consists of four phases: Proestrus, when follicles start to grow and the smears contain mostly nucleated epithelial cells; Estrus, the fertile phase when cornified epithelial cells are present; In the absence of conception, the metestrus phase begins, characterized by the presence of cornified epithelial cells and leukocytes in the smears; Diestrus, when the females are no longer receptive to males and smears contain a high amount of leukocytes[32]. Mice were considered to have ovarian failure when they remained in the metestrus or diestrus phase for the final five days of vaginal cytology.

Vaginal cytology was also performed daily for 3 weeks after transplantation to analyze the recovery of the estrous cycle.

Autotransplantation

After confirming ovarian failure, orthotopic transplantation of cryopreserved ovaries to the remaining ovarian bursa was performed based on a protocol by *Behringer*[33]. Briefly, the remaining non-functional ovary and uterus were surgically exposed under gas anesthesia. A small incision in the bursa was made using spring scissors on the site opposing the oviduct. The slow-frozen/thawed whole ovary was then inserted through the incision into the bursal sac next to the remaining ovary. The ovaries were then placed back into the peritoneal cavity followed by the suturing of the peritoneum and closing of the skin with surgical wound clips.

Fertility assessment

To analyze whether rapamycin increased fertility restoration, all female mice were mated after the transplantation with 8-week-old C57BL/6 males (1 male per 2 females) for a total of 4 months. Vaginal plugs were checked every morning and the females were weighed 3 times a week to determine pregnancies. The number of females giving birth, litter size, litter number, and live birth rate were recorded. When calculating the mean number of deliveries per mouse, only mice with a minimum of one delivery were included. To ensure that the absence of conception was not due to male subfertility, males used for mating with the control females were exchanged with the males used for mating with females in the rapamycin group every 4 weeks.

Superovulation

At the end of the experiment, mice were super-ovulated ($n = 8$ per group). They were injected IP with 5 IU (100 µl) of pregnant mare serum gonadotrophin (Folligon, MSD, Kenilworth, NJ, USA). Forty-nine hours later, 7.5 IU (100 µl) of human chorionic gonadotrophin (hCG, Merck, Darmstadt, Germany) was administered similarly. The mice were sacrificed fifteen hours after hCG administration and the ovary/oviduct complex was

collected and placed in M16 medium (Merck, Darmstadt, Germany). The ampulla of the oviducts was cut with spring scissors to release the cumulus-oocyte complexes, and oocytes were quantified.

Immunohistochemistry

To perform follicle quantification and analyze whether primordial follicles remained in the ovaries at the end of the experiment, ovarian sections were labeled with DEAD-box helicase 4 (DDX4; Abcam ab41519, Cambridge, UK) ($n = 8–10$ sections per mouse per group). DDX4 is a helicase involved in gamete generation and is localized in germ cells. It can therefore be used as a biomarker for easy follicle identification[34]. In short, ovarian sections were deparaffinized and rehydrated, followed by antigen retrieval using an autoclave (11 min, 126 °C, 1.3 Bar) in citrate buffer (Dako, Glostrup, Denmark). After cooling for 20 min, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 20 min at RT. Non-specific binding sites were blocked using Animal-Free Blocking Solution (Cell Signaling, Danvers, MA, USA) for 20 min at RT. The DDX4 primary antibody, diluted 1/600 in REAL antibody diluent (Dako, Glostrup, Denmark), was incubated for 1 h at RT. Next, the sections were incubated with the secondary antibody linked with horseradish peroxidase (ENVISION/HRP ready to use, Dako, Glostrup, Denmark) for 30 min at RT. Afterward, the revelation was performed using DAB+ (Dako, Glostrup, Denmark) followed by hematoxylin counterstaining, and sections were mounted using Entellan new mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Stained sections were scanned using the NanoZoomer 2.0 HT digital slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan).

Follicle quantification

Scanned DDX4 labeled sections were analyzed using the NDP.view2 software (Hamamatsu Photonics K.K., Hamamatsu, Japan). Follicles were classified into primordial, primary, and secondary or more growing according to morphological mouse follicle classification, to evaluate the presence (or not) and number of primordial follicles at the end of the experiment[35]. Every tenth section was analyzed, with a total of eight sections per ovary, to get a well-established representation of the whole ovaries. Total follicle density was defined as the number of primordial follicles per mm^2 after manually outlining the ovarian surface of each section, taking into account only mice with at least one remaining primordial follicle. Every section was individually analyzed, only using sections with a minimum of one primordial follicle, with the results being expressed as the mean of each section per ovary.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA). A two-way ANOVA with šídák's multiple comparisons test was used to compare the days the mice were in each phase of the estrous cycle. A Chi-square test was used to calculate the correlation between the absolute mice numbers of two groups, as well as between percentages of two groups. The Mann-Whitney test was used for comparison between the median of two experimental groups. For all tests, $P \leq 0.05$ was considered statistically significant.

Results

Rapamycin does not alter recovery of estrous cyclicity after ovarian transplantation

Vaginal cytology analysis was performed from the 10th week after the first VCD injection until and including the 13th week to analyze ovarian function. An absence of estrous cyclicity was found in all of the mice at the end of the 13th week, indicating that all mice had ovarian failure (data not shown). Subsequently, the frozen/thawed ovaries were autotransplanted into the remaining bursa of the non-functional ovary, followed by another round of vaginal cytology analysis starting one week after transplantation. No difference was found in the number of mice that recovered their estrous cyclicity after transplantation between the two experimental groups (SFct: 13 vs. SFrapa: 14) (Fig. 2A). Furthermore, the number of days the mice were in each of the 4 phases of the estrous cycle was calculated, and we found no significant differences between the group autotransplanted with ovaries slow-frozen with rapamycin and the control group (Fig. 2B).

Rapamycin in the freezing medium improved the recovery of fertility after autotransplantation

To investigate whether the addition of rapamycin to the freezing medium could improve fertility recovery in mice with ovarian failure, an ovarian orthotopic autotransplantation model was used. After transplantation, the female mice were mated with 8-week-old males for 4 months. The number of females giving birth, litter size, litter number, and live birth rate were recorded, and the results are summarized in Table 1. During this period, 8 out of 20 control mice gave birth, compared to 13 out of 20 mice whose ovaries were slow-frozen with rapamycin. Furthermore, twice as many pups were born from females with ovaries slow-frozen with rapamycin compared to control mice. Specifically, a total of 48 pups were born from females in the control group, while 102 pups were born from females in the rapamycin group. The live birth rate showed that the addition of rapamycin to the freezing medium resulted in a significantly higher percentage of pups born alive compared to the control ($^{**}P = 0.0025$). The live birth rate for pups born from control mice was 41.7%, compared to 67.7% for pups born from mice in the rapamycin group. Additionally, no difference in the number of litters per female was found between the two experimental groups (SFct: 2.38 ± 0.46; SFrapa: 2.31 ± 0.31), considering only mice with at least one litter. Although not statistically significant, a higher average litter size was observed in mice whose ovaries were slow-frozen with rapamycin (3.43 ± 0.37) compared to mice in the control group (2.53 ± 0.35, $P = 0.0837$). Since not all mice regained their estrous cyclicity post-transplantation, and not all were able to deliver offspring, we explored potential correlations between these factors. A chi-square test revealed a significant association between the restoration of estrous cyclicity and the capacity to produce offspring following transplantation ($^{**}P = 0.0011$, results contain pooled data from the control and rapamycin groups, see figure S1).

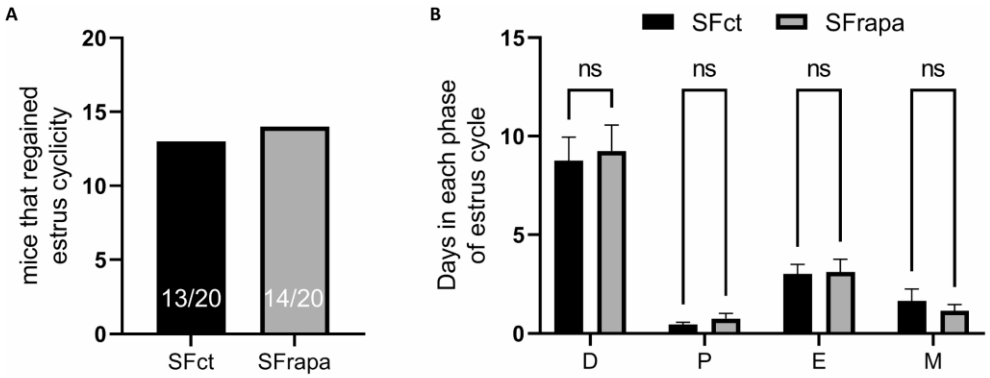

Fig. 2. Estrous cycle recovery after autotransplantation in mice. (**A**) Number of mice that recovered their estrous cycle after autotransplantation in both the control and rapamycin group. (**B**) Mean time (days) that mice were in each phase of the estrous cycle during the 3 weeks post-ovarian-transplantation (mean ± SEM). SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin, D = diestrus, P = proestrus, E = estrus, M = metestrus. Two-way ANOVA with šídák's multiple comparisons: ns = non-significant. $n = 20$ mice per group.

	SFct	SFrapa	Statistics
Total females	20	20	NA
No. of mice giving birth	8 (40%)	13 (65%)	$P = 0.2049^a$
No. of pups born	48	102	NA
Live birth rate	20/48 (41.7%)	69/102 (67.7%)	$P = 0.0025^a$
No. of litter per mouse (minimum 1) (mean ± SEM)	2.38 ± 0.46	2.31 ± 0.31	$P = 0.9739^b$
Litter size (mean ± SEM)	2.53 ± 0.35	3.43 ± 0.37	$P = 0.0837^b$

Table 1. Effects of adding rapamycin to the slow-freezing medium on the fertility of mice after orthotopic ovarian autotransplantation and subsequent mating. NA = not applicable, SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. [a] = Chi-square test, [b] = Mann-Whitney test.

Rapamycin did not affect oocyte numbers after superovulation

At the end of the experiment when the females were ten months old, 8 control mice and 8 mice in the rapamycin group were super-ovulated and oocytes were collected. Three mice in the control group ovulated at least 1 oocyte after superovulation, compared to one mouse in the rapamycin group (Fig. 3A). No significant difference in the mean number of ovulated oocytes was observed between the two experimental groups (Fig. 3B).

Ovarian cysts impact live birth rate regardless of rapamycin treatment

During the recovery of oocytes post-superovulation, the presence of ovarian cysts was examined. Rapamycin did not significantly affect the number of ovaries containing cysts. Specifically, 6 (75%) mice in the control group had ovaries with cysts, compared to 5 (62.5%) mice in the rapamycin group (figure S2). As the presence of ovarian cysts could impair fertility, a correlation assay was performed[36]. No correlation was observed between the presence of cysts and the possibility for mice to deliver offspring ($P = 0.1951$) (Fig. 4A). However, the presence of cysts was significantly correlated to the live birth rate ($^{**}P = 0.0032$) (Fig. 4B). Furthermore, the mean number of pups per dam was significantly lower in mice with cysts present (4 ± 1.29), compared to mice without cysts (10 ± 1.10, $^{*}P = 0.0119$) (Fig. 4C). Data from both control and rapamycin groups were pooled to perform the correlation tests and to calculate the mean number of pups per dam with/without cysts in the ovary.

Rapamycin preserved primordial follicle pool in mice

The use of rapamycin resulted in a higher number of mice still having a primordial pool at the end of the experiment compared to control mice. Besides the superovulation, 20 other females were sacrificed at the end of the experiment and their ovaries were collected to analyze whether they had any remaining primordial follicles. A significant correlation between adding rapamycin to the freezing medium and the presence of primordial follicles at the end of the experiment was found ($^{*}P = 0.0397$). Indeed, 8 mice whose ovaries were slow-frozen with rapamycin still had remaining primordial follicles compared to 3 mice in the control group (Fig. 5A–B).

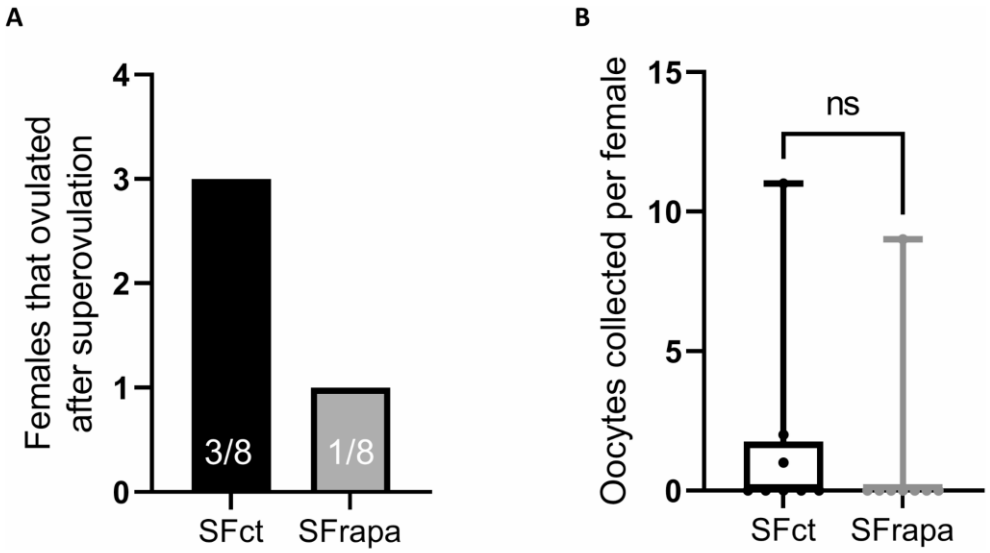

Fig. 3. Superovulation outcome in mice ± 6 months after orthotopic autotransplantation with ovaries slow-frozen in control medium or with ovaries slow-frozen in medium supplemented with rapamycin. (**A**) Comparison of the number of mice (SFct versus SFrapa groups) that ovulated at least one oocyte after stimulation. (**B**) The mean number of oocytes collected per female after superovulation (median + min to max). SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. Mann-Whitney test: ns = non-significant. $n = 8$ mice per group.

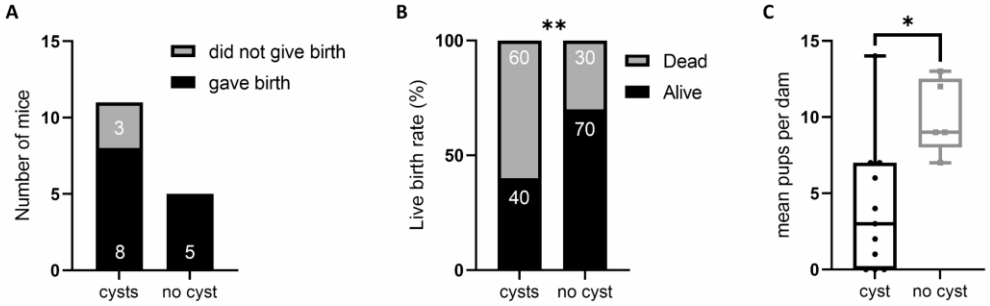

Fig. 4. Correlation between the presence of cysts and various fertility parameters in mice orthotopically autotransplanted with slow-frozen ovaries. The correlation between the presence of ovarian cysts and various fertility parameters was analyzed, including the ability to give birth (**A**), the live birth rate (**B**), and the mean number of pups per dam (**C**) (median + min to max). SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. Chi-square test (**A** and **B**) or Mann-Whitney test (**C**): * $P \leq 0.05$, ** $P \leq 0.01$. $n = 16$ total mice. Results contain pooled data from the control and rapamycin groups.

We then quantified the number of primordial follicles in ovaries from those mice. No difference in primordial follicle density was observed between the two experimental groups (figure S3).

Discussion

Female mice whose ovaries were cryopreserved with rapamycin in the freezing medium prior to chemically disabling the remaining ovary, and subsequent orthotopic autotransplantation gave birth to more pups with

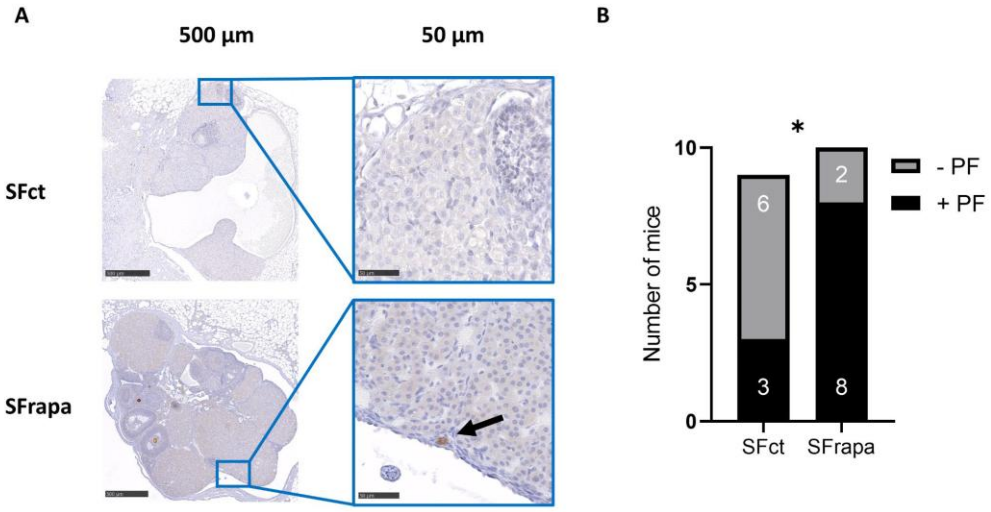

Fig. 5. Evaluation of the presence of primordial follicles at the end of the experiment in mice ovaries orthotopically autotransplanted to the remaining ovarian bursa of C57BL/6 mice for ± 6 months. (**A**) Representative images of DDX4 staining of SFct and SFrapa mice ovaries orthotopically transplanted. The arrow indicates primordial follicle. (**B**) Immunohistochemistry-assisted comparison of the number of mice still having primordial follicles at the time of sacrifice between the SFct and the SFrapa groups, including correlation analysis. SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin, - PF = mice without primordial follicles at the end of the experiment, + PF = mice with primordial follicles left at the end of the experiment. Chi-square test: * $P \leq 0.05$. $n = 9–10$ mice per group.

a significantly greater live birth rate after 4 months of mating compared to mice whose ovaries were frozen in control medium. Additionally, a significantly higher number of mice in the rapamycin group still had primordial follicles at the end of the experiment, and there was a trend indicating that more mice in the rapamycin group gave birth with a higher average litter size. However, no differences were observed in estrous cycle recovery, although a significant association between the restoration of estrous cyclicity and the capacity to produce offspring was found. Furthermore, the number of oocytes collected after superovulation and the number of litters per mouse were similar between the groups, as well as ovarian cyst formation. While rapamycin did not influence cyst formation, we found that mice with ovarian cysts gave birth to significantly fewer pups per dam and had significantly lower live birth rates compared to mice without ovarian cysts. However, no correlation was observed between the presence of cysts and the possibility for mice to deliver offspring.

Autotransplantation of ovarian tissue successfully restored estrous cyclicity in a portion of the mice, regardless of the use of rapamycin during the freezing process. This finding aligns with other studies that have evaluated the effectiveness of ovarian transplantation in restoring hormonal cyclicity. For instance, subcutaneous transplantation of ovarian tissue in bilaterally oophorectomized mice showed approximately 75% of the females regaining cyclicity within 3 weeks[37]. Another study found that orthotopic transplantation of cryopreserved/thawed ovaries could restore the estrous cycle within 2 weeks after grafting in all rats whose normal cyclicity was disrupted by chemotherapy treatment[38].

Interestingly, although we found no statistically significant difference in the proportion of mice recovering estrous cyclicity between the control and rapamycin groups, our results did reveal a significant correlation between restored cyclicity and improved fertility outcomes. This suggests that while returning to normal cyclicity is a key indicator of ovarian function, it is not the sole determinant of successful fertility restoration. Factors such as the quality and quantity of the primordial follicle pool, robust graft revascularization, and the prevention of rapid follicle "burn-out" also play critical roles. Our evidence indicates that rapamycin's protective effect on the primordial follicle reserve likely contributed to better long-term fertility outcomes, even if estrous cycle recovery itself was not significantly different between the groups.

Our previous work has demonstrated that adding rapamycin to the cryopreservation protocol can counteract slow-freezing-induced follicle activation in a heterotopic ovarian transplantation model, providing mechanistic insights into how rapamycin influences the Akt/mTOR pathway[24]. These earlier findings indicated that rapamycin modulates key signaling components involved in follicle survival and quiescence. However, we did not present these mechanistic data in the current manuscript because the experiments were conducted in a different transplantation model and at earlier timepoints more suitable for detecting transient molecular

changes. We chose to avoid conflating two distinct experimental settings and to maintain our focus on the long-term orthotopic transplantation outcomes described herein.

Performing additional molecular analyses in this long-term model would have required sacrificing mice at earlier timepoints to capture the immediate effects of rapamycin on the mTOR pathway. Given the ethical considerations and the study's primary objective of assessing long-term fertility restoration, we opted not to conduct these analyses. Thus, while we acknowledge that direct mechanistic data (e.g., Western blot or qPCR analyses for mTOR and apoptosis markers) would further strengthen the understanding of rapamycin's action, we emphasize that mechanistic aspects have been previously established. Future research could build upon both our earlier mechanistic studies and the current findings by integrating additional timepoints and interventions aimed at delineating the molecular changes across the transplantation timeline.

Our present results showed that adding rapamycin during the cryopreservation of ovaries prior to transplantation to mice with ovarian failure improved fertility preservation and increased graft survival. This was evident from the presence of primordial follicles approximately 6 months after grafting. To the best of our knowledge, the effect of including rapamycin during the slow-freezing process before orthotopic autotransplantation has not yet been studied. Recent studies have looked at various applications of rapamycin, such as injecting the inhibitor after ovarian transplantation, which resulted in higher primordial follicle numbers[19,39]. So far, only one in vivo study showed that the rapamycin pre-treatment before vitrification diminished the loss of follicles after grafting and increased graft survival[40]. All these studies show the potential of rapamycin to protect the follicle pool during cryopreservation, which is in line with the improved fertility preservation we found in our study.

While not significant, we found that the rapamycin group had a higher average litter size (3.43) compared to the control group (2.53). For C57BL/6 mice, the average number of pups per litter is approximately 7[41,42]. Thus, a litter size half of this, as observed in the rapamycin group, is close to the normal condition, considering that the mice have only one functioning ovary after transplantation.

At the end of the experiment superovulation was induced. Considering that our transplanted ovaries were ± 7.5 months old (time without storage in nitrogen), we obtained an average of 1.75 oocytes in the control and 1.13 in the rapamycin group. This superovulation efficiency is low, but aligns with the age-related decline reported in mice, where the oocyte number decreases from an average of 22.5 ± 3.8 oocytes/oviduct in 3-month-old females, to only 2.1 ± 0.2 oocytes/oviduct by 15 months[43]. Additionally, the OTCTP procedure itself can shorten the reproductive lifespan and thereby reduce the superovulatory response. Although rapamycin helps preserve the primordial follicle pool, it was not intended to improve superovulation efficiency. Moreover, we did not conduct direct oocyte quality assessments (e.g., in vitro maturation or fertilization tests) to determine whether rapamycin influenced oocyte competence. Future studies will be necessary to assess younger graft ages and incorporate developmental competence assays to determine whether altered superovulation responses or oocyte quality might be observed under different conditions.

Ovarian manipulation, such as ovarian transposition in women, often leads to cyst formation[28]. Consistent with this, one side effect observed in our study was the formation of ovarian cysts after whole ovary transplantation, irrespective of rapamycin treatment. Indeed, cyst incidence was similar between the groups, occurring in 6 out of 8 mice in the control group, compared to 5 out of 8 in the rapamycin group. This finding aligns with previous reports of cyst formation following subcutaneous/subfascial autotransplantation of fresh whole rat ovaries[29], suggesting that the OTCTP technique itself, rather than the brief exposure to rapamycin, is the primary contributor to cystogenesis. As the presence of ovarian cysts could lead to infertility, we performed a correlation analysis between the presence of cysts and different aspects of fertility[36,44]. No correlation was found between the presence of cysts and the ability to give birth. Females that developed ovarian cysts had fewer pups and a lower live birth rate compared to mice without ovarian cysts. Nonetheless, as the occurrence of cysts was similar in both the control and rapamycin groups, it is unlikely that cyst formation solely accounts for the observed differences in fertility outcomes. Thus, we remain confident that rapamycin's protective effects on the follicle pool are the main driver of the improved fertility measures observed in this study.

One of the strengths of our study is that rapamycin is only added during the cryopreservation process, meaning that there will be no contact between the inhibitor and the graft recipient, minimizing the possibility of side effects. The use of rapamycin during the OTCTP procedure in humans should be relatively simple to implement, as it is already used in clinical settings for preventing organ transplant rejection[21,45]. Additionally, both mice and humans have similar reproductive cycles exhibiting periodic fluctuations in progesterone and estrogen levels. These similarities make mice models suitable for addressing specific research questions about reproduction[46]. To further enhance translatability, a xenograft model, in which human ovarian tissue is transplanted into mice for several months, could be employed in future studies[47]. Such a model would allow for direct evaluation of the long-term safety and potential toxicological effects of rapamycin on human follicles, for instance by examining oocyte morphology. One concern is the potential for toxicity of rapamycin towards oocytes, potentially impairing offspring health. Conversely, rapamycin may also improve oocyte quality. Since we did not thoroughly assess these possibilities here, additional research is required. A future follow-up study could compare the fertility of pups born from females that underwent OTCTP with rapamycin-treated grafts to those from control groups[48,49]. Similar fertility indexes between pups born from mice that underwent OTCTP with rapamycin and those from the control group suggest that rapamycin is not toxic to oocytes and does not cause developmental issues in the pups. Furthermore, to determine whether rapamycin can enhance oocyte competence, in vitro maturation assays could be conducted, analyzing indicators such as first polar body morphology[50]. Because oocyte yield in our current study was low—likely due to the advanced age of the transplanted ovaries—superovulation and oocyte collection at an earlier time point might be necessary to obtain sufficient oocytes for detailed quality assessments.

In conclusion, our findings show that adding the mTOR inhibitor rapamycin during the OTCTP procedure significantly improved fertility restoration in mice. By addressing the main issue of the OTCTP technique - the excessive activation of primordial follicles after grafting - this method shows promise in prolonging the lifespan of the ovarian graft quality. This advancement could extend the window during which recovered patients can conceive, thereby increasing the likelihood of successful pregnancies following OTCTP usage.

Data availability

Data is provided within the manuscript or supplementary information file.

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Author contributions
C.M. and J.B. designed the study and methodology.; J.B. performed the experiments and statistical analysis with input and assistance from M.S. and L.B.; J.B. and C.M. drafted the manuscript.; J.B., C.M., M.S., L.B., and M.N. reviewed and edited the manuscript.; C.M. and M.N. acquired the funding.; C.M. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
This study was approved by the Animal Ethics Committee of the University of Liège (#2270). We confirmed that all experiments in this study were performed in accordance with the relevant guidelines and regulations. All the procedure of the study is followed by the ARRIVE guidelines.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-94588-9.

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Supplementary

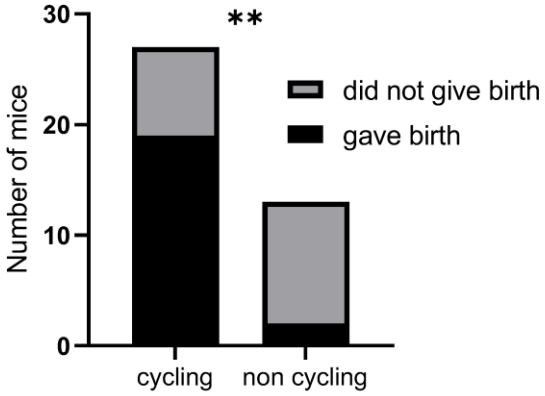

Figure S1. Correlation between having a regular estrus cycle and the ability to give birth for mice orthotopically autotransplanted with ovaries slow-frozen in control medium or with ovaries slow-frozen in medium supplemented with rapamycin after chemical disabling of the remaining ovary. Chi-square test: ** $P \leq 0.01$. n = 40 total mice. Results contain pooled data from the control and rapamycin groups.

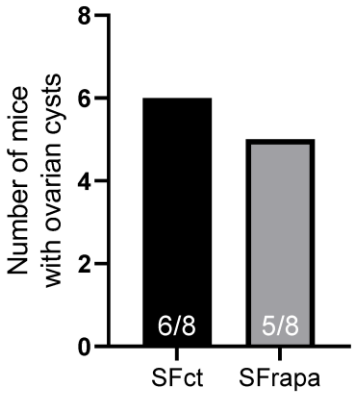

Figure S2. Comparison of cyst formation at the end of the experiment in SFct or SFrapa mice ovaries orthotopically autotransplanted to the remaining ovarian bursa of C57BL/6 mice for a total of ± 6 months. SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. n = 8 mice per group.

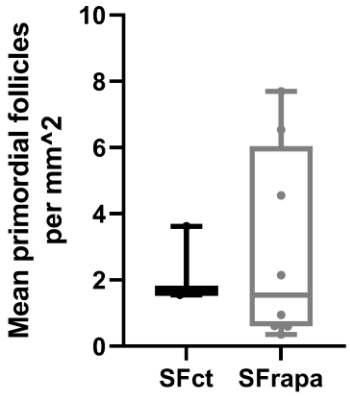

Figure S3. Primordial follicle density assessment at the end of the experiment in mice ovaries orthotopically autotransplanted to the remaining ovarian bursa of C57BL/6 mice for ± 6 months. Results are expressed as the number of primordial follicles per mm^2. SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. Only mice and ovarian sections with at least 1 remaining primordial follicle were taken into account. Each point represents the mean of all section analyzed per ovary. SFct: n = 3 mice, SFrapa: n = 8 mice.

Article 4

BEZ235-mediated PI3K/mTOR dual inhibition improves ovarian follicle survival in a preclinical model

Jules Bindels, Laëtitia Bernet, Marlyne Squatrito, Michelle Nisolle and Carine Munaut

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Reproductive Biology and Endocrinology

RESEARCH Open Access

BEZ235-Mediated PI3K/mTOR dual inhibition improves ovarian follicle survival in a preclinical model

Jules Bindels[1], Laëtitia Bernet[1], Marlyne Squatrito[1], Michelle Nisolle[2] and Carine Munaut[1*]

Abstract

Background Follicular loss after ovarian tissue cryopreservation and autotransplantation (OTCTP) remains a major challenge due to follicle activation and ischemia. We evaluated BEZ235, a dual PI3K/mTOR inhibitor, as a strategy to improve follicle survival in a preclinical model. Its effects were evaluated during ovarian culture, cryopreservation, and transplantation, including the potential benefit of post-grafting VEGF/G-CSF injections.

Methods Murine ovaries, organotipically cultured with chemotherapeutic treatment (4-HC, 2 µM), with or without supplementation with BEZ235 (1 µM), rapamycin (1 µM), LY294002 (25 µM), or AMH (200 ng/ml) were used to evaluate follicle activation. For cryopreservation studies, those inhibitors were added to the freezing medium, and pathways activation were assessed via Western blot. In vivo, ovaries cryopreserved with or without BEZ235 or rapamycin were autotransplanted under the kidney capsule of mice. A subset of mice received intraperitoneal VEGF/G-CSF injections for five days post-transplantation. Follicle quantification, proliferation and activation marker assessment, and fibrosis evaluation were performed three weeks post-grafting.

Results In vitro, BEZ235 significantly counteracted chemotherapy-induced activation of both Akt and mTOR pathways, whereas rapamycin and LY29400 inhibited only mTOR or Akt, respectively. Similarly, during cryopreservation, only BEZ235 significantly reduced activation of both pathways. AMH did not enhance BEZ235's protective effects. In vivo, ovaries slow-frozen with BEZ235 retained a higher percentage of primordial follicles and showed reduced follicle proliferation and activation compared to both control and rapamycin three weeks after transplantation. Additionally, post-grafting injection of VEGF/G-CSF did not further enhance follicle preservation or reduce fibrosis.

Conclusion Dual inhibition of PI3K/mTOR with BEZ235 provides superior protection of the primordial follicle pool by maintaining follicle dormancy, in both in vitro and in vivo models. These findings highlight BEZ235's potential to enhance OTCTP outcomes, extend graft longevity and improve fertility preservation strategies in women.

Keywords Follicle activation, Ovarian tissue cryopreservation, Fertility preservation, Ovarian transplantation, Organotypic ovarian culture, PI3K/PTEN/Akt signaling, mTOR inhibition, BEZ235, Preclinical mouse model

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Background

While high-dose chemotherapy and radiotherapy have drastically increased cancer cure rates [1, 2], a major side effect of these treatments is ovarian failure and infertility, a problem many young women express concerns about [3–6]. For prepubertal female cancer patients and young women requiring urgent oncological care, the only available fertility preservation option remains the

cryopreservation of cortical ovarian tissue followed by autotransplantation (OTCTP) [7]. A major advantage of the OTCTP technique is that it can be performed immediately, and will thus not lead to a delay in treatment [8]. Additionally, it can restore both natural fertility as well as the endocrine function of the gonads [9]. To date, over 200 live births have been reported following the use of OTCTP [10, 11].

Despite its success, OTCTP has several limitations, including significant follicular loss immediately after grafting, leading to premature ovarian failure [12, 13]. This rapid depletion of the follicular reserve is likely attributed to factors such as delayed neovascularization, apoptosis, and excessive follicle recruitment, a phenomenon termed follicular "burn-out"[14–16]. This process can substantially reduce the longevity of the graft. Under normal physiological conditions, the preservation of the primordial follicle reserve is regulated by an equilibrium between activation signals, such as those originating from granulosa cells, and inhibitory signals, including anti-Müllerian hormone (AMH), secreted by growing follicles. However, during the OTCTP process, a significant proportion of growing follicles do not survive. This loss reduces inhibitory signals, disrupting the balance and tipping it toward excessive follicle activation. Consequently, this imbalance accelerates follicle recruitment, ultimately depleting the primordial follicle reserve [14, 17, 18].

Two key pathways involved in follicle activation are the phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Akt pathway and the mammalian target of rapamycin (mTOR) pathway. Indeed, several inhibitors targeting these pathways have been investigated to mitigate excessive follicle activation [18–24]. Among them, rapamycin, a specific mTOR inhibitor, has shown promising results in protecting the primordial follicle pool in multiple experimental settings [22–24]. Our previous in vitro and in vivo studies demonstrated that adding rapamycin to the freezing medium suppresses slow-freezing and transplantation induced follicle activation and proliferation in mouse ovaries [25, 26].

While these findings suggest that rapamycin helps to maintain transiently primordial follicles in a quiescent state, this inhibitor was not able to prevent the decline of primordial follicles induced by slow-freezing and transplantation [26]. Since we previously observed that Akt activation increased when murine ovaries were slow-frozen with rapamycin, and subsequently subjected to whole organotypic culture, likely as a result of feedback activation of Akt following mTOR inhibition [25, 27]. Based on these findings, we now first aimed to identify alternative inhibitors of follicle activation pathways to better prevent follicular depletion. To this end, we compared the dual mTOR/PI3K inhibitor BEZ235, with previously investigated rapamycin and the PI3K inhibitor LY294002. Using whole murine ovary organotypic in vitro culture and ex vivo slow-freezing of 4–7-days-old mouse ovaries, we identified the most promising inhibitor, which was then tested against rapamycin in a heterotopic ovarian transplantation mouse model.

In addition to pharmacological inhibitors, the natural follicle inhibitor AMH has been examined in several in vivo studies, though its role in follicle preservation remains inconclusive [28–30]. A major benefit of AMH is that it acts ovary-specific and may thus reduce systemic side effects [21, 31]. Injection of AMH into mice before and/or after OTCTP showed no significant differences in the primordial follicle pool compared to the control [28]. Conversely, co-transplantation of ovarian tissue with endothelial cells constitutively secreting AMH resulted in a higher primordial follicle pool compared to the control group [30]. While previous studies yielded mixed results on AMH's protective effects during OTCTP, recent findings are more promising [21, 31]. One study reported that mice injected with AMH following ovarian transplantation retained a larger primordial follicle pool than controls [21]. Another study, in which human ovarian cortical biopsies were cultured with the active cyclophosphamide metabolite 4-hydroperoxycyclophosphamide (4-HC) and either rapamycin or AMH, found that AMH significantly reduced both follicle damage and activation induced by 4-HC, whereas rapamycin only counteracted follicle activation [31]. As these recent studies have highlighted the potential of AMH to protect the primordial follicles pool, our second objective was to compare and combine BEZ235 and AMH in a murine ovarian culture experiment.

While targeting follicle activation pathways offers a promising approach to preserving the ovarian reserve, optimizing graft survival also requires addressing ischemia and hypoxia—two other major contributors to post-transplantation follicular loss. Ischemic damage occurs during the first five days following transplantation, before revascularization is fully established, leading to significant follicle depletion [32, 33]. Given the importance of restoring oxygen supply in graft viability, we previously investigated angiogenic strategies to mitigate hypoxia-induced damage before shifting our focus to follicle activation pathways. Various angiogenic factors have been evaluated in cryopreservation and transplantation models [34–38]. As part of our earlier work, we investigated the role of vascular endothelial growth factor (VEGF) A, a key regulator of blood vessel formation that acts through VEGFR-1 and −2, both expressed in granulosa cells [35, 36, 39]. In one study sheep ovarian tissue was encapsulated with $VEGF_{111}$ in a collagen matrix

before xenotransplantation into SCID mice, resulting in improved revascularization and reduced loss of primary follicles 3 weeks post-grafting [35]. A follow-up study with $VEGF_{165}$ demonstrated its ability to enhance vascularization by significantly increasing the number of functional vessels as early as 3 days post-transplantation, though no significant difference in the primordial follicle pool was observed [36]. These findings underscored the importance of early graft revascularization but also highlighted the need for additional strategies to optimize ovarian tissue viability. This led us to shift toward targeting follicle activation pathways as a complementary approach to improve long-term graft survival.

In contrast, a study in which mice received VEGF in combination with granulocyte colony-stimulating factor (G-CSF) after orthotopic ovarian transplantation demonstrated significantly better preservation of the primordial follicle pool compared to saline control [40]. Building on these findings, we combined slow-freezing of murine ovaries with BEZ235, followed by post-grafting injection of VEGF and G-CSF, to evaluate whether this strategy could enhance primordial follicle pool protection.

Considering these observations, there is still a clear need for strategies maintaining the follicle pool throughout the OTCTP process. Therefore, we aimed to evaluate dual inhibition of PI3K and mTOR with BEZ235 and compare its effects with other inhibitors and angiogenic compounds in multiple in vitro and in vivo models. By systematically comparing these approaches, our study seeks to establish a robust method for keeping primordial follicles in a dormant state during cryopreservation, ultimately improving fertility preservation outcomes for patients undergoing OTCTP.

Methods

Ovarian retrieval and processing for in vitro studies

Eight-weeks-old C57BL/6 mice were obtained from Charles River and maintained at the accredited Mouse Facility of the University of Liège (Belgium). The mice were housed at ± 21 °C in a 12 h light/dark cycle with a maximum of five mice per cage, with food and water provided ad libitum. The mice were mated on a 1:2 male and female ratio. Pups were sacrificed at 4–7-days-old and ovaries were collected. Additionally, ovaries from C57BL/6 mice (4-weeks-old) that underwent oophorectomy from another project were collected for our studies. The ovaries were put in a transport solution composed of Leibovitz L-15 medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Gibco, Waltham, MA, USA). The adjacent oviduct and fat tissue were removed from the ovaries using a scalpel under a binocular microscope. Part of the adult ovaries were slow-frozen for later use, and the other adult ovaries were freshly used in culture experiment. All pup ovaries were slow-frozen to compare the effects of adding different inhibitors to the slow-freezing medium on follicle activation pathways. This study was approved by the Animal Ethics Committee of the University of Liège (#1934 and #2594). We confirmed that all experiments in this study were performed in accordance with the relevant guidelines and regulations.

Slow-freezing and thawing procedure

Designated ovaries were cryopreserved as described before [41]. Briefly, whole ovaries were placed in a cryopreservation solution containing Leibovitz L-15 medium supplemented with 10% FBS, 10% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany), and 0.1 M sucrose. After equilibration for 30 min at 4 °C, ovaries were placed in cryovial tubes (Simport, Montreal, QC, Canada) containing the cryopreservation solution and subsequently cooled in a programmable freezing machine (CL-8800i System; CryoLogic, Mulgrave, Victoria, Australia) as previously described and stored in liquid nitrogen [42]. For the pup ovaries, rapamycin (InvivoGen, Toulouse, France), LY294002 (InvivoGen, Toulouse, France), or BEZ235 (Selleckchem, Cologne, Germany) was added to the transport and cryopreservation solutions.

The thawing process was carried out by incubating the cryovials at room temperature (RT) for 2 min, followed by an additional 2-min incubation in a 37 °C water bath. Cryoprotective agents and/or inhibitors were removed by washing the ovaries three times in Leibovitz L-15 medium, with each wash lasting 5 min at 37 °C. Pup ovaries were immediately snap-frozen after thawing for Western blot analysis.

Whole ovary organotypic in vitro culture

Ovaries were organotypically cultured as described before [25]. Briefly, 4-weeks-old murine ovaries were cultured for a total of 25 h at 37 °C in a 12-well plate on inserts (ThinCerts 0.4 µm PET, Greiner Bio-One, Kremsmünster, Austria) with 2–3 ovaries per insert. The culture medium was composed of Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Gibco, Waltham, MA, USA) with 1% Bovine Serum Albumin (Thermo Fisher Scientific Gibco, Waltham, MA, USA), 1% penicillin–streptomycin (Thermo Fisher Scientific Gibco, Waltham, MA, USA), 1% L-Glutamine (Thermo Fisher Scientific, Gibco, Waltham, MA, USA), 1% insulin (1000 mg/l), transferrin (550 mg/l) and selenium (0.67 mg/l) mixture (Thermo Fisher Scientific, Gibco, Waltham, MA, USA), 2.5% hFSH (Sigma-Aldrich, St. Louis, MO, USA) and 0.5% ascorbic acid (Sigma-Aldrich, St. Louis, MO,

USA). The medium was further supplemented with rapamycin, LY294002, BEZ235 or AMH (R&D systems, Minneapolis, MN, USA). After 2 h, 4-HC (2 µM, Santa Cruz, Heidelberg, Germany) was added to the appropriate wells. At the end of the experiment, ovaries were either snap-frozen for Western blot analysis or fixed in 4% formaldehyde for immunohistochemistry (IHC) analysis.

In vivo ovarian autotransplantation under the kidney capsule

Under gas anesthesia (Isoflurane, Dechra, Northwich, UK), 4-weeks-old C57BL/6 mice (Charles river) underwent bilateral oophorectomy, and ovaries were placed in ovarian transport solution. The ovaries were prepared, and slow-frozen in medium supplemented with or without rapamycin or BEZ235. After thawing, ovaries were autotransplanted under the kidney capsule as described before [26, 43].

Following autotransplantation, part of the mice received daily intraperitoneal injections (IP) for 5 consecutive days, starting the day of grafting, with $VEGF_{165}$ (R&D systems, Minneapolis, MN, USA), G-CSF (R&D systems, Minneapolis, MN, USA), or their combination.

Grafted ovaries were collected 3 weeks after transplantation and fixed in 4% formaldehyde overnight, after which ovaries were put in 70% ethanol. Fixed ovaries were embedded in paraffin and cut into 5 µm sections using a microtome and mounted on slides for histological assessment.

Immunohistochemistry and sirius red staining

Follicle quantification and primordial follicle health assessment was facilitated by labeling ovarian sections with DEAD-box helicase 4 (DDX4; Abcam ab41519, Cambridge, UK), a helicase located in germ cells, and therefore useful as a follicle identifier [44]. Follicle proliferation and activation of the Akt and mTOR pathways were identified by immunostaining for Ki67 (Abcam ab16667, Cambridge, UK), phosphor-Akt (pAkt; Abcam ab81283, Cambridge, UK), and phosphor-Rps6 (pRps6; Cell signaling #2211, Danvers, MA, USA), respectively. A Sirius red staining was performed to identify fibrosis. For immunostainings, ovarian sections were deparaffinized and rehydrated prior to antigen retrieval, which was performed using an autoclave at 126 °C and 1.3 bar for 11 min in citrate buffer (Dako, Glostrup, Denmark). The sections were then allowed to cool for 20 min. To block endogenous peroxidase activity, the sections were incubated with 3% hydrogen peroxide for 20 min at room temperature (RT). Non-specific binding sites were subsequently blocked using Animal-Free Blocking Solution (Cell Signaling, Danvers, MA, USA) for 20 min at RT. Primary antibodies diluted in REAL Antibody Diluent (Dako, Glostrup, Denmark) were incubated for 1 h at RT. The following dilutions were used: DDX4 (1:600), Ki67 (1:100), pAkt (1:250), and pRps6 (1:400). Subsequently, sections were incubated with a secondary antibody conjugated to horseradish peroxidase (HRP; ENVISION/HRP, ready-to-use, Dako, Glostrup, Denmark) for 30 min at RT. For chromogenic detection, staining was developed with DAB+ (Dako, Glostrup, Denmark), followed by hematoxylin counterstaining, and sections were mounted using Entellan New Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). For fluorescent staining, the fluorescein tyramide kit (PerkinElmer, Waltham, MA, USA) was applied for 10 min, and sections were mounted with DAPI Fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL, USA). Stained sections were scanned using either the NanoZoomer 2.0 HT digital slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan) or the Olympus SLIDEVIEW VS200 high digital slide scanner (Olympus Corporation, Tokyo, Japan).

Histological follicle and fibrosis assessment

Sections labeled for DDX4 were examined using NDP.view2 software (Hamamatsu Photonics K.K., Hamamatsu, Japan). For each ovary, four to five sections, each 5 µm in thickness, were analyzed in a blinded manner. Successive sections were obtained at 50 µm intervals along the ovary. Follicles were classified into primordial, primary, or secondary or more growing based on morphological mouse follicle classification [45]. This classification was performed manually by examining each section, counting, and categorizing all follicles accordingly. Data were expressed as the percentage of each follicle type relative to the total follicle count per section. Individual sections were analyzed separately, and the mean values for follicle density were subsequently calculated across the analyzed sections for each ovary.

The health status of primordial follicles was classified based on morphological criteria as described previously [31, 46]. Both oocytes and granulosa cells were assessed for signs of degeneration. Oocytes were classified as unhealthy or degenerating if they exhibited condensed nuclear chromatin and/or overall compromised cellular morphology. Granulosa cells were deemed unhealthy if the majority within the follicle displayed irregular shapes and/or condensed chromatin. Follicles were classified as unhealthy if either the oocyte, the granulosa cells, or both demonstrated these characteristics.

To investigate follicle proliferation and activation, ovarian sections were double-stained for DDX4 in combination with Ki67, pAkt, or pRps6. Primordial and primary follicles were manually identified using DDX4 staining and subsequently classified as either positive or negative for the target protein based on visual assessment of the

staining (NDP.view software). Data were expressed as the percentage of protein-positive follicles relative to the total number of primordial and primary follicles.

To assess fibrosis formation, the QuPath 5.0 create pixel threshold function was used on sections stained for Sirius red. Using this function, the percentage of fibrotic area within the ovarian sections could be calculated.

For each ovary, every section was assessed individually, and the mean values from all analyzed sections were calculated to obtain the results for individual ovaries.

Western blot

For protein extraction from 4-weeks-old ovaries, one ovary was used for each sample, while for 4–7-days-old ovaries, four ovaries were pooled to obtain sufficient protein concentrations. Radioimmunoprecipitation assay (RIPA) buffer containing 4% of a protease and phosphatase inhibitor (Roche, Basel, Swiss) was used for the extraction, and lysate collected. The protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were denatured and separated by electrophoresis on 12% SDS–polyacrylamide gels. After the migration was complete, proteins were transferred onto a polyvinylidene difluoride membrane (PerkinElmer, Waltham, MA, USA) for 1 h at 100 v. After blocking for 2 h with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), proteins were incubated with respective primary antibodies diluted 1:1000 in blocking solution according to the manufacturer's protocol. Akt (Cell Signaling, #9272), pAkt (Cell Signaling, #9271), Rps6 (Cell Signaling, #2217), and pRps6 (Cell Signaling, #2211) primary antibodies were incubated overnight at 4 °C. The primary antibody for actin (Sigma, A2066) was incubated 1 h at RT. The appropriate HRP-conjugated secondary antibody was added to the membrane, followed by a 1 h incubation at RT. For (p)Akt and (p)Rps6, we used the goat anti-rabbit HRP-linked secondary antibody (1:2000; Cell signaling, #7074), and for actin, the swine anti-rabbit Immunoglobulins/HRP secondary antibody was used (1:1000; Dako, P0217). After sequential washing of the membranes to remove excess secondary antibody, signals were detected using an enhanced chemiluminescence (ECL) kit (PerkinElmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions in an Amersham ImageQuant 800 imager (Cytiva, Marlborough, MA, USA). The intensities of the protein bands were quantified using Quantity One Analysis software. To analyze Akt and Rps6 activation, the ratio of phosphorylated to total Akt and Rps6 was calculated. Data are expressed as the fold-change compared to the control group. Actin expression was used to verify equal loading and used for normalization when equal loading was not observed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA). The Anderson–Darling, D'Agostino & Pearson, Shapiro-Wil and Kolmogorov–Smirnov tests for normal distribution were performed. Ordinary one-way ANOVA with Tukey's post hoc test was applied for normal distributed multigroup comparisons. When normal distribution was not assumed, the Kruskal–Wallis test with Dunn's multiple comparison post hoc test was used when comparing three or more groups. A Chi-square test was performed on the percentages of healthy and unhealthy primordial follicles. For all tests, $P \leq 0.05$ was considered statistically significant. Data is shown as median + min to max.

Results

BEZ235 mitigates chemotherapy-induced activation of both Akt and mTOR pathways in organotypic ovarian culture

Fresh whole 4-weeks-old murine ovaries were organotypically cultured with BEZ235 (1 µM [47]), rapamycin (1 µM [48]), and LY294002 (25 µM [25]) to assess their ability to suppress chemotherapy-induced PI3K/Akt/mTOR pathway activation. While BEZ235 has not previously been tested in this specific setting, a dose–response study in ovarian cancer cell lines identified 1 µM as the minimum concentration that substantially inhibited multiple direct and indirect targets of PI3K and mTOR without inducing apoptosis [47]. Based on this evidence of effective dual pathway inhibition and lack of cytotoxicity, we selected this dose for our experiments. Western blot analysis was used to assess pathway activation by measuring the phosphorylated and total forms of Akt and Rps6. Rapamycin reduced mTOR activation without affecting Akt, LY294002 inhibited Akt only, while BEZ235 significantly suppressed both pathways (Fig. 1A-B). These findings suggest that BEZ235 is the most promising candidate for maintaining follicle quiescence in vitro.

BEZ235 in the slow-freezing medium prevents cryopreservation-induced follicle activation in thawed mouse ovaries

BEZ235 (1 µM), rapamycin (1 µM), or LY294002 (25 µM) were next added to the freezing medium during cryopreservation of 4–7-days-old mouse ovaries. After thawing, Western blot analysis was performed to assess Akt and mTOR pathway activation.

Ovaries cryopreserved with BEZ235 exhibited significantly lower activation of both the Akt and mTOR pathways compared to ovaries slow-frozen in control

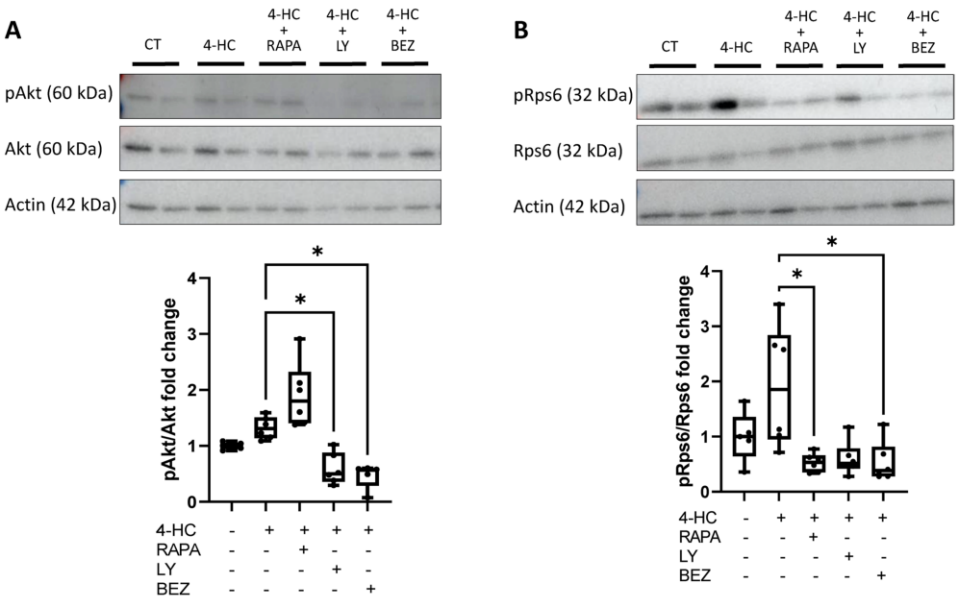

Fig. 1 Effects of In Vitro Organotypic Ovarian Culture under Chemotherapeutic Conditions on Follicle Pathway Activation. Fresh ovaries from 4-weeks-old C57BL/6 mice were cultured for 24 h in the presence of rapamycin (1 µM), LY294002 (25 µM), or BEZ235 (1 µM) under chemotherapeutic conditions with 4-HC (4-hydroperoxycyclophosphamide). **A** Fold change of phosphorylated to total protein form ratio of Akt and **B** Rps6, including representative blots. CT = control, LY = LY294002, RAPA = rapamycin, BEZ = BEZ235. $n = 6$ samples/group. * $p \leq 0.05$

medium. In contrast, LY294002 and rapamycin significantly reduced mTOR pathway activation, but both only showed a non-significant trend to lower Akt activation (Fig. 2A-B). These results confirm that among the tested inhibitors, BEZ235 is the most effective at inhibiting cryopreservation-induced activation of both pathways.

BEZ235 offers better protection of the primordial follicle pool against chemotherapy compared to AMH in an ovarian culture experiment

Frozen/thawed whole 4-weeks-old murine ovaries were cultured with the chemotherapeutic compound 4-HC in the presence of BEZ235 (1 µM), AMH (200 ng/ml [31]), or a combination of both inhibitors to compare their protective effects against chemotherapy-induced damage. Western blot analysis revealed that treatment with BEZ235 alone or in combination with AMH significantly counteracted chemotherapy-induced Akt activation, whereas AMH alone had no significant effect (Fig. 3A). Moreover, Rps6 activation was significantly lower in ovaries treated with BEZ235 compared to those treated with AMH (Fig. 3B). Follicle quantification and primordial follicle health assessment demonstrated 4-HC treatment in the presence of BEZ235 resulted in a significantly higher percentage of primordial follicles compared to controls. This effect was not observed with AMH alone or in combination with BEZ235 (Fig. 3C). Furthermore, 4-HC significantly increased the proportion of unhealthy primordial follicles, an effect that was significantly mitigated by BEZ235, AMH or their combination (Fig. 3D-E). Overall, these findings suggest BEZ235 is the preferred inhibitor over AMH for preserving primordial follicles under chemotherapeutic conditions in vitro. The combination of AMH with BEZ235 does not further enhance BEZ235's protective effect.

Cryopreservation with BEZ235 better preserves the follicle pool than rapamycin after transplantation

Having established BEZ235 as a promising inhibitor of follicle activation pathways during cryopreservation, we next compared its effects in vivo with rapamycin using a murine transplantation model. Ovaries from 4-weeks-old mice cryopreserved with either BEZ235 (1 µM) or rapamycin (1 µM) were autotransplanted under the kidney capsule and recovered three weeks later (Fig. 4A). Follicle quantification using

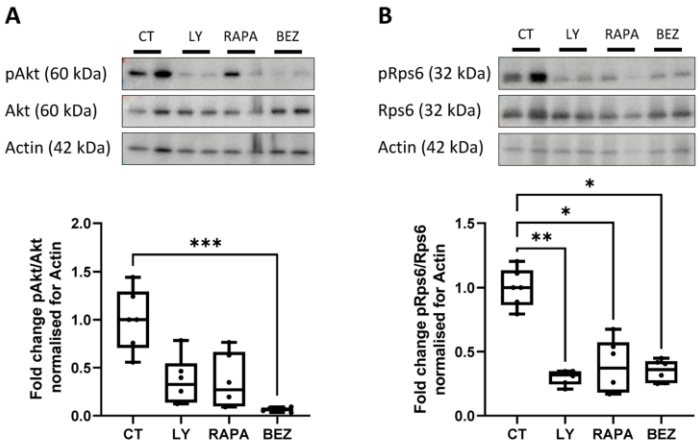

Fig. 2 Effects of Adding Rapamycin, LY294002, or BEZ235 During Cryopreservation on Follicle Activation in Murine Ovaries. Ovaries from 4–7-days-old C57BL/6 mice were cryopreserved with or without rapamycin (1 µM), LY294002 (25 µM), or BEZ235 (1 µM). **A** Fold change of phosphorylated to total protein form ratio of Akt and **B** Rps6, normalized for Actin, including representative blots. CT = control, LY = LY294002, RAPA = rapamycin, BEZ = BEZ235. $n = 6$ samples/group, with each sample comprising of 4 pooled ovaries. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

DDX4 labeling of ovarian sections revealed that slow-freezing with BEZ235 preserved a significantly higher percentage of primordial follicles compared to control ovaries slow-frozen without any inhibitor, whereas no significant difference was observed between the rapamycin and control groups. The percentage of primary follicles remained similar across all groups. However, the control group exhibited a higher percentage of secondary or more developed follicles compared to the BEZ235 group (Fig. 4B). To further compare the effects of BEZ235 and rapamycin on follicle proliferation and activation, ovarian sections were stained for Ki67, pAkt, and pRps6. Ovaries slow-frozen with BEZ235 contained significantly fewer Ki67-positive primordial and primary follicles compared to the controls, whereas rapamycin only showed a trend toward reduced proliferation (Fig. 4C). Significantly fewer primordial and primary follicles were pAkt-positive in the BEZ235 group compared to both the control and rapamycin groups (Fig. 4D). Additionally, cryopreservation with BEZ235 resulted in significantly fewer pRps6-positive primordial and primary follicles compared to the control, while this effect was not observed with rapamycin (Fig. 4E). Collectively, these results suggest that BEZ235 provides superior follicle pool protection during cryopreservation and transplantation compared to rapamycin.

Injection of VEGF/G-CSF after OTCTP does not enhance the protective effects of cryopreservation with BEZ235

We next evaluated whether post-transplantation injection of VEGF and G-CSF could enhance the protective effects of adding BEZ235 during cryopreservation in an in vivo setting. Using a 4-weeks-old murine transplantation model, we administered daily IP injections of $VEGF_{165}$ (8 µg/kg/day [40]), G-CSF (50 µg/kg/day [40]), or their combination for five consecutive days, starting on the day of ovarian autotransplantation under the kidney capsule. Ovaries were recovered three weeks later (Fig. 5A).

Primordial follicle quantification using DDX4 labeling revealed that, in ovaries slow-frozen in control medium, injection with a combination of VEGF and G-CSF resulted in a significantly higher percentage of primordial follicles compared to the other groups (Fig. 5B). However, in ovaries slow-frozen with BEZ235, post-grafting injection with VEGF, G-CSF or their combination had no additional effects on the percentage of primordial follicles (Fig. 5C).

To further assess follicle proliferation, Ki67 staining was performed on ovarian sections. Consistent with our previous findings, slow-freezing with BEZ235 significantly reduced percentage of proliferating primordial and primary follicles compared to control. However, post-grafting injection of VEGF, G-CSF or both did not

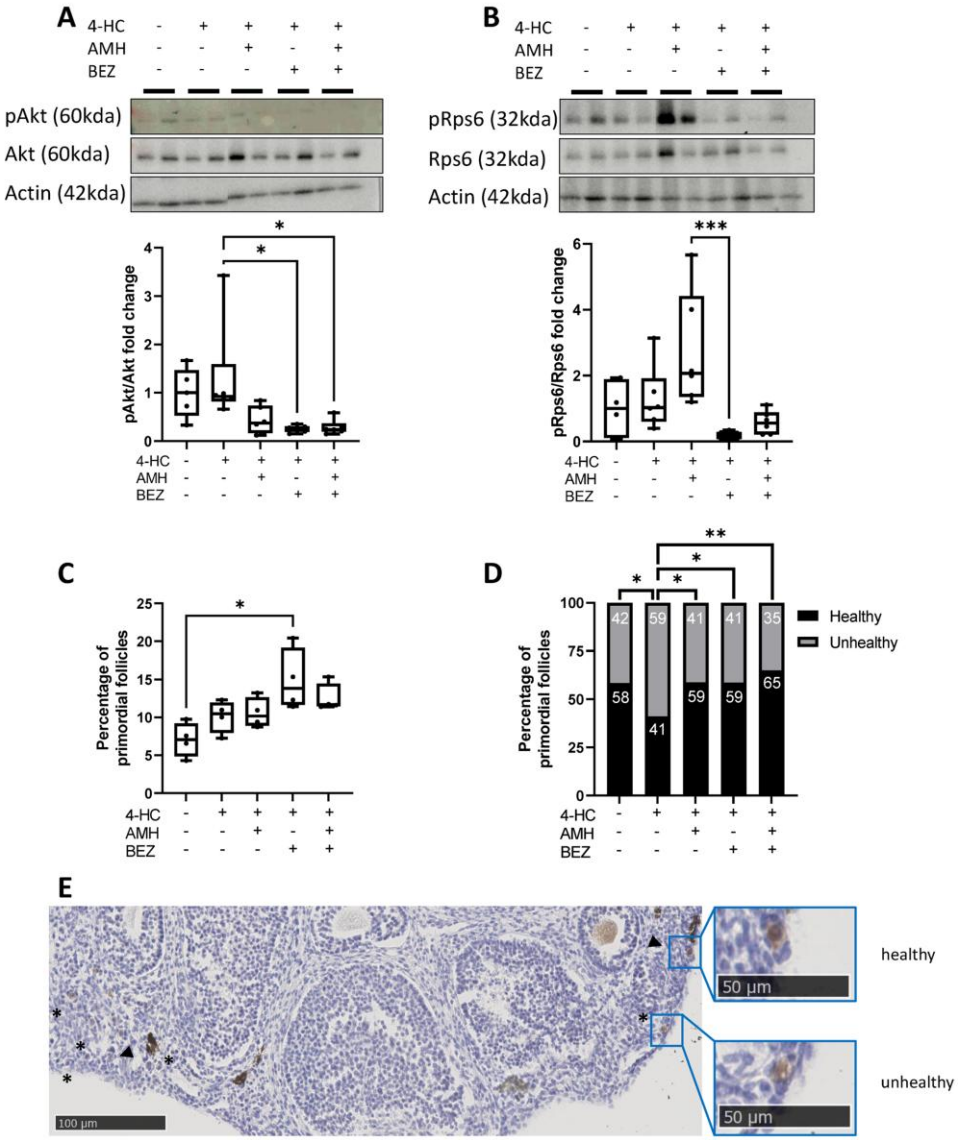

Fig. 3 Effects of BEZ235 and AMH on Follicular Preservation Under Chemotherapeutic Conditions in an Organotypic Ovarian Culture. Frozen/thawed ovaries from 4-weeks-old C57BL/6 mice were cultured for 24 h under chemotherapeutic conditions (4-HC) with or without AMH (200 ng/ml) or BEZ235 (1 µM). **A** Fold change of phosphorylated to total protein form ratio of Akt and **B** Rps6, including representative blots. **C** Quantifications of primordial follicles and **D** health assessment. **E** Representative image with unhealthy primordial follicles represented by asterisks and healthy primordial follicles by arrowheads. 4-HC = 4 hydroxy cyclophosphamide, AMH = Anti-Müllerian hormone, BEZ = BEZ235. $n = 4–6$ ovaries/group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

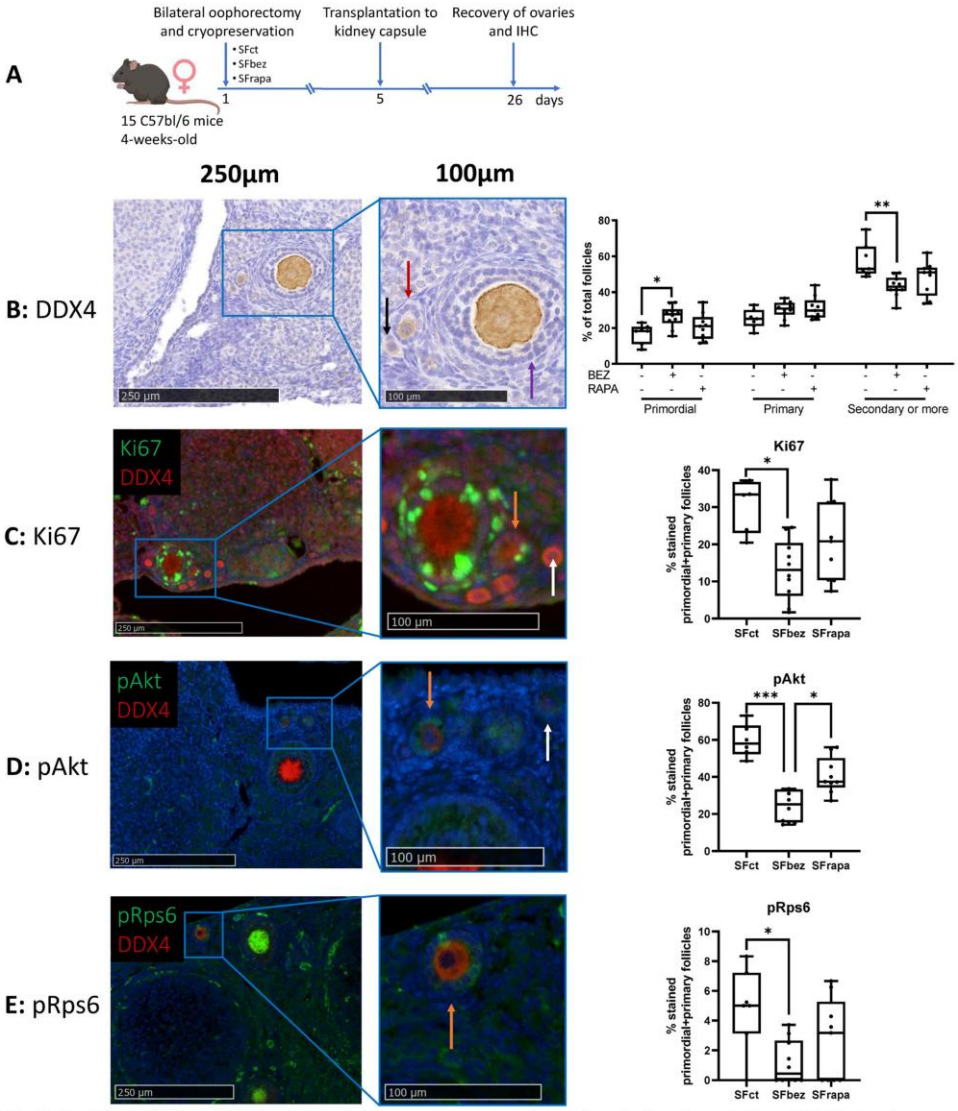

Fig. 4 In Vivo Effects of BEZ235 on Follicle Preservation After Cryopreservation and Transplantation. Experimental scheme (A). IHC assisted follicle quantification (B, DDX4), and analysis of follicle proliferation (C, Ki67) and activation (**D**, pAkt and **E**, pRps6), in ovaries slow-frozen in control medium or medium supplemented with rapamycin (1 µM) or BEZ235 (1 µM), autotransplanted under the kidney capsule of C57BL/6 mice, including representative images. SFct = slow-frozen/thawed ovaries in control medium, SFrapa = slow-frozen/thawed ovaries in medium supplemented with rapamycin, SFbez = slow-frozen/thawed ovaries in medium supplemented with BEZ235. $n = 7–10$ ovaries/group. Arrows: black = primordial follicle, red = primary follicle, purple = secondary or more growing follicle, white = negative staining, orange = positive staining. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

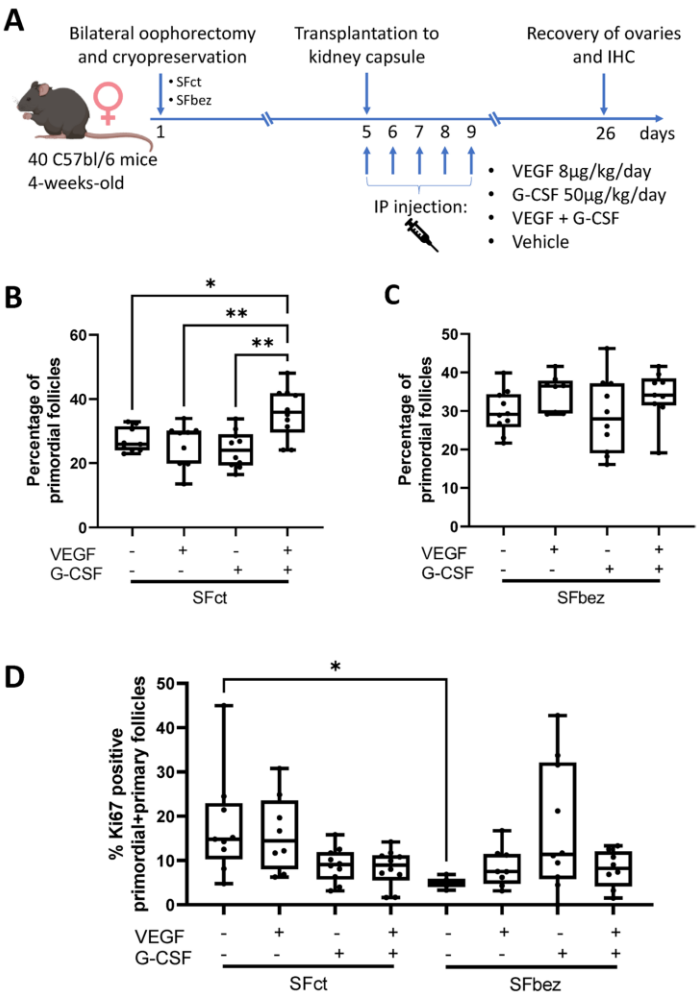

Fig. 5 Effects of VEGF and G-CSF on Follicle Preservation in BEZ235-Treated Cryopreserved Ovaries Post-Transplantation. **A** Experimental scheme. **B**, **C** IHC-assisted primordial follicle quantification in ovaries slow-frozen in control medium (SFct) or medium supplemented with BEZ235 (SFbez, 1 µM), autotransplanted under the kidney capsule of C57BL/6 mice, followed by intraperitoneal (IP) injection of VEGF (8 µg/kg/day), G-CSF (50 µg/kg/day), or their combination. **D** Analysis of primordial and primary follicle proliferation via Ki67 immunostaining. $n = 9–10$ ovaries/group. * $p \leq 0.05$, ** $p \leq 0.01$

significant affect follicle proliferation in either the control or BEZ235 groups (Fig. 5D). Furthermore, Sirius red staining was used to examine fibrosis, revealing no significant differences in ovarian fibrotic area among experimental groups (See Figure S1 in Supplementary material 1).

Collectively, these results suggest that post-grafting injection of VEGF and G-CSF does not enhance the protective effects of using BEZ235 during cryopreservation.

Discussion

Follicular loss immediately after grafting remains a major limitation of the OTCTP technique, largely due to increased follicle activation and ischemia [14–16]. In this study, we aimed to mitigate these effects by targeting the PI3K/PTEN/Akt and mTOR pathways, which are critical regulators of primordial follicle activation and growth through extracellular growth factor signaling. Indeed, manipulating these pathways produced significant changes in primordial follicle counts in cultured ovaries [25, 49, 50].

Our previous work has demonstrated that rapamycin can maintain primordial follicles in a quiescent state but failed to prevent follicle depletion following OTCTP [26]. Additionally, we also reported that Akt activation increased when murine ovaries were slow-frozen with rapamycin and subsequently cultured in vitro [25]. These findings highlighted the limitations of rapamycin, prompting us to explore alternative inhibitors with comprehensive pathway inhibition.

In order to find an alternative inhibitor of the follicle activation pathways, we compared the previously investigated mTOR inhibitor rapamycin and the PI3K inhibitor LY294002 with BEZ235, an inhibitor already being tested in clinical trials as a therapy for several cancers, especially in combination with other inhibitors, such as everolimus [51].

In our first in vitro experiment we cultured fresh 4-weeks-old murine ovaries to compare these inhibitors, which is a useful model as follicles are activated during culture [52]. The addition of 4-HC during culture, known to induce Akt pathway activation [31], tended to amplify follicle activation, thereby allowing a better visualization of the inhibitors' efficacy. Only BEZ235 counteracted chemotherapy induced Akt and mTOR pathways activation. While BEZ235 had not been tested before in a similar setting, culture of ovarian cancer cell lines with BEZ235 showed similar effects on PI3K/Akt/mTOR pathway activation [47, 53]. As we found before, rapamycin treatment tended to increase Akt activation, likely due to feedback activation following mTOR inhibition [27]. We next added the inhibitors during the cryopreservation of 4–7-day-old mouse ovaries. To the best of our knowledge, this experimental setting with BEZ235 has not been previously performed. Only BEZ235 significantly counteracted Akt and mTOR pathway activation. While rapamycin and LY294002 both significantly decreased Rps6 activation, they only showed a non-significant trend towards Akt inhibition.

While these results using pharmacological inhibitors show encouraging results, recent studies using the natural inhibitor AMH, and combining it with other inhibitors, had peaked our interest [31] [54]. Therefore, we compared and combined BEZ235 and AMH in a culture experiment using slow-frozen/thawed 4-weeks-old murine ovaries. We found that the combination of BEZ235 and AMH did not yield any additive or synergistic benefits beyond those observed with BEZ235 alone on maintaining follicles in a quiescent state. Surprisingly, in this setting, 4-HC had no effect on Akt and Rps6 activation, likely due to using frozen/thawed ovaries instead of fresh ones. Unlike in our other culture experiment using fresh ovaries, where a trend toward 4-HC-induced activation of Akt and Rps6 was observed, such an effect may have been masked here. As slow-freezing alone can upregulate these pathways, additional effects of 4-HC are potentially obscured [25]. Nonetheless, the consistency of BEZ235's effects across experiments supports the reliability of our findings.

Although the preceding results underscore the superior efficacy of BEZ235 over AMH in preserving the primordial follicle pool by inhibiting activation, follicle loss is also driven by apoptosis [55, 56]. We therefore assessed primordial follicle health and found that both AMH and BEZ235, alone or in combination, were associated with a higher proportion of morphologically healthy primordial follicles following chemotherapy exposure. This aligns with a study on human ovarian biopsies cultured with 4-HC and either rapamycin or AMH, which also reported that AMH significantly reduced chemotherapy-induced follicle damage. However, while that study found rapamycin ineffective in protecting follicles, we demonstrated that BEZ235 successfully counteracted chemotherapy-induced damage [31].

Building on our in vitro results, we compared the effects of adding BEZ235 to the cryopreservation medium with rapamycin in vivo, using a widely used murine transplantation model [26, 57]. The in vivo results were consistent with our in vitro findings. Ovaries slow-frozen with BEZ235 exhibited superior protection of the primordial follicle pool with reduced levels of follicle proliferation and activation compared to both control and rapamycin-treated groups. Similar results on cell proliferation and Akt activation were observed in other experimental cancer xenograft models comparing rapamycin and BEZ235 [58]. Furthermore, our results align with studies showing that dual PI3K/mTOR inhibitors more effectively reduce cell proliferation and Akt/mTOR pathway activation than mTOR inhibition alone, primarily by preventing PI3K feedback activation after mTOR inhibition [59, 60]. Interestingly, although our previous study using a comparable transplantation model demonstrated a significant reduction in Rps6 activation following rapamycin treatment [26], the present study revealed only a non-significant trend. A comparative analysis of both datasets suggests that while the proportion of pRps6-positive follicles in

the rapamycin group remained consistent, baseline activation in the control group was notably lower in the current study. This reduced baseline may have limited the dynamic range, thereby masking potential inhibitory effects. Furthermore, overall pRps6 expression was low across all groups, with average positivity not exceeding 5%, further constraining the ability to detect significant differences. It is also possible that analysis conducted three weeks post-grafting failed to capture transient early effects of rapamycin. In contrast, BEZ235 may exert more sustained pathway inhibition, potentially accounting for the significant reduction in pRps6-positive follicles observed. Collectively, these findings underscore the complexity of in vivo pathway dynamics and support the need for further mechanistic investigations to fully delineate the temporal and molecular effects of targeted inhibitors. In addition to inhibiting follicle activation with BEZ235 in vivo, we wanted to further improve graft survival by addressing another contributor to follicle loss: tissue ischemia [32, 33, 61]. Although previous studies have suggested that VEGF, particularly when combined with G-CSF, promotes revascularization and enhances graft survival [40, 62], our data indicated that post graft injection provided no additional benefit in ovaries slow-frozen with BEZ235. The percentage of primordial and proliferating follicles in the BEZ235 group remained unchanged despite the angiogenic treatment. This may reflect a ceiling effect, in which the strong preserving activity of BEZ235 limits any further improvement by VEGF/G-CSF. Alternatively, the timing and dosage of VEGF/G-CSF used in our study, based on a prior orthotopic transplantation model, may not have been optimal for our specific heterotopic setting [40]. However, the referenced study showed similar effects on primordial follicle density compared to those we observed in our control-frozen ovaries. Nonetheless, VEGF and G-CSF may still offer benefits in other contexts, particularly in orthotopic transplantation models. While angiogenic treatment did not further increase follicle pool preservation in our BEZ235 setting, it may still influence oocyte competence or fertility restoration, important parameters not evaluated here. Interestingly, several culture experiments have shown that VEGF promotes granulosa cell proliferation [63–65]. However, since we assessed its effects nearly three weeks after treatment, we may have missed an earlier impact on follicle proliferation. As fibrosis is associated with the OTCTP process, we investigated its formation in transplanted ovaries and we found no differences among experimental groups. However, previous studies indicate that post-grafting fibrosis is partly induced by cryopreservation, suggesting that improved revascularization may not necessarily reduce fibrosis formation [36, 66].

A key strength of our study is that the inhibitors were only in contact with the ovary, during the cryopreservation, eliminating direct exposure to the graft recipient. This minimized the risk of side effects, making it a safer option compared to systemic VEGF/G-CSF injection, *e.g.*, and facilitating its potential clinical implementation for human OTCTP procedures. Furthermore, we used multiple in vitro and in vivo models to compare BEZ235 with other inhibitors and angiogenic compounds, strengthening the evidence for its superior ability to protect the primordial follicle pool. Although mouse models are widely used in reproductive research due to similarities in certain physiological aspects of ovarian function, species-specific differences remain [67]. In addition, our study used whole murine ovaries, whereas clinical OTCTP protocols involve the transplantation of ovarian cortical strips. This difference in tissue structure and composition may influence drug diffusion, vascularization, and treatment efficacy. Although BEZ235 is currently being evaluated in clinical trials for cancer therapy, its application in fertility preservation introduces specific concerns, including potential impacts on oocyte quality and offspring health. To address these issues and bridge the translational gap, future studies should use xenograft models using human ovarian cortical tissue. Such experiments would provide crucial data on BEZ235's safety, efficacy and feasibility in a clinical relevant setting, including the need to meet regulatory standards for reproductive applications. Despite the promising outcomes of our study, several limitations should be acknowledged. Although we assessed the activation of Akt and Rps6, we did not evaluate downstream effectors such as 4E-binding protein 1 (4E-BP1) and Forkhead box O3 (FOXO3), which are key regulators of follicle dormancy and activation [47, 68]. Future studies examining *e.g.*, FOXO3 nuclear localization and 4E-BP1 phosphorylation could provide more comprehensive mechanistic insights into how BEZ235 contributes to primordial follicle preservation. While we focused on the use of BEZ235 during cryopreservation, we did not examine the potential benefits of incorporating AMH during this process. Furthermore, our culture experiments assessed follicle pathways activation using whole tissue Western blot, limiting insights into the direct effects of the inhibitors in individual follicles. While our current study demonstrates that BEZ235 can effectively preserve the follicle pool during OTCTP, we did not assess its direct impact on fertility restoration, representing an important limitation. In a previous publication, we showed that rapamycin improved fertility restoration using an orthotopic transplantation model in mice following chemically induced ovarian failure [69]. A

similar experimental approach should now be undertaken to evaluate BEZ235's potential in this regard. This would involve orthotopic transplantation of ovaries cryopreserved with BEZ235, followed by mating experiments to assess fertility outcomes (*e.g.,* number of offspring and live birth rate), and superovulation to examine oocyte competence. These studies are essential to determine whether the follicle preservation observed with BEZ235 translates into fertility restoration and to investigate possible long-term effects on oocytes and offspring health. Complementary in vitro maturation studies, including first polar body assessments, could further elucidate BEZ235's influence on oocyte viability [70].

Conclusion

In conclusion, BEZ235 emerges as a promising candidate for preserving the ovarian follicle pool during chemotherapy and cryopreservation by effectively inhibiting both Akt and mTOR pathways. Compared to rapamycin, BEZ235 offers superior protection of primordial follicles, both in vitro and in vivo. Additionally, while VEGF and G-CSF injections support follicle survival under ischemic conditions, they do not enhance BEZ235-mediated follicle protection.

By effectively addressing excessive follicle activation post-transplantation, this study highlights a potential strategy to extend ovarian graft longevity, thereby prolonging the reproductive window for patients undergoing OTCTP and, ultimately, improving the quality of life for patients requiring fertility preservation.

Abbreviations

4E-BP1	4E-binding protein 1
4-HC	4-Hydroperoxycyclophosphamide
AMH	Anti-Müllerian hormone
BEZ	BEZ235
BSA	Bovine serum albumin
CT	Control
DDX4	DEAD-box helicase 4
DMSO	Dimethylsulfoxide
ECL	Enhanced chemiluminescence
FBS	Fetal Bovine Serum
FOXO3	Forkhead box O3
G-CSF	Granulocyte colony-stimulating factor
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IP	Intraperitoneally
LY	LY294002
mTOR	Mammalian target of rapamycin
OTCTP	Ovarian tissue cryopreservation followed by autotransplantation
PI3K	Phosphatidylinositol-3-kinase
PTEN	Phosphatase and tensin homolog
RAPA	Rapamycin
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
SFbez	Slow-frozen/thawed ovaries in medium supplemented with BEZ235
SFct	Slow-frozen/thawed ovaries in control medium
SFrapa	Slow-frozen/thawed ovaries in medium supplemented with rapamycin
VEGF	Vascular endothelial growth factor

Supplementary Information

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Supplementary Material 1. Figure S1. (A) Computer assisted quantification of the percentage of ovarian area stained for Sirius red (w/o lumen) in ovaries slow-frozen in control medium or medium supplemented with BEZ235, autotransplanted under the kidney capsule of C57bl/6j mice, followed by injection with VEGF, G-CSF, or a combination of both. (B) Representative images of computer assisted quantification of the percentage of ovarian area stained for Sirius red. SFct = slow-frozen/thawed ovaries in control medium, SFbez= slow-frozen/thawed ovaries in medium supplemented with BEZ235. *n* = 9-10 ovaries/group.

Supplementary Material 2

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Authors' contributions
C.M. and J.B. designed the study and methodology.; J.B. performed the experiments and statistical analysis with input and assistance from M.S. and L.B.; J.B. and C.M. drafted the manuscript.; J.B., C.M., M.S., L.B. reviewed and edited the manuscript.; C.M. and M.N. acquired the funding.; C.M. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval and consent to participate
This study was approved by the Animal Ethics Committee of the University of Liège (#1934 and #2594). We confirmed that all experiments in this study were performed in accordance with the relevant guidelines and regulations. All the procedure of the study is followed by the ARRIVE guidelines.

Consent of publication
Not applicable.

Competing interest
The authors declare no competing interests.

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Supplementary

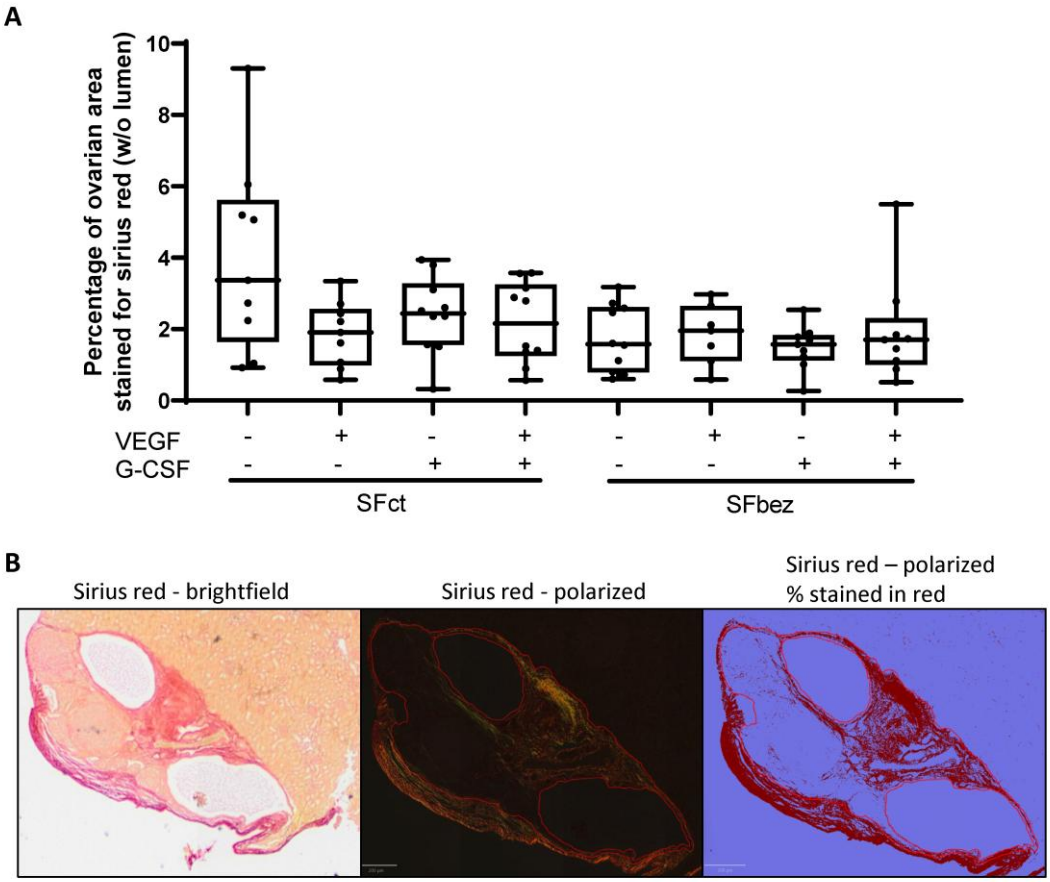
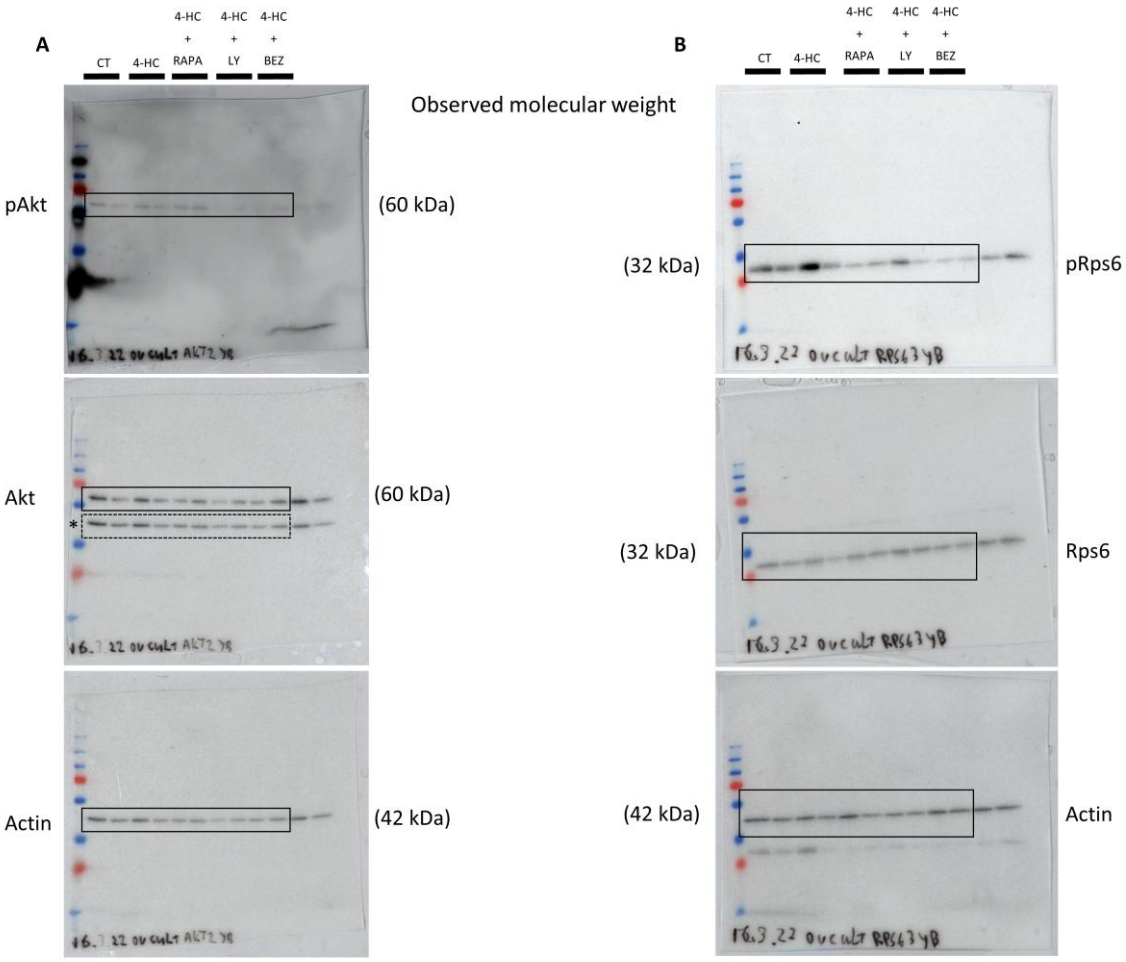


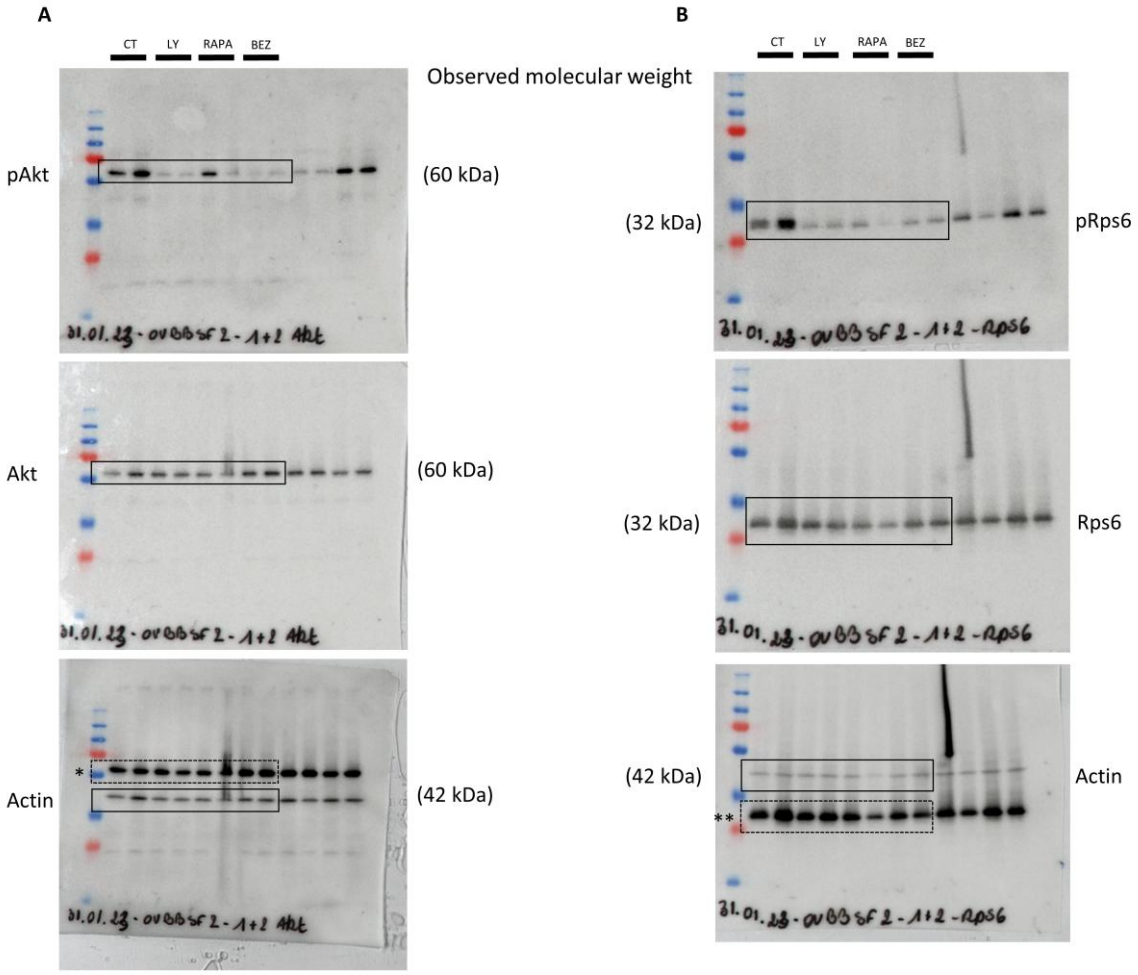
Figure S1. (A) Computer assisted quantification of the percentage of ovarian area stained for Sirius red (w/o lumen) in ovaries slow-frozen in control medium or medium supplemented with BEZ235, autotransplanted under the kidney capsule of C57bl/6j mice, followed by injection with VEGF, G-CSF, or a combination of both. (B) Representative images of computer assisted quantification of the percentage of ovarian area stained for Sirius red. SFct = slow-frozen/thawed ovaries in control medium, SFbez= slow-frozen/thawed ovaries in medium supplemented with BEZ235. *n* = 9-10 ovaries/group.

Figure 1 Full uncropped blots



* Stripping was not performed after Actin revelation.
Therefore, we observe Actin during Akt revelation.

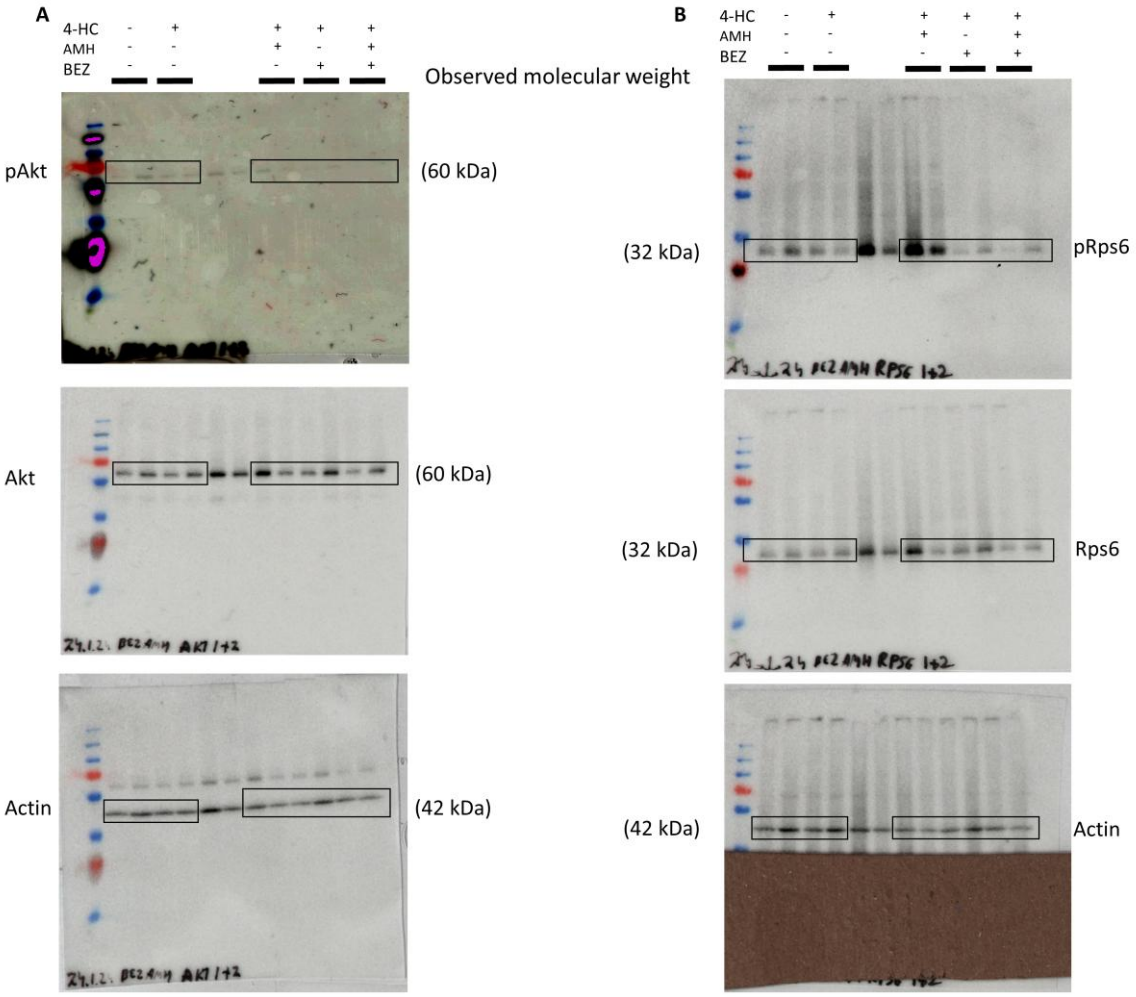
Figure 2 Full uncropped blots



* Stripping was not performed after Akt revelation. Therefore, we observe Akt during Actin revelation.

** Stripping was not performed after Rps6 revelation. Therefore, we observe Rps6 during Actin revelation.

Figure 3 Full uncropped blots



Chapter 4

Discussion and Conclusion

4.1 General discussion

While high-dose chemotherapy and radiotherapy have drastically improved cancer cure rates, one of their major long-term side effects is ovarian failure and infertility, a concern frequently expressed by many young female patients (4, 10). For prepubertal female cancer patients and young women requiring urgent oncological care, the only available fertility preservation option remains OTCTP. With over 300 live births reported to date, OTCTP has already demonstrated clinical success as a fertility preservation technique (51, 58, 81). Furthermore, 95% of women undergoing OTCTP experience a restoration of ovarian endocrine function with restored menses and physiological hormone levels, often persisting for over 5 years (51). However, its efficacy is limited by the rapid depletion of the follicle reserve directly after grafting, substantially reducing graft longevity, highlighting the need for further improvements of the technique (123).

This early follicular depletion is primarily driven by three mechanisms. One is ischemia in the first few days post-grafting, as ovarian tissue transplantation is avascular, leading to oxygen deprivation (126, 127). This is linked to the second mechanism, follicular loss through apoptosis (95, 128, 129). The third main factor contributing to the rapid depletion of the follicle pool is the massive recruitment of PMFs following the transplantation of ovarian tissue, often referred to as “burn-out” (123, 130, 131).

To address these challenges, various strategies have been investigated to suppress follicle activation and enhance neovascularization post-grafting. To comprehensively evaluate the efficacy of these interventions in both the cryopreservation and transplantation stages, *in vivo* models are required. Several murine ovarian transplantation sites have been employed, with a common disadvantage among these sites being the requirement for invasive surgical procedures (132, 143, 208, 209, 211-214).

Therefore, our **first objective** was to develop a minimally invasive transplantation model that would be easily accessible for local therapeutic interventions aimed at limiting follicle activation or improving neovascularization. To this end, we evaluated a novel heterotopic transplantation site located between the skin and cartilage of the ear, adapted from the ear sponge assay previously set up in our lab to study (lymph) angiogenesis (216-218). This site demonstrated ovarian revascularization, minimal fibrosis, and favorable conditions for follicle development, with no significant apoptosis. The model offers several advantages over traditional transplantation sites, including easier access for therapeutic delivery and reduced surgical invasiveness.

In order to further validate this study, our **second objective** was to compare this novel transplantation model with the more conventional but invasive kidney capsule transplantation site. Three weeks after ovarian transplantation, both sites showed similar PMF densities, vascularization, follicle proliferation, and mTOR pathway activation. At this time point, apoptosis was similarly absent in both models, suggesting that no persistent damage was present. However, as apoptosis is typically observed shortly after transplantation, early transient apoptosis in our model cannot be excluded. Interestingly, lower Akt pathway activation in the ear model suggests that follicles in this model may be maintained in a more quiescent state. These findings support the ear model as a less invasive alternative for ovarian transplantation research.

Following the initial publication of our novel transplantation model, another research group studied intra-auricular (IA) ovarian transplantation in dogs and goats (219-221). In their canine model, IA grafts showed better graft stability and follicle survival compared to the subcutaneous neck region 15 days post-grafting. However, unlike our findings in mice, they did not observe follicular development. This difference may be partially attributed to their use of ovarian fragments rather than whole ovaries, which are more vulnerable to ischemia and may experience higher oxidative stress, although xenograft studies have reported minimal oxidative stress levels following transplantation (222).

Additional factors such as species-specific ovarian physiology, the relative age of the animals at the start of the experiment, and the total duration the tissue was transplanted may further contribute to the lack of follicular growth (219). A follow-up study in goats found similarly favorable outcomes when comparing IA to intramuscular neck grafts (220). These findings support the potential of the ear as a transplantation site and highlight how species differences and graft composition may influence outcomes.

As mentioned before, one of the limitations of the OTCTP technique is the rapid depletion of the follicle pool directly after grafting, possibly due to the immense follicle recruitment (123, 130, 131). Among the signaling pathways involved, the PI3K/PTEN/Akt and mTOR pathways are particularly important regulators of follicular activation and growth (164, 166). In earlier *in vitro* work, our team demonstrated that culture-induced activation of the PI3K/PTEN/Akt pathway could be reversed by adding LY294002, a potent PI3K inhibitor, to the culture medium (167). This led us to test this inhibitor *in vivo*.

Taking advantage of the accessibility of the ear model, we next assessed LY294002 injection either locally (ear model) or systemically (kidney capsule model). No differences in weight gain were observed between LY294002-treated and untreated mice in both models three weeks post-injection, suggesting no adverse effects on growth. These findings are consistent with other studies using similar IP doses of LY294002, which also reported no impact on weight gain (223, 224).

However, behavioral analysis revealed that mice receiving IP injections of LY294002 were significantly less active than those receiving local injections or controls, indicating that systemic administration may have a greater physiological impact. This could be attributed to wider drug distribution following IP injection, whereas after local injection, the treatment mainly remains at the injection site and surrounding tissue (225). While LY294002 has been used in many studies, it is important to acknowledge its limitations, including the modest potency and lack of selectivity, affecting multiple kinases beyond PI3K (226). In addition, systemic administration of high doses of LY294002 has been associated with toxicity, including edema, weight loss, dry skin, and behavioral changes in animals (223, 227). These adverse effects, in addition to its poor solubility, have hindered its progression into clinical applications, with some researchers even stating it will never be feasible for clinical trials (228).

Notably, no differences in behavior or weight gain were observed between the two models, suggesting that even though the kidney model is more invasive, it does not affect the mice differently compared to the less invasive ear model. Additionally, both administration routes produced

comparable effects on follicle density, without any observation of apoptosis or DNA damage, indicating no long-term side effects, comparable to other studies involving murine LY294002 injection (229).

Injection of LY294002, either administered locally or IP, did not affect follicle proliferation or mTOR pathway activation compared to controls, suggesting that its effects may be transient and potentially undetectable three weeks after injection, which may also be related to LY294002's relatively short half-life (230). Interestingly, systemic administration resulted in a significant reduction in Akt activation, an effect not observed with local injection. Notably, baseline Akt activation appeared lower in ovaries from the local control group than in those from the systemic control group, suggesting that the transplantation site influences pathway activation. This could explain the route-specific effect of LY294002, as ovarian grafts under the kidney capsule may inherently exhibit higher Akt pathway activity than those in the ear model. While both the ear and kidney capsule models yielded comparable outcomes in terms of follicle density and proliferation, differences in Akt activation highlight that the local tissue environment and drug distribution dynamics may influence pathway-specific outcomes. Thus, both models are suitable platforms for testing pharmacological interventions targeting follicle activation, but site-specific variations should be carefully considered when drawing conclusions or comparing results across studies.

In addition to investigating the PI3K inhibitor LY294002, our team previously explored the use of rapamycin, a selective mTOR inhibitor. The team demonstrated that not only the transplantation, but the cryopreservation process itself induces follicle activation via the PI3K/PTEN/Akt and mTOR pathways, and that rapamycin could counteract this activation when added during cryopreservation (167). Building on these promising *in vitro* findings, our **third objective** was to evaluate the *in vivo* efficacy of rapamycin by incorporating it into the transport and freezing medium using a heterotopic ovarian transplantation mouse model. In our murine models, the ovaries were exposed to rapamycin continuously from the moment of extraction through the completion of the slow-freezing process.

We observed that rapamycin significantly reduced both follicle proliferation and activation, thus keeping PMFs in a dormant state. Notably, fewer pAkt-positive primordial and primary follicles were observed following rapamycin treatment, suggesting a bidirectional interaction between the PI3K/PTEN/Akt and mTOR pathways. While some studies have reported feedback activation of Akt following mTORC1 inhibition (167, 231), others suggest that the concentration of rapamycin may modulate this effect: lower doses may increase Akt activation via mTORC1 signaling, whereas higher doses suppress Akt phosphorylation, possibly through mTORC2 inhibition (232).

The mTOR pathway is additionally known for its function in cell proliferation (233), and our findings confirmed that rapamycin was able to counteract cryopreservation-induced follicle proliferation. Notably, our results showed that even though the contact time between rapamycin and the ovary is relatively short, its protective effects can still be observed three weeks after transplantation. Furthermore, rapamycin did not cause long-term tissue or DNA damage, and revascularization three weeks post-grafting was comparable to controls.

Although follicle quantification revealed that cryopreservation led to a decrease in PMF density, rapamycin did not prevent this loss. However, it remains possible that rapamycin improved the quality of the remaining follicles, an aspect that needs further investigation. Overall, our findings suggest that

rapamycin can transiently preserve PMFs in a quiescent state during OTCTP, supporting the strategy of limiting excessive follicle activation to improve graft longevity and fertility outcomes.

While this heterotopic transplantation model demonstrated the potential of rapamycin to maintain primordial follicles in a quiescent state, our ultimate goal is to improve the restoration of fertility and longevity of the ovarian graft. Therefore, our **fourth objective** was to investigate whether adding rapamycin to the transport and freezing medium could enhance fertility restoration in mice through an orthotopic autotransplantation after chemically-induced ovarian failure.

Our results indicated that orthotopic autotransplantation successfully restored estrous cyclicity in a subset of mice, regardless of rapamycin exposure. This finding aligns with other studies, where subcutaneous or orthotopic transplantation restored estrous cycles in 75-100% of rodents within 2-3 weeks post-grafting (234, 235).

We showed that mice in the rapamycin group demonstrated improved fertility preservation, with a significantly higher live birth rate after 4 months of mating compared to controls. Although the average litter size was not significantly different, mice in the rapamycin group gave birth to more pups overall. Given that C57BL/6 mice normally give birth to a litter of approximately seven pups (236, 237), the reduced litter size observed in our rapamycin model (3.43 pups per litter), with only one functional ovary, is consistent with physiological expectations.

In addition to improved fertility, rapamycin enhanced graft survival, as indicated by the presence of PMFs approximately 6 months after grafting. However, superovulation at this time point yielded few oocytes in both groups, with an average of 1.75 in controls and 1.13 in the rapamycin group. This superovulation efficacy is low but aligns with the age-related decline reported in mice, where the oocyte number decreases to only 2.1 ± 0.2 oocytes/oviduct by 15 months (238). Furthermore, the OTCTP procedure itself may contribute to a shortened reproductive lifespan and reduced superovulatory capacity.

A known side effect of ovarian manipulation, such as ovarian transposition in women, is cyst formation (239). Cyst formation was observed in both control and rapamycin groups following whole ovary transplantation, consistent with reports linking ovarian transplantation to cystogenesis (240). Although ovarian cysts are often associated with infertility (241, 242), no direct correlation was found between cyst formation and the ability to conceive. Nevertheless, females with cysts had fewer pups and a lower live birth rate. Since the incidence of cysts did not differ between the rapamycin and control groups, these outcomes likely reflect graft manipulation rather than treatment-specific effects.

In addition to our investigation of rapamycin, other studies have explored the addition of anti-apoptotic and antioxidant agents to the cryopreservation medium to protect ovarian tissue. For instance, Lee *et al*. supplemented the freezing medium with anti-apoptotic compounds S1P and Z-VAD-FMK, reporting that Z-VAD-FMK significantly increased PMF counts and enhanced graft angiogenesis four weeks after xenotransplantation of ovarian tissue into mice (128). Antioxidants like sericin and melatonin reduced oxidative stress, inhibited apoptosis, and preserved follicular morphology without cytotoxicity in cryopreserved murine ovaries. However, both treatments activated the PI3K/PTEN/Akt and mTOR pathways, potentially promoting premature follicle activation and depletion (243). Although various agents have been evaluated for addition to the cryopreservation medium, to the best

of our knowledge, the use of rapamycin during cryopreservation followed by orthotopic autotransplantation has not been previously studied. Most *in vivo* studies focused on rapamycin administration after transplantation, which resulted in higher PMF counts (178, 180). Only one *in vivo* study to date has shown that pre-treatment with rapamycin before vitrification reduced follicle loss and enhanced graft survival (179). Together, these findings support rapamycin's role in protecting the follicle pool during cryopreservation, which aligns with the improved fertility outcomes observed in our study.

While our *in vivo* studies showed the potential of rapamycin during the OTCTP process, the feedback activation of Akt remains a concern. As mentioned earlier, mTORC1 inhibition may result in Akt activation, either by relieving feedback suppression or promoting mTORC2 activity, which could undermine the maintenance of follicle quiescence and reinitiate follicular activation (34). Indeed, we previously observed increased Akt activation when murine ovaries were slow-frozen with rapamycin and subsequently subjected to culture (167).

To overcome this limitation, targeting both Akt and mTOR pathways simultaneously may offer enhanced protection, preventing feedback activation. BEZ235, a dual PI3K/mTOR inhibitor currently in clinical trials for cancer therapy (244), emerged as a promising candidate. Our **fifth objective** was, therefore, to evaluate whether BEZ235 could better preserve the PMF pool compared to LY294002, rapamycin, or the natural inhibitor AMH, and to assess the potential benefit of post-grafting VEGF/G-CSF injections.

Before progressing to murine transplantation models, we compared the inhibitors using a whole ovary organotypic *in vitro* culture model, a valuable tool for studying the effects of inhibitors on the follicle activation pathways, as follicles are activated during culture (245). Under chemotherapeutic conditions, only BEZ235 effectively counteracted chemotherapy-induced activation of both the Akt and mTOR pathways. Although its effect on follicle activation had not been previously studied, BEZ235 has been shown to suppress growth and proliferation in ovarian cancer cell lines through PI3K/PTEN/Akt and mTOR pathway inhibition (184, 246, 247).

Following these promising results, the addition of inhibitors during cryopreservation confirmed BEZ235's superiority: BEZ235 significantly counteracted Akt and mTOR pathway activation, while rapamycin and LY294002 both only significantly decreased Rps6 activation.

Although these pharmacological inhibitors showed encouraging results, recent studies highlighted the potential of AMH, especially in combination with other inhibitors, to protect the PMF pool (189, 248). However, our results indicated that combining BEZ235 and AMH in a murine culture experiment did not provide any additional or synergistic benefit beyond that achieved with BEZ235 alone, further emphasizing the superior efficacy of BEZ235 in preserving the follicle pool via decreased follicle activation. As follicle loss is also driven by apoptosis (249, 250), we assessed PMF health and found that both AMH and BEZ235, alone or combined, were associated with a higher proportion of morphologically healthy PMFs following chemotherapy exposure. These findings are consistent with a previous study showing that AMH can reduce chemotherapy-induced follicle damage in human ovarian biopsies cultured with 4-HC, although, unlike our findings with BEZ235, rapamycin was ineffective in that model (189).

The comparison between BEZ235 and rapamycin added to the cryopreservation medium in an *in vivo* transplantation model confirmed our *in vitro* findings. Indeed, BEZ235 more effectively preserved the follicle pool, with lower levels of follicle proliferation and activation compared to both control and rapamycin groups. These findings are in line with other experimental cancer xenograft models, which also reported superior efficacy of BEZ235 over rapamycin (251). Moreover, our results align with studies demonstrating that dual PI3K/mTOR inhibitors more effectively suppress cell proliferation and pathway activation than mTOR inhibition alone, largely by overcoming feedback activation of PI3K (184, 252).

Building on these promising results, we aimed to further improve graft survival by addressing another contributor to follicle loss: tissue ischemia (118, 132, 253). Despite previous studies suggesting that VEGF, particularly when combined with G-CSF, promotes revascularization and enhances graft survival (73, 254), our results showed no additional benefit when combined with BEZ235. Indeed, although VEGF is known to stimulate granulosa cell proliferation *in vitro* (255-257), follicle proliferation in the BEZ235-treated group remained unchanged despite angiogenic treatment. This may reflect a ceiling effect, in which the strong preserving activity of BEZ235 limits any further improvement by VEGF/G-CSF. In addition, no differences in fibrosis were observed among experimental groups, which is consistent with earlier findings suggesting that fibrosis is largely induced by cryopreservation itself and may not be modulated by improved revascularization (146, 258).

A key advantage of our *in vivo* models is that the pharmacological inhibitors were only in contact with the ovaries during cryopreservation, thereby avoiding direct exposure to the graft recipient. This significantly reduces the risk of systemic side effects, making the approach potentially safer than, *e.g.,* systemic VEGF/G-CSF injection*,* facilitating the potential clinical implementation of these inhibitors for human OTCTP procedures. However, several limitations should be addressed. Although mouse models offer valuable insights due to certain shared physiological characteristics with human ovarian function, species-specific differences remain (259). In addition, our studies used whole murine ovaries, whereas clinical OTCTP protocols involve the transplantation of ovarian cortical strips. This difference in tissue structure and composition may influence drug diffusion, vascularization, and treatment efficacy. Therefore, before clinical application can be considered, further studies using xenograft models are essential to better assess safety and efficacy under clinically relevant conditions.

While the pharmacological inhibitors were efficient in preserving the PMF pool, a limitation of our work was that their mechanistic effects were primarily assessed via proximal markers (*e.g.*, pAkt, pRps6). Downstream effectors such as FOXO3a nuclear localization or 4E-BP1 phosphorylation were not evaluated, limiting insight into the full signaling cascade. While rapamycin's impact on fertility restoration was validated in an orthotopic transplantation model, BEZ235 was not tested in this context. Future investigations should assess whether BEZ235 also improves fertility outcomes. This would involve orthotopic transplantation of ovaries cryopreserved with BEZ235, followed by mating experiments to assess fertility outcomes (*e.g.,* number of offspring and live birth rate), and superovulation to examine oocyte competence. These studies are essential to determine whether the follicle preservation observed with BEZ235 translates into fertility restoration and to investigate possible long-term effects on oocytes and offspring health. Moreover, functional assays such as *in vitro*

maturation or fertilization are needed to determine whether these interventions influence oocyte quality. Furthermore, while our angiogenic intervention did not enhance follicle pool preservation in combination with BEZ235, VEGF and G-CSF may still offer benefits in other transplantation models, particularly for improving oocyte competence or fertility restoration. Alternative delivery systems (*e.g.*, scaffolds) could optimize vascular support and should be explored in future studies.

4.2 Conclusion

Although several fertility preservation options exist, OTCTP remains the only strategy available to prepubertal girls and young women needing immediate cancer treatment. While clinically successful, OTCTP's long-term efficacy is limited by rapid follicle loss post-grafting due to ischemia, apoptosis, and excessive follicle activation.

To address these challenges, we developed a minimally invasive ear transplantation model and explored targeted inhibition of the PI3K/PTEN/Akt and mTOR pathways. The ear transplantation model provided a less invasive alternative to traditional sites, allowing for localized treatment delivery. LY294002 treatment post-grafting yielded comparable effects on follicle activation in both the ear and kidney capsule models, validating the ear model for testing pharmacological strategies.

Building on our prior findings with the mTOR inhibitor rapamycin, we demonstrated that its addition during cryopreservation maintains follicles in a quiescent state and improves fertility restoration in murine transplantation models. However, feedback activation of the Akt pathway remains a limitation of mTOR inhibition alone. We therefore investigated BEZ235, a dual PI3K/mTOR inhibitor, demonstrating its superiority in reducing follicle activation and preserving the follicle pool across *in vitro* and *in vivo* models. Furthermore, post-grafting VEGF/G-CSF injections offered no additional benefit when combined with BEZ235.

Beyond the experimental evidence, our findings carry important implications for patients. Improving OTCTP outcomes aligns with the growing demand for fertility preservation in young cancer patients. As cancer survival rates continue to improve, enabling patients to have biological children post-treatment reflects a more complete approach to cancer care, one that considers both medical treatment and future quality of life. By effectively addressing one of the main issues of the OTCTP technique, the excessive follicle activation post-grafting, our findings highlight a potential strategy to extend graft longevity and prolong the reproductive window for patients undergoing OTCTP, ultimately enhancing the likelihood of successful pregnancies and quality of life for patients requiring fertility preservation.

Chapter 5

Perspectives

Our studies provide novel insights into preserving the PMF pool and improving fertility preservation outcomes by keeping follicles in a dormant state during OTCTP. The demonstration that rapamycin improves fertility outcomes, and that dual inhibition of the Akt and mTOR pathways using BEZ235 significantly reduces follicle activation and preserves the PMF pool, represents promising therapeutic advances. While these results support the possibility of pharmacological interventions to improve OTCTP outcomes, several questions and concerns remain that warrant further investigation.

5.1 Ensuring safety and transgenerational integrity

Although drug exposure in our model was limited to the ovarian tissue during cryopreservation, the possibility of toxic effects towards oocytes and thus transgenerational consequences remains a key concern. Future studies should therefore compare the fertility and reproductive health of the offspring from females who underwent OTCTP with treated grafts to those from control groups (260, 261). Such comparisons will help determine whether exposure to rapamycin or BEZ235 compromises oocyte health or embryonic development. Additionally, IVM assays could be used to evaluate oocyte competence by analysing developmental markers such as first polar body morphology and meiotic progression (262). These approaches will be essential to validate the clinical safety of these treatments.

Beyond reproductive outcomes, it is essential to examine whether these treatments affect the epigenetic integrity of preserved follicles. Modulation of the follicle activation pathways may alter gene expression or DNA methylation critical for early development. High-throughput RNA sequencing and methylation analysis of oocytes and granulosa cells post-treatment could provide insight into subtle molecular disruptions with possible long-term consequences, establishing a more complete safety profile.

5.2 Combining angiogenesis and metabolic quiescence: integrated therapeutic approaches

While our findings suggest that post-grafting injection of VEGF/G-CSF does not provide additional benefit when combined with BEZ235, alternative strategies may yield improved outcomes. For example, bioengineered scaffolds would allow for a slow and controlled release of angiogenic agents, providing improved vascular support while reducing systemic exposure. It would be interesting to explore whether scaffold-based delivery of angiogenic agents combined with the use of pharmacological inhibitors during cryopreservation yields synergistic improvements in graft survival with minimal side effects.

In addition to promoting angiogenesis, another critical challenge is the period of acute hypoxia following ovarian grafting, contributing to oxidative stress and early follicle loss. However, as mentioned in the discussion, it is critical to note that recent xenograft studies have reported minimal oxidative stress levels following transplantation, suggesting that oxidative damage may play a less significant role in the depletion of the PMF pool than previously assumed (222). While VEGF-based strategies aim to improve neovascularization, studies suggest that the PI3K/PTEN/Akt and mTOR pathways also regulate hypoxia, mainly through regulation of HIF-1α (263). Given BEZ235's dual inhibition of these pathways, future studies should investigate whether it offers protective effects

under hypoxic conditions, alone or in combination with antioxidant therapies. These integrated strategies could offer a comprehensive solution to early post-transplant tissue damage.

Besides the PI3K/Akt/mTOR signalling cascade, other pathways have been implicated in PMF activation and may represent promising targets for therapeutic intervention. For instance, the Hippo pathway has been shown to regulate follicle growth through modulation of cell proliferation. Disruption of Hippo signalling, such as via ovarian fragmentation, can lead to nuclear translocation of YAP/TAZ and subsequent cell proliferation. Similarly, the JNK pathway, which responds to cellular stress and inflammation, has been linked to follicular activation and apoptosis. Pharmacological modulation of these pathways could offer alternative strategies to maintain follicular quiescence following OTCTP. Future studies should investigate whether combining these pathway inhibitors with others like BEZ235 further improves follicle preservation without compromising graft health or function.

Beyond the immediate context of OTCTP, the mechanisms explored in this work, such as PI3K/Akt/mTOR signalling and angiogenic modulation, are relevant to other ovarian pathologies such as POI, polycystic ovary syndrome, and age-related ovarian decline. Moreover, the use of localized pharmacological inhibitors for modulating ischemia and tissue integration could be extended to other organ transplantation or regenerative medicine contexts.

5.3 Improving experimental models for dynamic and translational research

To improve the clinical translatability of preclinical findings, further refinement of experimental models is needed. Our minimally invasive ear transplantation model offers considerable promise due to its vascularity, surface accessibility, and compatibility with real-time, non-invasive imaging. Unlike conventional transplantation sites, this model enables longitudinal visualization of graft physiology, including neovascularization, follicle dynamics, and fibrosis, beyond the resolution of traditional endpoint histology. Incorporating live imaging into this model could greatly accelerate the development of therapeutic strategies aimed at improving OTCTP outcomes.

Moreover, deeper investigations of the ovarian microenvironment could be performed. The stroma plays a crucial role in maintaining follicular dormancy through mechanical and biochemical support. Disruption of stromal integrity during the OTCTP process may alter extracellular matrix composition, immune cell recruitment, and local cytokine gradients, all factors known to influence PMF activation. Studies have shown that stromal damage can alter follicle fate and may initiate premature activation (264), underscoring the importance of preserving the microenvironment during OTCTP.

However, several limitations and assumptions in our models must be acknowledged. While murine models are useful for mechanistic studies, they do not fully replicate human ovarian physiology. Differences in follicle dynamics, immune responses, and tissue architecture may affect translational relevance. Additionally, our use of whole murine ovaries does not reflect the clinical use of human cortical strips. An implicit assumption in our approach is that pharmacological inhibitors penetrate ovarian tissue uniformly during cryopreservation, but this may not be true for larger, structurally distinct human tissue. To enhance translational relevance, future studies should incorporate human-specific platforms, including human ovarian tissue xenografts in immunodeficient mice (265), and *ex*

vivo models, such as 3D ovarian organoids or microfluidic-based ovarian culture systems (266). These human-specific systems allow for mechanistic and safety evaluations in a physiologically relevant context while reducing animal models.

5.4 Methodological reflections

In retrospect, several strategic refinements could have enhanced the scientific depth of our studies. First, although restricting pharmacological exposure to the cryopreservation phase minimized systemic toxicity, pharmacokinetic studies should have been included to confirm tissue retention and rule out rapid compound degradation. Second, while the heterotopic model provided valuable insight into follicle dynamics, incorporating orthotopic transplantation models earlier for BEZ235 would have allowed a direct comparison with rapamycin in terms of fertility restoration, the ultimate clinical endpoint. This could have strengthened conclusions regarding functional relevance. Finally, from a mechanistic point of view, the analysis could have benefited from a broader set of downstream molecular targets beyond pAkt and pRps6. Inclusion of additional downstream targets, including FOXO3a localization or 4E-BP1 phosphorylation, would have provided a more comprehensive view of the full signaling cascade.

5.5 Broader implications of the thesis results

Altogether, these future perspectives outline a research trajectory aimed at optimizing OTCTP by addressing safety, vascularization, and graft survival. These strategies, if validated, have the potential to transform clinical practice in fertility preservation. Beyond oncology, improvements in OTCTP may also benefit a broader population. A growing population of women is choosing to postpone childbearing for personal or professional reasons, and some may seek to preserve endocrine function as a means of delaying menopause. As such, the advancements explored in this thesis may contribute to broader reproductive and endocrine health applications, supporting reproductive autonomy.

Looking ahead, this research may serve as a foundation for broader translational advances. Over the next years, the field is likely to pivot toward more clinically relevant, human-specific platforms, including more xenotransplantation models and human ovarian organoids, which will enable patient-specific, high-throughput testing of novel interventions. Concurrently, integrated combination therapies that promote follicular dormancy while improving vascularization and cellular resilience could offer more robust, multifactorial solutions. With ongoing advances in molecular profiling and drug delivery, future interventions may be tailored to achieve greater specificity and fewer off-target effects. Ultimately, the principles established in this work could extend to other areas of regenerative medicine and transplant science, offering novel solutions to preserve tissue integrity and function under adverse conditions.

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