Development of Artificial Insemination Technique in Pig Production Based on the Evidence of Physiology and Molecular Biology in Reproductive Organs

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Abstract
Reproductive technology in the animal production field not only improves the reproductive performance of swine but also reduces the production costs. However, the diffusion of this technique has been limited in most countries including Japan due to the reproductive characteristics of pig, such as multiple ovulations in female and large volume of semen in male. In females, the key mechanisms of oocyte maturation in pig are not different as compared with that of the rodent model. However, the timing and regulation of gene expression and enzyme activity are characterized in pig, which results in the longer duration of oocyte maturation and ovulation. In semen, seminal plasma has dual roles in sperm quality and successful implantation. The contaminating of bacteria in semen increases endotoxin activity and reduces the sperm quality. On the other hand, the seminal plasma contains factors to regulate immune activity to reduce the number of leukocytes to make an optimal condition for implantation and to maintain the pregnancy. Based on basic scientific information, we developed novel techniques for oocyte in vitro maturation, freezing and thawing sperm and artificial insemination. These technologies will be expanded to commercial farms to increase the production-efficiency all over the world.

1. Introduction

Artificial insemination technique is useful in the pig production field because this technique not only solves the infection problem due to the contact of boar and swine during mating but also improves utilization rate of boar due to the dilution of semen. In western European countries, more than 70% of pig farms carry out artificial insemination using liquid storage semen, and this rate reaches more than 98% in the Netherlands (Feitsma 2009). However, the diffusion of this technique has been still less than half in other countries including Japan. The improvements of both insemination technique and diluent extender of boar semen
would increase the reproductive performance of artificial insemination. The low number of pups per delivery by this technique as compared with that by natural mating and the limited storage period of semen are the main problems to expand artificial insemination in the pig production field all over the world.

The prolificacy that is major reproductive characteristics of pig is caused by multiple ovulations. In recent improved breeds, more than 20 oocytes are ovulated and then fertilized, which provides the increased number of pups per delivery. The ovulation processes in each oocyte are induced by luteinizing hormone (LH) surge secreted from the pituitary gland of the brain, but don’t simultaneously proceed (Soede et al. 1995). Additionally, the fertilization ability in each ovulated oocyte is limited to within a few hours after ovulation (Soede et al. 1995, Almeida et al. 2000). Thus, to clear the timing of insemination by artificial insemination is a key factor to lead to maximum reproductive performance. The studies to identify how to induce ovulation in each follicle and how to maintain fertilization ability of oocytes after ovulation are required for the decision of insemination timing.

In cattle production field, artificial insemination using frozen-thawed semen is commonly used all over the world. Cryopreservation of sperm offers an effective means for long-term storage of important genetic material. This technique solves the problem of transporting animals or fresh semen over long distances. However, in the pig production field, cryopreservation of sperm is not available for artificial insemination since the conception rate, farrowing rate and litter size using this method spermatozoa have remained at low levels (Johnson 1981, Johnson et al. 2000). In the frozen-thawed sperm, the motility, membrane integrity and acrosome integrity are injured during freezing process (Curry 2000, Chauhan et al. 1994, Royerez et al. 1996; Courtens et al. 1989); it has been speculated that intracellular ice crystal formation is the major factor responsible for the freezing damage to spermatozoa (Johnson et al. 2000, Watson 2000). Thus, the liquid storage semen that is kept under 15–18°C for up to a week is mainly used in artificial insemination in pig. The dramatic improvement of frozen technique is essential to expand this technique in pig production.

Because the total number of pups per swine in a year is related to efficiency management in each pig farm, the methods to know which insemination timing is good and to improve the quality of frozen-thawed sperm have to be developed in artificial insemination techniques. In this monograph, we introduced the mechanisms of ovulation and oocyte fertilization ability. Additionally, the fertilization ability of sperm was also mentioned. Finally, we introduced the novel artificial insemination technique based on above basic biology.

2. The basic biological study to know how to induce ovulation

The surge of luteinizing hormone (LH) acts on granulosa cells of preovulatory follicles to induce luteinization, cumulus cell-oocyte complex (COC) expansion, oocyte maturation and follicle rupture (Richards 2005). During these dramatic changes, LH markedly induces the expression of genes (Cyp11a1, Star) regulating progesterone biosynthesis in mouse (Richards 1994), rat (Henderson et al. 1981, Ronen-Fuhrmann et al. 1998), pig (Ainsworth et al. 1980, Conley et al. 1994) and human (Duncan et al. 1999). In progesterone receptor (Pgr) knockout (PRKO) mice, follicle rupture is completely suppressed (Lydon et al. 1995, Mulac-Jericevic et al. 2000). Prostaglandin E2 (PGE2) that is derived from arachidonic acid by the rate-limiting enzyme prostaglandin synthase 2 (PTGS2; also known as cyclooxygenase 2, COX-2), is also increased by LH stimulation in mouse (Downs and Longo 1983), rat (Plas-Roser et al. 1985), rabbit (LeMarie et al. 1976), ewe (Murdock et al. 1986), pig (Evans et al. 1983) and human (Gelety and Chaudhuri 1992). In Ptg2 KO mice, or the PGE2 receptor (EP2) null mice, ovulation is impaired, and oocytes resume meiosis and reach the metaphase II stage in both in vitro and in vivo (Matsumoto et al. 2001, Segi et al. 2003). Thus, the production of progesterone and PGE2 is essential for the induction of the ovulation process.

In the LH-stimulated granulosa cells, the increase of cAMP level was markedly detected (Osterman et al. 1978, Hamberger et al. 1978, 1979), and then the targeted gene expression including Cyp11a1, Star and Ptg2 was induced by PKA-CREB dependent manner (Sayasith et al. 2005, Sher et al. 2007, Manna et al. 2009). Although the signaling pathway was transiently activated by LH in granulosa cells due to the degradation of LH receptor (Richards et al. 1976, Rao et al. 1977), the gene expressions were maintained in the cells during the ovulation process. The results suggest that other signaling pathway(s) is (are) also involved in granulosa cell differentiation during ovulation process. To identify the key signaling pathway(s), several groups generated mutant mice models that disrupted the signaling molecules activated in granulosa cells during the ovulation process. From these studies, ERK1/2 pathway is identified as signaling cascade in the change of the follicular development stage to the ovulation stage via the suppression of granulosa cell proliferation, the induction of final differentiation (luteinization) of granulosa cells and cumulus cell expansion (Fan et al. 2009).

ERK1/2 is activated by the EGFR-RAS-cRAF-MEK1 pathway in both granulosa cells and cumulus cells after LH surge (Fan et al. 2009, Richards and Pangas 2010). When COCs were cultured with the
EGFR tyrosine kinase inhibitor (AG14789) or MEK1 inhibitor (PD98059 or U0126), the phosphorylation of ERK1/2 in cumulus cells was blocked dramatically (Yamashita et al. 2007, 2009). In granulosa cells, these inhibitors also suppress the induction of Cyp11a1 and Star expression (Shimada et al. 2006), and progesterone production (Puttabyatappa et al. 2013), suggesting that the EGFR-dependent signaling pathway regulates ERK1/2 pathway, which is required for luteinization of granulosa cells and differentiation of cumulus cells at least in in vitro culture. To clarify the functional roles of ERK1/2 in in vivo, Fan et al. (2009) generated granulosa cell- and cumulus cell-specific ERK1/2 mutant mice using the Cre/LoxP technique. In ERK1/2 mutant mice in which both kinases are depleted in granulosa cells and cumulus cells, not only cumulus cell expansion and granulosa cell luteinization but also oocyte meiotic resumption are completely suppressed, suggesting that the ERK1/2 pathway in granulosa cells and cumulus cells works as a rate-limiting factor on the ovulation process.

As ligands for EGFR to activate the downstream signaling pathway, the EGF-like factors, amphiregulin (Areg), betacellulin (Btc) and epiregulin (Ereg) are transiently expressed after LH stimulation in granulosa cells and act on both granulosa cells and cumulus cells (Park et al. 2004; Ashkenazi et al. 2005; Shimada et al. 2006). Especially, Areg and Ereg are rapidly expressed within 1 hr after the onset of LH surge (Park et al. 2004). In mouse Areg gene promoter region, a putative cAMP responsible element (CRE) site is observed (Qin and Partridge 2005). The mutation of this region decreases the promoter activity in the luciferase promoter assay using primary culture of mouse granulosa cells, and CRE sequence binds to phosphorylated CREB (CRE binding protein) after LH stimulation (Fan et al. 2010). However, there is no CRE site in mouse Ereg promoter region, and the important role of Sp1 binding sites has been reported in the increase of its promoter activity in granulosa cells (Sekiguchi et al. 2002). The transcription factor, Sp1 has multiple phosphorylated sites including the target sites by PKA and the phosphorylation induces to transfer Sp1 to nuclei and to bind it to the consensus sequence of promoter region (Rohiff et al. 1997). Thus, the expressions of Areg and Ereg are directly regulated by the cAMP-PKA-CREB or cAMP-PKA-Sp1 cascades in granulosa cells during the ovulation process.

EGF like factors are transmembrane proteins that consist a signal sequence, a transmembrane domain and at least one EGF domain. To work as a ligand for EGFR, metallocprotease activity is critical for the release of the EGF domain. Several studies implied the existence of proteolytic enzyme that cleaved EGF domain of EGF-like factor to stimulate its own receptor during ovulation process (Ashkenazi et al. 2005; Jamnongjit et al. 2005; Conti et al. 2006). Firstly, Sahin et al. (2004) showed that tumor necrosis factor α-converting enzyme (TACE) was the main sheddase of AREG and EREG in mouse embryonic cells. The expression of TACE has been observed in many tissues, including heart (Sahin et al. 2004; Horiuichi et al. 2005), brain (Kikkainen et al. 2000; Hurtado et al. 2001), lung (Dijkstra et al. 2009), liver (Bourd-Boittin et al. 2009), muscle (Ohtsu et al. 2006), kidney (Goöz et al. 2006; Schramme et al. 2008) and testis (Lizama et al. 2010;
inhibitor, the enzyme activity of TACE was significantly decreased, which resulted in the reduction of ERK1/2 phosphorylation and target gene expression including Cyp11a1 (Fig. 2). Moreover, the knockdown of TACE by the specific siRNA suppressed the phosphorylation level of ERK1/2 and its target gene expression, and the down-regulations were overcome by the addition of AREG to culture media. Thus, the central dogma of the ovulation process is the sequential induction of EGF like factor, PGE2 and progesterone in LH-stimulated granulosa cells. The increase of PGE2 and progesterone to maximum levels is required for gene expressions directly induced granulosa cell luteinization, cumulus expansion and follicular wall rupture (Fig. 3).

3. The ovulation model in pig—The differences between pig and rodents—

The matured oocytes that resume meiotic from GV stage to progress to the metaphase II (MII) stage are released to oviducts at 36 to 44 hr after LH stimulation in pig (Ten Haff et al. 2002; Wongkaweewit et al. 2012). On the other hand, the ovulation is observed within 14 h after ovulation stimuli in mice (Fig. 4A). To clear which mechanisms are similar to and which are different as compared with those in mice model, we collected follicular fluid, granulosa cells, cumulus cells and oocytes from the hormone-stimulated swine at different time points after hCG injection to induce ovulation following eCG priming to induce follicular development (Kawashima et al. 2008). The expression of Areg, Ereg and Ptgs2 in granulosa cells, the phosphorylation of ERK1/2 and the secretion of progesterone in follicular fluid were detected in pig as similar to those in mice. However, the starts of cumulus expansion and oocyte meiotic resumption were delayed in pig model to 20 h after hCG injection. Interestingly, Areg and Ereg have already expressed in granulosa cells before hCG injection, however, the phosphorylation of ERK1/2 and Ptgs2 expression were induced at 12 hr after hCG injection (Fig. 4B). Thus, the results suggested that the enzyme activity of TACE to release EGF domain of EGF like factors works as a key regulator to initiate the ovulation process in pig. Strikingly, the expression of TACE was induced by hCG injection in porcine granulosa cells, and then activated by Ca2+-PKC-c-Src dependent mechanism (Yamashita et al. 2014) (Fig. 2A). In mice model, since the expression level of TACE is consistent during follicular development and ovulation process (Hernandez-Gonzalez et al. 2006), the enzyme activity is increased immediately by signaling pathway without transcription. Thus, it is estimated that the time lag to progress ovulation process in pig as compared with that in mice is caused by the time of transcription and translation of TACE in granulosa cells. The graduate increase of TACE en-

Fig. 2. The effect of PKC inhibitor (CalC) or Src inhibitor (PP2) on TACE/ADAM17 enzyme activity (A), phosphorylation of ERK1/2 (B) and Cyp11a1 mRNA expression in cultured granulosa cells. A: Effect of CalC or PP2 on TACE/ADAM17 enzyme activity in cultured granulosa cells. B: Effect of CalC or PP2 on phosphorylation of ERK1/2 in cultured granulosa cells. C: Effect of Cyp11a1 mRNA expression in granulosa cells in cultured granulosa cells. Republished with permission of The Endocrine Society, from Endocrinology, 155, Yamashita et al., Protein kinase C (PKC) increases TACE/ADAM17 enzyme activity in porcine ovarian somatic cells, which is essential for granulosa cell luteinization and oocyte maturation, 1080–1090, doi:10.1210/en.2013-1655, Fig. 4, © 2014; permission conveyed through Copyright Clearance Center, Inc.

Inoue et al. 2014). We revealed that TACE was expressed in granulosa cells and the enzyme activity was increased in granulosa cells during the ovulation process (Yamashita et al. 2007, 2014) (Fig. 1). The increased level of TACE enzyme activity was induced by the binding of TACE with PKC-induced phosphorylation of c-Src in granulosa cells. When the granulosa cells were cultured with PKC inhibitor or c-Src inhibitor, the enzyme activity of TACE was significantly decreased, which resulted in the reduction of ERK1/2 phosphorylation and target gene expression including Cyp11a1 (Fig. 2). Moreover, the knockdown of TACE by the specific siRNA suppressed the phosphorylation level of ERK1/2 and its target gene expression, and the down-regulations were overcome by the addition of AREG to culture media. Thus, the central dogma of the ovulation process is the sequential induction of EGF like factor, PGE2 and progesterone in LH-stimulated granulosa cells. The increase of PGE2 and progesterone to maximum levels is required for gene expressions directly induced granulosa cell luteinization, cumulus expansion and follicular wall rupture (Fig. 3).
zyme activity in granulosa cells after hCG injection would be a reason for the different time course of ovulation process in each follicle (Figs. 4A, B). Therefore, to synchronize the timing of ovulations and to judge the best timing of insemination, we have to examine the expression and activation of TACE in granulosa cells in each commercial breed of pig.

4. In vitro maturation technique of porcine oocytes

The positive roles of cumulus cells in oocyte maturation in vitro have been well known for the past four decades (Cross 1973; Thibault et al. 1975; Hillensjö et al. 1982; Eppig and Downs 1984). Recent microarray studies have identified novel activators including EGF like factors as mentioned above secreted from granulosa cells that induce the differentiation of cumulus cells (Hernandez-Gonzalez et al. 2006; Shimada et al. 2006). The gene targeting technology has clearly shown which signaling pathways are required in cumulus cells for oocyte maturation (Shimada et al. 2007; Lui et al. 2009). Most of them are only expressed in granulosa cells but not in cumulus cells during the ovulation process. Additionally, some of them are secreted from cumulus cells as well as granulosa cells in vivo, however in in vitro culture condition, the expression is not induced in cumulus cells of COCs cultured with any agonists. More importantly, their receptors are expressed in cumulus cells during follicular development process to preovulatory follicles. Because the COCs are collected from small antral follicles, with a diameter of 3 to 5 mm, of ovary of gilts collected from slaughterhouse, the preculture of COCs is required to

Fig. 3. Schematic diagram shows the simplified model of oocyte maturation and luteinization process. LH stimulus induces mRNA expression of EGF-like factor (Areg, Ereg and Btc) and Tada/Adam17 via cAMP-PKA-CREB or cAMP-PKA-SP1 dependent manner. Expressed TACE/ADAM17 protein is activated by LH-induced PKC-Src pathway, and sheds EGF-like factor. Soluble form of EGF-like factor stimulates EGFR-RAS-RAF-MEK1-ERK1/2 pathway in granulosa cells and cumulus cells. Activated ERK1/2 phosphorylates CEBP and AP1 transcriptional factor, and phosphorylated CEBP or AP1 induce granulosa cells specific genes, PtgS2 and progesterone production-related genes (Star, Cyp11a1 and Hsd3b1), which results in induction of granulosa lutenization and cumulus expansion.
acquire the ability to respond to EGF like factors and others that are secreted from granulosa cells during the ovulation process (Shimada et al. 2003; Kawashima et al. 2008).

The number of COCs cultured in 300 µl of maturation medium has significant correlation with the rate of oocyte maturation (Yamashita et al. 2003). When 1 COC was cultured in maturation medium, the rate of meiotic resumption was significantly lower than that when more than 20 COCs were cultured in the same volume of medium for 20 hr. Another group showed that, using condition medium where porcine cumulus cells or denuded porcine oocytes had cultured for 24 hr, the rate of meiotic resumption was increased when
COCs were cultured in condition medium where cumulus cells were cultured, but not oocyte (Procházka et al. 1998). The results suggested that the secretion factor(s) from cumulus cells play(s) an important role in oocyte maturation. The number of cumulus cells attached oocyte is increased during follicular development stage (Buccione et al. 1990; Okazaki et al. 2003). The proliferation of cumulus cells is dependent on the expression of Ccnd2 that is induced by FSH and estrogen (Robker and Richards 1998; Kawashima et al. 2008). In small antral follicles, the androgen is dominant and the level of estrogen is increased by FSH stimuli during follicular development (Leung and Armstrong 1980; Armstrong et al. 1991). Thus, preincubation with FSH and estrogen is required for cumulus cell proliferation before cumulus cell differentiation to acquire the responding ability to EGF like factors. Our study (Kawashima et al. 2008) revealed that the switch to change proliferation stage to differentiation is regulated by the ration of estrogen per progesterone. The decrease of the ration leads to the expression of EGF receptor and LH receptor in cumulus cells. The information should provide new methods for improving in vitro oocyte maturation. We have already reported a new system of porcine COC maturation which we briefly describe below and illustrate in Fig. 5.

1. COCs are recovered from small antral follicles (3–5 mm in diameter).
2. The recovered COCs are pre-cultured with 2 ng/ml FSH and 100 ng/ml estradiol 17β for 10 hr to induce cell proliferation.
3. At 10 hr, 20 ng/ml of progesterone is added to the FSH- and estradiol-containing medium to suppress cell proliferation and induce Egfr and Lhcgr mRNA expression.
4. After 20 hr of culture, COCs are moved to fresh medium with 1 μg/ml LH, 1 ng/ml EGF and 100 ng/ml progesterone for an additional 24 hr.

Using this novel culture system based on in vivo
changes in hormones and growth factor production, the matured porcine COCs exhibit full expansion, the cumulus cells remain healthy (low number of apoptotic cells) and when oocytes obtained from these COCs are used for in vitro fertilization, developmental competence to the blastocyst stage is significantly improved as compared with the conventional FSH+LH culture system (Kawashima et al. 2008). Although the successful rate of embryo transfer is still limited, the combination with in vitro maturation technique and embryo freezing will be contributed for conservation of genetic resources. In cattle production field, the system has been used in the commercial field. The numerous basic researches in this field promote the next generation reproduction technique to be available for pig commercial field.

5. Fertilization ability of oocytes

The matured oocytes are ovulated to oviduct and are then arrested at the MII stage until the penetration of sperm. The ability of oocytes to prevent the resumption of meiosis from the MII stage or the induction of fragmentation is called cytostatic factor (Masui et al. 1977), however the function is limited within several hours after ovulation (Blandau et al. 1952; Marston and Chang 1964). Our recent study revealed that the activity of cytostatic factor of matured oocytes is decided by granulosa cell-secreted neuregulin 1 (NRG1) during the ovulation process (Noma et al. 2011; Kawashima et al. 2014). NRG1 is one of the members of EGF like factor but does not act on EGFR as similar to AREG or EREG (Riese et al. 1995) (Figs. 6A, B). The ErbB3 and ErbB2 heterodimer that are expressed in granulosa cells and cumulus cells (Noma et al. 2011) are worked as receptor for NRG1 (Wallasch et al. 1995). The induction of Nrg1 mRNA in granulosa cells but not in cumulus cells was started within 2 hr after hCG injection (Fig. 6C). Western blot analyses showed that at least two high intensity immuno-reactive bands were detected in whole ovarian extracts after hCG stimulation (Fig. 6D). However the small molecular weight band was less intense in granulosa cells. In response to hCG, granulosa cells express specific cleav-

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Fig. 6. The expression of Neuregulin 1 (NRG1) in granulosa cells during ovulation process. (A) The image of murine Nrg1 gene; Because Nrg1 gene has three different transcription sites, three different types of transcripts are potentially expressed. (B) The functional mechanism of NRG1; EGF domain of NRG1 is released by Adam19 and then acts on ErbB2 and ErbB3 heterodimer. (C) The expression of Nrg1 in granulosa cells and cumulus cells during ovulation process. (D) The expression and modification of NRG1 in pre-ovulatory and peri-ovulatory follicles. Republished with permission of The Endocrine Society, from Mol. Endocrinol., 25; Noma et al., LH induces Neuregulin 1 (NRG1) type III specific transcripts that interact with EGF-like factor pathways to control granulosa cell differentiation and oocyte maturation, 104–116, Fig. 3, © 2011; permission conveyed through Copyright Clearance Center, Inc. Republished with permission of The Endocrine Society, from Mol. Endocrinol., 28(5), Kawashima et al., Targeted disruption of Nrg1 in granulosa cells alters the temporal progression of oocyte maturation, 706–721, Fig. 1, © 2014; permission conveyed through Copyright Clearance Center, Inc.
age enzymes, including Adam19 that processes extracellular growth factors (Shirakabe et al. 2001). Because the full length of type III NRG1 is more than 110 kDa (Hu et al. 2006; Savonenko et al. 2008), the present results suggest that the 75 kDa band is a secreted form including EGF domain and is worked as active ligand form. In fact, the specific antibody of EGF domain of NRG1 recognized both bands but the anti C-terminal domain of NRG1 antiserum revealed only 110 kDa. Thus, the small form is presumed to include the EGF domain converted by an enzyme that recognizes the N terminal region of type III NRG1. The positive signals by anti-EGF domain of NRG1 antibody were detected at granulosa cell layers, follicular fluid and cumulus cell layers (Kawashima et al. 2014).

To clear the role of NRG1 in in vivo, we tried to make an analysis of the oocyte maturation using Nrg1 mutant mice model (Kawashima et al. 2014). Whole body Nrg1 KO mice are lethal at day 10 during embryogenesis (Meyer and Birchmeier 1995). To overcome this severe limitation, we generated granulosa cell (GC)-specific Nrg1 gc–/– (gc Nrg1 KO) by mating Nrg1flox/flox mice to mice expressing Cre recombinase driven by Cyp19a1 (GC specific) promoter (Yang et al. 2001; Fan et al. 2008). Mating test with wild type male mice has been done to check the fertility in the mutant female mice. The average number of pups per delivery was significantly decreased in gc Nrg1 KO as compared with that in Nrg1flox/flox (WT) female mice due to the increase of abnormal fertilization. Although the fertilization rates of oocytes recovered at 14 and 16 h from gc Nrg1 KO were similar to those in Nrg1flox/flox (WT) female mice, the successful fertilization (2 pronuclei and sperm tail) rate was significantly lower in oocytes recovered at 18 h (Fig. 7A). Additionally, the rate of successful fertilization in in vivo was linearly decreased by delaying time points when plug was formed in gc Nrg1 KO mice (Fig. 7B). Thus, the reason for decreasing number of pups per delivery in gc Nrg1 KO is caused by the limiting time window of oocytes acquire fertilization competence.

In pig granulosa cells, Nrg1 is also expressed by hCG injection and the receptor, ErbB2 and ErbB3 is detected in both cumulus cells and granulosa cells of preovulatory follicles (Tabata et al., unpublished data). Moreover, the activation of ErbB3 was induced in cumulus cells when porcine COCs were cultured with NRG1, suggesting that NRG1 also plays an important role in fertilization ability of porcine oocytes. The NRG1-induced fertilization ability permits late timing of fertilization in in vivo, suggesting that further analysis of Nrg1 gene in pig leads to get a novel genetic marker to select a high reproductive performance line.

From the above study, we revealed that the induction of Nrg1 was observed in mouse and pig ovary during ovulation process. Although both species were members of prolificacy animals, we also detect the high level of Nrg1 expression in human granulosa cells collected from ovarian-stimulated cycles of infertility patients (Tabata et al., unpublished data). The preliminary data suggested that not only in multiple ovulation species such as pig and mouse but also in single ovulation species including human, NRG1 would play an important role in oocyte fertilization competence.

6. The fertilization ability of sperm

In ovulated COCs, a hyaluronan rich matrix was accumulated among cumulus cells called as cumulus expansion (Richards 2005). The expansion is essential, not only for ovulation but also for in vivo fertilization, and perhaps more specifically for sperm capacitation.
(Gutnisky et al. 2007). During fertilization process, the matrix is broken down by sperm-secreted hyaluronidase, which induces chemokine secretion from cumulus cells (Shimada et al. 2008). The secreted chemokine, such as CCL5 acts on sperm via the specific receptors (CCR1, CCR3 and CCR5) to induce hyperactivation status (Shimada et al. 2008, 2013). The high progressive motility and high frequency of sperm head are associated with the ability to pass through zona pellucida to detach the surface of oocyte cytoplasmic membrane (Suarez and Ho 2003a, b). Thus, the accumulation of hyaluronan rich matrix and the degradation of it by sperm-secreted hyaluronidase are an initial step of successful fertilization.

Hyaluronidase storages in sperm head region is called acrosome (Kim et al. 2008). The release of hyaluronidase is induced by follicular fluid-containing factor, such as progesterone that is emitted from follicles with matured oocytes (Calogero et al. 2000). Progesterone acts on sperm to increase Ca$^{2+}$ level in sperm cytoplasm, which induces exocytosis system that opens secret vesicle to release hyaluronidase (Roldan and Vazquez 1996; Publicover et al. 2008; Witte and Schäfer-Somi 2007). The loss or mutation of acrosome lost the fertilization ability, but the rate of acrosome integrity was very high, about 80% in boar and murine sperm recovered from epididymis (Lusignan et al. 2007; Yamashita et al. 2007) (Fig. 8). However, the rate was dramatically increased in bacteria-infected semen (Zan et al. 2008).

Bacterial infections of the genital tract increase the risk of male infertility and economical loss in domestic animal production (Althouse and Lu 2005; Maes et al. 2008; Goldberg et al. 2013). Because leukocytes are contaminated in bacteria-infected semen (El Feky et al. 2009; Gdoura et al. 2008) (Fig. 9), and because leukocytes express the Toll-like receptor (TLR) that is activated by bacteria-released endotoxin, LPS (Takeda and Akira 2005; Poltorak et al. 1998), the activation of leukocyte TLRs by LPS can induce the secretion of Tumor Necrosis Factor (TNF)-α thereby causing decreased sperm motility (Liu et al. 2012; Aggarwal et al. 2003; Perdichizzi et al. 2007). However, our previous study revealed that TLR2 and TLR4 were expressed in pig, mouse and human sperm (Fujita et al. 2011). TLR2 and TLR4 were localized at the acrosome regions and mid piece of pig, mouse and human sperm.

The function(s) of TLRs in sperm were then analyzed
in more detail by determining the effects of LPS on sperm in specific mutant mouse models (Fujita et al. 2011). Because LPS had high affinity binding for TLR4, and a high dose of LPS also activated TLR2, Tlr4−/− or Tlr2−/−; Tlr4−/− male mice were used for this study. Sperm motility decreased significantly in wild type (WT) mice exposed to 1.0 μg/ml of LPS for 6 hr, whereas LPS had little or no effect on sperm motility.
in Tlr4−/− mice or Tlr2/4 double knockout mice. Decreased acrosome integrity and the number of TUNEL positive and cleaved caspase-3 positive (apoptotic) sperm were increased in WT mice exposed to LPS but not in the Tlr mutant mice. These results in the Tlr4 and Tlr2/4 mutant mice, provide strong evidence that the bacterial endotoxin LPS mediates its apoptotic effects in sperm via activating the TLR pathways. Because apoptosis can be activated in sperm by the LPS-TLR4 pathways, we conclude that sperm respond to bacterial infection (LPS) in semen via a direct TLR4-dependent mechanism and do not require immune cell-secreted cytokines (Fig. 10).

Fertilization competence of mouse sperm was also impaired by bacterial release of LPS in both in vivo and in vitro and via a TLR4-dependent manner (Fujita et al. 2011). When the sperm samples were pre-cultured with or without bacteria and then injected into the uterus of female mice, the fertilization rate in control mice was about 60% whereas the fertilization rate in mice injected with sperm exposed to bacteria was less than 20%. However, the presence of polymyxin B (PMB) that neutralized LPS activity reversed the negative effects of bacteria and increased fertilization rates to 40%, suggesting that bacteria-secreted LPS reduced the in vivo fertilization ability of mouse sperm (Fujita et al. 2011).

In conclusion, sperm express some innate immune functions, including TLRs and functional responses to LPS. In semen samples infected with bacteria, sperm recognized the bacterial endotoxins via TLR-dependent pathways, inducing an apoptotic process. The apoptotic response induced by LPS may be required to ensure the removal of poor sperm that would be detrimental to fertilization and embryo development.

7. How to improve the quality of frozen-thawed boar sperm

7-1. Bacteria infection in boar semen decreases the frozen-thawed sperm motility and fertilization ability

Bacteria are frequently detected in boar semen and affect detrimental effects on sperm motility or survivability. In our recent study, the presence of bacteria in pig semen was determined first by collecting semen samples from 22 individual boars (pigs), and then culturing the seminal plasma in agar without antibiotics. Bacterial colonies were observed in 19 (13 gram-negative; 3 gram-positive; double positive 3) of the 22 samples but were absent in 3 samples (Table 1).

To examine the effects of bacteria on sperm motil-
ity, we incubated semen at 37°C for 1, 3 or 6 hr and then analyzed sperm motility rates. Before and 1 hr after incubation, sperm motility was not significantly correlated with the number of bacteria (Fig. 11). However, by 3 hr of incubation a significant negative correlation ($r^2 = 0.279$, $P < 0.05$) was observed between sperm motility and the number of bacteria (Fig. 11). It has been thought that the increasing number of bacteria in seminal plasma changes pH, metabolized source, or free radical due to their proliferation and metabolic activity (Althouse et al. 2000; Fraczek et al. 2007). Because sperm has a high responsibility to the environmental changes mentioned above, the motility and fertilization ability are decreased in the presence of bacteria. Additionally, the gram negative bacteria release lipopolysaccharide (LPS) that acts as endotoxin and sperm recognize the endotoxin by the specific receptor, TLRs to decrease sperm quality. Thus, to reduce the bacteria infection problem, Penicillin G that weakens the cell wall of the bacterium, or Amikamycin that suppresses protein synthesis in bacteria have been well-used for boar semen treatments (Okazaki et al. 2010). However, when the antibiotics 1,000 U/ml of Penicillin G and 100 µg/mL of Amikamycin were added to the pig semen samples containing high titers of bacteria (more than 5000 CFU/ml), neither Penicillin G nor Amikamycin significantly improved sperm motility 3 hr after incubation (Fig. 12). This is why the treatment with the antibiotics releases LPS from gram negative bacteria to semen due to the antibiotics-induced bacteriolysis. LPS directly bound to the sperm head region (Fig. 13) to reduce motility and induce apoptotic process as mentioned above. The negative effects of LPS on sperm function quickly started even if the sperm was only treated with LPS for 5 min and then removed. Because PMB neutralizes LPS activity, we sought to develop and improve the techniques for long storage of boar sperm by testing the beneficial effects of PMB.

In our study, the LPS activity in seminal plasma was increased by the addition of Penicillin G compared to that in seminal plasma without Penicillin G (endotoxin activity; 0.05 vs. 0.17 EU/mL). The addition of 100 µg/ml PMB to seminal plasma completely inactivated Penicillin G-increased LPS activity in boar semen. The inhibitory effect of PMB on the binding of LPS to sperm was examined using FITC-conjugated LPS. The results showed that in control (without PMB), the posi-

<table>
<thead>
<tr>
<th>Boar No.</th>
<th>G (-)</th>
<th>G (+)</th>
<th>Total</th>
</tr>
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<td></td>
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</tbody>
</table>

Fig. 11. The correlation between the motility of sperm at each culture point and the number of bacteria in the semen just after ejaculation.

Table 1. The number of gram positive and negative bacteria in each boar semen.
tive signals of LPS were localized at the head region and mid piece of sperm, and most of sperm were positive (Fig. 13). However, the binding was dramatically suppressed by PMB treatment in a dose dependent manner, and the maximum effect was observed at 100 μg/ml.

7-2. Removing and/or neutralizing endotoxin is (are) important for long storage and freezing of boar sperm

Because pig semen frequently contains high titers of bacteria and the treatment with antibiotics releases LPS to semen, which reduces sperm quality (Okazaki et al. 2009a), we reasoned that LPS released from bacteria would decrease sperm motility during long-term storage or freeze-thaw processes and consequently would account for the low success rate of artificial insemination in the pig. In fact, the treatment of sperm with PMB has been used to improve assisted reproductive technology protocols for domestic animals (Okazaki et al. 2010; Okazaki and Shimada 2012). However, only a few papers have documented the positive effects of PMB on sperm motility during short-term storage (Hakimi et al. 2006; Hosseinzadeh et al. 2003). Therefore to document this, a sperm-rich fraction was collected weekly from each of 22 boars and then treated with or without antibiotics. During long-term storage of pig sperm at 15°C for 2 days, sperm motility dramatically decreased in antibiotic free conditions. Decreased sperm motility was also observed when Penicillin G alone was present during a similar test period. However, when Penicillin G was combined with PMB, sperm motility improved significantly after 6 days of storage as compared with that in the Penicillin G group (Fig. 14). When sperm were stored for 10 days and then used for artificial insemination, the conception rate was significantly higher in the combined treatment group as compared to Penicillin G alone (Fig. 14). When pig sperm were treated immediately after ejaculation with both Penicillin G and PMB, and then frozen under liquid nitrogen, the motility of sperm after thawing was significantly higher than that of sperm treated with penicillin G alone (Fig. 15).

Fig. 12. The effects of antibiotics on sperm motility when semen containing high number of bacteria was incubated at 37°C.

Gram positive bacteria were also detected in 14% (3/22 boars) of semen (Table 1). Gram positive bacteria releases peptidoglycan or lipoprotein, and these factors stimulate TLR2 (Tsan and Gao 2004). When sperm was incubated with TLR2 ligand, Pam3Cys, the motility was markedly decreased in human (Fujita et al. 2011) and pig (Okazaki et al. 2009a) due to the decrease of membrane integrity. Therefore, it is necessary step to remove both LPS and gram positive bacterial factors from sperm incubation medium just after ejaculation.

Based on these findings, we designed the following novel handling method before the cooling and freezing process. Firstly, seminal plasma is removed from the sperm by centrifugation at room temperature immediately after semen collection. Secondly, the sperm pellets are resuspended in pre-treatment solution containing common antibiotics (e.g. Penicillin G and/or Amikamycin) and PMB. When semen was handled by novel extender before the freezing procedure, the success rate (borderline for preservation; post-thaw sperm motility >60%) of cryopreservation was significantly higher (88%) than that by conventional method.

7-3. Optimal combination of osmolality and glycerol in the freezing extender

In the conventional freezing method of boar sperm, the ejaculated semen is diluted to pretreatment solution and then kept at 15°C for at least 2 hrs. During the
first step, we added the additional process to remove bacteria-released endotoxin by centrifugation and the combined treatment with antibiotics as mentioned above. The incubation at 15°C reduces the metabolic activity of sperm. After 2-hr incubation, the dilution solution is removed by centrifugation, and then sperm is resuspended by the cooling extender containing egg yolk, and cooled from 15 to 5°C. At 5°C, the glycerol is added as cryoprotectant agents (CPAs) to sperm diluents to remove the surplus water from sperm or bind the water inside sperm. After 20-min of glycerol equilibration, the sperm diluents are put into straws, and are frozen under liquid nitrogen.

Glycerol is known to be good CPAs in freezing extenders to protect sperm from the freezing damages. It has been reported that the addition of glycerol improves motility and acrosomal integrity of frozen-thawed spermatozoa (Johnson et al. 2000; Almilid and Johnson 1988; Fiser and Fairfull 1984). However, because boar sperm are highly susceptible to glycerol (Almilid and Johnson 1988), more than 4% of glycerol affects the membrane integrity and reduces the fertilization rate after artificial insemination (Wilmut and Polge 1974). The concentration of glycerol for sperm freezing is much lower in pig as compared with that in other species, such as cattle (8 to 10%) and human (10 to 12%). Therefore, in pigs, it is necessary to develop a novel extender containing a lower concentration of glycerol than that used in other species to protect sperm from freeze-injury and maintain motility and fertilization competence after thawing.

The positive effects of glycerol for sperm freezing

Fig. 13. The binding of LPS on boar sperm, modified from Okazaki et al. 2010, Theriogenology LPS bound to some of sperm surface, but the treatment with PMB completely suppressed the binding of LPS to sperm. A; control bright, b; control immunofluorescent microscope, c; 100 mg/mL PMB bright, d; 100 mg/mL, PMBl immunofluorescent microscope. Reprinted with permission from Theriogenology, 74, Okazaki et al., Polymyxin B neutralizes bacteria-released endotoxin and improves the quality of boar sperm during liquid storage and cryopreservation, 1691–1700, Fig. 3, © 2010, Elsevier.
are thought to reduce the damage caused by water ice crystals. Sperm have little cytoplasm, but nonetheless a sufficient volume of water to form intracellular ice during the freezing procedure, estimating that the decrease of water in cytoplasm before the freezing process might improve the quality of frozen-thawed sperm. Liu et al. (1998) showed that the exposure of bull spermatozoa to hyperosmotic solution caused sperm shrinkage and dehydration, which prevented intracellular ice crystal nucleation during the freezing process. The positive effects of the treatment with hyperosmotic extender before freezing on motility and membrane integrity have also been reported in ram sperm (Fiser et al. 1981; Watson and Duncan 1988) and boar sperm (Zeng et al. 2001).

To determine the optimal combination of hyperosmotic extender and glycerol treatment, boar sperm is divided to 3 × 4 (total 12) treatment groups with varying osmolalities (300 to 500 mOsm/kg) during cooling process and glycerol concentration (0.5 to 3%) treating before freezing in 3 boars. The ANOVAs analysis showed that significant difference was not detected in sperm motility among 3 boars, however in parameter, osmolality and glycerol concentration, significant differences are detected (Table 2). More than 2% glycerol is required to keep the sperm high motility after thawing. 400 mOsm is optimal osmolality for treatment of sperm during cooling process. The novel hyperosmotic (400 mOsm/kg) and low-glycerol (final concentration 2%) freezing extender not only improved the motility of sperm just after thawing but also maintained the high motility after long incubation at 37°C. The continuous motility after thawing in vitro predicts high motility of sperm in uterus after artificial insemination. Additionally, the novel combinational treatment increased acrosome integrity of frozen-thawed sperm. The integrity will be contributed for high fertilization ability in vivo as described above.

7-4. Ca²⁺ uptake in sperm during thawing process damages sperm quality

The frozen-thawed sperm has exhibited abnormal capacitation and lost acrosome region due to an increase of Ca²⁺ level in sperm (Bailey and Buhr 1993). When the frozen semen was made by our novel technique to suppress the risk of bacteria released endotoxin and formation of ice crystal during freezing process, the acrosome integrity and motility of sperm after long incubation were still lower than those in sperm just after ejaculation. Because the inductions were observed in a time dependent manner after thawing, we hypothesized that the induction of abnormal capacitation and the loss of acrosome would be caused by the damage during the thawing process but not the cooling and freezing process. The capacitated sperm...
showed high motility but was not maintained for a long time culture after thawing. The capacitation is induced by several extracellular factors, however in sperm cytoplasm, the increased level of Ca\(^{2+}\) is a trigger of capacitation (Arnoult et al. 1999; Baldi et al. 2000). It has been known that during the thawing process, the membrane integrity is decreased due to release of cholesterol and phospholipid from cell membrane (De Leeuw et al. 1990; Plummer and Watson 1985; Müller et al. 1999; Ollero et al. 1998; Pena et al. 2003; Bwanga et al. 1991). From these reports, we estimated that unstabilization of cell membrane during thawing process would allow the uptake of Ca\(^{2+}\) in sperm cytoplasm to induce the abnormal capacitation and the loss of acrosome cap. To clear this hypothesis, we investigated the level of Ca\(^{2+}\) in sperm after thawing and the additional effects of Ca\(^{2+}\) chelator, EGTA to thawing solution on [Ca\(^{2+}\)] level and post-thawed sperm functions (Okazaki et al. 2011).

Strikingly, the intercellular level of Ca\(^{2+}\) in sperm was dramatically increased in frozen-thawed sperm after incubation. Firstly, the increased signals were detected in the sperm mid piece and the signals were expanded to sperm head region. Capacitation of sperm is referred to be the change of metabolic activity, and the ATP production was dramatically increased in capacitated sperm. The induction of Ca\(^{2+}\) to activate TCA cycle and electronic chain transfer in mitochondria that localize on the mid piece of sperm. The loss of acrosome cap is caused by the exocytosis of hyaluronidase and other secreted factors, which is induced by Ca\(^{2+}\). dependent formation of SNAP25, Synaptotagmin, and synaptobrevin (Schulz et al. 1998; Tomes et al. 2002). Thus, the increase of Ca\(^{2+}\) after thawing is thought to be a trigger of abnormal capacitation and loss of acrosome cap.

The freezing extender and thawing solution don’t contain Ca\(^{2+}\), however it is very difficult to make Ca\(^{2+}\) free solution without any contaminations. We used Ca\(^{2+}\) chelator, EGTA to suppress the uptake of Ca\(^{2+}\) to sperm after thawing. The addition of EGTA (10 mM) to thawing solution completely suppressed the increase of Ca\(^{2+}\) in frozen-thawed sperm. The tyrosine phosphorylation of sperm protein that was a marker of capacitation was also significantly suppressed by the addition of EGTA. Moreover, the treatment increased the acrosome integrity and the thawed sperm motility similar to those in sperm just after ejaculation.

8. The novel artificial insemination using frozen-thawed boar sperm

8-1. Seminal plasma improves uterus condition after artificial insemination

It is known that the injection of seminal plasma into the female genital tract suppressed the increase of polymorphonuclear neutrophilic granulocytes in the uterus (uterine clearance) and enhanced the rate of disappearance of uterus inflammation (Matthijs et al. 2000; Rozeboom et al. 1998, 2001). Moreover, O’Leary et al. (2004) reported that differentiation of endometrial cells was induced by seminal plasma and that these cells expressed cytokine and chemokine mRNA for embryo development and implantation preparation in pigs. Taken together, these data indicate that seminal plasma plays an important role in the uterine environment conducive to the success of conception and pregnancy. However, when sperm was injected into the uterus in pig artificial insemination, the factors included in seminal plasma were not or less taken into uterus because the diluted semen or frozen-thawed
sperm were injected to uterus. Thus, it is possible that low reproductive performance in artificial insemination as compared with that in natural mating is caused by the dilution of semen or removing seminal plasma during the process of preparing frozen semen.

To clear this hypothesis, we identified the factors in seminal plasma and then developed a novel pig artificial insemination method using cryopreserved sperm (Okazaki et al. 2009b) as shown in Fig. 16. The sperm-rich fraction was collected weekly from each boar using the gloved-hand technique. The seminal plasma was removed just after collection by centrifuge and then was frozen as described in above section. The frozen sperm was thawed using the thawing solution with or without 10% (v/v) of seminal plasma. The addition of seminal plasma significantly increased the conception rate as compared with that of frozen-thawed sperm without the addition of seminal plasma to the thawing solution. The increased rate was comparable to that of fresh semen (Table 3). When frozen-thawed sperm was artificially inseminated into natural estrus sows, the conception rate was 67%, and the total number of fetuses was 9.7±3.5. The rates were not significantly different from those for artificial insemination using fresh semen (Table 3).

8-2. Chemically defined solution to improve uterus condition and increase the conception rate after artificial insemination

Since it has been known that seminal plasma contains various bacteria and/or virus (e.g. PRRS, AD, PCV2, PPV etc.) (Kim et al. 2003; Guérin and Pozzi 2005; Althouse and Rossow 2011), it is estimated that the use of seminal plasma as thawing solution increases the risk of infection. Furthermore, the quality of seminal plasma depends on seasonal or individual characteristics, suggesting that it is necessary to develop the thawing solution without animal derived materials (without seminal plasma). The treatment with EGTA increased the fertilization rate in in vivo artificial inseminations, similar to that in sperm with seminal plasma. The same number of blastocyst embryo were collected from the uterus by artificial insemination using post thawed sperm with EGTA, however the number of leukocytes in uterus was increased and implantation was prevented. Because the co-injection of seminal plasma with frozen-thawed sperm decreased the number of leukocytes in uterus, it is suggested that the factors to repress inflammation in the uterus are contained in seminal plasma.

Cortisol is well known to be the factor to reduce inflammation and decrease the number of leucocytes (Whitehouse 2011). Although it is no report to mention cortisol in seminal plasma, it has been reported that the circular level of cortisol is increased at the time of mating (Borg et al. 1991). Thus, we examined the level of cortisol in seminal plasma and the effects of injection of cortisol in uterus condition after artificial insemination. The results showed that cortisol was detected in seminal plasma (Table 3). When the sows of natural estrus were twice artificially inseminated with or without cortisol, the injection of cortisol into the uterus with sperm significantly decreased the number of leukocytes in the uterus and endometrium at 24–36 h after artificial injection. The low number of leukocytes in the uterus was similar to that in the uterus injected with fresh semen. The cortisol injection significantly increased the implantation rate and litter size of sows as compared to artificial insemination without cortisol (implantation rate; 83% vs. 51%, litter size; 10.6 vs. 7.3). From these results, we concluded that the injection of 5 mg cortisol with frozen-thawed spermatozoa by EGTA-containing solution was a novel method of pig artificial insemination using cryopreserved spermatozoa.

9. Discussion

The rodent model is commonly used to analyze how to induce ovulation and oocyte maturation (Richards 2005). The characteristic of multiple ovulations are similar to that in pig and most factors to regulate ovarian function in mice are also expressed in pig ovaries. However, it takes a long time to induce ovulation in pig compared with mice, and especially the synchronization of ovulations is not observed in pig even if under hormone-stimulation condition. To clear the reasons why the ovulation process is not synchronized and takes a long time to induce the ovulation process in pig compared with those in rodent model, we examined the kinetic changes of gene expression and acti-

Table 3. The reproductive performance of artificial insemination using fresh semen and frozen-thawed semen (from Okazaki et al., 2011).

<table>
<thead>
<tr>
<th>Artificial insemination</th>
<th>Number of inseminated sows</th>
<th>Conception rate (%)</th>
<th>Percentage of aborted sows (%)</th>
<th>Litter size</th>
<th>Number of live born</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh semen</td>
<td>57</td>
<td>84.2 (48/57)</td>
<td>0</td>
<td>11.0±0.3</td>
<td>10.4±0.3</td>
</tr>
<tr>
<td>Frozen-thawed semen</td>
<td>17</td>
<td>82.4 (14/17)</td>
<td>0</td>
<td>11.8±0.4</td>
<td>11.1±0.4</td>
</tr>
</tbody>
</table>
Table 4. The concentration of cortisol in boar seminal plasma.

<table>
<thead>
<tr>
<th>Sample</th>
<th>cortisol (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma</td>
<td>7.11+/−0.89</td>
</tr>
<tr>
<td>seminal plasma</td>
<td>0.92+/−0.01</td>
</tr>
</tbody>
</table>

Evolution of signaling pathway in granulosa cells during the ovulation process. In mice, the pattern of gene expression was sequentially induced in granulosa cells after hCG (ovulation stimuli) injection. Firstly, LH-targeted genes were expressed within an hour after hCG injection via LH-induced cAMP-PKA-CREB or cAMP-PKA-Sp1 dependent mechanisms (Fan et al. 2010; Sekiguchi et al. 2002; Rohiff et al. 1997). EGF like factors, AREG and EREG were included in the initial expression genes, and activated the expression of the next group of genes, such as Ptgs2 and Cyp11al (Shimada et al. 2006; Yamashita et al. 2007). These genes are involved in PGE2 production and progesterone production, respectively, which also induce the gene expressions involved in cumulus expansion, granulosa cell luteinization and follicular wall rupture (Richards 1994; Matsumoto et al. 2001; Segi et al. 2003). In porcine granulosa cells, the initial expression of genes that was observed in mice just after hCG injection was also induced within a few hours (Kawashima et al. 2008). However, the phosphorylation of ERK1/2 that was a target signaling pathway of EGF like factor and the expression of Ptgs2 were delayed for 12 hr after hCG injection (Kawashima et al. 2008; Yamashita et al. 2009). The different time courses were associated with the different mechanism of the release of EGF like factor (Yamashita et al. 2007; Kawashima et al. 2008). Thus, the wide variation to induce ovulation in each follicle and different individuals is a logical consequence of characteristic induction of ovulation in pig. Therefore, it is very difficult to know the timing of ovulation and to synchronize the timing of ovulation for efficient artificial insemination technique in pig.

To solve the above problem of artificial insemination in pig, we think of two different approaches; one is to prolong the period that ovulated oocytes can be successfully fertilized in the oviduct, the other is to prolong the period that injected sperm is alive in uterus and oviduct, and can be fertilized in oviduct. In the first strategy, the limited period of fertilization ability of oocytes is caused by the spontaneous resumption of oocyte meiosis from the MII stage (Komar 1982). The arrested period at the stage is thought to be less than 5 to 10 hours, and the ovulation is observed from 30 to 44 hours after hCG injection (Branden and Austin 1954). Thus, several times of injection are required to penetrate sperm to all of the oocytes arrested at the MII stage. A recent study using a mutant mouse model revealed that the ability to arrest at the MII stage was decided to be the granulosa cell-secreted NRG1 during ovulation process (Kawashima et al. 2014). NRG1 acted on cumulus cells to regulate the AREG-EGFR-induced PKC activity to a moderate level. The superb regulation system controls the timing of closing gap junctional communication between cumulus cells and oocyte, which induces meiotic resumption after oocyte gets the ability to be arrested at the MII stage. It is estimated that the period between LH surge and the onset of meiotic resumption is required for polyadenylation of maternal RNA that is stably accumulated in oocyte during follicular development stage (Goldman et al. 1987; Mutter et al. 1987). In granulosa cell-specific Nrg1 mutant mice, the stabilization of phosphorylated ERK1/2 was limited in the MII oocytes, suggesting that Mos protein synthesis during meiotic progression was low level in oocytes due to the limited polyadenylation of Mos RNA. The polyadenylation in oocytes is initiated after EGF like factors act on cumulus cells, and is stopped at the MI stage (Chen et al. 2013). In other words, the tight control of the level of polyadenylatein of maternal mRNA is regulated by the timing of EGF like factors and NRG1 expressions. In our unpublished data, NRG1 and its receptor (ErbB3) were expressed in porcine granulosa cells and cumulus cells. However, there is no report about the differences of their expressions among the breeds, individuals and follicles. It has been known that the genes encoding Nrg1 and ErbB3 have a numerous single nucleotide polymorphism (SNP) in not only promoter region but also exon to change the expression levels or function of their translation products in the human genome (Li et al. 2006; Garcia-Barcelo et al. 2009; Betcheva et al. 2009). Current genome project of pig finds SNP using the next generation sequencer, and challenges to clear the relationship between each SNP and function. The genome project of pig and molecular study about porcine ovary coordinately progress to breeding to select the high reproductive performance.

Another strategy to reduce the times of artificial insemination is to prolong the period that sperm is alive and has the fertilization ability in uterus and oviduct. The survival of sperm in uterus and oviduct is destined by sperm themselves and the condition of female reproductive tracts (Katila 2001; Rodriguez-Martinez et al. 2005). In artificial insemination, especially using frozen-thawed sperm, the morphological integrity such as acrosome and tail before injection predicts the motility in uterus and oviducts (Roca et al. 2006; Zeng and Terada 2000). We developed the method to freeze and thaw boar semen and described it in this monograph. Briefly, the sperm was separated from seminal
plasma just after ejaculation and then treated with antibiotics to remove the risk of bacteria-released endotoxin. The sperm was re-suspended by hyperosmotic extender to remove the free water from cytoplasm of sperm to reduce the risk of ice crystal formation during the freezing process. During the thawing process, to suppress the uptake of Ca\(^{2+}\) to sperm cytoplasm, Ca\(^{2+}\) chelater, EGTA was added to thawing solution. The frozen-thawed sperm can be alive for at least 6 hours after thawing in vitro, however the conception rate by artificial insemination has still been limited.

We also revealed the increase number of leukocytes in the uterus after the injection of frozen-thawed sperm. Leukocytes are accumulated to the region of inflammation, and then excludes non-self or repair the wound (Borregaard et al. 2005). Because sperm is non-self for female, a factor is required for the reduction of inflammation status in uterus when semen is injected not only by artificial insemination but also natural mating. We cleared that cortisol that is a component of seminal plasma reduced the number of leukocytes to make an optimal condition for implantation and maintain the pregnancy. The combinational approach to keep the good morphology of sperm and to induce the optimal condition in uterus improved the conception rate and litter size by artificial insemination using frozen-semen. In the current data, the conception rate was more than 85% and litter size was more than 10, when 3 to 5 billion sperm were injected twice per estrus. The reproductive performance by frozen-thawed semen was similar to that by fresh semen and natural mating. If we induced synchronization of ovulation, the one-time injection of frozen-thawed sperm would predict good reproductive performance. The genetic breeding and the method to induce ovulation by pharmacological approach will progress to develop a more efficient technique for artificial insemination.

In conclusion, the basic biological approaches to identify the mechanisms of ovulation, oocyte maturation and sperm fertilization ability are required to develop a novel technique of reproduction. Each species has common mechanisms but also has a unique system to survive and leave offspring. In our model to develop reproductive technology of pig, we examined the molecular mechanisms using knockout mice model and comprehensive gene expression analysis. The basic data from rodent model is adjusted to pig model to develop the reproductive technology. Our novel artificial insemination technique using frozen-thawed sperm would be expanded to commercial pig farm to improve the genetics and increase the production-efficiency all over the world.

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