Markers of growth and development in primate primordial follicles are preserved after slow cryopreservation

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Objective: To investigate the effect of slow cryopreservation on the morphology and function of primate primordial follicles within ovarian tissue slices.
Design: Fresh monkey ovarian tissue was frozen by slow cryopreservation and thawed for analysis of morphologic and functional parameters.
Setting: University-affiliated laboratory.
Animals: Rhesus monkey ovarian tissue.
Intervention(s): None.
Main Outcome Measure(s): Histologic analysis, follicle counting, assessment of protein abundance and localization.
Result(s): After freezing and thawing, 89% of the primordial follicles maintained their laminar-based architecture, with sizes close to those of fresh fixed follicles. Molecular markers of early follicle health (activin subunits and the phosphorylated form of the signaling protein Smad2 [pSmad2]) were present in fresh and frozen-thawed primordial follicles. Stroma cells, but not follicles, had a higher level of TUNEL staining. Granulosa cells within the follicles of frozen-thawed ovarian tissue cultured for 48 hours had the capacity to proliferate and sustained expression of the activin subunits and nuclear pSmad2.
Conclusion(s): This study provides evidence that markers of early follicle growth and development are preserved after slow cryopreservation and thaw, with little effect on follicle morphology and function. (Fertil Steril® 2010;■:■–■. ©2010 by American Society for Reproductive Medicine.)

Key Words: Slow cryopreservation, primordial follicles, rhesus monkey, stromal cell, activin subunits, pSmad2

Cryopreservation of oocytes or ovarian tissue provides a potential option for fertility preservation of women facing the loss of reproductive function, due to either diseases, such as cancer, or treatments, including radiation and chemotherapy (1–5). Cryopreserved and thawed ovarian tissue has been successfully reimplanted and has produced live births, with lower success rates for frozen tissue versus fresh transplant (4–9). Ovarian tissue is cryopreserved using conventional freezing (slow) or vitrification (rapid). Although studies on each of these techniques report varying levels of success, there is evidence suggesting that slow cryopreservation is more efficient and produces a greater number of surviving and functional follicles after thawing (10, 11). The viability of primordial follicles has been confirmed after slow cryopreservation and thawing, whether they had been frozen within ovarian tissue or as individually isolated follicles (12, 13).

Activin is involved in mouse and human follicle formation and promotes germ cell and granulosa cell proliferation (14–17). The null gene mutation of activin results in gonadal dysgenesis or inappropriate follicle function (18). Smad2 is a downstream target of activin and is phosphorylated as a consequence of activation. Also, activin subunits and pSmad2 are detected in the primordial follicles of cats (19). Thus, the activin-pSmad2 signaling pathway may play an important role in primordial follicle development. We tested the effects of an ovarian tissue cryopreservation technique on molecular markers and the developmental capacity of nonhuman primate primordial follicles.

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MATERIALS AND METHODS

Collection of ovarian tissue

Four female monkeys (0–5 years old) were used. The general care and housing of rhesus monkeys was provided by the Division of Animal Resources at the Oregon National Primate Research Center (ONPRC). Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the ONPRC Institutional Animal Care and Use Committee.

Cryopreservation and Thawing of Ovarian Tissue

Freezing of monkey ovarian tissue strips was performed according to the method described by Gosden et al. (20) with some modifications. Pieces of

ovarian cortical tissue (5 × 1 × 1 mm) were transfered into 1.5-mL cryovials containing 1 mL 1.5 mol/L ethylene glycol (EG), 0.1 mol/L sucrose, and 10% (v/v) serum substitute in Leibovitz L-15 media. Ovarian tissue was equilibrated for 30 minutes at 4°C. The cryovials were frozen using a programmable freezer with a cooling rate of 2°C/min from 4°C to −7°C. At −7°C, seeding was manually performed, then cooling was resumed at a rate of 0.3°C/min from −7°C to −40°C, and then 10°C per min from −40°C to −140°C. Finally, the cryovials were plunged into liquid nitrogen for storage.

For thawing, the cryovials were exposed to room temperature for 30 sec and immersed in a water bath at 37°C until the ice completely melted. Then media and pieces of tissue were poured into a culture dish. Ovarian cortical tissues were rehydrated in four steps, 10 minutes per step, at room temperature in the following thawing solutions: thawing solution I: 10% serum protein substitute (SPS), 0.1 mol/L sucrose, 5.57% EG; thawing solution II: 10% SPS, 0.1 mol/L sucrose, 2.79% EG; thawing solution III: 10% SPS, 0.1 mol/L sucrose, 0% EG; and thawing solution IV: 10% SPS, 0 mol/L sucrose, 0% EG.

Histologic and Immunologic Analysis and Follicle Classifications

Monkey ovarian tissue from the fresh group, frozen-thawed group, or cultured frozen-thawed group were fixed, and 5-μm sections were cut for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). A healthy primordial follicle was defined as previously described (21, 22), consisting of an oocyte completely encapsulated by one layer of flattened pregranulosa cells. The H&E images were acquired on a Nikon E600 microscope using a Spot Insight Mosaic 11.2 color digital camera (Diagnostic Instruments, Sterling Heights, MI) and Advanced Spot Imaging software (version 4.6; Universal Imaging, Downington, PA). The basement membrane surrounding the granulosa cell layer was considered to be the outer boundary of the follicle. IHC was performed and visualized as previously described (15).

Antibodies

Primary antibodies used were rabbit polyclonal antihuman β_A- and β_B-subunit antibodies (a gift from W. Vale and J. Vaughn, Salk Institute, La Jolla, CA) and a rabbit polyclonal anti-pSmad2 antibody (Zymed Laboratories, South San Francisco, CA). The antibody for detection of proliferating cell nuclear antigen (PCNA) was a rabbit polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA). The laminin antibody was from Sigma. Dilutions of the primary antibodies against the βA- and βB-subunit, pSmad2, PCNA, and laminin were 1:100, 1:100, 1:50, 1:100, and 1:50, respectively. The secondary antibody used was biotinylated goat antirabbit (Vector Laboratories, Burlingame, CA). Negative control was obtained by omitting the primary antibody.

Identification of Apoptotic Cells

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the TACS.XL in situ apoptosis detection kit (catalog no. TA200; R&D, Minneapolis, MN) following the manufacturer's protocol. Negative control consisted of omitting the terminal deoxynucleotidyl transferase enzyme.

Counting of Healthy and Immunopositive Follicles

Only follicles that contained an oocyte nucleus were counted. Follicles were counted in four different areas (same power field) per section, six random sections per sample, and two or three ovarian tissue samples per animal were used. Follicle counts were repeated in four different animals. The percentage of healthy follicles was calculated as the number of healthy follicles out of the total number of follicles. The percentage of immunopositive follicles was relative to the total number of healthy follicles. Follicles with either an oocyte or single granulosa cell stained positive by IHC were considered to be immunopositive follicles (23, 24).

Culture of Frozen-Thawed Ovarian Cortical Tissues

Frozen ovarian cortical tissues were thawed and cultured as described previously (21, 25). Tissues were cut into small thin pieces of approximately 0.5 mm^3 and transfered in a culture plate insert (Millicell-CM, 0.4-um pore size; Millipore Corp., Billerica, MA) in a 24-well cell culture plate. Ovarian tissue slices on the membrane were covered with a thin film of medium, and 400 μL culture medium was added in the compartment below the membrane insert. Up to four ovarian slices were placed in each well. The culture medium was minimal essential medium (αMEM) supplemented with 10 mIU/mL recombinant human (rh) FSH (A. F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases), 3 mg/mL bovine serum albumin, 1 mg/mL bovine fetuin (Sigma-Aldrich, St. Louis, MO), 5 ng/mL insulin, 5 ng/mL transferrin, and 5 ng/mL selenium. The ovarian tissue was incubated at 37°C in 5% CO_2 for 48 hours.

Statistical Analysis

At least three replicates were performed for each experiment on each animal. Comparisons for diameter of follicle, oocyte, and nucleus were made between fresh and frozen-thawed groups using the Student t test, with $P<.05$ considered to be statistically significant. The percentage of healthy follicles, PCNA-positive follicles or cells, and TUNEL-positive follicles or cells were analyzed using the Fisher exact test, with $P<.05$ considered to be statistically significant. Values are given as mean ± SEM.

RESULTS

Morphologic Assessment of Primordial Follicles Before and After Slow Cryopreservation

Fresh or frozen-thawed monkey ovarian tissues were sectioned for H&E staining to determine the effect of slow cryopreservation and thawing on the morphology of primordial follicles. In the fresh group, 98% (n = 540) of primordial follicles had a complete architecture, and the oocyte was surrounded by a single layer of pregranulosa cells with round nuclei (Figs. 1A and 1E). After cryopreservation, 89% (n = 470) of thawed primordial follicles had a complete architecture and compact structure (Figs. 1B, 1C, and 1E). Moreover, the laminin-stained basement membrane (Fig. 1D) was maintained in primordial follicles after cryopreservation. For the healthy follicles from the fresh group (n = 530) or the frozen-thawed group (n = 419), the average diameters of nuclei (13.4 μm vs. 13.2 μm), oocytes (29.3 μm vs. 29.4 μm), and primordial follicles (35.9 μm vs. 35.1 μm) were not significantly different ($P < 0.05$; Fig. 1F).

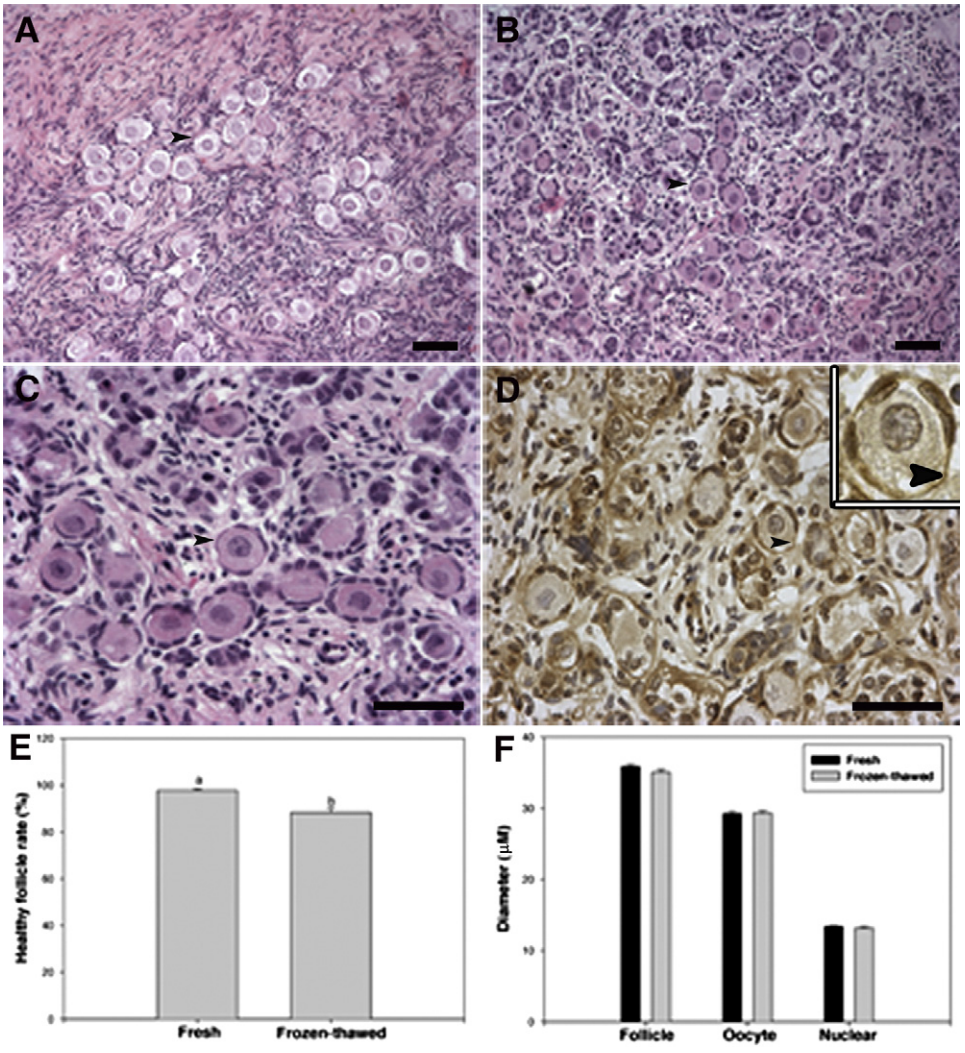
Effect of Cryopreservation on Apoptosis and Expression of Activin and pSmad2 of Primordial Follicle Cells

To determine the amount of apoptosis in monkey primordial follicles after slow cryopreservation and thawing, TUNEL staining was performed. Sections of fresh and frozen-thawed monkey ovarian tissue showed very little TUNEL-positive staining in the primordial follicles (Figs. 2A and 2B), with no difference between the two groups (5 ± 1.1% vs. 8 ± 1.9%; $P>.05$; Fig. 2C). However, many more stromal cells were positive by TUNEL staining after cryopreservation (25 ± 2.4% vs. 5 ± 0.9%; $P<.05$; Figs. 2A–2C).

To investigate whether cryopreservation and thawing affected the localization of activin signaling pathway proteins in early follicles, activin β_A-subunit, activin β_B-subunit and pSmad2 were measured in fresh and frozen-thawed monkey ovarian tissues. All three proteins were present in primordial follicles from both groups (Fig. 2D). The activin β_A-subunit was detected in oocytes and pregranulosa cells in fresh and frozen-thawed tissues; however, the staining was weaker in the cytoplasm of oocyte in frozen-thawed tissue (Fig. 2D, panels a and d). Activin β_B-subunit expression had a similar pattern in primordial follicles in fresh and frozen-thawed ovarian tissue (Fig. 2D, panels b and e). Phosphorylated Smad2 immunostaining was detected in the nuclei of oocytes and the

FIGURE 1

Morphologic assessment of primordial follicles in monkey ovarian tissue before and after cryopreservation. (**A**) In fresh tissue, the normal primordial follicle indicated by the *arrowhead* contains a germinal vesicle-stage oocyte surrounded by a layer of flattened pregranulosa cells. (**B**) In frozen-thawed monkey ovarian tissue, the primordial follicle indicated by the *arrowhead* is similar in appearance to that of follicles in the fresh group at the same magnification and (**C**) at high magnification. (**D**) Laminin expression was detected in frozen-thawed monkey ovarian tissues, and a primordial follicle with a complete basement membrane is indicated by the *arrowhead*; the **inset** shows a laminin-stained follicle at high magnification. Each image is a representative section from each group. Bar = 50 μm. The healthy rate (**E**) and morphologic parameters (**F**) of primordial follicles between fresh and frozen-thawed groups were compared. Values are from four animals. Different superscripts indicate statistically significant differences ($P<.05$).



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pregranulosa cells of primordial follicles in both groups (Fig. 2D, panels c and f).

Effect of Cryopreservation and Thawing on Proliferation and Abundance of Activin/pSmad2 in Rhesus Primordial Follicles After Culture

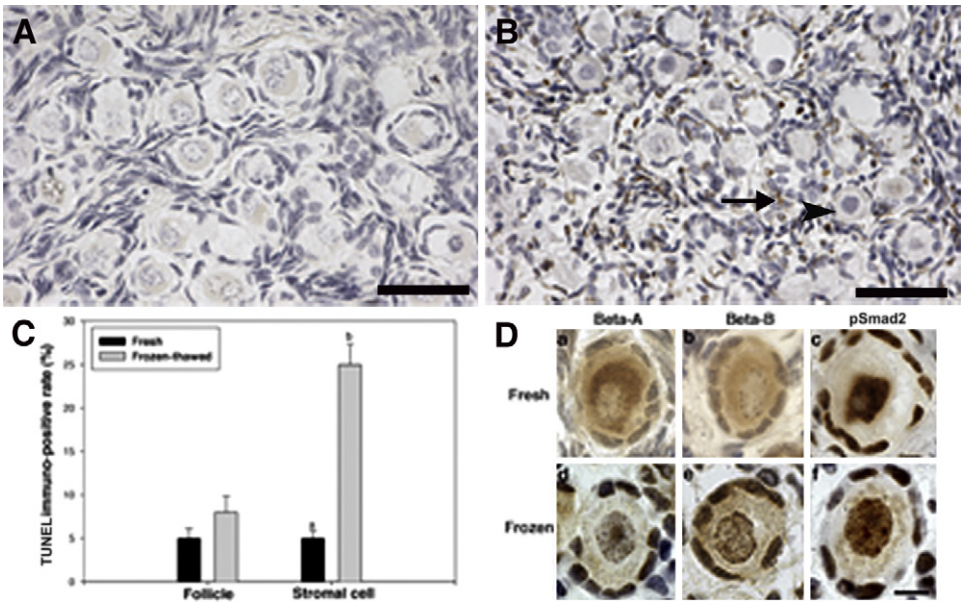
We investigated differences in follicle or stromal cell proliferation before and after culture of frozen-thawed tissue. PCNA was detected in some oocytes and many granulosa cells in transiting primordial follicles in frozen-thawed monkey ovarian tissue after 48 hours of culture (Fig. 3B), and more follicles had PCNA-positive staining after culture compared with the noncultured group (78 ± 9.2% vs. 7 ± 0.7%; $P<.05$) (Figs. 3A–3C). Moreover, more PCNA-staining positive stromal cells were found in cultured tissue (25 ± 4.1% vs. 4 ± 0.6%; $P<.05$; Figs. 3A–3C). Furthermore, the activin β_A-subunit (Fig. 3D), activin β_B-subunit (Fig. 3E), and pSmad2 (Fig. 3F) were expressed in early-stage follicles after 48 hours of culture.

DISCUSSION

This study demonstrates that nonhuman primate primordial follicles can undergo slow cryopreservation and thawing with little effect on their morphology or markers of early follicular development. Most primordial follicles in frozen-thawed tissue maintained complete and healthy follicle structure, with round oocytes surrounded by

FIGURE 2

Apoptotic activity and localization of activin subunits and phosphorylated (p) Smad2 in fresh and frozen-thawed primordial follicles. Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining was detected in (**A**) fresh and (**B**) frozen-thawed monkey ovarian tissues. No difference was seen between the groups regarding staining in primordial follicles (*arrowhead*), but more TUNEL-positive staining was observed in stromal cells of the frozen-thawed group (*arrow*) as shown in (**C**). Activin β_A-subunit, activin β_B-subunit, and pSmad2 were detected in fresh and frozen-thawed ovarian tissues (**D**). All three proteins localized mainly in both oocytes and pregranulosa cells. Panels a–c are fresh, d–f are frozen-thawed. Each image is a representative section from each group. Bars: 50 μm (A and B); 10 μm (D).



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a layer of flattened pregranulosa cells and an intact laminar membrane, and had nuclear, oocyte, and follicle diameters similar to those of primordial follicles in fresh ovarian tissue. The abundance and localization of proteins important in early follicle development (activin subunits and pSmad2) were also similar in fresh and frozen-thawed tissues. Finally, after short-term in vitro tissue culture, primordial follicles in frozen-thawed ovarian tissue exhibited a high level of follicle survival and proliferation.

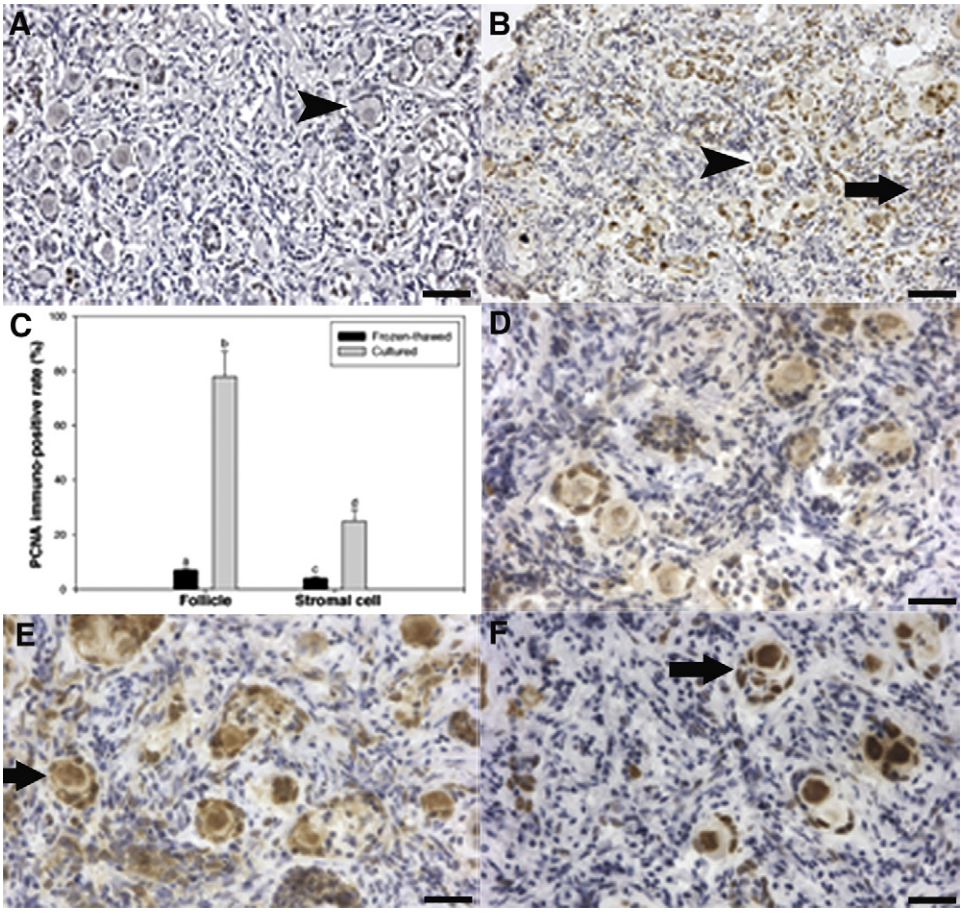
A controlled-rate freezing process has been successfully applied in rodent, human, and other species and has proven to be an effective method for preservation of ovarian tissue (26–29). The present study examined the application of this slow cryopreservation technique to nonhuman primate (rhesus monkey) ovarian tissue. After thawing, 89% of the primordial follicles had complete follicle architecture, consistent with the findings of others applying this technique in other species. Chen et al. (30) reported that 88% of the primordial follicles were morphologically normal after slow freezing in mice, and Jeremias et al. (31) reported that 91% of primordial follicles survived after cryopreservation. Also, no difference was reported in oocyte or follicle size before and after freezing human ovarian tissue (32). Our observation of low apoptotic activity in frozen-thawed ovarian tissue is similar to previous findings in mice and humans (32–34). Moreover, after short-term culture, most follicles not only survive and proliferate, they also sustain activin-pSmad2 expression, which phenocopies normal fresh follicles. These results suggest that slow cryopreservation does not induce primordial follicle apoptosis or disrupt the three-dimensional architecture and somatic-germ cell interactions that are crucial for oocyte growth and development.

To date, the majority of studies of ovarian tissue cryopreservation have described the effects on follicle morphology and survival (12, 32, 34). We further investigated whether cryopreservation affects primordial follicle function, specifically the activin-pSmad2 pathway, which has been shown to play an important role in regulating the formation and development of rodent ovarian follicles in the female (14–19, 36). Previous studies in our laboratory showed that the activin β_A- and β_B-subunits are expressed in both oocytes and somatic cells in primordial follicles, and that activin A promotes primordial follicle formation when administered to neonatal mice (15). The present study demonstrated that the activin signaling pathway is present in primordial follicles and remains intact after slow freezing and thawing of macaque ovarian tissue. There was a modest reduction in activin β_A-subunit staining in oocyte cytoplasm after freezing, which was restored during the short culture period. The expression patterns of the activin β_B-subunit and pSmad2 were similar in fresh, thawed, and cultured tissues. One potential conclusion from this study is that the activin signaling pathway is a relevant marker for follicle health that can be used as cryopreservation methods are improved in coming years.

In contrast, TUNEL staining of stromal cells was higher in frozen-thawed ovarian tissue compared with fresh tissue, and the tissue responded with an increase in proliferation. Keros et al. (35) reported that the stroma was not preserved as expected by slow-programmed freezing of human ovarian tissue. Ovarian tissue is composed of different cell types, and it might be expected that each cell type has different requirements for optimal survival. Stromal cells not only support the three-dimensional environment necessary for maintaining follicle architecture, they also produce various

FIGURE 3

Proliferation detection and expression of the activin β_A- and β_B-subunits and phosphorylated (p) Smad2 in cultured monkey ovarian cortical tissue. Frozen ovarian cortical pieces were thawed and cultured for 48 hours in vitro, then fixed and sectioned at 5 μm for proliferating cell nuclear antigen (PCNA) staining and immunohistochemistry. Most follicles showed no PCNA staining in granulosa cells and oocytes in frozen-thawed uncultured tissue (**A**, *arrowhead*), but strong PCNA staining was measured in cultured tissue (**B**). More PCNA-immunostaining follicles (*arrowhead*) and stromal cells (*arrow*) were detected after culture (**C**). Immunostaining of activin β_A-subunit (**D**), activin β_B-subunit (**E**), and pSmad2 (**F**) (*arrows*) was seen in growing follicles in cultured tissue. Activin β_A- and β_B-subunits localized in the oocyte and granulosa cells, and pSmad2 localized in the nuclei of both oocytes and granulosa cells. Each image is a representative section from each group. Bars: 50 μm (A and B); 25 μm (D–F).



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factors that promote follicle growth and development (36). Although changes in stromal cell health after freezing may not affect follicle function after short-term culture, they may negatively affect the growth and development of follicles over the long term, which may partially explain the observed follicle loss and low efficiency of implantation using follicles from frozen-thawed tissue (26, 37–40). These data suggest that better methods for protecting the stroma cells from programmed cell death and inappropriate proliferation need to be developed.

In conclusion, the present results suggest that after slow cryopreservation and thawing, monkey primordial follicles retain their morphology as well as markers of functional development during short-term culture. The goal of these studies is to develop an ovarian tissue cryopreservation technique that will allow thawed primordial follicles to be matured in vivo, through retransplantation of thawed tissue, or in vitro, and produce oocytes competent for fertilization and culminating in live births (41). Identifying good molecular and functional markers for primordial follicles in nonhuman primate ovarian tissue is the first step toward this goal.

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