

## Allogeneic and Xenogeneic Transplantation of Cryopreserved Ovarian Tissue to Athymic Mice<sup>1</sup>

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### ABSTRACT

Cryopreserved ovarian tissue has major applications for female oncology patients and for the development of genome resource banks. The objective of the present study was to develop a bioassay of cryopreserved ovarian tissue function after allogeneic and xenogeneic transplantation to ovariectomized athymic nude (*nu/nu*) Balb/C mice. Transplant function was assessed by examination of vaginal smears, number of live births, and posttransplant histology. Animals were sham operated (group I; *n* = 4) or ovariectomized (group II; *n* = 5) or were given transplants of either fresh (group III; *n* = 3) or cryopreserved (group IV; *n* = 4) Institute of Cancer Research-strain mouse ovarian tissue or cryopreserved sheep ovarian tissue (group V; *n* = 7). Vaginal smears were examined 5–7 times per week; the number of days between visualizations of epithelial cells in smears was  $4.3 \pm 0.6$  for group I,  $8.6 \pm 3.8$  for group II,  $3.4 \pm 0.4$  for group III,  $3.3 \pm 0.5$  for group IV, and  $4.6 \pm 0.6$  for group V. Epithelial cells were seen for 1.2–1.7 consecutive days; this value was significantly different between groups III and V. Live births were recorded from 3 of 4 animals from group I, 0 of 5 animals from group II, 2 of 3 animals from group III, and 1 of 4 animals from group IV. In vivo function and long-term survival of cryopreserved ovarian tissue after allogeneic or xenogeneic transplant were confirmed by the examination of vaginal cytology, and offspring were derived from allografts.

### INTRODUCTION

Many female oncology patients suffer iatrogenic loss of ovarian function resulting in infertility, premature menopause, and associated effects on general health (e.g., increased likelihood of heart disease, osteoporosis) as a result of radiation and/or chemotherapy treatment [1–3]. Men undergoing similar treatments have the option of cryopreserving and banking sperm samples for future use, thereby protecting their gametes [4, 5]. A similar option for women is not currently available, as cryopreserved oocytes are developmentally compromised, infrequently progressing beyond the embryo stage [6–9]. Ovarian tissue from oncology patients could be cryopreserved prior to treatment for autologous transplant after remission. This would provide a therapeutic option through which endogenous steroidogenic function could be protected and restored, obviating the need for exogenous hormone replacement therapy.

Efforts are underway worldwide to develop genome resource banks of cryopreserved sperm and embryos from endangered species, which can be used to propagate and protect biodiversity [10, 11]. Successful oocyte cryopreservation is still a considerable challenge, even in well-studied laboratory species such as mice [6, 8, 12]. Therefore, the chances of successfully cryopreserving oocytes from endangered animals, in which even the reproductive cycles are often not defined, is more remote still. Cryopreservation of ovarian tissue from these endangered species could be used to bank follicle-enclosed oocytes, which are quiescent and less susceptible to chromosomal aberrations and spindle depolymerization [12]. However, the recovery and continued growth of these oocytes in vitro, from this very early stage to a “mature” metaphase II (MII) stage at which fertilization can occur, present a considerable challenge. Currently, the in vitro growth and maturation of primordial follicle mouse oocytes from germinal-vesicle to MII stage has been successfully reported by only one group [13].

Early studies evaluating cryopreserved ovarian tissue were carried out by Deansley [14], Parkes [3, 15, 16], and Parrott [17, 18]. Cooled or frozen-thawed tissues were transplanted allogeneically to the site of the recipient ovary (previously sterilized by x-ray irradiation), and a few pregnancies were established after inclusion of glycerol in the cryopreservation media [3, 16, 18]. Allogeneic orthotopic transplantation of nonfrozen ovarian tissue resulted in two litters from an individual mouse [19]. A few more recent studies evaluating cryopreserved ovarian tissue function after syngeneic or autologous transplant have demonstrated retention of some degree of estrogenic function [20–23]. Some graft development after xenotransplantation of marmoset, cat, or sheep ovarian tissue to immune-incompetent mice has been recently reported by two groups [24, 25].

Cryopreserved ovarian tissue has been shown to recover steroidogenic function and fertility in vivo after allogeneic or autologous transplantation [21, 22]. Recently, we obtained live births from 8 of 11 animals after autologous transplant of cryopreserved mouse ovaries [26]. However, in vivo functioning is currently the only method of determining cryopreserved ovarian tissue function, limiting evaluation to mice or other laboratory animals. In order to develop the two prospective applications for the use of cryopreserved ovarian tissue (i.e., for female oncology patients and genome resource banking) alternate methods to assess postthaw viability are necessary. Cells lining the vagina are particularly sensitive to estrogens and display characteristic changes on each day of the 4- to 5-day estrous cycle in rodents [27, 28]. Nucleated epithelial cells are seen prior to ovulation (concomitant with rising estradiol levels), at which time they keratinize and become cornified epithelial

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cells; there is then a decrease in the number of cornified cells and appearance of leukocytes before the nucleated epithelial cells are seen again [27].

The present study was undertaken to evaluate the feasibility of examining vaginal cytology of athymic Balb/C nude (*nu/nu*) mice as an *in vivo* bioassay of cryopreserved ovarian tissue function after allogeneic (from Institute of Cancer Research [ICR] mice) or xenogeneic (sheep) transplant. The ovarian bursa was chosen as the transplant site so that pregnancies could be established by oocytes ovulated from allografts. Ovarian tissue from ICR mice and sheep was cryopreserved using the protocol of our previous study [26]. Transplant function was assessed by examination of vaginal cytology 5–7 times per week, number of pregnancies, and number of live births in animals with allografts. Evaluation of the reproductive tract and graft site and histology of grafted tissues were performed upon completion of the follow-up period.

## MATERIALS AND METHODS

### *Animals and Experimental Groups*

Approval for the project was obtained from the Institutional Animal Care and Use Committee of Methodist Hospital. Athymic Balb/C nude (*nu/nu*) mice females (4–6 wk) and males (6–8 wk) were housed in a high-efficiency particulate air-filtered, positive-pressure room. Cages were filter topped, and animals had free access to sterilized food and water under 14L:10D cycles. All procedures, tests, and surgeries were performed in the positive-pressure room or under sterile conditions in a surgery suite. Outbred female mice (4–6 wk) of the ICR strain (Harlan Sprague-Dawley, Indianapolis, IN) were housed under 14L:10D cycles and given free access to food and water. Before use, nude mice were allowed to acclimate for 1 wk and then assessed for estrous cyclicity by examination of vaginal smears for 10 days to 2 wk. Smears were taken using sterile flame-polished pasteur pipettes and sterile PBS (Life Technology, Grand Island, NY). Animals showing estrous cyclicity were randomly assigned to one of the following experimental groups: sham operated (group I); ovariectomized (group II); fresh (nonfrozen) ICR mouse ovarian tissue (group III); cryopreserved/thawed ICR mouse ovarian tissue (group IV); cryopreserved/thawed sheep ovarian tissue (group V).

### *Ovarian Tissue Collection and Storage*

Ovarian tissue was collected after cervical dislocation of ICR mice for immediate transplant to group III or was cryopreserved (using the protocol described below) prior to transplant to group IV. Biopsies of Dorsett sheep ovarian tissue were collected while the animal was under general anesthesia and were cryopreserved using the protocol described below. All cryopreserved ovarian tissue was stored in liquid nitrogen ( $-196^{\circ}\text{C}$ ) until use, a period of 2–3 mo (ICR mouse tissue) or 4–6 mo (sheep tissue).

### *Surgical Procedure*

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. General anesthesia was induced with 5% 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane; Fort Dodge Laboratories, Fort Dodge, IA) and maintained with 1–2% Halothane in oxygen. Mice were kept on a warming plate ( $35$ – $37^{\circ}\text{C}$ ), covered with a sterile towel, during surgery. Ovaries were externalized through a small dorsolateral incision and kept

moist with sterile Hepes-buffered Tyrode's lactate (TL-Hepes) medium [29]. Sham-operated (group I) animals received no further treatment; the ovaries were replaced, the body wall incisions were sutured, and the skin was closed using 3–4 wound clips. Both ovaries of animals in all other experimental groups were removed by making a slit along about one third of each bursa at one of the ovoid edges. Group II (ovariectomized) animals received no further surgery, and the incisions were closed as described above. Animals in the other experimental groups (III, IV, and V) received transplants of fresh ovarian ICR mouse tissue, cryopreserved/thawed ovarian ICR mouse tissue, or cryopreserved/thawed ovarian sheep tissue, respectively. Donor ovarian tissue was thawed during the removal of the ovaries from the recipient nude mouse using the protocol described below. Donor ovarian tissue was cut into pieces that would fit comfortably in the host bursa, about  $1\text{ mm} \times 1$ – $1.5\text{ mm}$ . Cortical sections of thawed ovine ovarian tissue were cut to the same size at the time of transplant. Transplants were performed bilaterally. Ovarian tissue was placed in the pocket of the bursa, covered by the membrane, which was secured with 8–0 vicryl suture (Ethicon, Somerville, NJ). The ovarian fat pad was replaced and the incisions were closed as described above.

### *Cryopreservation and Thawing Protocol*

Ovarian tissue from ICR mice or sheep was cryopreserved using a protocol modified from Harp et al. [22]. A whole ICR mouse ovary or  $3 \times 3$ -mm biopsies of ovine ovarian tissue were placed in a 1.2-ml cryovial (Corning, Corning, NY) with 1 ml of 1.4 M dimethyl sulfoxide (DMSO) in TL-Hepes and held at room temperature for 5 min. The vials were sealed by screwing on the lid, placed in a programmable rate freezer (Planer, Sunbury-on-Thames, England, UK), and cooled from  $25^{\circ}\text{C}$  to  $10^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ , then at a rate of  $0.5^{\circ}\text{C}/\text{min}$  to  $-7^{\circ}\text{C}$ , and finally held at  $-7^{\circ}\text{C}$  for 5 min. Ice nucleation was induced manually using pre-cooled forceps, and the temperature was held at  $-7^{\circ}\text{C}$  for a further 5 min for release of latent heat fusion. The tissue was then cooled to  $-55^{\circ}\text{C}$  at a rate of  $0.5^{\circ}\text{C}/\text{min}$ , plunged in liquid nitrogen at  $-196^{\circ}\text{C}$ , and stored for 24 h. Thawing was performed by removing the cryovial to room temperature until all ice had melted (15–20 min) and then transferring the tissue to 5 ml fresh TL-Hepes at room temperature for 10 min, shaking gently to promote efflux of DMSO from the tissue. Tissues were then placed in TL-Hepes at  $37^{\circ}\text{C}$  until transplant, performed as described above. Thermocouples connected to a computer data-sampling program were placed in cryovials containing 1 ml TL-Hepes medium and were cryopreserved and thawed using the protocols described above. The thawing rate was  $48.8^{\circ}\text{C}/\text{min}$  for the first 2 min,  $9.1^{\circ}\text{C}/\text{min}$  for the next 8 min, and  $0.2^{\circ}\text{C}/\text{min}$  for the last 10 min, by which time all the ice had melted.

### *Evaluation of Transplant Function*

Animals were allowed to recover for at least 10 days before vaginal cytology was examined. Smears of animals in groups I, II, III, and IV were evaluated for 5–8 wk prior to mating with male nude mice. Females were exposed to males beginning when epithelial cells were seen in vaginal smears, except for ovariectomized animals, which were exposed to males 4–6 wk after surgery. Matings were confirmed by the presence of a vaginal plug, at which time females were housed individually. If no vaginal plug was

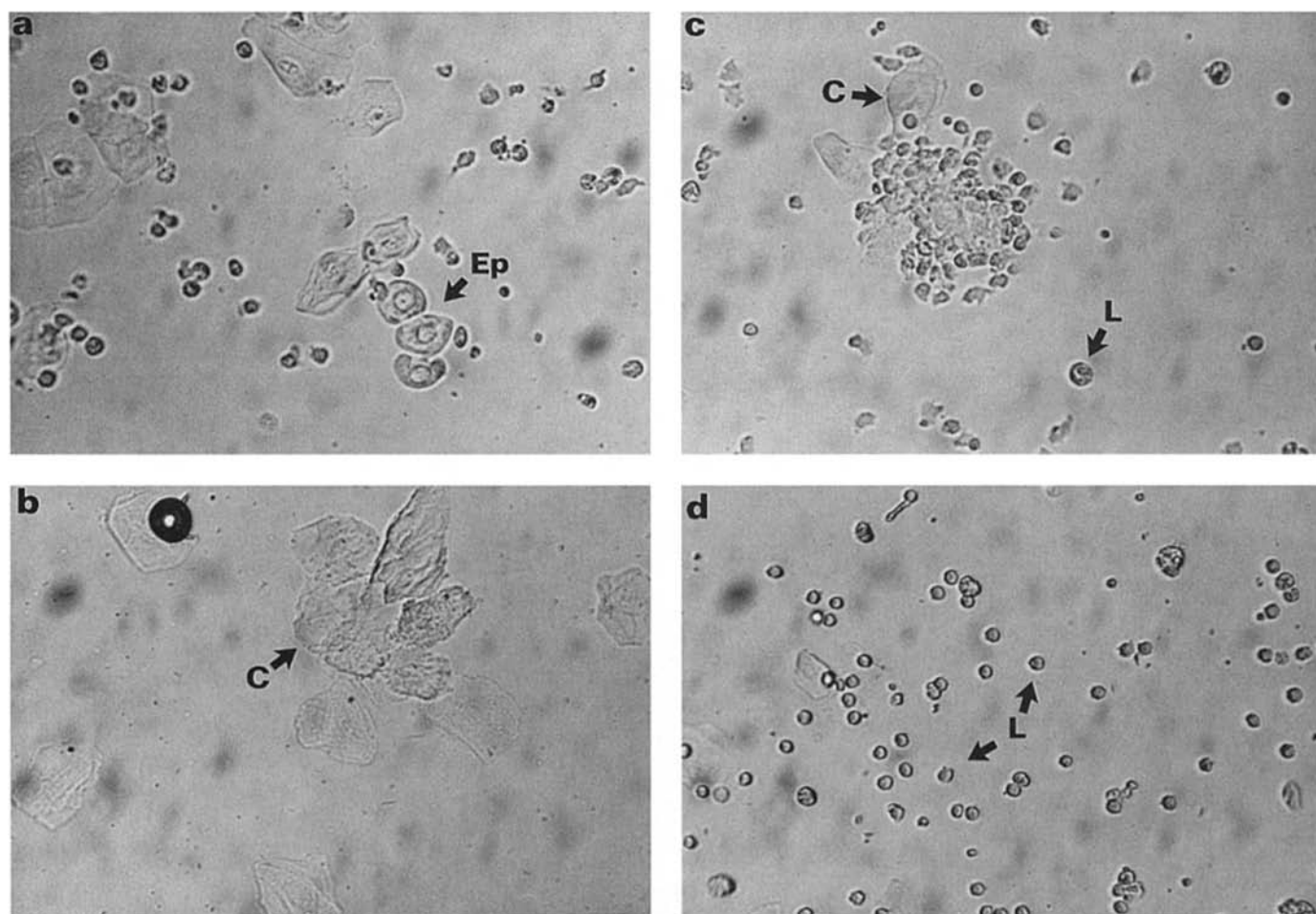


FIG. 1. Vaginal smears taken from control animals showing each stage of the murine estrous cycle: proestrus (a), estrus (b), metestrus (c), and diestrus (d). Arrows indicate cell types: epithelial (Ep), cornified epithelial (C), or leukocytes (L). Magnification  $\times 200$  (reproduced at 54%).

seen, females were left in continuous pairings with males for 21 days and then separated and monitored for a further 21 days. If no pregnancy resulted after the first mating, females were exposed to males once more and monitored in the same manner. Live births and number of pups were recorded. Animals in group V were evaluated with vaginal smears 5–7 times per week, for at least 60 days posttransplant, and were not mated.

### Histology

Ovarian tissue superfluous to transplants was placed in fixative (Prefer; Anatech Ltd., Battle Creek, MI) and processed as described below. Upon completion of the follow-up period, animals were killed by cervical dislocation. The appearance of the reproductive tract, degree of adhesion, graft site, and the quantity and quality of ovarian tissue recovered were noted. Recovered grafts (enclosed by the bursa) or the graft site (fluid filled bursa or fat pad) was placed in fixative. Fixed tissues were mounted in paraffin blocks, and 5- $\mu$ m sections were cut before staining with hematoxylin and eosin.

### Data Analysis

Data were examined using a standard analysis of variance approach via the General Linear Models procedure of the Statistical Analysis System [30] and are expressed as mean  $\pm$  SEM, and coefficients of variation (CV) were cal-

culated. Means separation was conducted using a least significant difference approach; a value of  $p < 0.05$  was considered significant.

### RESULTS

All animals recovered from surgeries and survived the postoperative period with the exception of one mouse that developed an infection and died after an attempted mating.

#### Vaginal Cytology

**Group I.** Smears from sham-operated animals demonstrated cyclic changes in cytology characteristic of murine estrous cycles (e.g., epithelial cells at proestrus) (Fig. 1). Estrous cyclicity was seen 10–14 days after sham surgeries. A variable number of leukocytes were present in all smears from nude mice. Table 1 shows for all groups the interval between days on which epithelial cells were observed as well as the number of consecutive days on which epithelial cells were seen in smears. In sham-operated animals ( $n = 4$ ), epithelial cells were seen at intervals of  $4.3 \pm 0.6$  days (mean  $\pm$  SEM; CV 57.3%), for durations of  $1.4 \pm 0.1$  consecutive days (CV 41.9%), characteristic of estrous cyclicity [27].

**Group II.** One ovariectomized animal did not demonstrate any epithelial cells in smears throughout the monitoring period, whereas the other animals ( $n = 4$ ) demonstrated sporadic incidence of epithelial cells at intervals of

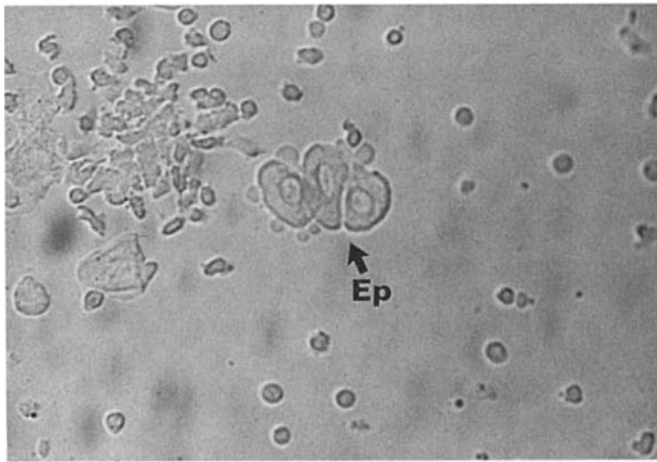


FIG. 2. Vaginal smear from an animal with cryopreserved sheep ovarian tissue (group V) showing epithelial cells (Ep). Magnification  $\times 200$  (reproduced at 53%).

1–30 days ( $8.6 \pm 3.8$ ; CV 115.7%) lasting for 1–2 days ( $1.3 \pm 0.2$ ; CV 37.2%). Most smears from ovariectomized animals were composed of leukocytes and occasional cornified epithelial cells. Upon examination of the transplant site, no evidence of ovarian tissue from an incomplete ovariectomy was found in any ovariectomized animals.

Groups III and IV. Epithelial cells were seen in all animals in groups III ( $n = 3$ ) and IV ( $n = 4$ ) 10 to 28 days after transplant (Table 1). The intervals between the appearance of epithelial cells in smears was 1–8 days in group III ( $3.4 \pm 0.4$ ; CV 52.7%) and 2–8 days in group IV ( $3.3 \pm 0.5$ ; CV 66.7%). The duration of appearance of epithelial cells was similar in the two groups. There were no significant differences ( $p > 0.10$ ) between the interval or the duration of appearance of epithelial cells after allogeneic transplant of fresh or cryopreserved ICR ovarian tissue and these values in the sham-operated animals (Table 1).

Group V. Ovarian tissue from 3 sheep were transplanted to 7 nude mice recipients following complete ovariectomy. These animals were monitored 5–7 times per week for 3–

TABLE 1. The interval between, and duration of, visualization of epithelial cells in vaginal smears (mean  $\pm$  SEM).

Group	No. of days	
	Interval between (CV)	Duration (CV)
I: Sham	$4.3 \pm 0.6$ (57.3%)	$1.4 \pm 0.1$ (41.9%)
II: Ovariectomized	$8.6 \pm 3.8$ (115.7%)	$1.3 \pm 0.2$ (37.2%)
III: Fresh ICR transplant	$3.4 \pm 0.4$ (52.7%)	$1.2 \pm 0.1^*$ (31.85%)
IV: Cryopreserved ICR transplant	$3.3 \pm 0.5$ (66.7%)	$1.6 \pm 0.2$ (46.4%)
V: Cryopreserved sheep transplant	$4.6 \pm 0.6$ (85.8%)	$1.9 \pm 0.2^*$ (62.8%)

\* Values within a column significantly different from each other ( $p < 0.05$ )

4 mo. Epithelial cells were seen in vaginal smears of 4 recipients (at least one from each donor) 17–34 days after transplant (Fig. 2). Epithelial cells were seen at intervals of 1–14 days ( $4.6 \pm 0.6$ ; CV 85.8%) for up to 5 consecutive days ( $1.9 \pm 0.2$ ; CV 62.8%), a pattern that was not seen in any of the other groups. The duration of appearance of epithelial cells in the animals grafted with sheep ovarian tissue was significantly higher ( $p < 0.05$ ) than in the fresh ICR transplant group (group III). Upon necropsy, no evidence of transplanted tissue could be found in the 3 animals not demonstrating any epithelial cells in smears, suggesting loss or resorption of grafts.

#### Pregnancies and Live Births

Live births were recorded from 3 of 4 sham-operated (2, 7, and 9 pups), 0 of 5 ovariectomized, 2 of 3 fresh tissue transplant (4 and 5 pups), and 1 of 4 cryopreserved tissue transplant (2 pups) animals. Pups delivered by animals in groups III and IV developed white fur upon weaning (Fig. 3), confirming that they were heterozygous and were derived from the ICR allograft tissue implanted in the ovarian bursa.



FIG. 3. Animal from group III (fresh ICR transplant) with her pups. Note that pups have white fur and are derived from allogeneic transplants of ICR ovarian tissue.

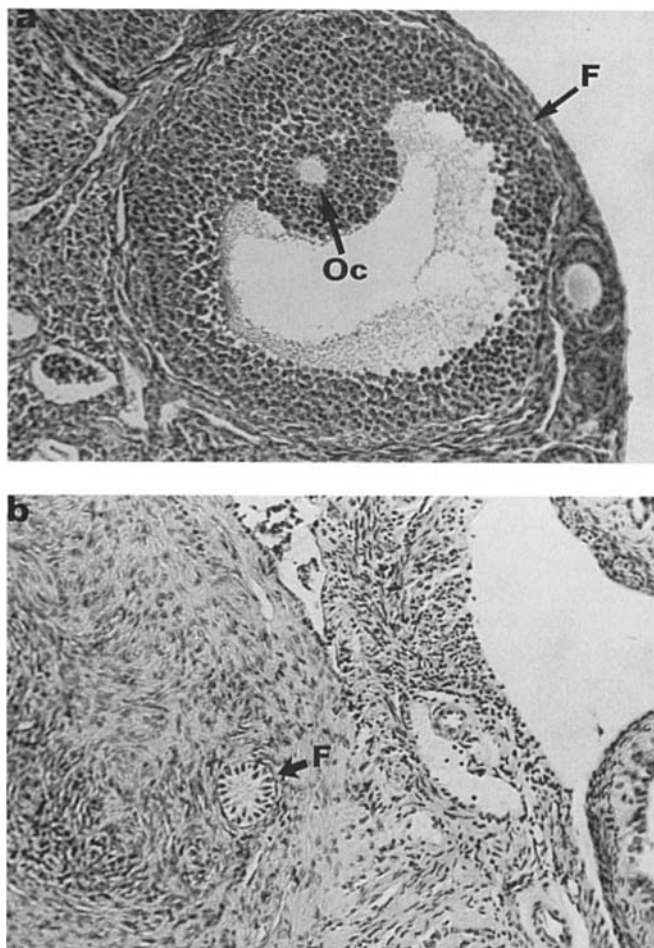


FIG. 4. Histological sections of grafted ovarian tissue from (a) group III (fresh ICR transplant) and (b) group V (cryopreserved sheep transplant). An antral follicle (F) and oocyte (Oc) are clearly visible in a, and a primordial follicle (F) without an oocyte in b. Sections were stained with hematoxylin/eosin. Magnification  $\times 100$  (reproduced at 53%).

### Histology

Moderate adhesions were seen in 3 of the 22 animals, 1 from group IV and 2 from group V. The uteri of animals in groups I (sham), III (fresh ICR transplant), and IV (cryopreserved ICR transplant) were normal or slightly hypertrophied, whereas those in group II (ovariectomized) and V (cryopreserved sheep transplant) were visibly atrophied. Some remaining graft tissue was collected from all animals, except those in groups II, in which only fluid-filled bursas or small necrotic spots were visible. Well-developed antral follicles were seen in graft tissues recovered from group I, III, and IV animals (Fig. 4a). Histological evaluation of ovine ovarian tissues before transplant and after grafting did not show any antral follicles and only occasional primordial follicles (Fig. 4b). When animals in group V were killed, no epithelial cells had been seen in smears for the previous 21 days.

### DISCUSSION

Viability and function of ovarian tissue biopsies allogeneically transplanted to the ovarian bursa, as opposed to beneath the kidney capsule, were confirmed in this study. Allografts recovered estrous cyclicity as early as 10 days after transplant, as seen in the 4-day interval and 1-day duration of appearance of epithelial cells in vaginal smears.

Cryopreservation did not have a detrimental effect on ovarian allograft function, and both estrous cyclicity and pregnancies were established. Viability of cryopreserved ovine ovarian tissue for up to 4 mo after xenografting was also confirmed by examination of vaginal cytology. Examination of vaginal cytology can thus be used as an effective, noninvasive method of assessing the postthaw viability of cryopreserved ovarian tissue after allogeneic or xenogeneic transplantation. Transplant to the ovarian bursa can allow sufficient revascularization of the grafts to enable follicle growth, ovulation, and in situ pregnancies to be established, with delivery of live offspring. In vivo-matured oocytes from xenografts could then be recovered for in vitro fertilization, embryo culture, and transfer to an appropriate surrogate. Xenotransplantation to nude mice has been shown to provide a valuable tool for assessing postthaw viability of cryopreserved ovarian tissue and could be a potential system for recovery of "banked" oocytes from within these tissues.

In this study we present the first report of live births from fresh (nonfrozen) and cryopreserved ovarian tissue after allogeneic transplant to the nude mouse model. Previous studies evaluating allogeneic transplant of cryopreserved ovarian tissue in other mice strains resulted in some pregnancies (1 of 8 animals), together with significant graft rejection [18] or limited function [23]. Autologous transplantation of cryopreserved ovarian tissue has resulted in recovery of estrous cyclicity and live births in mice [22] and sheep [21]. Cryopreserved mouse primordial follicles generated live births after syngeneic transplantation to the ovarian bursa of ovariectomized recipients, followed by superovulation, embryo recovery, and transfer to another recipient [20]. Although this technique would prove valuable for recovery of fertility, transplantation of primordial follicles alone would have a limited contribution to steroidogenic activity. However, transplant of ovarian tissue biopsies would enable sufficient steroidogenic, and potential gametogenic, function to be retained.

Viability of ovine xenografts was confirmed by examining vaginal cytology. Two groups have previously evaluated xenotransplantation of ovarian tissue to immune-incompetent nude mice [24, 25]. Grafts were placed beneath the kidney capsule, and function was assessed by histology, vaginal cytology, and vaginal patency. Antral follicles developed within xenografts of cryopreserved-thawed marmoset (a primate) ovarian tissue [24]. The ability of a rodent host to support limited development of follicles within a primate ovary is more evidence that the immune-incompetent mouse can be used for an effective bioassay of cryopreserved ovarian tissue xenograft function. Development of appropriate superovulation regimens would be necessary before mature oocytes from xenografts could be recovered, but this could be possible and could be facilitated by grafting to the ovarian bursa.

The patterns of epithelial cells seen in vaginal smears from mice with ovine xenografts were not significantly different from the pattern for sham or allografted animals. However, patterns of estrogenic activity did not unambiguously follow that of either the donor or the host species. The estrous cycle of the Dorsett sheep, a domesticated breed, is 16–17 days. During the estrous cycle, three waves of follicular growth, associated with increased estradiol secretion, have been characterized [27]. The first two waves occur at 3- to 5-day intervals, and the final wave occurs just before ovulation, about 9–11 days later. Intervals between estrogenic effects on vaginal cytology of mice with



ovine xenografts ranged from 1 to 14 days, suggesting that the intrinsic estrous cyclicity of the host (sheep) was not completely lost to that of the recipient (mouse). Estrogenic effects on vaginal cytology diminished 4 mo after transplant, and loss or deterioration of the grafts was confirmed on examination of the transplant site at necropsy. In a previous study, nonfrozen ovine ovarian tissue showed low levels of estrogenic activity and small antral and preantral follicles 8–20 wk after xenotransplant to the kidney capsule of immune-incompetent mice [25]. In the present study, evidence of graft function was seen as early as 17 days after transplant of cryopreserved-thawed ovine ovarian tissue. Therefore, cryopreservation does not seem to adversely affect the steroidogenic activity of ovine ovarian tissue, and the nude mouse can be used to confirm viability and functioning of frozen-thawed xenografts.

Use of the nude mouse for xenogeneic transplants of cryopreserved ovarian tissue would be a valuable tool for the postthaw assessment of these tissues. If human ovarian tissue is cryopreserved therapeutically for autologous use by patients at risk of sterility following cytotoxic treatments, a mechanism to evaluate the tissue prior to transplant is essential. Furthermore, the potential to induce development and recovery of "mature" oocytes using the nude mouse system is exciting. Although human assisted reproductive techniques have made great advances, there is still a paucity of donated oocytes that are needed by women with severe ovarian dysfunction. Oocyte donation currently involves extensive clinic visits; expensive, uncomfortable, and hazardous superovulation therapies; and surgical aspiration of mature oocytes. If donated ovarian tissue could be utilized to recover mature oocytes (e.g., with the nude mouse model), the inconveniences and hazards of oocyte donation, which often result in withdrawal of donors from programs, could be eliminated. However, these applications are fraught with ethical concerns that should be addressed and clarified before such techniques are used in clinical practice.

Ovarian tissue from endangered species could be cryopreserved in genome resource banks as part of the worldwide effort to protect biodiversity. The primary reason for cryopreserving ovarian tissue is to bank female gametes, although their recovery is currently limited, if not impossible. Recovery of oocytes induced to develop in a nude mouse system would be a major addition to the resources needed to protect and increase biodiversity. We have recently used this nude mouse model to successfully support the development of follicles within cryopreserved xenografted elephant ovarian tissue to the antral stage. In addition to this application for conservation, evaluation of estrogenic activity of xenografts could provide additional basic scientific information about many uncharacterized species.

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