Journal of Reproduction and Development, Vol. 59, No 4, 2013

—Technology Report—

Selection of *In Vitro*-Matured Porcine Oocytes Based on Localization Patterns of Lipid Droplets to Evaluate Developmental Competence

Kou HIRAGA[1)], Yumi HOSHINO[1)], Kentaro TANEMURA[1)] and Eimei SATO[1)]

[1)]*Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*

Abstract. Localization patterns of lipid droplets in the cytoplasm of porcine oocytes were evaluated as a novel marker for *in vitro* maturation (IVM) of oocytes with high developmental competence. Porcine oocytes were cultured in TCM-199, which is a complete synthetic medium, for 44 h at 38.5 C. Localization patterns were divided into 2 classes: lipid droplets localized uniformly in the whole cytoplasm (class I) and those that were centrally located (class II). After IVM in TCM-199, 60% of matured oocytes exhibited the class II pattern. To investigate the relation between the distribution of lipid droplets and the developmental rate of the oocyte, the developmental rates of class I and class II oocytes were compared after *in vitro* fertilization (IVF). Class II oocytes showed a significantly higher rate of blastocyst development than class I oocytes. These results suggest that porcine oocytes with high developmental competence can be selected based on the localization patterns of lipid droplets.
Key words: Lipid droplet, Novel marker, Porcine oocyte

(**J. Reprod. Dev. 59:** 405–408, 2013)

Porcine embryos from *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) develop into blastocysts under *in vitro* conditions [1–3]; however, the developmental rates are very low compared with those of oocytes matured and fertilized *in vivo* [1, 3–6]. One factor contributing to this low developmental rate has been thought to be incomplete cytoplasmic maturation of the oocytes that mature *in vitro* [7]. Therefore, considerable improvement has been made to the IVM medium in which porcine oocytes are matured [7, 8]. A frequently used standard medium for IVM of porcine oocytes is North Carolina State University (NCSU)-23 [5] with porcine follicular fluid (pFF) supplementation [9–11]; however, follicular fluid contains numerous undefined factors and can be contaminated with viral pathogens [12–15]. Application of a fully defined IVM system eliminating follicular fluid from the medium could decrease the risk of viral contamination during *in vitro* production (IVP) of embryos. Tissue culture medium (TCM)-199, a complete synthetic medium for oocyte maturation, has been used in many laboratories [2, 16–18]. Blastocyst development [1, 2, 16, 17] and the birth of piglets [16] from oocytes matured in TCM-199 have been reported, although the efficiency has remained low.

To make the IVP system more stable for porcine oocytes using a complete synthetic medium, blastocyst production rate and quality had to be improved. Selection of IVM oocytes with high grades of cytoplasmic maturation for IVF would make further steps more easy by removing low-potential oocytes, saving time and materials and thus costs. Also, the quality of blastocysts, and developmental rate may improve. To do so, a simple, quick, and highly precise method for the evaluation of cytoplasmic maturity is needed. Using stereomicroscopic evaluation, immature oocytes appeared uniform in term of lipid droplet distribution in the cytoplasm (Fig. 1). We observed and classified the localization patterns of lipid droplets in matured oocytes after IVM in TCM-199 with stereomicroscope observation. In "class I" oocyte the lipid droplets were uniformly distributed throughout the entire cytoplasm, whereas in "class II" oocytes, the lipid droplets were centrally located in the cytoplasm (Fig. 2). As a result, significantly ($P<0.01$) more class II oocytes (% ± SEM = 60.9 ± 1.6) were observed after IVM than class I oocytes (27.3 ± 2.6). The remaining 11.8% of oocytes were degraded. The accuracy of classification of lipid droplet distribution by stereomicroscopy was verified by fluorescent lipid-specific staining (Fig. 2). To investigate the relationship between the distribution of lipid droplets and developmental rate, we compared developmental ability of class I and class II oocytes after IVF (Table 1). The blastocyst developmental rate of class II oocytes was significantly ($P<0.05$) higher than that of class I oocytes. However, cell number of the blastocyst was no different. That is, after IVM in TCM-199, the oocytes in which lipid droplets were centrally located had a higher developmental rate. The results suggest that the localization pattern of lipid droplets in porcine IVM oocytes can be an important indicator for selecting oocytes with high developmental competence.

The change in the localization of the cortical granules or mitochondria in IVM had previously been reported as a morphological marker of cytoplasmic maturation [19–21]. However, for such investigations, oocytes have to be fixed and dyed during evaluation, and they then cannot be used for *in vitro* fertilization and *in vitro* culture after IVM. The localization pattern of lipid droplets after IVM that became clear in our study is a morphological character that is observation without dye, enabling simple and quick evaluation of live oocytes. Evaluation of lipid droplet localization pattern in porcine IVM oocytes may also be a useful tool for clarifying the relationship between cytoplasmic maturation and developmental competence.

Sturmey and Leese reported that the level of triglycerides, which are main ingredients in lipid droplets in immature porcine oocytes decreased during IVM [22]. In addition, Somfai *et al.* reported that the

Received: August 29, 2012
Accepted: March 10, 2013
Published online in J-STAGE: April 18, 2013

Correspondence: K Hiraga (e-mail: b2ad1209@s.tohoku.ac.jp)

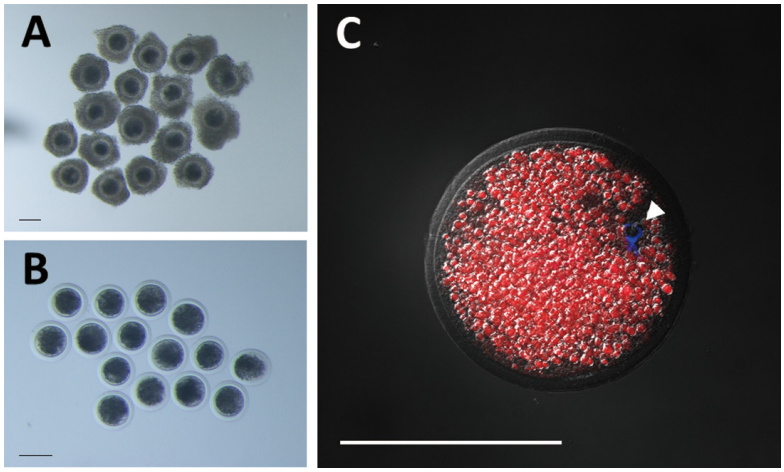

Fig. 1. Status of lipid droplets in porcine germinal vesicle (GV) oocytes. A: Selected COCs for IVM. B: Denuded GV oocyte. C: Immunostaining of lipid droplets. Intracellular lipids and DNA were stained by Nile red (red) and Hoechst 33342 (blue; arrowhead), respectively. Scale bar = 100 µm.

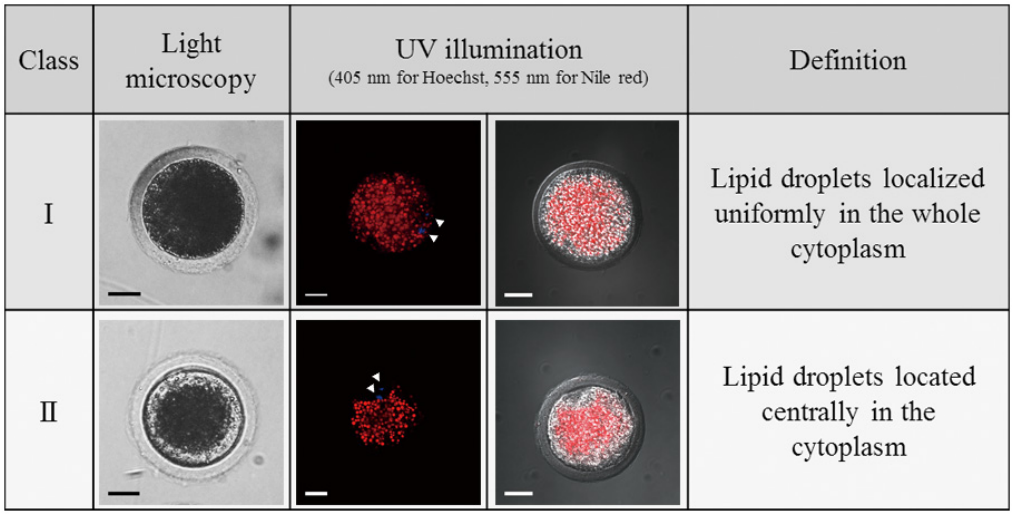

Fig. 2. Classification of localization patterns of lipid droplets in porcine oocytes after IVM. Intracellular lipids and DNA were stained by Nile red (red) and Hoechst 33342 (blue; arrowheads), respectively. Scale bar = 30 µm.

Table 1. Developmental competence of matured porcine oocytes with different localization patterns of lipid droplets

Lipid droplet distribution	No. of oocytes	Cleavage at Day 2 (%)	Blastocyst at Day 7 (%)	Cell number/ blastocyst
Class I	77	64 (85.3 ± 3.4)	3 (3.3 ± 2.1)[b]	48.7 ± 14.3
Class II	138	110 (80.6 ± 3.8)	23 (16.4 ± 3.6)[a]	50.6 ± 4.9

%: Mean ± SD. Day means day after fertilization. Different superscripts within columns indicate significant differences ($P<0.05$; 6 replicates).

addition of L-carnitine to the maturation medium, which enhances lipid metabolism, decreased intracellular lipids in porcine oocytes and improved maturation rate and division capability [23]. In accordance with these reports, our research suggests that lipid metabolism in porcine oocytes had an effect on cytoplasmic maturation in IVM and subsequent embryonic growth.

Methods

In vitro maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in a container within 2 h of

extraction. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3–6 mm) by using a 10-ml syringe attached to an 18-gauge needle. Compact cumulus-oocyte complexes (COCs) with uniformly granulated cytoplasm were selected in PBS supplemented with 0.1% polyvinyl alcohol (PVA; Sigma, St. Louis, USA). After washing in 0.1% PBS-PVA, the COCs were cultured in TCM-199 supplemented with 2.2 mg/ml sodium bicarbonate (Sigma), 570 μM cysteamine (Sigma), 10 ng/ml EGF (Sigma), 100 mIU/ml FSH (Sigma), 100 IU/ml penicillin G (Gibco, Grand Island, USA), and 100 mg/ml streptomycin (Gibco). Groups of 25 oocytes were cultured in 250 μl of medium microdroplets for 44 h in an atmosphere containing 5% CO_2 at 38.5 C. Each droplet of medium was overlaid with liquid paraffin (Nacalai Tesque, Kyoto, Japan) in a 35-mm dish (Sumitomo Bakelite, Tokyo, Japan). At the end of culture, cumulus cells were removed from oocytes by glass micropipette after treatment with 0.1% hyaluronidase (Sigma) at room temperature.

Selection of lipid droplet localization patterns

After IVM, matured oocytes exposing the first polar body were selected under a stereomicroscope (Olympus, SZ-40, Tokyo, Japan) and used for subsequent experiments. Matured oocytes were observed with a stereomicroscope and classified according to the localization patterns of the lipid droplets. The standard classification of lipid droplet distribution was performed by using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij). Matured oocytes were photographed using an inverted microscope (Olympus, IX70) and photography software (Olympus, DP2-BSW), and the pictures were transformed into 8-bit grayscale images. The area of the oocyte measured in the number of pixels, and the percentage of the total oocyte area occupied by lipid droplets was computed. An area showing a pixel intensity of 50 or less was assumed to be occupied by lipid droplets. In "class I" oocytes, the lipid droplets were uniformly distributed throughout the entire cytoplasm, whereas in "class II" oocytes, the lipid droplets were centrally located and covered less than 70% of the cytoplasm.

Evaluation of lipid droplets and chromosomes by confocal laser scanning microscopy

Oocytes were stained by the method of X-W Fu *et al*. with some modifications [24]. Denuded oocytes were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PBS-PVA) for at least 90 min at room temperature. The fixed oocytes were then rinsed three times in PBS-PVA, for 5 min each, and treated overnight with 10 μg/ml Nile red solution (Invitrogen, Grand Island, USA) dissolved in PBS-PVA at room temperature. The Nile red stock solution (1 mg/ml) was prepared by dilution in dimethyl sulfoxide (DMSO; Sigma) and stored at room temperature in the dark. Final concentrations were obtained by diluting the stock with saline solution. The following morning, they were briefly washed in PBS-PVA and stained with 5 μg/ml Hoechst 33342 (Sigma) in PBS-PVA for 20 min at room temperature. After being washed two additional times in PBS (5 min each), oocytes were mounted on nonfluorescent slides and observed under a confocal laser-scanning microscope (Zeiss LSM700, Oberkochen, Germany). Each treatment was repeated at least three times.

In vitro fertilization and culture

The methods for IVF and IVC were based on those described by Kikuchi *et al.* (2002) [11]. Epididymides from a Landrace boar were obtained, and epididymal spermatozoa were collected and frozen. Spermatozoa were thawed and preincubated for 30 min at 38.5 C in TCM-199 adjusted to pH 7.8. Fertilization medium (Pig-FM) for porcine oocytes consisting of 90 mM NaCl, 12 mM KCl, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate (Kanto Chemical, Tokyo, Japan), and 10 mM HEPES was modified further by adding 8 mM CaCl2, 2 mM sodium pyruvate (Sigma), 2 mM caffeine, and 5 mg/ml BSA (Fraction V; Sigma). A portion (10 μl) of the preincubated spermatozoa was introduced into 90 μl of fertilization medium containing approximately 10 denuded oocytes. The final sperm concentration was adjusted to 1×10^5/ml. *In vitro* fertilization was carried out at 38.5 C under 5% CO_2. After IVF for 3 h, all putative zygotes were freed from the attached spermatozoa and transferred into IVC medium. The day of insemination was defined as day 0. The basic IVC medium was NCSU-37 containing 4 mg/ml BSA (Sigma) and 50 mM β-mercaptoethanol. Two types of IVC medium were prepared: 1) basic supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate (IVC-PyrLac) and 2) basic with 5.55 mM D-glucose (IVC-Glu; Wako, Osaka, Japan), as originally reported [5]. Groups of 20 oocytes were cultured in 500 μl of IVC-PyrLac for 48 h, and then incubated in the IVC-Glu for an additional 120 h in an atmosphere containing 5% CO_2 at 38.5 C. Each droplet of medium was overlaid with liquid paraffin (Nacalai Tesque) in 4-well dishes (Nunc). These experiments were repeated at least five times.

Evaluation of embryo development

To examine their ability to develop into the blastocyst stage *in vitro*, all embryos and oocytes were cultured for 7 days, fixed and stained with 5 μg/ml Hoechst 33342 in PBS supplemented with 0.1% PVA; embryos were mounted on nonfluorescent slides and observed under a confocal laser-scanning microscope (Zeiss LSM700). An embryo with a clear blastocoel was defined as a blastocyst for the purposes of this study. The rate of blastocyst formation was evaluated, and the total number of cells in each blastocyst was evaluated as an indicator of embryo quality.

Statistical analysis

Statistical analyses were carried out using analysis of variance (ANOVA) and Fisher's protected least significant difference test using StatView. Differences of $P<0.05$ were considered significant.

Acknowledgments

We thank the staff of the Meat Inspection Office, Sendai City, Japan, for supplying the porcine ovaries. This work was supported by a Japan Society for the Promotion of Science grant to ES (No. 21248032).

References

1. **Nagashima H, Nagai T, Yamakawa H.** *In vitro* development of *in vivo* and *in vitro* fertilized pig zygotes. *J Reprod Dev* 1993; **2**: 163–168.
2. **Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN.** *In vitro* development of *in vitro*-matured porcine oocytes following chemical activation or *in vitro* fertilization. *Biol Reprod* 1994; **50**: 1072–1077. [Medline]
3. **Rath D, Niemann H, Torres CR.** *In vitro* development to blastocysts of early porcine embryos produced *in vivo* or *in vitro*. *Theriogenology* 1995; **43**: 913–926. [Medline]
4. **Beckmann LS, Day BN.** Effects of media NaCl concentration and osmolarity on the culture of early-stage porcine embryos and the viability of embryos cultured in a selected superior medium. *Theriogenology* 1993; **39**: 611–622. [Medline]
5. **Petters RM, Wells KD.** Culture of pig embryos. *J Reprod Fertil Suppl* 1993; **48**: 61–73. [Medline]
6. **Dobrinsky JR, Johnson LA, Rath D.** Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development. *Biol Reprod* 1996; **55**: 1069–1074. [Medline]
7. **Niwa K.** Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. *J Reprod Fertil Suppl* 1993; **48**: 49–59. [Medline]
8. **Nagai T.** *In vitro* maturation and fertilization of pig oocyte. *Anim Reprod Sci* 1996; **42**: 153–163.
9. **Funahashi H, Kim NH, Stumpf TT, Cantly TC, Day BN.** Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation porcine oocyte. *Biol Reprod* 1996; **54**: 1412–1419. [Medline]
10. **Funahashi H, Cantley TC, Day BN.** Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilization. *Biol Reprod* 1997; **57**: 49–53. [Medline]
11. **Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T.** Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. *Biol Reprod* 2002; **66**: 1033–1041. [Medline]
12. **Sur JH, Doster AR, Galeota JA, Osorio FA.** Evidence for the localization of porcine reproductive and respiratory syndrome virus (PRRSV) antigen and RNA in ovarian follicles in gilts.*Vet Pathol* 2001; **38**: 58–66. [Medline]
13. **Galik PK, Givens MD, Stringfellow DA, Crichton EG, Bishop MD, Eilertsen KJ.** Bovine viral diarrhea virus (BVDV) and anti-BVDV antibodies in pooled samples of follicular fluid. *Theriogenology* 2002; **57**: 1219–1227. [Medline]
14. **Devaux A, Soula V, Sifer C, Branger M, Naouri M, Porcher R, Poncelet C, Neuraz A, Alvarez S, Benifla JL, Madelenat P, Brun-Vazinet F, Feldmann G.** Hepatitis C virus detection in follicular fluid and culture media from HCV+ women, and viral risk during IVF procedures. *Hum Reprod* 2003; **18**: 2342–2349. [Medline]
15. **Pogranichniy R, Lee K, Machaty Z.** Detection of porcine parvovirus in the follicular fluid of abattoir pigs. *J Swine Health Prod* 2008; **16**: 244–246.
16. **Mattioli M, Bacci ML, Galeati G, Seren E.** Developmental competence of pig oocytes matured and fertilized *in vitro*. *Theriogenology* 1989; **31**: 1201–1207. [Medline]
17. **Yoshida M, Ishizaki Y, Kawagishi H.** Blastocyst formation by pig embryos resulting from *in-vitro* fertilization of oocytes matured *in vitro*. *J Reprod Fertil* 1990; **88**: 1–8. [Medline]
18. **Wang WH, Niwa K, Okuda K.** *In-vitro* penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. *J Reprod Fertil* 1991; **93**: 491–496. [Medline]
19. **Wang WH, Sun QY, Hosoe M, Shioya Y, Day BN.** Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. *Biol Reprod* 1997; **56**: 1376–1382. [Medline]
20. **Sun QY, Wu GM, Lai L, Park KW, Cabot R, Cheong HT, Day BN, Prather RS, Schatten H.** Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development *in vitro*. *Reproduction* 2001; **122**: 155–163. [Medline]
21. **Sha W, Xu BZ, Li M, Liu D, Feng HL, Sun QY.** Effect of gonadotropins on oocyte maturation *in vitro*: an animal model. *Fertil Steril* 2010; **93**: 1650–1661. [Medline]
22. **Sturmey RG, Leese HJ.** Energy metabolism in pig oocytes and early embryos. *Reproduction* 2003; **126**: 197–204. [Medline]
23. **Somfai T, Kaneda M, Akagi S, Watanabe S, Haraguchi S, Mizutani E, Dang-Nguyen TQ, Geshi M, Kikuchi K, Nagai T.** Enhancement of lipid metabolism with L-carnitine during *in vitro* maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod Fertil Dev* 2011; **23**: 912–920. [Medline]
24. **Fu XW, Wu GQ, Li JJ, Hou YP, Zhou GB, Suo L, Wang YP, Zhu SE.** Positive effects of Forskolin (stimulator of lipolysis) treatment on cryosurvival of *in vitro* matured porcine oocytes. *Theriogenology* 2011; **75**: 268–275. [Medline]