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RESEARCH

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

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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

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

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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

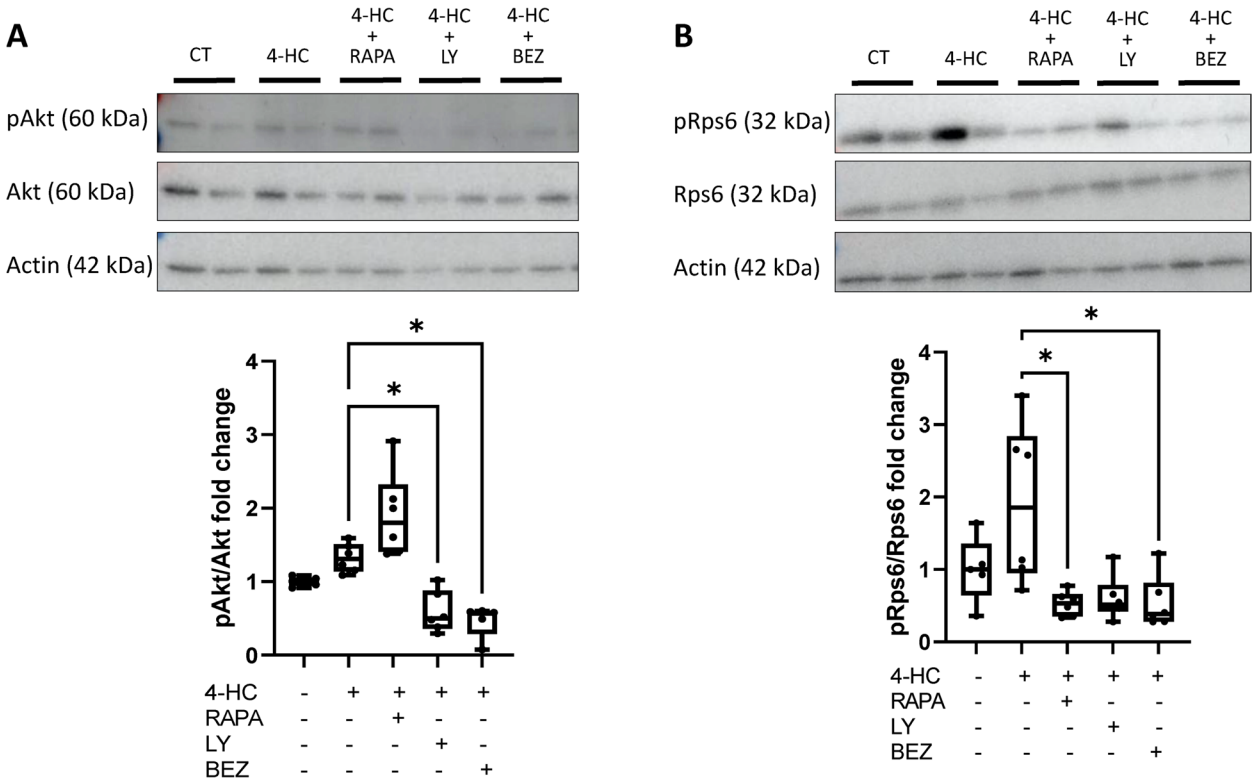

Fig. 1 Effects of In Vitro Organotypic Ovarian Culture under Chemotherapeutic Conditions on Follicle Pathway Activation. Fresh ovaries from 4-weeks-old C57BL/6 mice were cultured for 24 h in the presence of rapamycin (1 µM), LY294002 (25 µM), or BEZ235 (1 µM) under chemotherapeutic conditions with 4-HC (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

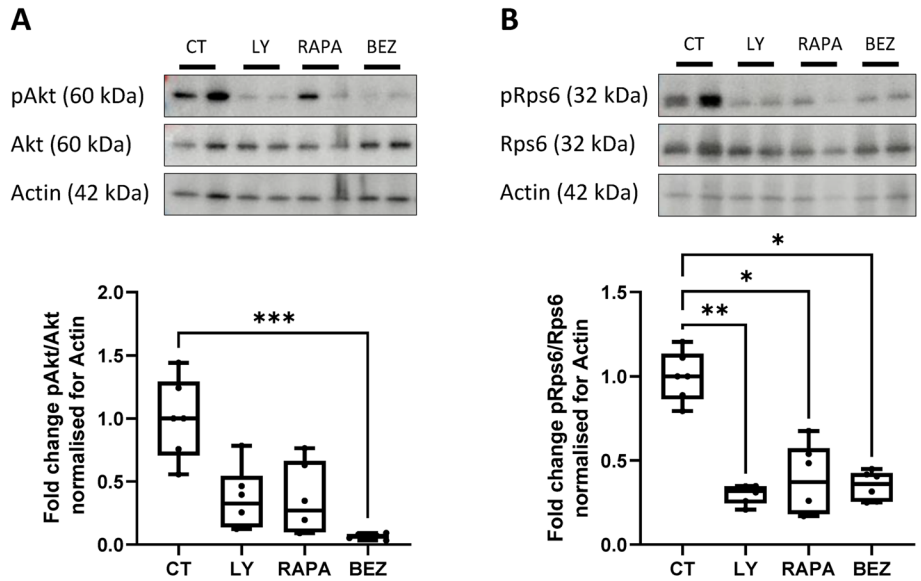

Fig. 2 Effects of Adding Rapamycin, LY294002, or BEZ235 During Cryopreservation on Follicle Activation in Murine Ovaries. Ovaries from 4–7-days-old C57BL/6 mice were cryopreserved with or without rapamycin (1 µM), LY294002 (25 µM), or BEZ235 (1 µM). **A** Fold change of phosphorylated to total protein 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

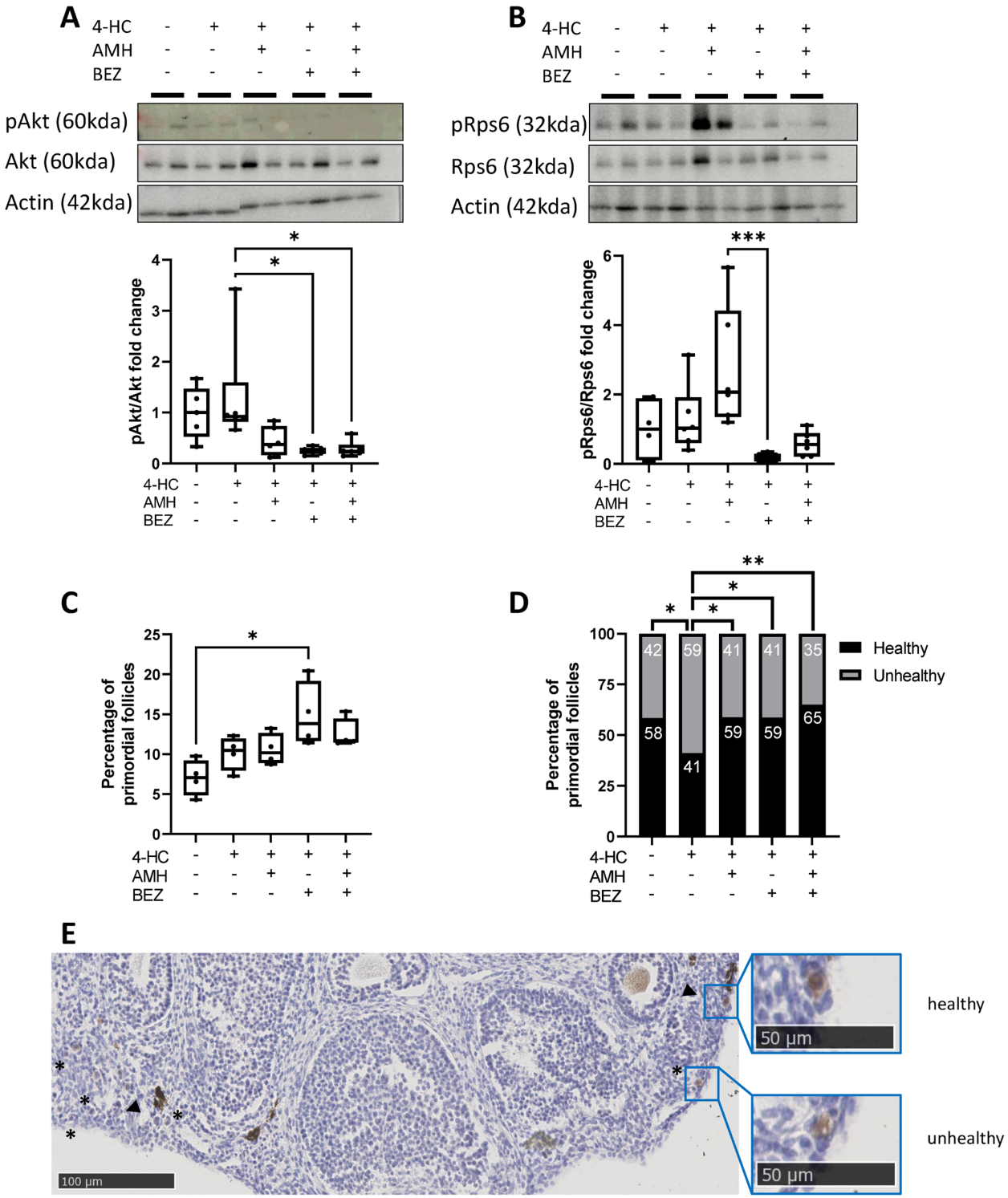

Fig. 3 Effects of BEZ235 and AMH on Follicular Preservation Under Chemotherapeutic Conditions in an Organotypic Ovarian Culture. Frozen/thawed ovaries from 4-weeks-old C57BL/6 mice were cultured for 24 h under chemotherapeutic conditions (4-HC) with or without AMH (200 ng/ml) or BEZ235 (1 µM). **A** 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. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

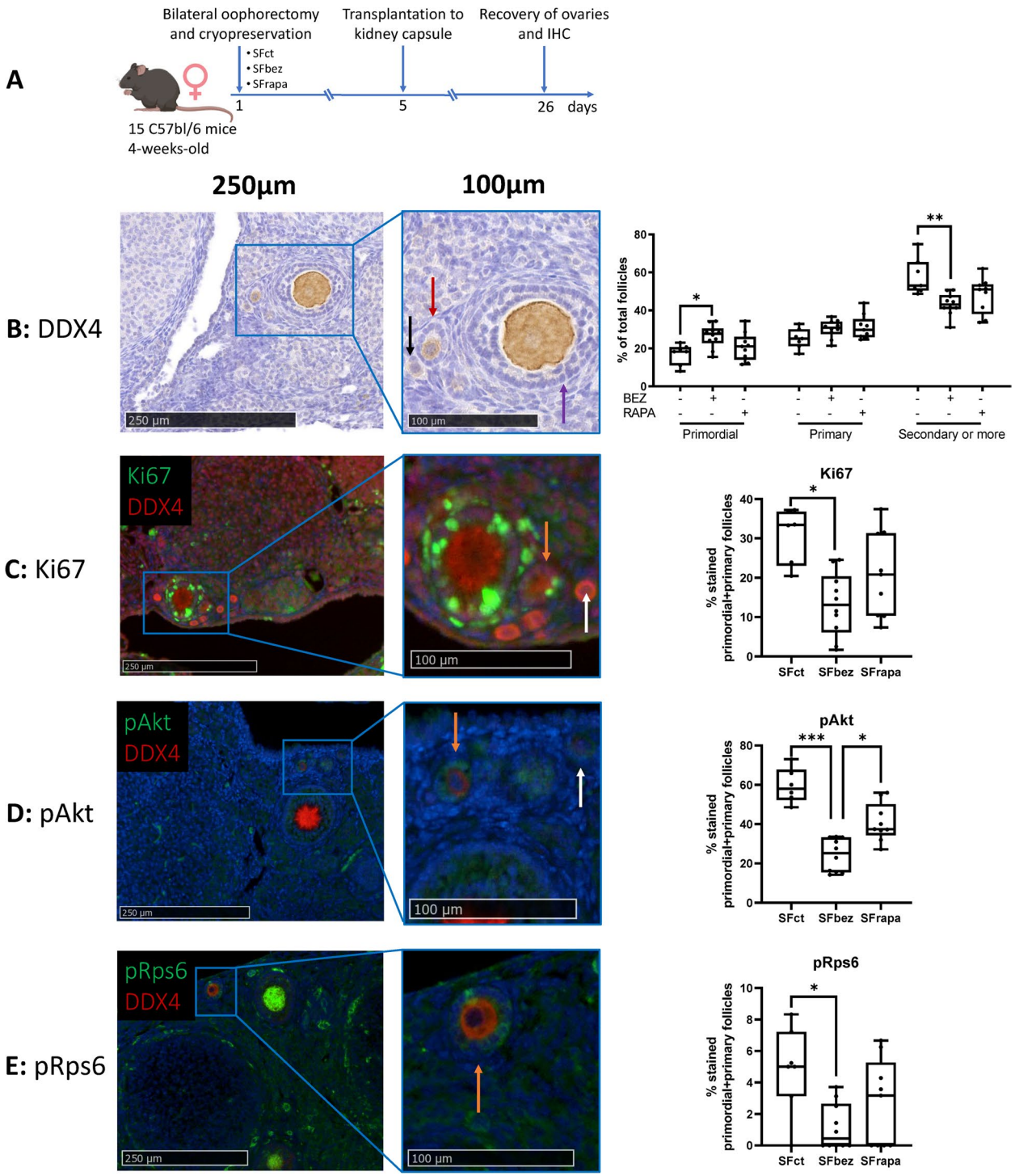

Fig. 4 In Vivo Effects of BEZ235 on Follicle Preservation After Cryopreservation and Transplantation. Experimental scheme (A). IHC assisted follicle quantification (B, DDX4), and analysis of follicle proliferation (C, Ki67) and activation (**D**, pAkt and **E**, pRps6), in ovaries slow-frozen in control 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. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

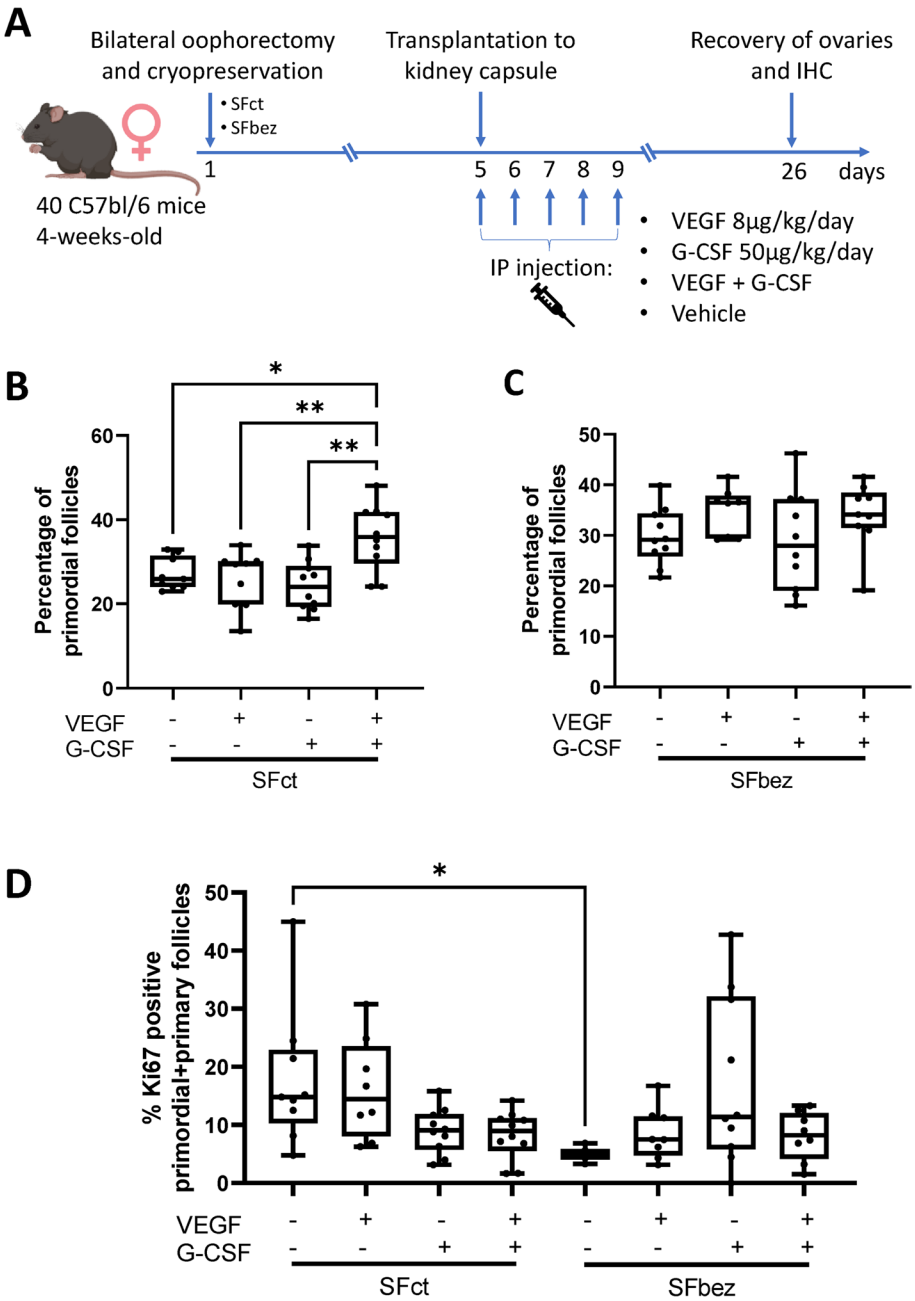

Fig. 5 Effects of VEGF and G-CSF on Follicle Preservation in BEZ235-Treated Cryopreserved Ovaries Post-Transplantation. **A** Experimental scheme. **B**, **C** IHC-assisted primordial follicle quantification in ovaries slow-frozen in control medium (SFct) or medium supplemented with BEZ235 (SFbez, 1 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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