Multiple aster formation is frequently observed in bovine oocytes retrieved from 1 day-stored ovaries

H. Hara[2,3], M. Tagiri[4], M. Hirabayashi[3] and S. Hochi[1,2,4,5]

[1]All correspondence to: S. Hochi. Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan. Tel: +81 268 215350. Fax: +81 268 215830. e-mail: shochi@shinshu-u.ac.jp

[2]Interdisciplinary Graduate School of Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan

[3]National Institute for Physiological Sciences, Okazaki, Aichi 444-8787, Japan

[4]Graduate School of Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan

[5]Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan

Running title: Ovary storage and aster formation

Abstract. We have recently reported that multiple aster formation after IVF was one of the factors negatively affecting to developmental competence of vitrified-warmed bovine matured oocytes, and that a short-term culture of the post-warm oocytes with an inhibitor of Rho-associated coiled-coil kinase (ROCK) suppressed the multiple aster formation and improved the blastocyst yield. The present study was conducted to investigate whether increased multiple aster formation following IVF can be involved in impaired developmental competence of stored ovary-derived bovine oocytes. Oocytes retrieved from 1-day stored ovaries had lower developmental potential to Day 8 blastocysts when compared with those from fresh ovaries (37 vs 63%). Immunostaining against α-tubulin 10 h post-IVF revealed that the higher incidence of multiple aster formation occurred in oocytes retrieved from stored ovaries than fresh ovaries (31 vs 15%). Treatment of post-IVM oocytes with ROCK inhibitor for 2 h significantly suppressed the incidence of multiple aster formation (10 vs 32% in control group). However, the suppressing effect of ROCK inhibitor on multiple aster formation in IVM/IVF oocytes did not improve blastocyst yield from stored ovary-derived oocytes (41 vs 37% in control group). These results suggest that higher incidence of multiple aster formation by bovine ovary storage was not responsible for decreased developmental competence of IVF oocytes**.**

Key words: Blastocysts, Bovine IVF, Multiple asters, Ovary storage, ROCK inhibitor (Y-27632)

Introduction

Since an outbreak of bovine spongiform encephalopathy (BSE) in Japan at 2001 September, all the slaughtered animals have been screened to confirm the BSE infection-negative. In addition, hazard analysis and critical control point (HACCP)-based approach for food safety promoted to eliminate small-scale abattoirs, forcing long distance transportation from HACCP criteria-satisfying large-scale abattoirs to the laboratories. The time-consuming screening assay and the long distance transportation of ovaries to laboratories have compelled us to conditioning transient storage of bovine ovaries, although the BSE screening assay was obligated only to the cattle aged at > 48 months old (2013 April). It seemed common that developmental competence of bovine oocytes retrieved from stored ovaries was lower than that of those retrieved from fresh ovaries (Nakao & Nakatsuji, 1992; Matsukawa *et al*., 2007). Recommended conditions for storage of bovine ovaries include decreasing the temperature of storage solution from 30-37 ºC to 15-20 ºC (Nakao & Nakatsuji, 1992; Matsukawa *et al*., 2007; Nagao *et al*., 2010), and exposing to anti-oxidants (Nagao *et al*., 2010), magnesium and raffinose (Iwata *et al*., 2005) in the storage solution.

During the process of fertilization, a bull spermatozoon brings a centrosome into an oocyte and a single sperm aster is formed by polymerization of microtubule components (α- and β-tubulin) from the centrosome (Schatten, 1994). The microtubule network contributes to migration of male and female pronuclei (PN) to the center of a fertilized oocyte and facilitates subsequent fusion of the 2PN and mitotic cleavage (Kim *et al*.,

1996; Terada *et al*., 2004). We reported that impaired development of sperm aster in bovine oocytes was caused by a procedure for intracytoplasmic sperm injection (Hara *et al*., 2011), and that frequent multiple aster formation after *in vitro* fertilization (IVF) was induced by vitrification of *in vitro*-matured bovine oocytes (Hara *et al*., 2012), with impaired migration and development of pronuclei prior to the first cleavage. Recently, we have reported that a short-term culture of vitrified-warmed bovine oocytes with an inhibitor of Rho-associated coiled-coil kinase (ROCK) suppressed the incidence of multiple aster formation, resulting in an improvement of the blastocyst yield (Hwang *et al*., 2013).

The purpose of the present study was to investigate (1) whether multiple aster formation is frequently observed in bovine oocytes retrieved from 1 day-stored ovaries after *in vitro* maturation (IVM) and IVF, (2) whether incidence of multiple aster formation can be suppressed by a short-term ROCK inhibition after IVM, and (3) whether the normalized multiple aster formation rate can contribute to improve the blastocyst yield from stored ovary-derived oocytes.

Materials and Methods

Oocyte preparation

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Abattoir-derived bovine ovaries were transported to the laboratory in saline within 2-6 h at 21-26 ºC (defined as “fresh ovaries”)

or 22-26 h at 12-14 ºC (defined as “stored ovaries”). The contents of 2-8 mm follicles were aspirated and cumulus-oocyte complexes (COCs) surrounded with at least two layers of compact cumulus cells were collected from the follicular fluid. IVM were conducted in Hepes-buffered TCM-199 (Earle’s salt; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/ml FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 μg/ml 17β-estradiol and 50 μg/ml gentamycin sulfate for 22 h at 38.5 ºC in 5% CO_2 in air (8-12 COCs per 100-μL microdrop covered with mineral oil). Cumulus cells were then removed by a brief vortex-mixing in Hepes-buffered TCM-199 supplemented with 3 mg/ml bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1,000 IU/ml hyaluronidase and 50 μg/ml gentamycin sulfate. Denuded oocytes extruding first polar body were defined as matured.

Treatment with ROCK inhibitor

Hepes-buffered TCM-199 supplemented with 5% (v/v) FBS, 0.2 mM sodium pyruvate, and 50 μg/ml gentamycin sulfate (TCM-199/5%FBS) was used as the base medium for ROCK inhibition of stored ovary-derived oocytes prior to the IVF, as reported previously (Hwang *et al*., 2013). Matured oocytes were incubated in the TCM-199/5%FBS containing 10 μM ROCK inhibitor (Y-27632; (R)-(+)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate) for 2 h at 38.5 ºC in 5% CO_2 in air (15-30 oocytes per 100-μL microdrop), while oocytes incubated for the same 2 h in ROCK inhibitor-free medium were served as

controls.

In vitro fertilization and culture

Spermatozoa were prepared from commercially available frozen semen of a Japanese Black bull. The content of a 0.5-mL straw was layered on the top of Percoll density gradient consisting of 2 mL of 45% Percoll above 2 mL of 90% Percoll in a 15-mL conical tube, and centrifuged for 20 min at 700 *g*. The sperm pellet was suspended in modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) supplemented with 5 mM theophylline, washed twice (5 min at 300 *g* each) and then re-suspended in the mBO medium supplemented with 5 mg/ml BSA and 10 µg/ml heparin (IVF medium) to yield a concentration of 1.5×10^7 sperm cells/ml. Twenty µl of the sperm suspension was added to 80 µl of IVF medium containing 10-12 matured oocytes (final sperm concentration; 5×10^6 sperm cells/ml) and kept for 6 h at 38.5 ºC under 5% CO_2 in air. Presumptive zygotes were cultured in modified synthetic oviduct fluid (Holm *et al*., 1999) supplemented with 30 µl/ml essential amino acids solution (× 50, Gibco-11130), 10 µl/ml non-essential amino acids solution (× 100, Gibco-11140) and 5% (v/v) FBS for 8 days at 39.0 ºC in 5% CO_2, 5% O_2 and 90% N_2. Cleavage rate was recorded 2 days after IVF, and number of expanding to hatched blastocysts was recorded 7 and 8 days after IVF.

Assessment of aster formation

Oocytes released from IVF medium were cultured for an additional 4 h in the

TCM-199/5%FBS at 38.5 ºC in 5% CO_2 in air, and subjected to immunostaining as reported previously (Hara *et al*., 2011; Hara *et al*., 2012; Hwang *et al*., 2013). Briefly, the oocytes were extracted for 15 min by buffer M (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH6.8) containing 5% (v/v) methanol and 1% (v/v) Triton X-100 detergent after the zonae pellucidae had been removed with 0.75% protease in M2 medium. The oocytes were then fixed with cold methanol for 10 min and permeabilized overnight in PBS (-) containing 0.1% (v/v) Triton X-100. Sperm aster were labeled with a monoclonal antibody against α-tubulin and the primary antibodies were detected by FITC-conjugated goat anti-mouse IgG. Nuclear DNA was visualized by counterstaining with 2.5 μg/ml DAPI. The preparations were mounted with coverslips in 100 mg/ml 1,4-diazabicyclo[2.2.2]octane dissolved in glycerol:PBS (9:1; v/v) and digital images were taken with a confocal laser scanning microscope (FV1000-D; Olympus, Tokyo, Japan) at a distance of 2 μm and stacked. Normally fertilized oocytes, defined by the presence of 2PN, with detectable microtubule network were selected and classified into either those with single sperm aster (Fig. 1A) or multiple asters (Fig. 1B) because polyspermically fertilized oocytes exhibit more than two asters.

Statistical analysis

Percentage data in each replicate were arcsine-transformed and subjected to the Student's *t*-test or one-way ANOVA. When ANOVA reached significance, differences among means were analyzed by post hoc Tukey's tests. Data for aster formation (single or

multiple) were analyzed by chi-square test. *P* values less than 0.05 were considered significant.

Results

Under our conventional IVM system for 22 h, proportion of denuded oocytes extruding the first polar body, defined as matured oocytes, was 83 ± 1% (659/795) and 64 ± 4% (730/1,138) in fresh ovary group and stored ovary group, respectively ($P < 0.05$). When these denuded mature oocytes were subjected to IVF, cleavage rate of the IVF oocytes retrieved from stored ovaries was significantly lower than that of fresh ovary-derived IVF oocytes (71 vs 85%, Table 1). Significant difference between the stored ovary group and the fresh ovary group was notable in the blastocyst yields at Day 7 (33 vs 51%) and Day 8 (37 vs 63%).

Proportion of IVF oocytes with 2PN in stored ovary group was significantly lower (P=0.03) than that in fresh ovary group (Fig. 2A). In contrast, incidence of multiple aster formation was more than double in stored ovary group (31%) compared with fresh ovary group (15%), while proportions of aster-forming 2PN zygotes were comparable between the two groups (95 and 99%, respectively). Such a higher incidence of multiple aster formation in stored ovary-derived IVF oocytes was significantly suppressed by ROCK inhibition during 2 h incubation after 22 h IVM culture (10 vs 32% in control group; Fig. 2B).

An additional 2 h incubation of post-IVM oocytes had no harmful influence on

cleavage rate and blastocyst yield (Table 2). Cleavage rate of IVF oocytes in ROCK inhibitor-treated group (80%) was not different from that in the control group (71%), in addition to yield of the blastocysts on Day 7 (both 31%) and Day 8 (41 vs 37%). Blastocyst yields from IVF oocytes in the 3 stored ovary groups were significantly lower than that in fresh ovary group (61%).

Discussion

As reported previously (Nakao & Nakatsuji, 1992), developmental competence of stored ovary-derived oocytes was lower than that of fresh ovary-derived oocytes (Table 1). One possible explanation for the decreased developmental competence is a lower rate of normally fertilized oocytes (Fig. 2A), which may be caused by zona-hardening. Relatively higher yields of bovine blastocysts 7 to 8 days after IVF in the fresh ovary group, compared with general yields as 20-40% reported in conventional bovine IVF (Iwata *et al*., 2005; Rizos *et al*., 2002; Sripunya *et al*., 2010), may be due to the selected use of maturation-confirmed oocytes after removal of cumulus cells for subsequent IVF, instead of COCs with unknown maturation rate.

As shown in Fig. 2A, stored ovary-derived oocytes showed frequent multiple aster formation after IVF compared with fresh ovary-derived ones. The frequent multiple aster formation was suppressed by ROCK inhibition during 2 h incubation after 22 h IVM culture (Fig. 2B). This result was consistent with our previous report (Hwang *et al*., 2013) in which 2 h treatment of vitrified-warmed bovine matured oocytes with the ROCK

inhibitor was found to normalize their aster formation. ROCK was characterized as a down stream target of the small GTP-binding protein Rho and regulates amount of actin filaments and cellular contractility through actin polymerization and stress fiber organization (Riento & Ridley, 2003). It is also reported that ROCK can regulate microtubule acetylation via phosphorylation of the tubulin polymerization promoting protein 1, resulting in microtubule unstabilization (Schofield *et al*., 2012; Schofield *et al*., 2013). Because the stored ovary-derived oocytes showed abnormal gene expression (Somfai *et al*., 2011), over-expressed ROCK signaling might disturb microtubule network for sperm aster in stored ovary group. Therefore, treatment with the ROCK inhibitor may be useful to suppress the multiple aster formation by stabilizing astral structure.

Hwang *et al*. (2013) reported that ROCK inhibition-assisted suppression of multiple aster formation in vitrified-warmed bovine oocytes led to a significant improvement of blastocyst yield from 14 to 21%. However in the present study, it may be reasonable to conclude that multiple aster formation is not directly involved in the developmental potential of bovine oocytes impaired by ovary storage (Table 2). Difference in multiple aster formation rate between fresh and stored ovary-derived oocytes was small despite of the presence of significant difference (Fig. 2B) when compared to the difference described for vitrified-warmed oocytes with or without ROCK inhibitor treatment (Hwang *et al*. 2013). Therefore, this may explain the failure of ROCK inhibition-mediated improvement in blastocyst yield from stored ovary-derived oocytes. Otherwise, in the bovine oocytes surviving vitrification by a short-term recovery culture

(Hwang *et al.*, 2013), another cellular functions correlating (or happening simultaneously) with the normalization of aster formation through ROCK inhibition might be effective in improvement of their developmental competence. Matsukawa *et al*. (2007) speculated that oocyte nucleus is sensitive to ovary storage because production of bovine blastocysts by somatic cell nuclear transfer, unlike parthenogenetic activation or intracytoplasmic sperm injection, was not influenced by the use of stored ovaries. Somfai *et al.* (2011) proposed that mRNA of maternal ATP1A1 gene is a possible target for damages induced by storage of bovine ovaries.

Thus, multiple aster formation frequently induced by ovary storage was suppressed by ROCK inhibition of the IVM oocytes. However, the normalized rate of multiple aster formation did not contribute to improve the decreased blastocyst yield from stored ovary-derived IVF oocytes. In conclusion, multiple aster formation was not responsible for the low blastocyst yield from stored ovary-derived bovine oocytes.

Acknowledgments

This study was supported in part by Grant-in-Aids for Scientific Research from JSPS (No. 24580407 to S.H.) and for the JSPS Fellow (No. 25.10439 to H.H.).

References

Hara, H., Abdalla, H., Morita, H., Kuwayama, M., Hirabayashi, M. & Hochi, S. (2011).

Procedure for bovine ICSI, not sperm freeze-drying, impairs the function of the microtubule-organizing center. *J. Reprod. Dev.* **57**, 428-32.

Hara, H., Hwang, I.S., Kagawa, N., Kuwayama, M., Hirabayashi, M. & Hochi, S. (2012). High incidence of multiple aster formation in vitrified-warmed bovine oocytes after *in vitro* fertilization. *Theriogenology* **77**, 908-15.

Holm, P., Booth, P.J., Schmidt, M.H., Greve, T. & Callesen, H. (1999). High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **52**, 683-700.

Hwang, I.S., Hara, H., Chung, H.J., Hirabayashi, M. & Hochi, S. (2013). Rescue of vitrified-warmed bovine oocytes with Rho-associated coiled-coil kinase inhibitor. *Biol. Reprod.* **89**, 26.

Iwata, H., Hayashi, T., Sato, H., Kimura, K., Kuwayama, T. & Monji, Y. (2005). Modification of ovary stock solution with magnesium and raffinose improves the developmental competence of oocytes after long preservation. *Zygote* **13**, 303-8.

Kim, N.H., Simerly, C., Funahashi, H., Schatten, G. & Day, B.N. (1996). Microtubule organization in porcine oocytes during fertilization and parthenogenesis. *Biol. Reprod.* **54**, 1397-404.

Matsukawa, K., Akagi, S., Adachi, N., Kubo, M., Hirako, M., Watanabe, S. & Takahashi, S. (2007). Effect of ovary storage on development of bovine oocytes after intracytoplasmic sperm injection, parthenogenetic activation, or somatic cell nuclear transfer. *J. Mamm. Ova Res.* **24**, 114-9.

Nagao, Y., Harada, Y., Yamaguchi, M., Igarashi, A., Ooshima, Y. & Kato, Y. (2010). Antioxidant treatment during preservation of bovine ovaries increased the development potential of embryos. *Zygote* **18**, 315-21.

Nakao, H. & Nakatsuji, N. (1992). Effects of storage conditions of bovine ovaries and oocytes on the success rate of *in vitro* fertilization and culture. *J. Reprod. Dev.* **38**, 11-3.

Riento, K. & Ridley, A.J. (2003). Rocks: Multifunctional kinases in cell behaviour. *Nat. Rev. Mol. Cell. Biol.* **4**, 446-56.

Rizos, D., Ward, F., Duffy, P., Boland, M.P. & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: Implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234-48.

Schatten, G. (1994). The centrosome and its mode of inheritance: The reduction of the centrosome during gametogenesis and its restoration during fertilization. *Dev. Biol.* **165**, 299-335.

Schofield, A.V., Garmell, C., Suryadinata, R., Sarcevic, B. & Bernard, O. (2013). Tubulin polymerizing protein 1 (TPPP1) phosphorylation by Rho-associated coiled-coil kinase (ROCK) and cyclin-dependent kinase 1 (Cdk1) inhibits microtubule dynamics to increase cell proliferation. *J. Biol. Chem.* **288**, 7907-17.

Schofield, A.V., Steel, R. & Bernard, O. (2012). Rho-associated coiled-coil kinase (ROCK) protein controls microtubule dynamics in a novel signaling pathway that regulates cell migration. *J. Biol. Chem.* **287**, 43620-9.

Somfai, T., Imai, K., Kaneda, M., Akagi, S., Watanabe, S., Haraguchi, S., Mizutani, E., Dang-Nguyen, T.Q., Inaba, Y., Geshi, M. & Nagai, T. (2011). The effect of ovary storage and *in vitro* maturation on mRNA levels in bovine oocytes; A possible impact of maternal *ATP1A1* on blastocyst development in slaughterhouse-derived oocytes. *J. Reprod. Dev.* **57**, 723-30.

Sripunya, N., Somfai, T., Inaba, Y., Nagai, T., Imai, K. & Parnpai, R. (2010). A comparison of Cryotop and solid surface vitrification methods for the cryopreservation of *in vitro* matured bovine oocytes. *J. Reprod. Dev.* **56**, 176-81.

Terada, Y., Nakamura, S., Simerly, C., Hewitson, L., Murakami, T., Yaegashi, N., Okamura, K. & Schatten, G. (2004). Centrosomal function assessment in human sperm using heterologous ICSI with rabbit eggs: A new male factor infertility assay. *Mol. Reprod. Dev.* **67**, 360-5.

Table 1. *In vitro* development of bovine IVF oocytes retrieved from fresh versus 1-day stored ovaries.

	No. (%) of oocytes			
		Cleaved	Developed to blastocysts	
Ovaries	Inseminated	on Day 2	on Day 7	on Day 8
Fresh	100	85 (85 ± 2) [a]	51 (51 ± 4) [a]	63 (63 ± 5) [a]
Stored	117	83 (71 ± 5) [b]	39 (33 ± 4) [b]	44 (37 ± 4) [b]

Percentages are expressed as the mean ± SEM of 5 replicates in each group.

[a,b] Significantly different within columns ($P < 0.05$).

Table 2. Effect of ROCK inhibition during 2 h post-IVM on developmental competence of stored ovary-derived oocytes following IVF.

Ovaries	ROCK inhibitor	No. (%) of oocytes			
		Inseminated	Cleaved on Day 2	Developed to blastocysts on Day 7	Developed to blastocysts on Day 8
Fresh	– (0 h)	103	84 (82 ± 4)	48 (47 ± 3) [a]	63 (61 ± 5) [a]
Stored	– (0 h)	94	71 (75 ± 8)	28 (30 ± 3) [b]	36 (38 ± 4) [b]
Stored	– (2 h)	93	66 (71 ± 4)	29 (31 ± 6) [b]	35 (37 ± 5) [b]
Stored	+ (2 h)	94	75 (80 ± 3)	29 (31 ± 2) [b]	39 (41 ± 3) [b]

Percentages are expressed as the mean ± SEM of at least 4 replicates in each group.

[a,b] Significantly different within columns ($P < 0.05$).

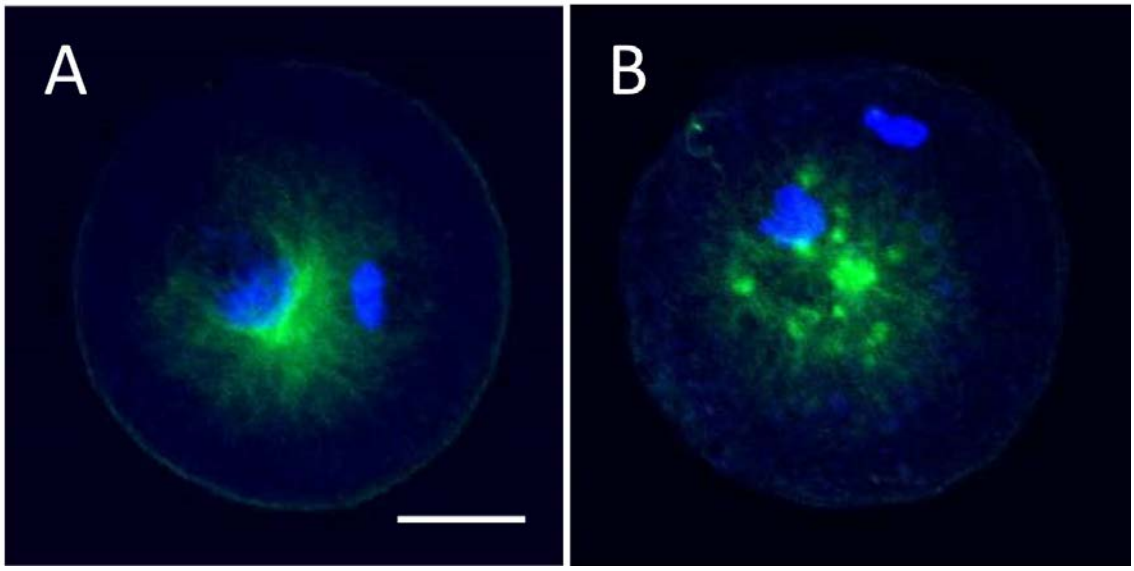

Figure 1 Confocal microscopic observation of bovine IVF oocytes. (A) An oocyte forming a single sperm aster. (B) An oocyte forming multiple asters. Nuclei and microtubules were stained with DAPI (blue) and α-tubulin antibody (green), respectively. The scale bar represents 30 μm.

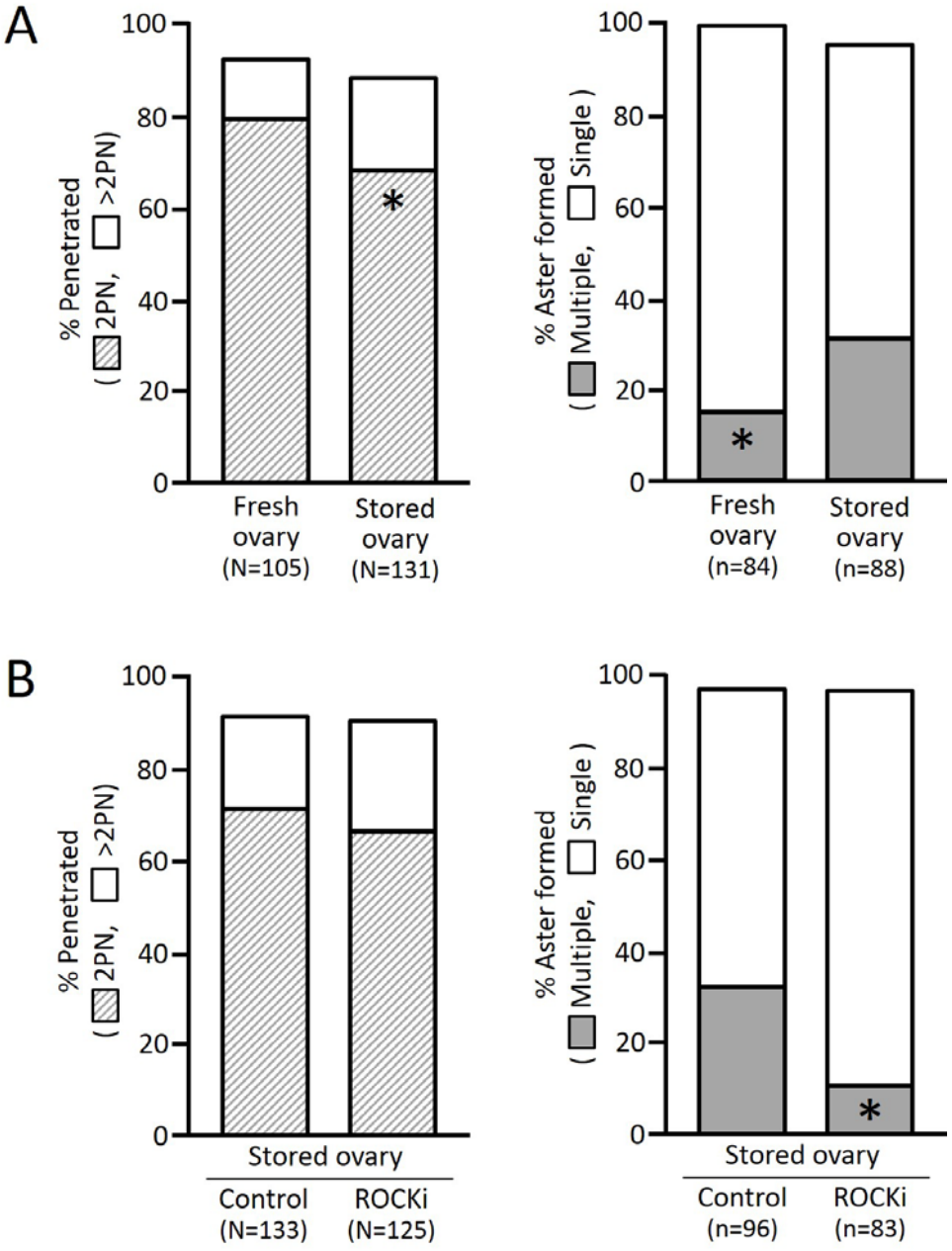

Figure 2 Normal fertilization and aster formation. (A) IVM oocytes retrieved from stored versus fresh ovaries. (B) Stored ovary-derived IVM oocytes with or without ROCK inhibitor (ROCKi) treatment for 2 h. Ten hours after IVF, the oocytes were immunostained against α-tubulin and counterstained with DAPI. N: number of oocytes evaluated. n: number of 2PN oocytes evaluated. Asterisks in the columns denote the significantly lower proportions from the values of counterparts ($P < 0.05$).