Assessment of follicular development in cryopreserved primate ovarian tissue by xenografting: prepubertal tissues are less sensitive to the choice of cryoprotectant

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Abstract

Improvements in cancer survival rates have renewed interest in the cryopreservation of ovarian tissue for fertility preservation. We used the marmoset as a non-human primate model to assess the effect of different cryoprotectives on follicular viability of prepubertal compared to adult ovarian tissue following xenografting. Cryopreservation was performed with dimethylsulfoxide (DMSO), 1,2-propanediol (PrOH), or ethylene glycol (EG) using a slow freezing protocol. Subsequently, nude mice received eight grafts per animal from the DMSO and the PrOH groups for a 4-week grafting period. Fresh, cryopreserved–thawed, and xenografted tissues were serially sectioned and evaluated for the number and morphology of follicles. In adult tissue, the percentage of morphologically normal primordial follicles significantly decreased from 41.2 ± 4.5% (fresh) to 13.6 ± 1.8 (DMSO), 9.5 ± 1.7 (PrOH), or 6.8 ± 1.0 (EG) following cryopreservation. After xenografting, the percentage of morphologically normal primordial follicles (26.2 ± 2.5%) and primary follicles (28.1 ± 5.4%) in the DMSO group was significantly higher than that in the PrOH group (12.2 ± 3 and 5.4 ± 2.1% respectively). Proliferating cell nuclear antigen (PCNA) staining suggests the resumption of proliferative activity in all cellular compartments. In prepubertal tissues, primordial but not primary follicles display a similar sensitivity to cryopreservation, and no significant differences between DMSO and PrOH following xenografting were observed. In conclusion, DMSO shows a superior protective effect on follicular morphology compared with PrOH in cryopreserved tissues. Xenografting has confirmed better efficacy of DMSO versus PrOH in adult but not in prepubertal tissues, probably owing to a greater capacity of younger animals to compensate for cryoinjury.

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Introduction

Cryopreservation of ovarian tissue has been under investigation for many years, mainly for use in domestic animals. Improvements in the diagnosis and therapy of childhood malignancies accompanied by a significantly increased life expectancy, however, have renewed interest in the cryopreservation of human ovarian tissue. This is mainly due to the severe side effects of therapeutic regimens often including subfertility and premature ovarian failure (Meirow 2000). Among the promising strategies for fertility preservation is the laparoscopic removal and cryopreservation of ovarian cortical tissue fragments prior to gonadotoxic therapy followed by retransplantation (von Wolff et al. 2009). This approach facilitates the storage of a substantial number of primordial follicles without interfering with the oncological treatment, since it can be performed at any time of the menstrual cycle. To date, ten live births have been reported after orthotopic transplantation of frozen–thawed ovarian tissue (Donnez et al. 2004, Meirow et al. 2005, Demeestere et al. 2007, Rosendahl et al. 2008, Silber et al. 2008, Ernst et al. 2010, Roux et al. 2010, Sanchez-Serrano et al. 2010), and potential long-term survival of grafted tissue has been documented (Ernst et al. 2010, Silber et al. 2010). In spite of these encouraging results, the cryopreservation and subsequent use of ovarian tissue thus far remains experimental and requires further research. There are still concerns about the safety of tissue retransplantation, as a potential reintroduction of malignant cells at least in some cancer subtypes cannot be fully excluded (Dolmans et al. 2010). Moreover, none of these live births originate from ovarian tissue cryopreserved at childhood or adolescence, and the developmental potential of juvenile ovarian tissue is unknown (Feigin et al. 2007). Due to the limited availability of donated human ovarian tissue, systematic approaches for the
comparison of different cryopreservation protocols have mainly been conducted in domestic animals. Early studies in rodents suggested that the restoration of fertility following the cryopreservation of ovarian tissue in glycerol and subsequent transplantation was possible (Parrott 1960). Since then, the preservation of reproductive potential after cryopreservation using slow-freezing methods has been demonstrated successfully in various mammalian species with differing cryoprotectants (Sztein et al. 1998, Candy et al. 2000, Salle et al. 2002, Bosch et al. 2004, Lucci et al. 2004, Gook et al. 2005).

The main purpose of ovarian cryopreservation is the storage of developmentally competent follicles. Histological evaluation may be employed to investigate the effects of cryopreservation procedures on follicular morphology: atresia is usually accompanied by nuclear pyknosis, cytoplasmic disorder, detachment of granulosa cells (GCs) from the oocyte, and changes to the basement membrane (Hulshof et al. 1995, Demirci et al. 2002). However, since not all morphologically intact follicles may convey viability, xenografting of cryopreserved ovarian tissue can demonstrate developmental potential by the resumption of follicular growth.

In this study, the common marmoset (Callithrix jacchus) served as a non-human primate model for the cryopreservation and subsequent xenografting of ovarian tissue fragments into immunodeficient nude mice. We analyzed the cryoinjury inflicted by different cryoprotective agents (CPAs) on prepubertal versus adult ovarian tissues. The aims of this study were to i) optimize the cryopreservation procedure for adult and prepubertal primate ovarian cortical tissue fragments with respect to the cryoprotectant used and ii) to analyze the relationship between pre-freeze histology and follicular development following xenografting of thawed tissue.

Results

Histological analysis of adult ovarian tissues frozen with dimethylsulfoxide, 1,2-propanediol, or ethylene glycol

Typical pre- and post-cryopreservation morphology is shown in Fig. 1. In sections of freshly fixed tissue, cellular density of the follicles is high, instances of misshapen oocytes or the apparent loss of structural integrity of GCs are rather rare, and small vacuoles are found in only a few oocytes (Fig. 1A). In frozen–thawed tissue fragments (Fig. 1B–D), more oocytes have lost their spherical shape and displayed large vacuoles in the cytoplasm. GCs are more often unevenly distributed.

Cryopreservation, largely irrespective of the cryoprotectant used, challenges the morphological integrity of ovarian tissue fragments (Fig. 2): the percentage of morphologically normal primordial follicles significantly decreases from 41.2 ± 4.5% (fresh) to 13.6 ± 1.8 (dimethylsulfoxide, DMSO), 9.5 ± 1.7 (1,2-propanediol, PrOH), or 6.8 ± 1.0 (ethylene glycol, EG), respectively, following cryopreservation. DMSO displayed slightly better protective abilities compared with EG (P<0.04), whereas the difference between DMSO and PrOH and between PrOH and EG was not statistically significant (P=0.83 and 0.68 respectively). Within the class of primary follicles, the percentage of morphologically normal follicles decreased from 41.8±5.1% in fresh

Figure 1 Typical morphology of freshly fixed marmoset ovarian tissue (A) and tissues fixed after freeze-thawing in DMSO (B), PrOH (C), and EG (D); unevenly distributed granulosa cells are indicated by asterisks and non-spherical oocytes are indicated by arrowheads.
tissue to 13.3 ± 3.1 (DMSO), 8.4 ± 1.9 (PrOH), or 4.9 ± 1.5 (EG). The difference between the CPAs did not reach statistical significance (P > 0.05 for DMSO compared with PrOH, P > 0.05 for DMSO compared with EG, and P = 0.08 for PrOH compared with EG respectively). Morphologically intact preantral or antral follicles were not observed after cryopreservation.

**Histological analysis of adult ovarian tissues frozen with DMSO or PrOH after xenografting**

To assess the effects of different CPAs on the functionality of ovarian tissue fragments, thawed tissues not directly fixed for histological analysis were grafted to ovariectomized nude mice for a grafting period of 4 weeks. Due to the results of the post-freeze evaluation, six tissue fragments from each adult donor (18 fragments in total) were included in the experiment from both the DMSO and the PrOH groups, but not the EG group. For group assignment, see Table 1. Histological evaluation of all treatment groups (fresh tissues and tissues frozen with one of the CPAs before and after grafting) comprised a total number of 16 235 primordial and primary follicles in adult marmoset tissues. The grafting procedure is commonly known to result in high follicular loss rates, and a one to three ratio of cryopreserved–thawed versus grafted tissues was chosen to achieve in each experimental group absolute follicle numbers adequate for further histological analysis (Table 1). At the end of the experiment, the graft retrieval rate was 72.9% (adult), without significant differences between treatment groups. All grafts displaying no follicles were excluded from the analysis.

The typical post-grafting morphology of adult tissue exhibits a reduced cellular density of the interstitial area compared with fresh controls. In addition to primordial and primary follicles, secondary and preantral follicles were detected in the freshly grafted control tissues and in both the DMSO (Fig. 3A) and the PrOH groups. However, due to the small overall numbers of advanced follicles in grafted tissues post-cryopreservation, the differences were not statistically analyzed. Even though the percentage of morphologically normal follicles significantly decreases after cryopreservation and grafting compared with freshly grafted controls, proliferating cell nuclear antigen (PCNA) staining of GCs, oocytes, and stromal cells suggests the initiation or resumption of proliferative activity in all xenografts (fresh and frozen, Fig. 3B).

Comparing the proportion of morphologically normal primordial and primary follicles after grafting of adult tissue with regard to the CPA used, the DMSO group displays a significantly higher percentage of primordial (26.2 ± 2.5%) and primary follicles (28.1 ± 5.4%) than the PrOH group (12.2 ± 3 and 5.4 ± 2.1%, respectively, Fig. 4A). The ratio of primordial to primary follicles does not differ between the DMSO and the PrOH groups (Fig. 5). However, in comparison to freshly grafted tissues, tissues grafted after freeze–thawing show a shift towards primary follicle activation with a lower proportion of primordial follicles (66.5 ± 2.5% (DMSO) and 71.4 ± 4.6% (PrOH) versus 86.1 ± 2.4% (freshly grafted); Fig. 5).

**Table 1** Assignment of adult and prepubertal marmoset ovarian cortical tissue fragments to treatment groups.

<table>
<thead>
<tr>
<th>No. of donor animals (n)</th>
<th>Treatment</th>
<th>Overall no. of tissue fragments per donor/total</th>
<th>No. of grafted tissue fragments per donor/total</th>
<th>Total no. of analyzed follicles Before grafting</th>
<th>After grafting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>3</td>
<td>34/102</td>
<td>20/60</td>
<td>11 349</td>
<td>4886</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
<td>10/30</td>
<td>8/24</td>
<td>2422</td>
<td>2011</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>8/24</td>
<td>6/18</td>
<td>2609</td>
<td>2296</td>
</tr>
<tr>
<td></td>
<td>PrOH</td>
<td>8/24</td>
<td>6/18</td>
<td>1382</td>
<td>579</td>
</tr>
<tr>
<td></td>
<td>EG</td>
<td>8/24</td>
<td>–</td>
<td>4945</td>
<td>–</td>
</tr>
<tr>
<td>Prepubertal</td>
<td>3</td>
<td>24/72</td>
<td>12/36</td>
<td>31 584</td>
<td>6737</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
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<td>4/12</td>
<td>17 729</td>
<td>4635</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
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<td>4/12</td>
<td>6706</td>
<td>1599</td>
</tr>
<tr>
<td></td>
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<td>4/12</td>
<td>804</td>
<td>503</td>
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<tr>
<td></td>
<td>EG</td>
<td>6/18</td>
<td>–</td>
<td>6345</td>
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</table>
Cryopreservation of prepubertal ovarian tissue with DMSO, PrOH, and EG and subsequent grafting

The effects of DMSO, PrOH, and EG on the percentage of morphologically normal primordial and primary follicles in prepubertal marmoset ovarian tissue are shown in Fig. 6: the proportion of morphologically normal follicles is highest in the DMSO group (84.6 ± 3.4%). However, morphologically normal follicles were not found in the PrOH group, whereas the EG group showed a rather low number of follicles with a normal morphology (1.4 ± 0.6%).

Due to the small size of prepubertal marmoset ovaries, four tissue fragments of each prepubertal donor were included into the grafting experiment (from both the DMSO and PrOH groups), yielding a total of 24 grafts. In total, 83.3% of all (prepubertal) grafts were retrieved, without statistical difference between treatment groups. For group assignment and absolute follicle numbers, also see Table 1. When grafted to immunodeficient nude mice, unlike in adult tissues, the effects of the CPAs analyzed (DMSO and PrOH) on normal morphology of primordial and primary follicles are not significantly different in prepubertal tissues (36.3 ± 2.8 (DMSO) and 28.8 ± 3.9 (PrOH)). However, the shift towards primary follicle activation in grafted tissues post-freezing when compared to freshly grafted tissues is also visible in prepubertal tissue and even more pronounced in tissues frozen with PrOH than with DMSO (Fig. 7). In freshly grafted ovarian tissue fragments, primordial follicles hold a proportion of 97.3 ± 1.0% among the pool of total primordial and primary follicles, whereas in tissues grafted after freeze–thawing this proportion is significantly reduced to 78.3 ± 3.2% in the DMSO and 61.4 ± 5.0% in the PrOH groups respectively.

Discussion

Regular ovarian function comprises a natural maturation process, during which consecutive hormonal cycles permit the development of follicles to more advanced

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**Figure 3** Typical post-graft morphology of frozen–thawed marmoset ovarian tissue (A). Advanced follicle post-grafting (arrowhead). PCNA staining of granulosa cells (arrows), oocytes (asterisks), and interstitial cells (arrowheads) suggest proliferative activity in xenografts (B); negative control for PCNA staining (C).

**Figure 4** Confirmative superior effect of DMSO compared to PrOH following xenografting. The relative number of morphologically normal primordial (A) and primary follicles (B) in xenografted adult marmoset ovarian tissue is significantly higher following cryopreservation with DMSO. Different letters indicate significant differences (P<0.05) between treatments.
stages, while others within the same cohort become atretic and degenerate (Gougeon 1996). Against this background of natural follicular change in the adult ovary, we investigated the effects of different cryoprotectants on non-human primate ovarian tissue from adult and juvenile donor animals. In accordance with the earlier studies for a number of different species (Gook et al. 1999, Paynter et al. 1999, Gandolfi et al. 2006, Maltaris et al. 2007, Borges et al. 2009), we found a pronounced decrease in the percentage of morphologically normal primordial and primary follicles following cryopreservation.

Among the cryoprotectants investigated, DMSO overall had a superior protective effect compared with PrOH and EG. In human ovarian tissue, comparisons between DMSO and PrOH proved both the cryoprotectants’ ability to preserve morphological integrity (Hovatta et al. 1996, Van den Broecke et al. 2001, Maltaris et al. 2007) to a certain degree; however, DMSO was suggested to yield more viable follicles after freeze–thawing than PrOH (Hovatta et al. 1996). Other investigations found DMSO and PrOH to induce significant structural damage in human and bovine primordial follicles in particular (Gandolfi et al. 2006), whereas both agents preserve porcine primordial follicles well. It was also shown by one group that for PrOH, best results were achieved with a prolonged pre-freezing equilibrium period of 90 min (Gook & Edgar 1999). Possibly, better results not only for PrOH but also for EG could be obtained by prolonging equilibrium periods beyond the period used in our investigation. Also, a significant effect of post-thaw short-term tissue incubation (2–4 h) on the percentage of morphologically normal follicles has been described, suggesting a recovery of some follicles (Borges et al. 2009). During this period, cells might be able to re-establish metabolic activity, cellular volume control, and cell–cell communication. A positive effect of *in vitro* culture before transplantation on viability and development of human fetal follicles in ovarian grafts has recently been demonstrated (Lan et al. 2010). Given that in our study tissues were either grafted or fixed as soon as possible after thawing, prolonged post-thaw incubation might improve results for all CPAs analyzed.

A significantly faster penetration of DMSO and EG versus PrOH in human ovarian tissue has been described previously (Newton et al. 1998). Furthermore, the higher tissue permeability coefficient of DMSO has been suggested to account for a better preservation of bi-directional interactions between germ cells and their somatic neighbors (Navarro-Costa et al. 2005). This communication is mediated by transzonal projections (TZPs) emanating from neighboring GCs (Anderson & Albertini 1976). DMSO maintains a higher post-thaw density of filamentous actin, which sustains TZPs (Navarro-Costa et al. 2005). In our study, DMSO has shown an increased protective effect compared with PrOH, but also to EG. As previously observed, differences in penetration rates have been temperature dependent (Newton et al. 1998); this may be due to the fact that dehydration conditions (as cooling and warming rates) have to be chosen specifically for different CPAs. Supplementing the permeating cryosolutions with components which do not penetrate the cell membrane, such as sucrose, promotes dehydration and thus improves the overall performance of the CPA solution (Kuleshova et al. 1999). Furthermore, in whole tissue cryopreservation as opposed to oocyte cryopreservation, optimal conditions for one cell type may not be ideal for other cellular components. One recent study on primate ovarian tissue
cryopreservation has highlighted the cryoinjury incurred by interstitial cells, which may be of importance for long-term graft survival and the full developmental competence of retrieved oocytes (Jin et al. 2010). In tissue fragments, unlike in cell suspensions, the dense packing of cells creates a thermal gradient across the tissue, resulting in different cooling rates for individual cells. Particularly in primordial follicles, where pre-GCs and oocytes differ remarkably in volume, a different sensitivity to the cryopreservation procedure compared to other follicular populations can be expected. In our study, this effect would not only account for differing results between the primordial and the primary follicle compartment but may also explain differences observed between adult and prepubertal tissues, as the proportion of primordial follicles is higher in the latter.

Although morphological evaluation has proven useful to estimate the extent of injury in tissues submitted to cryopreservation, morphological integrity does not necessarily correspond to viability or developmental competence. We have, therefore, decided to assess the effects of different cryopreservation protocols by xenografting the ovarian tissue fragments to nude mice. Our results show a beneficial effect of DMSO on the percentage of morphologically normal primordial and primary follicles compared with PrOH in adult tissues. This is in accordance with the results of other groups, who found DMSO to allow for better preservation of primary follicles than PrOH in human tissue (Gandolfi et al. 2006), although no effects were found in the population of primordial follicles in that study. Other studies suggest that even though EG is known for its low toxicity when applied as a cryoprotectant of bovine and caprine ovarian tissue (Santos et al. 2006b, Celestino et al. 2008), DMSO preserves the ultrastructure of ovine early-staged follicles better (Santos et al. 2006a).

Despite a decreased percentage of morphologically normal follicles after cryopreservation compared with fresh controls, PCNA staining of interstitial cells, GCs, and oocytes suggests ongoing proliferative activity within all cellular compartments of xenografted tissues after cryopreservation.

The evaluation of tissues post-grafting as a functional test of follicular viability, however, did not show an advantage of DMSO in prepubertal tissues. In a recent paper by Abir et al. (2009), follicular development of xenografted frozen–thawed human fetal ovarian tissue could only be documented following cryopreservation with PrOH but not with DMSO. It is of note that contrary to our protocols, sucrose was added to the PrOH cryoprotectant solution only. The observed lack of difference between DMSO and PrOH in comparison to adult tissues in our study may be attributed to the fact that throughout life cohorts of primordial follicles are recruited to go down the differentiation pathway, leading to a steady decline of the ovarian reserve. Accordingly, ovaries of younger individuals should have a greater follicular capacity to compensate for cryoinjury and be less sensitive. Thus, the ability of the cryoprotectants to preserve morphological integrity and functionality of at least some follicles within the treated tissue fragments also seems to be dependent on the age of the tissue donor.

It has previously been shown that ovarian tissue grafting, even without cryopreservation, induces a dramatic follicular loss (Mussett & Parrott 1961, Newton et al. 1996, Nisolle et al. 2000, Shaw et al. 2000, Liu et al. 2002, Borges et al. 2009), irrespective of grafting site (Wang et al. 2002), the age of donors and recipients (Liu et al. 2002), and even species (Salle et al. 2003, Gook et al. 2005). This is associated with a reduced graft size and significant fibrosis in most grafts (Agca et al. 2002). These phenomena have been related to initial ischemia induced by the harvesting procedure and the prolonged time until neovascularization after reimplantation allows a sufficient supply of oxygen and nutrients (Baird et al. 1999, Laschke et al. 2002, Aubard 2003, Van Eyck et al. 2009). In this study, the superior effect of DMSO on both primordial and primary follicle morphology in adult tissue was to the full extent distinguishable only following xenografting.

Moreover, in prepubertal and adult tissues, xenografting revealed remarkable dynamics in the transfer of follicles from the primordial to the primary follicle compartment. Female fertility of most mammals is determined by the primordial follicle pool size and by the rate at which primordial follicles deplete from this reservoir to go down the maturation pathway. The local production and action of several factors are supposed to mediate cell–cell interactions required during follicular development (Skinner 2005). A disruption of GC–oocyte cross talk induced by the cryopreservation procedure has also been suggested to account for the initial follicular
loss following grafting (Navarro-Costa et al. 2005). Recently, it has been demonstrated that as an effect of cryopreservation, stromal cell health might be severely affected (von Wolff et al. 2009). Stromal cells are not only involved in producing factors regulating follicular development (Nilsson & Skinner 2003) but also provide the three-dimensional environment required for follicle maintenance. Our results show a shift towards primary follicle activation from the resting primordial follicle pool when grafting frozen–thawed tissue, whereas we do not find this effect in freshly grafted tissues. This may rather reflect a decrease in inhibitory signals on follicular activation following cryodamage of interstitial cells (Jin et al. 2010) than an increase in activating signals deriving from a permissive recipient milieu.

In conclusion, both the morphological appearance of follicles and any condition facilitating their recruitment in the pool of growing follicles will ultimately define the oocyte’s developmental competence and long-term graft survival. The present data suggest that DMSO has a slightly advantageous effect compared to other cryoprotectives during conventional slow-freezing procedures of marmoset ovarian tissue. The whole extent of structural and functional damage inflicted on ovarian tissue by cryopreservation becomes apparent only following the grafting of thawed tissue fragments. In prepubertal tissue, significant differences between DMSO and PrOH concerning follicular morphology are abrogated following xenografting, probably owing to a greater capacity of younger individuals to compensate for cryoinjury. The propagation of these results from non-human primate tissue can assist in further developing ovarian tissue cryopreservation and grafting into a routinely successful option for fertility preservation in children and young adults.

Materials and Methods

Animals

All handling and experimental procedures concerning the animals utilized in this study were in accordance with the German animal protection law (license no. AZ 50.0835.1.0).

Ovarian tissues were taken from three healthy female common marmosets with proven fertility aged between 2 and 3 years (adult group) and three healthy females aged between 6 and 20 weeks (prepubertal group) originating from the breeding colony of our institute’s animal research unit. Directly upon removal, ovaries were rinsed briefly in PBS (Gibco), transferred into fresh PBS supplemented with human serum albumin (HSA, 4 mg/ml, Irvine Scientific, Santa Ana, CA, USA), and dissected under microscopic control at room temperature (RT). Fragment sizes of 1–2 mm³ were used in all experiments. Control tissues were fixed immediately; the remaining tissues were either grafted freshly or assigned to cryopreservation with DMSO, PrOH, or EG; see also Table 1 for group assignment.

Cryopreservation

The effects of three different cryopreservation protocols were investigated as follows:

1. DMSO: PBS supplemented with DMSO (1.5 mol/l, Merck), sucrose (0.5 mol/l, Sigma–Aldrich), and HSA (10 mg/ml).
2. PrOH: PBS containing PrOH (1.5 mol/l, Sigma–Aldrich), sucrose (0.5 mol/l), and HSA (10 mg/ml).
3. EG: PBS containing EG (1.5 mol/l), sucrose (0.5 mol/l), and HSA (10 mg/ml).

In these solutions, fragments were gently agitated at RT for 20 min, transferred into cryostraws (Consarctic, Schoellkrippen, Germany) with 0.5 ml of cryoprotectant solution, sealed, and cryopreserved following a slow-freezing protocol (Gook et al. 1999) in a controlled rate programmable freezing system (Consarctic): starting at RT, the cooling rate to −7 °C was 2 °C/min. Seeding was induced manually at −7 °C. Cooling then continued at 0.3 °C/min to −40 °C followed by −10 °C/min to −150 °C. Cryostraws were subsequently immersed in liquid nitrogen and stored for a minimum time period of 2 weeks.

On the day of transplantation, ovarian tissue fragments were thawed following a rapid procedure: straws were warmed at RT for 40 s and then immersed in a water bath at 30 °C until all ice crystals vanished. The contents of the straws were then released into cryoprotectant solution. The cryoprotectant was removed by washing the tissue fragments in PBS containing decreasing concentrations of cryoprotectant (75, 50, and 25% of the original concentration) at RT for 5 min each followed by PBS (with 10 mg/ml HSA) and either grafted as quickly as possible or fixed for histological evaluation (see below). Cooling rates, manual seeding, and rapid thawing procedures in preparation for grafting were alike in all cryoprotocols.

Surgical procedures

Totally, eight Nu/Nu nude mice (Crl:NU-Foxn1<sup>nu</sup>) were obtained (Charles River Germany, Sulzfeld, Germany) and housed in the central animal facility of the university for at least 1 week prior to procedures. The environment was air filtered with a 12 h light:12 h darkness cycle at 23 °C; all animals had free access to sterilized food and water. Ovariectomies were carried out 2 weeks prior to grafting through a dorsomedian incision; surgical procedures were performed under a laminar flow hood in aseptic conditions. The animals were anesthetized with 0.15 mg/g body weight ketamine and 2% (v/v) xylazine intraperitoneally. They were housed in the central animal facility of the university for at least 1 week prior to procedures. The environment was air filtered with a 12 h light:12 h darkness cycle at 23 °C; all animals had free access to sterilized food and water. Ovariectomies were carried out 2 weeks prior to grafting through a dorsomedian incision; surgical procedures were performed under a laminar flow hood in aseptic conditions. The animals were anesthetized with 0.15 mg/g body weight ketamine and 2% (v/v) xylazine intraperitoneally. They were kept on a warming plate; eye protection was carried out with humidified sterile swabs. On the day of transplantation, mice received eight grafts per animal. For best accessibility and to achieve a maximum number of grafts per host animal, the tissue was placed in subcutaneous pouches formed with a blunt probe in a small incision in the dorsal skin. Each graft was placed in an individual subcutaneous pouch. The grafting experiments have been carried out in multiple rounds to ensure optimal technical handling and minimize the number of animals to be killed. Grafting procedure was...
scheduled to the availability of freshly donated ovarian tissue. For cryopreserved tissues, storage time was a minimum of 2 weeks and could also be longer without effect on later analysis. To avoid bias inflicted by different endogenous hormonal milieu of the recipients, cryopreserved fragments from both treatment groups along with fresh control tissues were transplanted to every mouse except in the first round of experiments, when only fresh tissue was grafted. The grafting pattern (four grafts were placed on either side of the spinal column) was retained in all animals for easy identification of the grafts at the end of the experiment. Pouches were sutured with 5/0 vicryl sutures. No animal died during surgery or was lost prior to the end of the experiment.

**Graft retrieval and procession of tissue**

On the day of explantation of the grafts, the mice were killed by cervical dislocation. Grafts were retrieved 4 weeks after the transplantation procedure and immediately fixed in 4% paraformaldehyde (Sigma–Aldrich). Following fixation, tissues were dehydrated in a graded ethanol series, clarified with xylene, and routinely embedded in paraffin wax. Serial sections of 5 μm thickness were cut; every fifth section was stained with hematoxylin–eosin (HE) and analyzed with a light microscope (Axiophot, Zeiss, Oberkochen, Germany) under a magnification of 400×. The size of the tissue sections was determined based on follicle sizes and previous work with marmoset and primate ovarian tissue (Gougeon 1986, Candy et al. 1995). Marmoset primordial follicles have a diameter of 30 μm, increasing up to 45–85 μm in primary follicles (Buse et al. 2008). On the opposite end, small follicles with a nucleus <19 μm are considered non-growing in primates (Gougeon & Bussó 2000). Given these numbers, the aforementioned tissue section size is adequately chosen to detect the large majority of follicles while at the same time prevent double-counting of follicles. An additional oocyte staining technique has, therefore, not been employed.

**Histological evaluation**

All sections were analyzed by a single investigator blinded to the experimental groups. In the analyzed sections, all follicles were counted. To avoid overcounting, follicles were only counted when the nucleus of the oocyte was observed in the section. The developmental stages of the follicles were classified according to Gougeon (1996): the oocyte of a primordial follicle is surrounded by a single layer of flattened GCs or a mixture of flattened and cuboidal GCs. In a primary follicle, the single GC layer is cuboidal; a secondary follicle displays two or more layers of GCs; in a preantral follicle, small fluid-filled cavities are observed between the GCs, and in the antral follicle, an antrum is distinguishable.

Follicular morphology was evaluated based on variables such as integrity of the oocyte, the GCs, and the basement membrane. Follicles were classified as morphologically normal if they showed no pyknosis of the oocyte nucleus, no disorganized or detached GCs, or shrunken ooplasm.

**Immunohistochemistry**

Tissue sections were immunohistochemically stained for PCNA using an indirect method with a peroxidase-labeled secondary antibody according to the manufacturer’s instructions. Briefly, sections were deparaffinized in paraclear and rehydrated in a graded series of ethanol. For antigen retrieval, sections were heated in a microwave oven in glycine/HCl buffer (50 mM, pH 3.5) for 10 min at 100 °C. Endogenous peroxidase activity was quenched by treatment with hydrogen peroxide (3% v/v) for 5 min) followed by blocking of nonspecific antibody binding with 5% (v/v) normal goat serum supplemented with BSA (0.1% v/v) for 20 min at RT. All antibodies were diluted in TBS/BSA (0.1% v/v). The slides were incubated with primary rabbit-anti-PCNA antibody (1:100, Dako, Glostrup, Denmark) at RT in a humidified chamber for 1 h and rinsed in TBS (10 mM TBS, 150 mM NaCl, pH 7.6) for 3 × 5 min between each of the following incubations. Sections incubated in TBS/BSA without primary antibody served as negative control. All tissues were immunostained with HRP-conjugated goat-anti-rabbit IgGs (1:50; Dako) for 30 min. The label was visualized by incubation in 3,3-diaminobenzidine tetrahydrochloride in urea buffer for 5–12 min (Sigma–Aldrich). Positive staining appeared as a brown precipitate in the cells. All sections were counterstained in hematoxylin, mounted, and analyzed.

**Statistical analysis**

Comparisons between experimental groups were carried out using ANOVA, and in case of statistical significance, Tamahane was used for post hoc comparisons. Values were considered statistically significant with \( P < 0.05 \) and are presented as mean ± S.E.M. Data analysis was carried out utilizing SPSS for Windows (Version 15.0; SPSS Inc, Chicago, IL, USA).

**Declaration of interest**

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

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