Study on the efficient selection of porcine in vitro produced embryos and their expression of pluripotency-associated genes

ブタの体外生産胚の効率的な選択とその多能性関連遺伝子の発現に関する研究

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LIST OF ABBREVIATIONS

AD-MSC	adipose-tissue-derived mesenchymal stem cells
BM-MSC	bone-marrow-derived mesenchymal stem cells
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
DAPI	4′, 6-diamidino-2-phenylindole
dbcAMP	dibutyryl cAMP
DNA	deoxyribonucleic acid
eCG	equine chorionic gonadotropin
EGA	embryonic genome activation
EGF	epidermal growth factor
ES	embryonic stem
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FSH	follicle-stimulating hormone
GFAP	glial fibrillary acidic protein
GJ	gap junction
GSH	glutathione
GV	germinal vesicle
hCG	human chorionic gonadotropin
HEPES	hydroxyethyl-piperazineethane-sulfonic acid buffer
Hoechst 33342	bisBenzimide H33342 trihydrochloride
Immunoglobulin	*IgH*

heavy chain

IVC	in vitro culture
IVEP	in vitro embryo production
IVF	in vitro fertilization
IVM	in vitro maturation
IVP	in vitro production
LH	luteinizing hormone
MET	maternal to embryonic transition
MII	metaphase II
MPN	male pronucleus
mRNA	messenger RNA
NCSU	North Carolina State University
POM	porcine Oocyte medium
PPARG	peroxisome proliferator-activated
PSCs	pluripotent stem cells
PZM	Porcine zygotic medium
RNA	ribonucleic acid
SCNT	somatic cell nuclear transfer
ZGA	zygotic genome activation
ZP	zona pellucida

SUMMARY

In vitro embryo production (IVEP) of porcine embryos is an important tool not only for porcine gene banking but also for human biomedical research. However, in pigs, *in vitro* fertilization (IVF) is characteristic with a high frequency of polyspermy, causing chromosomal abnormalities in embryos. Although various approaches have been tried for reduction of polyspermy in pigs, it still remains as major obstacle. Therefore, a reliable selection system for high quality embryos under polyspermy condition is essential for reproduction studies in pigs of which high polyspermy is often unavoidable.

Study on gene expression especially pluripotency-associated genes and their repositioning during embryonic genome activation (EGA) is important for embryonic development and stem cell research. However, the information about allelic expression patterns, location and repositioning of pluripotency-associated genes in mammalian embryos other than mice is little known. Although DNA/RNA fluorescence in situ hybridization (FISH) have been established in mouse embryos, they have not been set up in porcine embryos yet. Besides, the efficiency of FISH assays can be affected by frequency of abnormalities in embryos.

Therefore, the first study (Chapter 2) was aimed to examine the efficiency of embryo selection based on morphological features and timing of early cleavage in order to select good quality embryos under polyspermy condition. The embryos were produced by IVF under moderate and high polyspermy conditions. The 4-cell embryos were selected at 48 hr after IVF (single selection) and 8-cell embryos were selected at 79 hr after IVF from the collected 4-cell embryos (double selection). Both of single and double

selection embryos showed high developmental competence to blastocyst under both moderate and high polyspermy conditions. However, blastocysts derived under high polyspermy condition had significantly fewer cells than those produced under moderate polyspermy condition. Moreover, the frequency of nuclear and chromosomal abnormalities in 4- and 8-cell embryos produced under high polyspermy condition were significantly higher in comparison to those under moderate polyspermy condition. These findings suggest that although high polyspermy affects the frequency of anomalies in nucleus and number of chromosome in porcine embryos produced by IVF, subsequent selection based on morphological features of 4- and 8-cell embryos even under high polyspermy condition, could be an alternative option for selecting porcine embryos with high developmental ability. Furthermore, the 4- and 8-cell embryos produced under moderate polyspermy condition showed low rate of abnormalities. It would be associated improving the efficiency of DNA/RNA FISH assays which were utilized to examine allelic gene expression.

The second study (Chapter 3) was designed to evaluate the allelic expression and positioning of two pluripotency-associated genes, *OCT4* and *SOX2*, and two housekeeping genes, *ACTB* and *TUBA*, in 4- and 8-cell porcine embryos which coincide with embryonic genome activation (EGA), utilizing RNA and DNA FISH assays. The expression of *SOX2* in bi-allelism increased from 45% at the 4-cell stage to 60% at the 8-cell stage. Moreover, *SOX2* was expressed bi-allelically in 8-cell embryos in significantly more blastomeres than those of *OCT4*. Also, this was associated with a tendency that *SOX2* alleles move toward the nuclear interior during 4- to 8-cell transition. However, *OCT4* alleles did not change significantly radial location during this transition. The locations of active and inactive alleles also were measured based on DNA/RNA FISH

assays. Active *OCT4* alleles were more centrally disposed in the nucleus meanwhile inactive *OCT4* alleles located in very close to the nuclear membrane. Nevertheless, active and inactive *SOX2* alleles did not change location in the nucleus in either 4- or 8-cell blastomeres. The present results provide novel information on the allelic expression patterns and positioning of pluripotency-associated genes, *OCT4* and *SOX2*, during EGA in pigs.

In conclusion, the first study demonstrated that although polyspermy affects the frequency of abnormalities in embryos, subsequent selection of 4- and 8-cell embryos based on morphological features and timing of early cleavage would be effective. This knowledge will contribute as an alternative option for selection of high developmental competence embryos produced by IVF even under high polyspermy condition. The second study revealed that the repositioning of *SOX2* alleles coincided with an increase in the percentage of blastomeres with bi-allelic expression during these stages of EGA, and the expression of *OCT4* correlated with its nuclear location. To my knowledge, it is the first study on allelic expression in mammalian embryos other than mice. The information should be useful for improvement of embryonic development as well as stem cell research.

Key words: abnormalities, allelic expression, chromosome, gene positioning, polyspermy, porcine embryos, pluripotency-associated genes.

CHAPTER 1 GENERAL INTRODUCTION

1.1 IVEP IN PIGS

IVEP is the technology of production of embryos from gametes under laboratory conditions. In pigs, IVEP is an important technology for the utilization of frozen sperm kept in gene banks (Kikuchi et al., 2016). Also, IVEP is a basic technology necessary for the production of genetically modified pigs, which have great importance for human biomedical research (Prather et al., 2003). The first successful piglet production by the transfer of in vitro produced blastocyst-stage embryos was reported in 2001 by Marchal et al. The most common source of oocytes for IVEP is ovaries of slaughtered pigs from commercial slaughterhouses; however, harvesting immature oocytes from live pigs by ovum pick up is also possible (Yoshioka et al., 2020). Although fresh sperm can be utilized to generate embryos, it is more common to use frozen sperm, both from ejaculation and epididymis. IVEP consists of three steps; 1) in vitro maturation (IVM) of immature oocytes; 2) in vitro fertilization (IVF) of matured oocytes and; 3) the subsequent in vitro culture (IVC) of fertilized oocytes to embryos, usually to the blastocyst stage. Aspects of IVM, IVF and IVC will be discussed in this chapter.

1.1.1 IVM in pigs

The basis of IVM is the phenomenon of "spontaneous maturation" which was first described by Pincus and Enzmann (1935). In antral follicles, mammalian oocytes stay arrested at the first meiotic prophase (also known as germinal vesicle (GV) stage) until the gonadotropins resume meiosis (Motlik and Fulka, 1976). However, Pincus and

Enzmann observed that once removed from the follicles (and hence from the meiosis-suppressing factors from the ovary) and cultured in isotonic solutions, rabbit oocytes resumed meiosis spontaneously. Based on this phenomenon, mammalian oocytes have been successfully matured to the Metaphase-II (MII) stage and embryos/offspring have been produced from such oocytes in rabbit (Chang, 1959), mice (Mukherjee and Cohen, 1970), sheep (Cheng et al., 1986), pig (Mattioli, 1989), and cattle (Bracket et al., 1982; Fukuda et al., 1990).

During IVM, the oocytes must acquire the ability to be fertilized and to develop subsequently to healthy embryos and offspring which requires both nuclear and cytoplasmic maturation. Nuclear maturation is a term that refers to the resumption of meiosis and the progression of the oocyte to the MII stage at which it remains temporarily arrested until fertilization. The failure of oocyte nuclear maturation normally leads to abnormal development after fertilization which results in the formation of embryos with abnormal chromosome numbers. Such abnormal embryos are very unlikely to develop to offspring (Kikuchi et al., 2009). In another word, the success of nuclear maturation has key importance for the normality of chromosome numbers in the developing embryo. Nuclear maturation of oocyte can be evaluated by morphology based on the presence of the first polar body or by nuclear staining such as with orcein or DAPI. (reviewed by Dang-Nguyen et al., 2011).

Cytoplasmic maturation is a broad term that refers to all cytoplasmic events occurring during maturation that prepare the oocyte for fertilization and preimplantation development. It includes the accumulation of cytoplasmic glutathione (GSH) (Yoshida et al., 1993a,b), the redistribution of organelles (Ferreira et al., 2009), and after reaching the MII stage, the adjustment of levels of cytoplasmic protein kinases to a level that will allow

oocyte activation by the fertilizing sperm (Kikuchi et al., 1995). GSH compounds major non-protein sulfhydryl which exists in mammalian cells (Abeydeera et al., 1998a) and it is very important for cytoplasmic maturation (Eppig, 1996). GSH protects cells against the effects of reactive oxygen species (Meister, 1983) as well as supports the male pronucleus (MPN) formation after fertilization (Yoshida, 1993a, b). Also, GSH extends the transportation of amino acids, and stimulates DNA and protein synthesis (Grupen et al., 1995). Therefore, the cytoplasmic maturation can be improved by increasing the concentration of GSH in the oocyte such as supplementation composition of maturation medium with cystein (Yoshida et al., 1993a), cysteamine (Yamauchi and Nagai, 1999), β-mercaptoethanol, glutamine (Abeydeera et al., 1998a, Jeong and Yang, 2001) and epidermal growth factor (EGF) (Abeydeera et al., 2000). The cytoplasmic maturation could not be analyzed directly, but indirectly by examination of GSH content, the formation of MPN after fertilization or the competence to develop to blastocyst and cell number in blastocysts (reviewed by Dang-Nguyen et al., 2011).

The cumulus cells surround and connect with the mammalian oocyte via gap junctions (GJ) in mammals (Anderson and Albertini, 1976). During IVM, cumulus cells are essential since they play important roles in the initiation of nuclear maturation and the process of cytoplasmic maturation of the oocyte in cattle and pigs (Yamauchi and Nagai, 1999; reviewed by Nagai, 2001; Tanghe et al., 2002) and also for the process of fertilization (Kikuchi et al., 1993). Cumulus cells synthesize and transport GSH into the oocytes through GJ (Maedomari et al., 2007).

The success of IVM to provide oocytes with the competence to embryonic development depends on many factors such as 1) the initial capacity of oocytes to mature and develop at the time of oocyte collection (i.e. “oocyte quality”) (Mermillod et al.,

2008) and 2) the culture conditions during IVM (Nagai, 2001). In slaughterhouse-derived ovaries from commercial pig breeds, fully grown oocytes obtained from 3-6 mm follicles with at least 2 layers of cumulus cells are known to have acquired the ability to mature and develop (Marchal et al., 2002). The morphology and behavior of cumulus cells during IVM affects nuclear and cytoplasmic of the oocytes and thus affect IVF results (Somfai et al., 2004). Besides, age of pigs also has effects on blastocyst development since young gilts oocytes have lower competence than mature sows (Bagg et al., 2006).

Among the culture conditions, the medium has a great importance for the success of maturation. Immature porcine oocytes were traditionally cultured in media such as Whitten's, Waymouth MB 752/1, Tissue Culture Medium 199, North Carolina State University (NCSU)-23 or NCSU-37, usually enriched with a non-defined protein supplementation such as fetal calf serum (FCS) or follicle fluid (FF) and other supplements, such as gonadotropins and growth factors (reviewed by Abeydeera, 2002; Grupen, 2014). Later, a chemically defined medium called Porcine Oocyte Medium (POM) was developed (Yoshioka et al., 2008). Since a chemically defined medium eliminates undefined factors present in biological materials, its application for IVM of oocytes (and later embryo culture) has various great advantages, especially for studying the effects of chemicals on embryonic development (reviewed by Dang-Nguyen et al., 2011).

Porcine IVM media are generally supplemented with gonadotropins, follicle-stimulating hormone (FSH), luteinizing hormone (LH); or its analogues such as equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). The gonadotropins improve nuclear and cytoplasmic maturation of porcine oocytes (Mattioli et al., 1991; Funahashi et al., 1994). Evidence shows that gonadotrophins contribute to

the synthesis of GSH in cumulus cells, which is transferred to the oocytes via GJs (Ozawa et al., 2010). The supplementation of gonadotropins together with EGF improve MPN formation (Ding et al., 1994), indicating the beneficial effects of EGF on cytoplasmic maturation (Abeydeera, 2002). The treatment improves the subsequent synchrony of oocyte nuclear and cytoplasmic maturation and increases the subsequent blastocyst development rates (Funahashi et al., 1997). Also, elevating cAMP levels during the first half of IVM have other various positive effects on the oocyte developmental ability (reviewed by Appeltant et al., 2016). IVM of porcine oocytes is normally performed under 38.5-39ºC in the humified atmosphere of 5% CO_2 (Kikuchi et al., 2002a).

1.1.2 IVF in pigs

In IVEP systems, IVF is achieved by the co-incubation of matured cumulus-enclosed oocytes with spermatozoa. Boar spermatozoa can be frozen-thawed ejaculated (Wang et al., 1991) or epididymal (Kikuchi et al., 1998; Kikuchi et al., 1999b). Fertilization medium of in vitro systems is designed to mimic the in vivo systems. Several different media have been used for IVF in pigs such as TCM199 (Nagai and Moor, 1990; Yoshida et al., 1990; Funahashi et al., 2000), Brackett and Oliphant (BO) medium (Kikuchi et al., 1993; Wang et al., 1995), modified tris-buffered medium (Abeydeera and Day, 1997) and Pig fertilization medium (PigFM) (Suzuki et al., 2002). It was found that mammalian spermatozoa need extracellular calcium for capacitation and maximal acrosomal exocytosis (Fraser, 1995). Bovine serum albumin (BSA) and caffeine are also crucial modulators of sperm penetration. The addition of BSA into IVF medium increased the number of sperm penetrating the oocyte (Abeydeera and Day, 1997) whereas caffeine

promoted the capacitation of boar spermatozoa and fertilization (Wang et al., 1991; Nagai et al., 1993).

The efficiency of IVF is affected by sperm concentration and interval of sperm and oocyte co-incubation (Nagai, 1996). In traditional porcine IVF systems, it was found that sperm penetration takes places around 3 hr after insemination and is completed by 6 hr. Therefore, numerous laboratories incubate gametes for about 6 hr (reviewed by Dang-Nguyen et al., 2011). However, the incidence of polyspermy increases as the co-culture duration is extended (Funahashi et al., 2000). The incubation with sperm for 6 hr doubles the average number of sperm per oocyte in comparison with 3 hr thus increased polyspermy whereas did not improve the MPN formation (Kikuchi et al., 2006). The optimum sperm concentration for frozen-thawed epididymal sperm, was 1 x 10^5 sperm/ml, and co-incubation with oocytes for 3 hr gave the best results (Kikuchi et al., 2002a). Furthermore, when shorter co-culture periods were applied, the penetration and polyspermy rates were not affected (Gil et al., 2004). Since then 3 hr gametes incubation has been suggested.

However, the effectiveness of a brief gamete co-incubation in decreasing polyspermy also dependent on the sperm:oocyte ratio used (Gil et al., 2007), which must be optimized based on boar and/or storage on sperm fertilizing capacity (Gil et al., 2008; Alminana et al., 2005). The optimal duration and sperm concentration for successful penetration with low incidences of polyspermy may differ among samples from different sources (ejaculate or epididymal), preservation (fresh, stored or frozen/thawed), boar or even the lot (i.e. ejaculate) even when taken from the same boar. Therefore, it is recommended to set up the optimal concentration and duration of gametes incubation specifically for each frozen lot (reviewed by Dang-Nguyen et al., 2011).

Polyspermy occurs as a result of simultaneous penetration of an oocyte by two or more spermatozoa (Kikuchi et al., 2009). It is one of the major obstacles of porcine IVF systems (reviewed by Nagai et al., 2006; Dang-Nguyen et al., 2011; Grupen, 2014), which significantly interfere embryonic development (reviewed by Kikuchi et al., 2009). Therefore, multiple of studies have been carried out to improve IVF conditions in order to reduce polyspermy in pigs (reviewed by Nagai et al., 2006; Dang-Nguyen et al., 2011; Grupen, 2014). The degree of polyspermy was closely linked with the number of sperm per oocyte at fertilization (Rath, 1992). However, simply reducing the sperm concentration within a fertilization droplet containing oocytes also results in a decrease in the overall penetration rates. Also, various methods have been examined to restrict the number of sperm reaching the oocytes during fertilization such as the climbing-over-a-wall method (Funahashi and Nagai, 2000); straw IVF (Li et al., 2003); biomimetic microchannel IVF system (Clark et al., 2005); modified swim up method (Park et al., 2009) or microfluidic sperm sorter (Sano et al., 2010) to be ensured that only highly motile sperm are able to make contact with the oocytes. Each of systems attempts to mimic the in vivo selection of the best sperm by forcing sperm to overcome some sort of ratification obstacle. Although this approach can reduce the incidence of polyspermy, it does not eliminate the problem completely.

Another method to reduce polyspermy in pigs is ‘zona hardening’ in which the ZP is hardened by pre-treating oocytes with an amine-reactive cross-linker (Coy et al., 2008). By this method, the oocytes were pre-treated with the cross-linker. As the results, the monospermy rate increased and the number of penetrated sperm per oocyte reduced significantly, and the IVF efficiency improved 45% (Coy et al., 2008). Besides, the modified IVF method established by Grupen and Nottle (2000) also supports reducing

polyspermy in pigs. In this method, the oocytes are briefly co-incubated with sperm before transfer to fresh insemination droplets. It was based on the report that the sperm which binds to the zona pellucida (ZP) within the first 10 minutes of insemination effectively penetrate a high proportion of oocytes (Alminana et al., 2008; Gil et al., 2004; Grupen and Nottle, 2000). Removing the oocytes and bound sperm from the excess sperm also increased the sperm penetration rate and the subsequent embryonic development (Grupen and Nottle, 2000). Similarly, a brief gamete co-incubation in the presence of caffeine, followed by insemination culture in the absence of caffeine, reduced the rate of polyspermic penetration (Funahashi and Romar, 2004).

Another question is the necessity and essential of cumulus cells for the successful of IVF in pigs. In cattle IVF system, cumulus cells play an important role during fertilization since removal cumulus cells from oocytes reduced efficiency of IVF systems (Cox et al., 1993). The role of cumulus cells on IVF of in vitro-matured oocytes was contradicting. Early research has reported the importance of follicle cells on MPN formation and efficiency of IVF in vitro-matured porcine oocyte (Kikuchi et al., 1993). However, later research showed that removal of cumulus cells before IVF did not reduce the penetration rate when using certain lots of frozen sperm (reviewed by Nagai et al., 2006; Dang-Nguyen et al., 2011; Grupen, 2014).

An issue that confounds the problem of polyspermic fertilization in porcine embryos is that polyspermic embryos are still able to develop to the blastocyst stage (Han et al., 1999a, Somfai et al., 2008). However, only mixoploid embryos could develop to term and most of them fail to develop to offspring (Han et al., 1999b). Therefore, it is important to select normal and good quality embryos for the successful production of piglets.

1.1.3 IVC in pigs

Early attempts to culture porcine embryos in vitro throughout the pre-implantation stages were unsuccessful. In vivo embryos could develop to the blastocyst stage in various media only when cultured from the four-cell stage, but the development was arrested the four-cell stage when cultured from the one-cell stage (Davis, 1985). To solve this problem, porcine embryos after IVF were transferred into the oviducts of recipient pigs (reviewed by Grupen, 2014). Early stage porcine embryos transferred to the ligated oviducts of other species could develop to the morula, blastocyst stages, and give rise to offspring after subsequent transfer to recipient pigs (Prather et al., 1991, Kikuchi et al., 1999a). Therefore, embryo culture media had to be modified to more closely mimic the composition of oviductal fluid. The approaches such as supplementing medium with oviductal fluid, co-culturing with oviductal epithelial cells improve in vitro porcine embryonic development (reviewed by Petters and Wells, 1993).

Until now many culture systems have been developed for in vitro produced porcine embryos (reviewed by Grupen et al., 2014). In early studies, embryos were cultured in media such as Whitten's medium (Menino and Wright, 1982), modified Kreb's Ringer bicarbonate medium (Krisher et al., 1989), NCSU-23 medium (Petters and Wells, 1993) modified NCSU-23 (Abeydeera and Day, 1997) or NCSU-37 (Kikuchi et al., 2002a) media. Differences in the presence and abundance of glucose, pyruvate, and lactate were considered to be the primary cause for the observed variance in results between media. It was suggested that porcine embryos did not require pyruvate or lactate, because NCSU-23 medium only contained high levels of glucose, and that lactate

inhibited the development of porcine embryos in the presence of glucose (reviewed by Grupen, 2014). However, the concentration of glucose in porcine oviductal fluid was found to decrease markedly from the pre- to post-ovulatory period via an unidentified systemic mechanism (Nichol et al., 1998; Nichol et al., 1992). Furthermore, analysis of IVEP porcine embryo metabolism revealed that glucose utilization increases from the one-cell to the blastocyst stage (Gandhi et al., 2001), as it occurs in the embryos of other species (reviewed by Gardner, 1998). Therefore, changing the culture medium composition after 2 days to simulate the changing in vivo conditions seems a valid rationale (reviewed by Grupen, 2014). Kikuchi et al. (2002) found that culture of porcine embryos in NCSU-37 medium lacking glucose and containing low concentrations of pyruvate (0.17mm/L) and lactate (2.73 mm/L) for the first 48 hours, followed by NCSU-37 with glucose (5.55 mm/L), improved blastocyst development. This is because the by-product of glucose metabolism is hydrogen peroxide (H_2O_2) which is very toxic at high levels to pig embryos (Karja et al., 2006). However, embryos at later stages, from Day 2 (the day of IVF is Day 0) need glucose. Therefore, embryo culture is normally performed in glucose-free medium for the first 2 days after IVF, then in glucose-containing medium on Day 2.

However, a more recently described single-step culture medium, porcine zygote medium (PZM) was also effective (Yoshioka et al., 2002). Its composition is based on the concentration of inorganic elements and energy substrates in porcine oviducts (Nichol et al., 1992; Iritani et al., 1974). Wang et al. (2009) found that PZM-3 medium supported porcine early embryonic development more efficiently than NCSU-23 medium. Also, the later developed PZM-4 and PZM-5 media supported the in vitro development of in vivo-derived zygotes and IVP embryos for 5 days and the embryos could develop to term

(Yoshioka et al., 2002; Yoshioka et al., 2012; Yoshioka et al., 2003). Besides, the supplementation of glutamine and hypotaurine to PZM media improved the blastocyst formation (Suzuki and Yoshioka, 2006).

Despite these advances, porcine embryo culture media will continue to be considered suboptimal while the blastocyst development of porcine zygotes cultured in vitro remains poorer than that obtained in vivo (Kikuchi, 2004). The development of fully defined cultured media has been essential to future progress in this area.

1.2 SELECTION OF GOOD QUALITY EMBRYOS BASED ON MORPHOLOGY AT EARLY STAGE IN IVEP SYSTEMS

The quality of embryo affects the embryonic developmental ability in human and mammals. The good embryos had high competence to develop to blastocyst and to induce pregnancy (Hardarson et al., 2001; Magli et al., 2007; Dang-Nguyen et al., 2010; Sugimura et al., 2012; Ochota and Nizanski, 2016). It was found that timing of developmental stages and morphology of early embryos were linked with the quality of embryos in human and other mammalian species (Edirisinghe et al., 1992; McKiernan and Bavister, 1994; Lonergan et al., 1999; Alikani et al., 2000; Hardarson et al., 2001; Magli et al., 2007; Ulloa et al., 2008a,b; Dang-Nguyen et al., 2010; Sugimura et al., 2012; Ochota and Nizanski, 2016). The time for selection of good quality embryos is different between species and normally early cleaved embryos are good (Lundin et al., 2001; Dang-Nguyen et al., 2010). For example, bovine embryos were recommended to be selected on Day 2 (Day 0 = IVF), at the 5- to 8-cell stage (Ulloa Ulloa et al., 2008b). Meanwhile, porcine embryos were good at the 3- to 4-cell stage and 5- to 8-cell stage selected 52 h

after IVF (Ulloa Ulloa et al., 2008a), or 2-cell stage embryos selected 30 h after IVF (Dang-Nguyen et al., 2010). Those embryos showed lower incidences of chromosomal abnormalities and high developmental competence (Dang-Nguyen et al., 2010).

Beside timing of cleavage and morphology of embryos, evenness of division and degree of fragmentation could also be useful criteria to predict blastocyst formation ability of early embryos in pigs (Mateusen et al., 2005; Booth et al., 2007; Dang-Nguyen et al., 2010). Blastocyst from evenly cleaved embryos had higher cell number in blastocyst than those from unevenly cleaved embryos (Dang-Nguyen et al., 2010). Similarly, in human, the evenly cleaved embryos showed higher implantation and pregnancy rate compare with unevenly cleaved embryos (Hardarson et al., 2001). It can partly be explained by a higher rate of chromosomal abnormalities in unevenly cleaved embryos (Hardarson et al., 2001). However, it has not been examined if such morphological evaluation also effective for selecting high quality embryos under high polyspermy condition.

For this reason, one of the objectives of my study was to find out, whether such embryonic morphology selection method at specific time points, a simple and non-invasive method, is still effective under high polyspermy condition (Chapter 2). A reliable selection system for high quality embryos under polyspermy condition is essential for reproduction studies in pigs since dealing with high polyspermy rate is often unavoidable.

1.3 GENE EXPRESSION IN MAMMALIAN EMBRYOS

1.3.1 Genome activation

An oocyte and sperm fuse during fertilization to form a zygote. Embryonic development starts after syngamy, when the genome of embryo is formed. Interestingly,

in all animal embryos, early stages of preimplantation development occur in the absence of transcription (Bogliotti and Ross, 2015). During this period, development relies on maternal proteins and mRNAs stored in cytoplasm of oocyte during oogenesis (Tadros and Lipshitz, 2009). The transition from maternal to embryonic control of development happens in successive waves of increasing intensity until the major activation of the embryonic genome occurs. This transition has been known with different names through the literature such as maternal to embryonic transition (MET), zygotic genome activation (ZGA), or embryonic genome activation (EGA). It was found that EGA starts at different time point depending on species such as between the 1- and 2-cell stage in mice (Flach et al., 1982; Schultz, 1993); 4-cell stage in pigs (Jarrell et al., 1991); by 4- to 8-cell stages in humans (Braude et al., 1998); by 8- to 16-cell stage in bovine (Memili and First, 2000). Despite the differences in timing of EGA across species, its major features, such as the degradation of maternal mRNA and proteins and the massive transcriptional activation of the embryonic genome are conserved in all metazoan (Schultz, 2002; Schier, 2007; Walser and Lipshitz, 2011).

In cattle, the largest proportion of up-regulated genes was found at the 8-cell stage, coinciding with EGA. Among the first embryonic genes to be expressed was heterogeneous nuclear ribonucleoprotein A2/B1 (*HNRNPA2B1)*, which is known to interact with *SOX2*, a key transcription factor for embryonic stem cell pluripotency (Masui et al., 2007), as well as *KLF17*, which can activate/suppress transcription (van Vliet et al., 2006), and the Nanog homeobox (*NANOG*) coinciding with previous reports (Khan et al., 2012). In pigs, EGA occurred at 8-cell-stage in somatic cell nuclear transfer (SCNT) embryos, one cycle delayed compared to in vivo fertilized embryos (Cao et al., 2014). Hierarchical clustering of DNA binding protein transcripts, characteristic of EGA,

appeared at the 4-cell stage for in vivo embryos, and at the 8-cell stage for SCNT derived embryos (Cao et al., 2014). Maternal genes are largely conserved among the divergent species, while the earliest embryonic genes are not, suggesting species-specific functions of embryonic genes during EGA and central conserved maternal program across species (Heyn et al., 2014).

EGA is a key event during preimplantation development, as demonstrated by the development block when transcription is inhibited from the embryonic genome (Bogliotti and Ross, 2015); however, the specific mechanisms involved in this process are still poor understood. A better understanding about the activities of genes during EGA may lead to a clear conception of the achievement of totipotency and later differentiation. The next parts will be discussed about housekeeping genes, pluripotency-associated genes and their expression in mammalian embryos and stem cells.

1.3.2 Housekeeping genes

In molecular biology, housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function. They are considered to produce the essential transcripts necessary for normal cellular physiology (Butte et al., 2001). Among housekeeping genes, beta (β)-actin (*ACTB*) and α-tubulin (*TUBA*) are good candidates for control genes in gene expression research.

ACTB gene provides instructions for making a protein beta (β)-actin which is a part of the actin protein family. These proteins are organized into a network of actin cytoskeleton which makes up the structural framework inside cells. (β)-actin play important roles in determining cell shape and controlling cell movement. It was suggested that (β)-actin may also be involved in relaying chemical signals within cells (according

to United State, National library of medicine).

Whereas, *TUBA* gene provides instruction for making alpha-tubulin (α-tubulin) protein. This protein is part of tubulin family that form and organize structures named microtubules. Microtubules are rigid, hollow fibers that make up the cell's structural framework (the cytoskeletal). Microtubules are necessary for cell division and movement (according to United State, National library of medicine).

1.3.3 Pluripotency-associated genes and their expression in mammalian embryos and embryonic stem (ES) cells

The pluripotency-associated genes have important roles in maintenance of pluripotency in early embryos and ES cells. In human and mouse, Octamer-binding transcription (*OCT4*), Sex-determining region Y-box 2 (*SOX2*) and *NANOG* were considered as core transcription factors in maintenance ES cells state. ES cells are isolated from the ICM of blastocyst embryos. They have capacity for self-renewal and pluripotency. They can remain undifferentiated in long term culture and can differentiated into cells of all three germ layers (Ivan et al., 2010). Therefore, ES cells are used extensively not only in biomedical research but also as a model to study early mammalian development (Hou et al., 2016).

Likewise, early mammalian embryogenesis is controlled by mechanisms governing the balance between pluripotency and differentiation. The dynamic expressions of core pluripotency-associated genes such as *Oct4*, *Nanog*, and *Sox2* were considered to be associated with large-scale changes of embryonic genome activation and essential to maintain totipotency of the ICM (Boyer et al., 2005).

Sox2, a member of the *SoxB1* transcription factor family, is essential in the formation of pluripotent cells in early embryos. It is also a critical factor for embryonic development, differentiation of pluripotent stem cells (PSCs) as well as in somatic cell reprogramming (Keramari et al., 2010; Pan and Schultz, 2011; Zhang and Cui, 2014; Liu et al., 2015). *Sox2* is together with octamer-binding transcription factor 4 (*Oct4*) and *Nanog* are the core factors for regulating pluripotency, control gene expression PSCs (Boyer et al., 2005; Chen et al., 2008) as well as maintain self-renewal in human and mouse ES cells (Wang et al., 2012).

OCT4 expressed specifically in the ICM of blastocyst and ES cells of mice and humans (Kirchhof et al., 2000). Moreover, *Oct4, Sox2*, Kruppel-like factor 4 (*Klf4*) and *c-Myc* are common factors used to generate induced pluripotent stem cells (iPSCs) from somatic cell (Takahashi et al., 2007). Besides, Oct4 and Sox2 protein maintains ES cells identity and arranges germ layer fate selection in mouse (Thomson et al., 2011). In addition, *Oct4* and *Sox2* co-occupy a large number of enhancers/promoters and regulate the expression levels of their target genes (reviewed by Zhang and Cui, 2014). However, the expression pattern and regulation mechanism of *OCT4* in porcine embryos are quite different from those in mice (reviewed by Han et al. 2019).

The ES cells have been established successfully in mouse (Evans and Kaufman 1981), human (Thomson et al., 1998) and rat (Li et al., 2008). However, in pigs, although porcine embryonic stem-like cells have established from 1990, until now, they are still not satisfied for the real ES cells because germline transmission is not successful yet (Hou et al. 2016). Therefore, more information about gene expression of such key transcription factors *OCT4, SOX2* and *NANOG* would be helpful for establishment of ES cells in pigs in the future.

1.3.4 Allelic gene expression

Allelic gene expression is considered as one of approaches to study gene expression. It was revealed that during differentiation of mouse ES cells to neural progenitor cells, mono-allelic expression increased 5.6-fold, from 67 genes expressed in mono-allelic in mouse ES cells to 376 genes expressed in mono-allelic in neural progenitor cell (Eckersley-Maslin et al., 2014). Also, *Nanog* can control the ground-state pluripotency by allelic regulation (Miyanari and Torres-Padilla, 2012). It was revealed that *Nanog* expressed in mono-allelic in 4- and 8-cell stage embryos in mice. *Nanog* then undergoes a progressive switch to bi-allelic expression during the transition towards ground-state pluripotency in the naive epiblast of the late blastocyst. Similarly, *Nanog* expressed in mono-allelic in undifferentiated ES cells and switches to bi-allelic expression when ES cells become differentiated. Besides, *Nanog*-heterozygous blastocysts have fewer ICM and delayed primitive endoderm formation, indicating a role for the bi-allelic expression of *Nanog* in the timely maturation of the ICM into a fully reprogrammed pluripotent epiblast. Therefore, allelic regulation of *Nanog* can control the ground state pluripotency in both in vivo and in vitro in mice (Miyanari and Torres-Padilla, 2012). However, allelic expression of pluripotency-associated genes in human and porcine embryos are not known!

Allelic expression of genes has another advantage for the investigation of the position of those genes of interest. Genes expressed in mono-allelism can be used as models to study the relationship between gene activity and its localization in the nucleus (Takizawa et al., 2008). In mouse, the astrocyte marker glial fibrillary acidic protein

(GFAP) is of mono-allelic expression and the functionally distinct alleles occupy differential radial positions within the cell nucleus and differentially associate with intranuclear compartments (Takizawa et al., 2008).

Allelic gene expression also links with its movement during differentiation (Stachecka et al., 2019). In pigs, proper expression of the peroxisome proliferator activated *(PPARG)* gene, which encodes a key transcription factor of adipogenesis, is important for the formation of mature adipocytes. The adipocytes were established via adipose-tissue-derived mesenchymal stem cells (AD-MSC) or bone-marrow-derived mesenchymal stem cells (BM-MSC). *PPARG* moved from nuclear periphery to the interior during differentiation of adipocytes. Using DNA/RNA fluorescent in situ hybridization, it was found that transcription of *PPARG* begins with one allele, but both alleles are active in later stages of differentiation.

Allelic gene expression has been studied in humans and mice, but not yet in other mammalian embryos, especially with pluripotency-associated genes that affect the establishment and maintenance of pluripotency in early embryonic development as well as in stem cells.

1.3.5 Gene positioning and expression

The genome of high eukaryotes is organized nonrandomly in the nucleus of a cell (Misteli, 2007; Schneider and Grosschedl, 2007). The chromatin of eukaryotes contains euchromatin and heterochromatin. Heterochromatin is a tightly packed form of DNA or condensed DNA and associated with the di- and tri-methylation of H3K9 and regarded as inactive. Whereas, euchromatin is an uncoiled packed form of chromatin and are genetically active. The degree of chromatin condensation is thought to be linked to

transcriptional activity (reviewed by Fedorova and Zink., 2008). It has been suggested that nuclear position can affect expression of heterochromatic regions (Jachowicz et al., 2013). Also, the repositioning occurs during physiological processes such as differentiation (reviewed by Tanizawa et al., 2008). In certain genes, position of a gene in the nucleus changes when they become highly expressed. Several genes move from the peripheral area into interior part of the nucleus upon their activation during differentiation such as *IgH* and *c-maf* of B cells and T cells, respectively (Kosak et al., 2002; Hewitt et al., 2004); β-*globin and Mash1* of erythroid cells and neurons, respectively (Ragoczy et al., 2006; William et al., 2006) as well as *NANOG* and *OCT4* in human ES cells compared with their location in differentiated lymphoblastoid cells (Wiblin, 2005).

The change of location toward a more internal area of the nucleus also happens with *GFAP* in murine astrocytes or *HoxB1* and *HoxB9* in mouse embryos upon activation. Similarly, the *PPARG* gene moved from the nuclear periphery to the nuclear center as its transcriptional activity increased during the differentiation from mesenchymal stem cells to matured adipocytes in pigs (Stachecka et al., 2019). Active alleles preferentially occupy the central part of the nucleus, while the inactive alleles are found on the nuclear periphery. Study on genes position and their activities may be useful in gene controlling and stem cell research.

DNA/RNA FISH is a useful tool for studying allelic gene expression and the relationship between their activities and position in the nucleus. Consequently, FISH technique will be discussed in the following part of the chapter.

1.3.6 Fluorescent in situ hybridization (FISH) as a tool to study allelic gene expression

and gene positioning in single cells

FISH is a molecular cytogenetic technique that uses fluorescent probes that bind to only those parts of a nucleic acid sequence with a high degree of sequence complementarity. DNA FISH can detect and localize the presence or absence of specific DNA sequences on chromosomes whereas RNA FISH can be used to detect and localize specific RNA targets such as mRNA. The RNA/DNA FISH method involves a hybridization reaction between a labeled nucleotide probe and complementary target RNA or DNA sequences. These probes are labeled with fluorescent-labeled bases (Jensen, 2014). The binding of complementary probes to the target of interest enables its detection and can be visualized by fluorescent microscopy (Reviewed by Jin and Lloyd, 1997; Jensen, 2014, Huber et al., 2018).

The procedure of RNA FISH requires cells to be fixed rapidly to prevent deterioration of the RNA, which is then hybridized to a hapten-labelled probe. The type of probe will determine whether only nascent transcript is detected (intronic probe) or whether later forms of the mRNA are also visualized (genomic or exonic probes). Such hybridizations require careful controls to exclude background signal, non-specific hybridization of the probe. The probes are then annealed to matching sequences in fixed cells or tissue (Reviewed by Jin and Lloyd, 1997; Jensen, 2014, Huber et al., 2018).

RNA-FISH permits the sensitive detection of specific transcripts within individual cells while preserving the cellular morphology (Brown and Buckle, 2010). Also, RNA FISH technique can enable the maximum use of a tissue that is difficult to obtain such as embryos and clinical biopsies (reviewed by Jensen, 2014). Recently, RNA FISH becomes an extremely powerful tool when used in combination with the detection of specific regions of DNA (RNA-DNA-FISH) which can be used to investigate gene activity and

its position in nuclear organization simultaneously (Takizawa et al., 2008; reviewed by Jensen, 2014). Therefore, DNA and RNA FISH are useful tools to study allelic expression and gene positioning in single cells.

AIMS OF RESEARCH

As mentioned above, IVF in pigs is associated with a high frequency of polyspermy which causes abnormalities in embryos (Abeydeera & Day, 1997; McCauley et al., 2003). Those embryos with abnormalities normally have low developmental competence and fail to develop to terms (Han et al., 1999b). To date, polyspermy still remains as difficult obstacle in pigs (reviewed by Nagai et al., 2006; Grupen, 2014). Therefore, the establishment of reliable selection system for high quality embryos under polyspermy condition is necessary for reproduction studies in pigs.

Pluripotency-associated genes have important roles in maintenance of pluripotency in stem cells and early embryonic development, and mono-allelic expression might be one possible mechanism for this in mouse (Miyanari and Torres-Padilla, 2012). Moreover, mono-allelic gene expression increased 5.6-fold during differentiation from ES cells to neural progenitor cells in mouse. Also, it was reported that some genes can change position when gene activities change especially during embryonic genome activation (reviewed in Takizawa et al., 2008). However, until now, there is no information reported about allelic expression patterns, location and repositioning of pluripotency-associated genes in other mammalian embryos other than mice. In mouse embryo, DNA/RNA FISH have been established successfully. However, they have not set up in porcine embryos yet and the frequency of abnormalities in embryos can affect the

efficiency of FISH assays.

As a consequence, the first study (Chapter 2) was aimed to clarify whether selection of 4- and 8-cell stage embryos as single- and double-selected embryos at specific time points might be effective for selection of good quality embryos, and how degree of polyspermy might affect the developmental competence, nuclear status and karyotype of those selected embryos. The data from this study would lead to an alternative option for selection of good embryos based on their morphological features at fixed time points under high polyspermy conditions. It would be essential for reproduction studies in pigs where a high polyspermy rate is often unavoidable.

The second study (Chapter 3) was designed to examine the allelic expression and positioning of two pluripotency-associated genes, *OCT4* and *SOX2*, and two housekeeping genes, *ACTB* and *TUBA*, in 4- and 8-cell porcine embryos, utilizing RNA and DNA FISH in single blastomeres. The expected results would give better understanding on allelic expression patterns, location and repositioning of such important genes. It would be potentially useful for embryonic development and stem cell research in pigs.

CHAPTER 2 SELECTION BASED ON MORPHOLOGICAL FEATURES OF PORCINE EMBRYOS PRODUCED BY IN VITRO FERTILIZATION: TIMING OF EARLY CLEAVAGES AND THE EFFECT OF POLYSPERMY

2.1 ABSTRACT

The aim of this study was to examine whether a morphological approach is efficient for selecting high-quality porcine embryos produced by IVF under high

polyspermy condition. Frozen-thawed Meishan epididymal spermatozoa showing moderate and high polyspermy were subjected to IVF (1×10^5 sperms/ml). Under condition of moderate polyspermy, 4-cell embryos were selected at 48 hr after IVF (single selection) and 8-cell embryos were selected at 79 hr after IVF from the collected 4-cell embryos (double selection) showed high developmental competence. Likewise, 4- and 8-cell embryos produced by IVF under high polyspermy condition also showed high competence for development to blastocysts. However, blastocysts derived from high polyspermy condition had significantly fewer cells than those produced under moderate polyspermy condition. Furthermore, the frequency of nuclear and chromosomal abnormalities in 4- and 8-cell embryos produced under condition of high polyspermy was significantly ($p < 0.05$) higher in comparison to moderate polyspermy condition. These findings suggest that although high polyspermy affects the frequency of nuclear and chromosomal anomalies in porcine IVF embryos, subsequent selection based on morphological features of 4- and 8-cell embryos even under high polyspermy condition, could be an alternative option for selecting porcine IVF embryos with high development ability.

2.2 INTRODUCTION

In vitro production of porcine embryos is an important tool for porcine gene banking (Kikuchi et al., 2016) as well as for human biomedical research (Niemann & Rath, 2001). However, IVF in pigs is associated with a high frequency of polyspermy, causing chromosomal abnormalities in embryos (Abeydeera & Day, 1997; Herrick et al., 2003; Koo et al., 2005; Nagai et al., 2006). Polyspermy also occurs during IVF in humans (Rudak et al., 1984; Kola et al., 1987) and cattle (Iwasaki et al., 1989; Iwasaki et al., 1992)

but at lower frequency. Although various approaches have been tried for reduction of polyspermy in pigs, production of normal porcine embryos *in vitro* remains a challenge (Nagai et al., 2006; Grupen, 2014).

Although IVP embryos are able to develop to term after embryo transfer (ET) (Yoshida et al., 1993; Kikuchi et al., 2002), their quality is still lower than those of embryos produced *in vivo* (Han et al., 1999a; Kikuchi et al., 1999b; McCauley et al., 2003). A higher incidence of chromosomal abnormalities has been reported in IVP embryos relative to their *in vivo* counterparts, especially in pigs (van der Hoeven et al., 1985; McCauley et al., 2003; Ulloa Ulloa et al., 2008a). Although some polyspermic zygotes have been reported to develop to piglets (Han et al., 1999b), most embryos with chromosomal abnormalities fail to develop to term (Plachot, 1989; Causio et al., 2002, reviewed in Yoshizawa, 2003). Therefore, it is important to select embryos with a low frequency of chromosomal abnormalities utilizing simple and reliable procedures.

In humans and many other mammalian species it has been shown that the developmental competence of embryos is linked with the timing of early cleavages after IVF (Edirisingle et al., 1992; McKiernan & Bavister, 1994; Lonergan et al., 1999; Alikani et al., 2000; Magli et al., 2007; Ulloa Ulloa et al., 2008a, 2008b; Dang-Nguyen et al., 2010). Those previous studies showed that good quality embryos could be selected based on their morphological features and the timing of early cleavages. Bovine embryos at the 5- to 8-cell stage selected on Day 2 (Day 0 = IVF) had lower incidences of chromosomal abnormalities (Ulloa Ulloa et al., 2008b). Similarly, porcine embryos at the 3- to 4-cell stage and the 5- to 8-cell stage selected 52 hr after IVF (Ulloa Ulloa et al., 2008a), or 2-cell stage embryos selected 30 hr after IVF (Dang-Nguyen et al., 2010) were proven to have lower incidences of chromosomal abnormalities and high developmental

competence. Other studies have also shown that the timing of cleavage, evenness of division, and the degree of fragmentation can also be useful criteria for predicting the blastocyst formation ability of early porcine embryos (Mateusen et al., 2005; Booth et al., 2007; Dang-Nguyen et al., 2010). However, whether or not such morphological evaluation could also be effective for selecting high-quality embryos under high polyspermy condition has not been investigated.

In a preliminary experiment using the current IVP system in our laboratory, with the support of time lapse cinematography, It was found that single selection of 4-cell embryos and double selection of 8-cell embryos showed a high potential for rapid development to good quality blastocysts. Consequently, the present study was conducted to clarify whether selection of 4- and 8-cell stage embryos as single- and double-selected embryos at specific time points might be effective for ensuring good quality embryos, and the degree to which a high rate of polyspermy might affect the developmental competence, nuclear status and karyotype of the selected embryos. It was considered that the data from this study would lead to an alternative option for selection of embryos at fixed time points based on their morphological features, and furthermore yield insight into whether a selection approach based on embryonic morphology would still be effective under high polyspermy condition, an aspect that is essential for porcine reproduction studies where a high polyspermy is often not able to be avoided.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

All reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO,

USA) unless otherwise stated.

2.3.2 Oocyte collection and IVM

The ovaries were collected from prepubertal cross-bred gilts (Landrace × Large White × Duroc) at a local slaughterhouse, and carried to the laboratory in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 35–37◦C within 1 hr. Cumulus-oocyte complexes (COCs) were collected from follicles 3–6 mm in diameter in collection medium consisting of Medium 199 (with Hanks' salts) supplemented with 10% fetal bovine serum (Gibco; Thermo Scientific, Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), and antibiotics (100 units/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate). IVM of oocytes was carried out as reported previously (Kikuchi et al., 1999b). In brief, about 50 COCs were cultured in each 500 μl of maturation medium, a modified NCSU-37 solution (Petters & Wells, 1993) containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 mM β-mercaptoethanol, 1 mM dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP), 10 IU/ml equine chorionic gonadotropin (Serotropin; ASKA Pharmaceutical Co. Ltd., Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (Gonatropin; ASKA) in four-well dishes (Nunclon Multidishes, Nunc; Thermo Fisher Scientific) for 22 hr in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5◦C. The COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for an additional 24 hr under the same atmosphere.

2.3.3 Preparation of epididymal spermatozoa

Epididymal spermatozoa were collected and frozen according to Kikuchi et al. (1998, 1999a). Briefly, epididymides from four Meishan boars were brought to the laboratory just after the slaughter at Institute of Livestock and Grassland Science, NARO (NILGS) at room temperature. Luminal fluid containing spermatozoa was extruded from the distal portion of the cauda epididymidis by air pressure using a syringe. The fluid was diluted with 30 ml of collection solution at room temperature. The sperm suspension was then cooled to 15ºC over about 3 hr. The solution containing the spermatozoa was centrifuged at 1,200 × *g* in for 10 min at 4ºC and the supernatant was discarded. The precipitated spermatozoa were gently resuspended with Niwa and Sasaki Freezing (NSF)-I extender at 4ºC (Niwa 1989), then diluted with the same volume of NSF-II at the same temperature. The concentrations of sperm were diluted to 1×10^9 (Boar A) or 5×10^8 sperm/ml (Boars B–D) before freezing. Whereas, motility after collection was higher than 80% in all the four examined boars. The sperm suspension was then transferred into 0.25-ml plastic straws (IMV, L'Aigle, France), which were placed in liquid nitrogen vapor for 10 min and finally stored in liquid nitrogen.

2.3.4 IVF and IVC

COCs were treated with 0.1% (w/v) hyaluronidase to remove part of the cumulus using a glass pipet. *In vitro* fertilization was performed according to the 2-step IVF method of Grupen and Nottle (2000) with some modifications. The medium used for IVF was a modified Pig-FM medium (Suzuki et al., 2002) containing 10 mM HEPES, 2 mM

caffeine and 5 mg/ml bovine serum albumin (BSA). The oocytes were washed 3 times in IVF medium. They were then transferred into 90-μl IVF droplets (approximately 20 oocytes in each droplet) covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan). Frozen-thawed epididymal spermatozoa from each of Meishan boars were preincubated at 38.5°C in Medium 199 (with Earle's salts, Gibco, pH adjusted to 7.8) for 15 min (Kikuchi et al., 1998; Ikeda et al., 2002). Matured oocytes were co-incubated with preincubated sperm at 1×10^5 sperms/ml (Kikuchi et al, 2002; Ikeda et al., 2002) for 30 min at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2. The oocytes with the zona-bound sperm were then transferred to other fresh droplets of the IVF medium and subsequently incubated for 2.5 hr. At the end of IVF, spermatozoa were removed from the surface of the zona pellucida by gentle pipetting with a fine glass pipette. The day of IVF was defined as Day 0. The basic IVC medium was NCSU-37 medium containing 4 mg/ml BSA and 50 mM β-mercaptoethanol. The putative zygotes were cultured in 500 μl drops of IVC-PyrLac, basic medium supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate, for Days 0–2 and in IVC-Glu, basic medium with 5.55 mM D-glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for Days 2–6 (Kikuchi et al., 2002) in four-well dishes in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39°C.

2.3.5 Polyspermy evaluation

Putative zygotes were fixed at 10 hr after IVF in a mixture of acetic acid and absolute ethanol (1:3) for at least 3 days, stained with 1% aceto-orcein and examined for sperm penetration and male pronucleus formation (MPN) under a phase-contrast microscope. Zygotes with one female pronucleus and a single sperm head or MPN in the

cytoplasm were considered to be monospermic (Fig 1A). Zygotes with more than one sperm and/or MPNs were considered to be polyspermic (Fig 1B). In the present study, polyspermy rates of approximately 60% or lower and 90% or higher were considered as "moderate" and "high" polyspermy conditions, respectively.

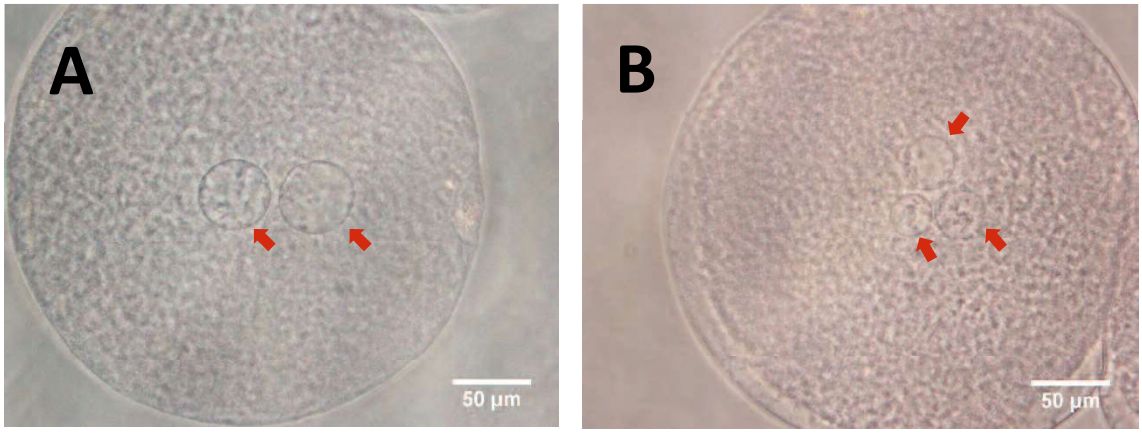

Figure 2.1. Pronucleus(ei) in zygotes at 10 hr after IVF. A monopsermic (A) and a polyspermic (B) zygote. Arrows show pronucleus(ei).

2.3.6 Embryo evaluation

An embryo with more than 10 cells and a clear blastocoel was defined as a blastocyst. The rate of blastocyst formation and the total number of cells per blastocyst were examined on Day 6 (Day 0 = IVF). The blastocysts were fixed and stained in ethanol containing 25 μg/ml Hoechst 33342 (Calbiochem; EMD Biosciences Inc., San Diego, CA, USA) and examined under an epifluorescence microscope (Olympus, Tokyo, Japan). The total number of cells per blastocyst was evaluated as an indicator of embryo quality.

2.3.7 Nuclear status evaluation

The zona pellucida was removed from single- and double-selected embryos using 1% pronase (protease, P8811). They were then fixed in 4% paraformaldehyde (PFA) for at least 3 hr, stained with 4′, 6-diamidino-2-phenylindole (DAPI) in a mounting medium (Vectashield; Vector Laboratories Inc., Burlingame, CA, USA) and visualized using an epifluorescence microscope (Olympus). Embryos in which all blastomeres contained exactly one nucleus were considered normal (Fig 2A, B). Embryos with one or several blastomere(s) carrying no or more than one nucleus(ei) were considered abnormal (Fig 2C–F).

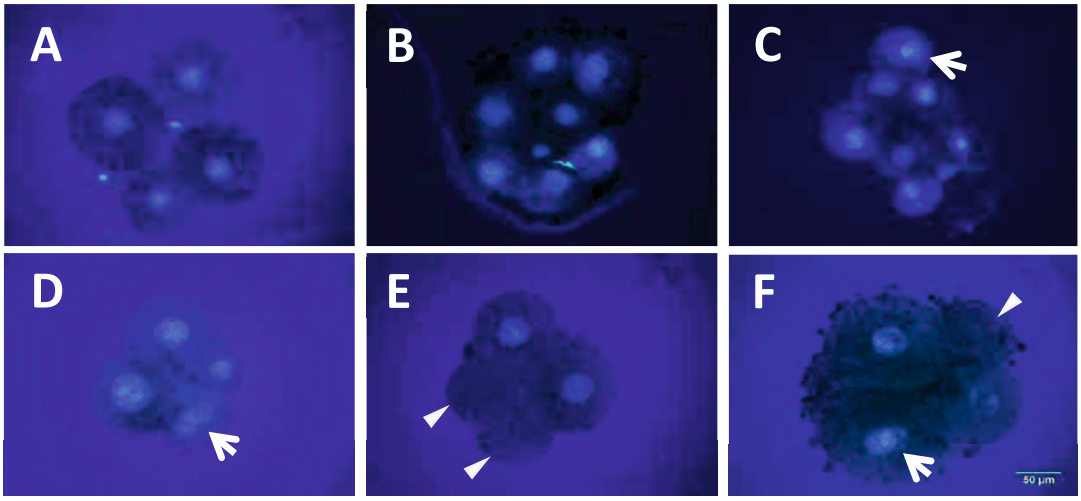

Figure 2.2. Nuclear staining of 4- and 8-cell stage embryos. Normal embryos at the 4-cell stage (A) and 8-cell stage (B). Nuclear abnormalities in 4- and 8-cell stage embryos: an 8-cell embryo carrying a binuclear blastomere (C), a 4-cell embryo with a binuclear blastomere (D), a 4-cell embryo containing two anuclear blastomeres (E) and 4-cell embryos with a binuclear and an anuclear blastomeres (F). Arrows show binuclear blastomeres and arrowheads show anuclear blastomeres.

2.3.8 Chromosome analysis

Chromosome samples from single- and double-selected embryos, respectively, were prepared according Yoshizawa et al. (1998) with some modifications. Briefly, the single- and double-selected embryos were treated with 20 ng/ml colcemid in IVC Glu for 17–20 hr. The embryos were then washed and incubated in 0.4 ml of 1% (w/v) hypotonic sodium citrate solution for 15 min and fixed mildly by adding 0.02 ml of acetic acid: methanol (1:1) to the sodium citrate solution for 2 min. A single embryo was placed on a glass slide with a minimal volume of hypotonic solution, immediately covered with a very small droplet of acetic acid to separate the cells, and then re-fixed with several drops of acetic acid: ethanol (1:3). After being allowed to dry completely, chromosome samples were stained with DAPI in Vectashield and then visualized under an epifluorescence microscope (Olympus). Only embryos containing at least two well-spread metaphases

plates (intact and non-overlapping) were analyzed. Embryos that had two sets of chromosomes (2n = 38) in all analyzable metaphase plates were defined as being diploid (Fig. 3A), whereas those with only one set of chromosomes (n = 19) in all analyzable metaphase plates were defined as being haploid (Fig. 3B). Embryos containing more than two sets of chromosomes in all countable metaphase plates were defined as being polyploid (3n, 4n, etc.) (Fig. 3C, D). Embryos containing a mixture of diploid and haploid cells (n/2n), triploid (2n/3n), tetraploid (n/4n) or other types of polyploid cells were defined as being mixoploid.

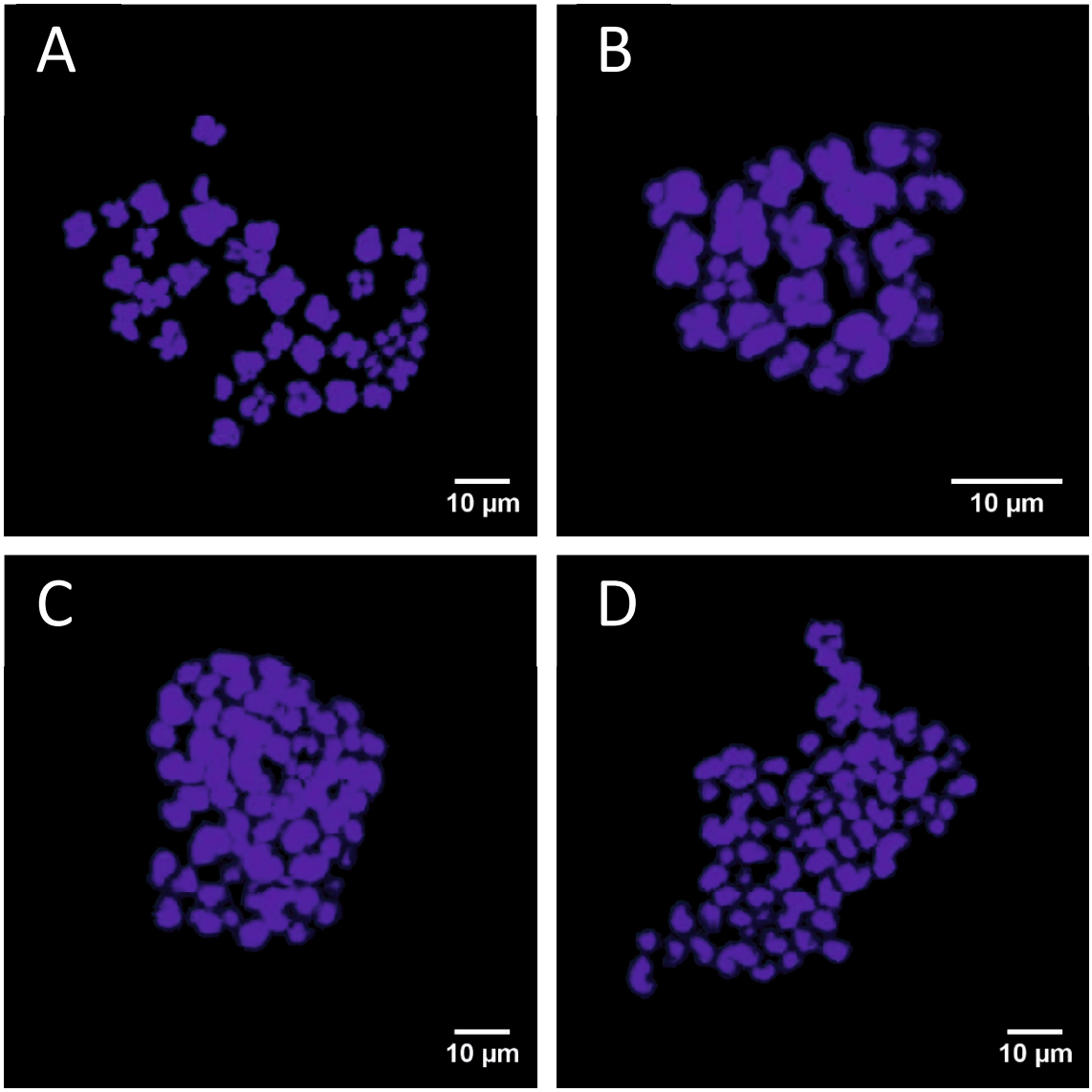

Figure 2.3. Blastomere ploidy in porcine embryos. Metaphase spread from a diploid (2n) cell with two chromosome sets (A). Ploidy abnormalities: haploid (n) (B), triploid (3n) (C) and tetraploid (4n) (D).

2.3.9 Experimental design

2.3.9.1 Experiment 1

I first aimed to establish moderate and high polyspermy conditions. Firstly, four Meishan boars (A-D) have been examined to select "moderate" and "high" polyspermy

rate for the purpose of the present study. In the system of our laboratory, sperm acceptable for reasonable in vitro embryo production show polyspermy rate is 50%-100% and the penetration rate is 80% to 100%. The low polyspermy rate could be obtained when reduce sperm concentration. However, it also means low penetration rate, and therefore resulting in low embryonic development. In the present study, the polyspermy rates at 50-70% and >80% to be consider as "moderate" and "high" polyspermy rate, respectively.

2.3.9.2 Experiment 2

In order to compare the developmental competence of embryos produced under moderate and high polyspermy conditions (evaluated and selected in Experiment 1), in vitro matured oocytes were fertilized with each type of sperm. Blastocyst formation rates and the numbers of cells per blastocyst were recorded after 6 days of IVC. The experiment was replicated six times.

2.3.9.3 Experiment 3

In order to compare the effect of selection on developmental competence, three embryo groups were set. I have separated 4-cell embryos and the other "remaining" cleaved (2-3-cell) embryos at 48 hr after in vitro fertilization under moderate and high polyspermy conditions (as evaluated in Experiment 2). Both groups of embryos were cultured separately. Subsequently, from a group of the 4-cell embryos, 8-cell embryos as "double selected" embryos and the other cleaved (4–7-cell) embryos were re-separated at 79 hr after IVF. Those embryos, as well as "remaining" embryos, were cultured until Day 6 after IVF. In this experiment, data of double-selected and the other cleaved embryos are

pooled and analyzed as "single-selected" embryos. Blastocyst formation rates calculated from the selected embryos and cell numbers per blastocyst in the respective groups (single-selected, double-selected and remaining embryos) were analyzed. Three to six replications with each sperm line were carried out.

2.3.9.4 Experiment 4

In order to compare nuclear abnormalities of single- and double-selected embryos produced under moderate and high polyspermy conditions (evaluated in Experiment 3), in vitro-matured oocytes were fertilized with each type of sperm. The single- and double-selected embryos were subjected to nuclear staining to evaluate nuclear abnormalities as described above. Three to five replications with each sperm line were carried out.

2.3.9.5 Experiment 5

In order to compare chromosomal abnormalities in single- and double-selected embryos produced under moderate and high polyspermy conditions (evaluated in Experiment 3), in vitro-matured oocytes were fertilized with each type of sperm. The single- and double-selected embryos were then used for chromosome spreads as described above. Three to six replications with each sperm line were carried out.

2.3.10 Statistical analysis

All data were expressed as mean ± SEM values. The data were analyzed by one-way ANOVA followed by Bonferroni correction by using the Stata/SE 15.0 software

package (StataCorp., College Station, TX, USA). Differences at $p < 0.05$ were considered statistically significant when it is not identified in the text.

2.4 RESULTS

2.4.1 Experiment 1

All four Meishan boars (A–D) were informed at the slaughter that they were over 1.5 years of age and used for this study. Penetration and polyspermy rates of these boar sperm lines were presented in Table 1. Zygotes derived from oocytes fertilized by Boar B sperm (90.4 ± 1.7%) had a significantly higher ($p < 0.05$) polyspermy rate than zygotes derived from oocytes fertilized by Boars D, C, and A sperm (80.3 ± 1.9%; 65.8 ± 4.3% and 54.4 ± 6.9%, respectively) (Table 1). In addition, the percentage of oocytes penetrated by Boar B sperm (97.1 ± 1.0%) was equal to that of Boar A sperm (83.8 ± 5.3%). As a result, sperm from Boars A and B were used for IVF to assess in the following experiments. Boar A was defined as “moderate” polyspermy rate, since the proportion of polyspermy produced from Boar A was lowest in the all samples. Boar B was selected to produce high polyspermy conditions.

Table 1. Penetration and polyspermy rates of different boar sperm lines

Boars	Total IVF	Penetration (% total)	Polyspermy (% penetration)
A	145	122 (83.8 ± 5.3)[ab]	69 (54.4 ± 6.9)[a]
B	172	167 (97.1 ± 1.0)[a]	151 (90.4 ± 1.7)[c]
C	88	73 (83.0 ± 3.3)[b]	48 (65.8 ± 4.3)[a]
D	91	76 (83.5 ± 3.2)[b]	61 (80.3 ± 1.9)[b]

Epididymal sperm were collected from Boars A-D.
[a-c]Values with different superscripts in the same column are significantly different ($P < 0.05$).
Three to ten replications of each sperm line were carried out.
Results are presented as mean percentage ± SEM.

2.4.2 Experiment 2

The data for development of embryos produced under moderate and high polyspermy conditions are shown in Table 2. There were no differences in cleavage rate between embryos produced under moderate (77.9 ± 3.3%) and high (77.0 ± 3.0%) polyspermy conditions. However, the proportion of embryos that formed blastocysts and the total number of cells in blastocysts formed under moderate polyspermy condition (28.6 ± 4.1% and 48.8 ± 1.8, respectively) were significantly higher ($p < 0.05$) than the corresponding figures for high polyspermy conditions (14.8 ± 2.7% and 38.5 ± 0.9, respectively).

Table 2. Development of porcine embryos produced by IVF under moderate and high polyspermy conditions

Polyspermy condition	No. of embryos	Cleavage (% IVF)	Blastocyst (% IVF)	Cell no./blastocyst
Moderate	582	446 (77.9 ± 3.3)	143 (28.6 ± 4.1)[a]	48.8 ± 1.8[a]
High	1276	976 (77.0 ± 3.0)	218 (14.8 ± 2.7)[b]	38.5 ± 0.9[b]

Sperm with moderate and high polyspermy were collected from Boars A and B, respectively.

[a,b]Values with different superscripts in the same column are significantly different ($P < 0.05$).

Six replications of each sperm line were carried out.

Results are presented as mean percentage ± SEM.

2.4.3. Experiment 3

There were no differences in the numbers and percentages of selected 4-cell embryos in total cleaved embryos produced under moderate (21.1 ± 1.0%) and high (17.0 ± 2.6%) polyspermy conditions (Table 3). However, the percentage of double selection under 8-cell embryos produced under moderate polyspermy condition (6.3 ± 0.6%) was significantly higher than that of selected 8-cell embryos produced under high polyspermy condition (3.4 ± 0.8%) (Table 3).

Table 3 shows the developmental competence of single-, double-selected and remaining embryos derived under moderate and high polyspermy conditions. Both sets of embryos showed a high potential for development to blastocysts under both moderate (50.2 ± 6.6% and 70.7 ± 11.8%, respectively) and high (40.8 ± 9.1% and 57.9 ± 4.1%, respectively) polyspermy conditions, and no significant difference was evident between them (Table 3). However, the total numbers of cells in blastocysts derived from single-

and double-selected embryos produced under moderate polyspermy condition (60.9 ± 2.5 and 63.5 ± 2.9, respectively) were higher ($p < 0.05$) than those produced under high polyspermy condition (36.9 ± 1.6 and 37.7 ± 3.0, respectively) (Table 3).

Under moderate polyspermy condition, the single- and double-selected embryos showed higher ($p < 0.05$) competence for blastocyst development (50.2 ± 6.6% and 70.7 ± 11.8%, respectively) than remaining embryos at the time of selection (24.0 ± 5.6%) (Table 3). Meanwhile, the rate of development of blastocysts derived from the double-selected embryos (57.9 ± 4.1%) was significantly higher than that of blastocysts derived from remaining embryos (19.3 ± 4.4%) under high polyspermy condition (Table 3).

Under high polyspermy condition, there were no differences in the number of cells per blastocyst derived from single-, double-selected or remaining embryos (36.9 ± 1.6, 37.7 ± 3.0 and 37.5 ± 1.3, respectively). However, the numbers of cells in blastocysts derived from single- and double-selected embryos (60.9 ± 2.5 and 63.5 ± 2.9, respectively) were similar and significantly higher than that of blastocysts derived from remaining embryos (45.1 ± 2.2) (Table 3).

Table 3. Comparison of developmental competence and cell number in porcine blastocysts derived from single-, double-selected and remaining embryos produced under moderate and high polyspermy conditions after IVF

Polyspermy condition	Total no. oocytes for IVF	No. cleaved embryos[$]	Embryo selection group	48 hr after IVF			79 hr after IVF			No. blastocysts (% of selected embryos)	Cell no./blastocyst
				2-3-cell	4-cell	4-cell (% cleaved)	4-7-cell	8-cell	8-cell (% cleaved)		
Moderate	1045	769	Remaining	606			NA	NA		144 (24.0 ± 5.6)[a]	45.1 ± 2.2[aA]
			Single*		163	21.1 ± 1.0	115[†]	48[#]		80 (50.2 ± 6.6)[ab]	60.9 ± 2.5[bA]
			Double					48	6.3 ± 0.6[A]	35 (70.7 ± 11.8)[c]	63.5 ± 2.9[bA]
High	1074	824	Remaining	687			NA	NA		137 (19.3 ± 4.4) [a]	37.5 ± 1.3[B]
			Single*		137	17.0 ± 2.6	110[†]	27[#]		59 (40.8 ± 9.1)[bc]	36.9 ± 1.6[B]
			Double					27	3.4 ± 0.8[B]	16 (57.9 ± 4.1)[c]	37.7 ± 3.0[B]

Sperm with moderate and high polyspermy were collected from Boars A and B, respectively.

[$]Mono cell embryos were discarded at 48 hr after IVF.

*Data of single-selected embryos pooled from both double-selected[#] and other cleaved[†] embryos separated at 79 hr after IVF

[a-c]Values with different superscripts are significantly different among embryo selection categories in the same polyspermy condition ($P < 0.05$).

[A,B]Values with different superscripts are significantly different between polyspermy conditions in the same embryo selection group ($P < 0.05$).

Three to four replications with each sperm line were carried out.

Results are presented as mean percentage ± SEM. NA= Non applicable.

2.4.4. Experiment 4

The incidences of nuclear abnormalities in both single- and double-selected embryos produced under moderate polyspermy condition (20.5 ± 4.2% and 30.1 ± 4.5%, respectively) were significantly ($p < 0.05$) lower than in those produced under high (38.8 ± 4.7% and 67.6 ± 9.0%, respectively) polyspermy condition (Table 4).

Table 4. Comparison of nuclear abnormalities of single- and double-selected porcine embryos produced under moderate and high polyspermy conditions after IVF.

Polyspermy condition	No. (%) of single-selected embryos			No. (%) of double-selected embryos		
	Total	Normal nuclear	Abnormal nuclei	Total	Normal nuclei	Abnormal nuclei
Moderate	106	83 (79.5 ± 4.2)[a]	23 (20.5 ± 4.2)[a]	39	27 (69.9 ± 4.5)[a]	12 (30.1 ± 4.5)[a]
High	105	66 (61.2 ± 4.7)[b]	39 (38.8 ± 4.7)[b]	28	10 (32.4 ± 9.0)[b]	18 (67.6 ± 9.0)[b]

Sperm with moderate and high polyspermy were collected from Boars A and B, respectively.

[a,b]Values with different superscripts in the same column are significantly different in each column ($P < 0.05$).

Three to six replications of each sperm line were carried out.

Results are presented as mean percentage ± SEM.

2.4.5. Experiment 5

The results of chromosome analysis of single-selected embryos obtained under moderate and high polyspermy conditions are shown in Table 5. The proportion of diploid embryos produced under moderate polyspermy condition (72.5 ± 9.9%) was significant higher ($p < 0.05$) than that under high polyspermy condition (16.7 ± 12.5%). Moreover,

the incidences of mixoploidy and haploidy in embryos obtained under moderate polyspermy condition (18.5 ± 9.0% and 2.4 ± 2.4%, respectively) were significantly lower ($p < 0.05$) than those obtained under high polyspermy condition (57.6 ± 8.7% and 19.0 ± 5.5%, respectively). Triploidy was found in 4-cell embryos produced under both moderate and high polyspermy conditions (6.6 ± 4.2% and 6.7 ± 6.7%, respectively).

The proportion of diploid double-selected embryos obtained under moderate polyspermy condition (82.4 ± 5.6%) was significantly higher ($p < 0.01$) than that under high polyspermy condition (31.0 ± 9.7%), (Table 6). Moreover, the incidence of mixoploidy in embryos obtained under moderate polyspermy condition (17.6 ± 5.6%) was lower ($p < 0.05$) than that under high polyspermy condition (53.4 ± 10.3%). Haploid and triploid embryos were not found among the double-selected embryos produced under moderate polyspermy condition. Nevertheless, a number of haploid and triploid embryos were produced among the double-selected embryos in the high polyspermy group (4.2 ± 4.2% and 11.4 ± 1.1%, respectively) (Table 6).

Mixoploid 4- and 8-cell embryos were produced under both moderate and high polyspermy conditions. Among mixoploid embryos, the frequency of (n/2n) was highest among single- and double-selected embryos produced under high polyspermy condition (57.6 ± 8.7% and 53.4 ± 10.3%, respectively) (Tables 5 and 6).

Table 5. Comparison of chromosomal abnormalities of single-selected porcine embryos produced under moderate and high polyspermy conditions after IVF

Polyspermy condition	No. (%) of single-selected embryos				
	Total	Normal (Diploid)	Mixploidy	Haploid	Triploid
Moderate	31	22 (72.5 ± 9.9)[a]	6 (18.5 ± 9.0)[a]	1 (2.4 ± 2.4)[a]	2 (6.6 ± 4.2)
High	20	3 (16.7 ± 12.5)[b]	12 (57.6 ± 8.7)[b]	4 (19.0 ± 5.5)[b]	1 (6.7 ± 6.7)

Sperm with moderate and high polyspermy were collected from Boars A and B, respectively.

[a,b]Values with different superscripts in the same column are significantly different ($P < 0.05$).

Five to six replications of each sperm line were carried out.

Results are presented as mean percentage ± SEM.

Table 6. Comparison of chromosomal abnormalities of double-selected porcine embryos produced under moderate and high polyspermy conditions after IVF

Polyspermy condition	No. (%) of double-selected embryos				
	Total	Normal (Diploid)	Mixploidy	Haploid	Triploid
Moderate	24	20 (82.4 ± 5.6)[a]	4 (17.6 ± 5.6)[a]	0 (0)	0 (0)[a]
High	27	9 (31.0 ± 9.7)[b]	15 (53.4 ± 10.3)[b]	2 (4.2 ± 4.2)	3 (11.4 ± 1.1)[b]

Sperm with moderate and high polyspermy were collected from Boars A and B, respectively.

[a,b]Values with different superscripts in the same column are significantly different ($P < 0.05$).

Three replications of each sperm line were carried out.

Results are presented as mean percentage ± SEM.

2.5 DISCUSSION

The aim of the present study was to clarify whether selection of 4- and 8-cell stage embryos as single and double selection at specific time points might be an alternative for selection of good quality embryos, and how the degree of polyspermy rate might affect the development competence, nuclear status and karyotype of the selected embryos.

First, I set up IVF systems under moderate and high polyspermy conditions utilizing two different lots of frozen epididymal sperm (Boars A and B). These sperm lots had similar motility (40-50%) after warming; however, when used for IVF at the same concentrations, the rates of polyspermy differed significantly (54.4% vs 90.4%) whereas the total oocyte penetration rates did not. Consequently, these lots were used for in vitro production of embryos under moderate and high polyspermy conditions, respectively. This result confirmed the variability of polyspermy in pigs among sperm lots frozen by the same method.

When presumptive zygotes produced under moderate and high polyspermy conditions were cultured, I observed a significant decrease in the number of cells per blastocyst under high polyspermy condition relative to those produced under moderate polyspermy condition.

Previous studies have shown that selection of good quality porcine embryos depends on the timing and patterns of the first cleavage (Ulloa Ulloa et al., 2008a; Dang-Nguyen et al., 2010). On this basis, in the present study I focused on 4-cell embryos selected at 48 hr after IVF and 8-cell embryos selected at 79 hr after IVF from 4-cell embryos as single- and double-selected embryos, respectively. I found that the single- and double-selected embryos had similarly high competence (p >0.05) for development to the blastocyst stage under both moderate and high polyspermy conditions (Table 3).

Moreover, the selected 4- and 8-cell embryos produced under both moderate and high polyspermy conditions developed to blastocysts at significantly higher rates compared with relative to embryos at different stages. The results of my study are in agreement with previous reports (Ulloa Ulloa et al., 2008a; Dang-Nguyen et al., 2010); 4- and 8-cell embryos chosen at 48 hr and 79 hr after IVF, respectively, had high competence for development to blastocysts even under high polyspermy conditions, suggesting that this morphological approach could be an alternative method for selection of embryos with high developmental ability in pigs. In this study I observed no significant difference between single and double selection groups in terms of developmental competence and embryo normality, suggesting that single selection of embryos at the 4-cell stage at 48 hr after IVF would be sufficient for delineation of embryos with high developmental competence.

However, I also found that the total numbers of cells in blastocysts derived from single- and double-selected embryos produced under moderate polyspermy condition were significantly higher than in those produced under high polyspermy condition (Table 3). As mentioned previously, embryos produced under moderate polyspermy condition had significantly higher developmental competence, in terms of blastocyst rate and total cell number per blastocyst, than those produced under high polyspermy condition (Table 2). These results confirm previous reports suggesting that high polyspermy condition likely affect the developmental competence of embryos (Han et al., 1999a; Somfai et al., 2008). I then performed a more detailed examination of nuclear status and chromosome numbers of single- and double-selected embryos produced under moderate and high polyspermy conditions to determine whether the incidences of nuclear and chromosomal abnormalities were increased in embryos produced under high polyspermy condition.

Nuclear staining with DAPI revealed that the incidences of nuclear abnormalities in both single- and double-selected embryos produced under moderate polyspermy condition were significantly lower than those produced under high polyspermy condition (Table 4). The incidences of nuclear abnormalities in embryos produced under both moderate and high polyspermy conditions were lower than those reported previously in 4-cell embryos selected at 48 hr after IVF (Wang et al., 1999).

Similarly, chromosome analysis revealed that the incidences of chromosomal abnormalities were elevated in single- and double-selected embryos produced under high polyspermy condition compared with those in single- and double- selected embryos produced under moderate polyspermy condition (Tables 5 and 6). Significant increases in percentages of the mixoploid 4- and 8-cell embryos produced under high polyspermy condition were also recorded (Tables 5 and 6). The results of my study confirmed that higher frequency of chromosomal abnormalities is observed in polyspermic embryos compare with monospermic ones (Han et al.,1999b; Ulloa Ulloa et al., 2008a; Somfai et al., 2008). Altogether, the present results suggest that high frequency of nuclear and chromosomal abnormalities in single- and double-selected embryos produced under high polyspermy condition could be responsible for the reduced the quality of the derived blastocysts.

Interestingly, among the embryos produced under moderate polyspermy condition, I recorded only very low incidences of haploidy and triploidy in single-selected embryos (Table 5) and found no haploid or triploid embryos among the double-selected embryos (Table 6). However, a degree of haploidy and triploidy was found among both single-selected embryos and double-selected embryos produced under high polyspermy condition (Tables 5 and 6). A large proportion of single- and double-selected embryos

produced under high polyspermy condition were mixoploid, and approximately half of them showed haploid/diploid (n/2n) mosaicism. It has been suggested that mixoploid and triploid embryos result from polyspermy: haploid/diploid (n/2n) mosaicism occurs when one female and one male pronucleus are close but the other is eccentrically located, whereas triploid embryos are formed when all pronuclei are centrally located (Han et al., 1999b; reviewed in Funahashi, 2003). The high frequencies of triploid and haploid/diploid (n/2n) mosaic embryos produced under high polyspermy condition in my study confirm the link between polyspermy and chromosomal anomalies.

In conclusion, 4- and 8-cell embryos selected at 48 and 79 hr after IVF have been shown to have high competence for development to blastocysts under both moderate and high polyspermy conditions. Such selection of 4- and 8-cell embryos at these respective time points could be used as an alternative option for embryo selection based on morphology even under high polyspermy condition in IVF in pigs. The polyspermy rate affects the frequency of nuclear and chromosomal abnormalities of 4- and 8-cell embryos and the quality of the derived blastocysts.

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CHAPTER 3 PLURIPOTENCY-ASSOCIATED GENES REPOSITION DURING EARLY EMBRYONIC DEVELOPMENTAL STAGES IN PIGS

3. 1 ABSTRACT

I examined the allelic expression and positioning of two pluripotency-associated genes, *OCT4* and *SOX2*, and two housekeeping genes, *ACTB* and *TUBA*, in 4- and 8-cell porcine embryos utilizing RNA and DNA fluorescence in situ hybridization (FISH) in single blastomeres. The proportion of blastomeres expressing *SOX2* bi-allelically increased from 45% at the 4-cell stage to 60% at the 8-cell stage. Moreover, in 8-cell embryos, *SOX2* was expressed bi-allelically in significantly more blastomeres than was the case for *OCT4*, and this was associated with a tendency for *SOX2* alleles to move toward the nuclear interior during 4- to 8-cell transition. However, the radial location of *OCT4* alleles did not change significantly during this transition. The locations of active and inactive alleles based on DNA and RNA FISH signals were also calculated. Inactive *OCT4* alleles were located in very close proximity to the nuclear membrane, whereas active *OCT4* alleles were more centrally disposed in the nucleus. Nevertheless, the nuclear location of active and inactive *SOX2* alleles did not change in either 4- or 8-cell blastomeres. The RNA and DNA FISH data provide novel information on the allelic expression patterns and positioning of pluripotency-associated genes, *OCT4* and *SOX2*, during EGA in pigs.

3.2 INTRODUCTION

In eukaryotic cells, DNA is contained in the nucleus with heterochromatin

concentrated at the periphery and around the nucleolus, and gene-rich regions are preferentially located in the interior of the nucleus (Gilbert et al., 2004; Cremer and Cremer, 2006; Misteli, 2007). Heterochromatin is a tightly packed form of chromatin and is gene-poor, possessing markers of closed chromatin such as di- and tri-methylated H3K9 or H3K27 (Guelen et al., 2008). It has been suggested that positioning within the nucleus can affect the expression of heterochromatic regions (Jachowicz et al., 2013). Several studies have also reported that some genes, but not all, undergo repositioning when gene activity changes (reviewed in Takizawa et al., 2008). Such genes are those whose activity is tightly linked to differentiation and developmental events, such as *IgH* and *β-globin*, which are activated during the differentiation of B cells and erythroid cells, respectively (Kosak et al., 2002; Ragoczy et al., 2006), and genes of the hoxB cluster that are involved in embryonic development (Chambeyron and Bickmore, 2004). Similarly, *NANOG* is located at a more internal position within the nucleus of human ES cells, where it shows high expression in comparison to its state within the nucleus of differentiated lymphoblastoid cells (Wiblin, 2005). Likewise, *OCT4* shifts its localization from the interior to the surface of its chromosome territory in lymphoblastoid cells (Wiblin, 2005). These observations suggest that a large number of genes likely undergo repositioning during early embryonic development, especially during genome activation when the expression of many genes is significantly up- or down- regulated. In fact, using DamID technology, Borsos et al. (2019) have observed nuclear reorganization in mouse embryos and this process is not inherited from the maternal germline but is established de novo shortly after fertilization. The two parental genomes establish lamina-associated domains (LADs) with different features that converge after the 8-cell stage (Borsos et al., 2019). Positional changes in specific genes during EGA in other animals are not well understood.

Maintenance of pluripotency in stem cells or early embryos requires tight regulation of key pluripotency-associated genes, and one possible mechanism for this may be mono-allelic expression (Miyanari and Torres-Padilla, 2012). Studies of mouse ES cells have revealed that *Nanog* expression is subject to mono-allelic firing (Chambers et al., 2007), possibly through transcriptional bursts (Navarro et al., 2012; Hansen and van Oudenaarden, 2013; Filipczyk et al., 2013). Research on mouse embryos has suggested that the transcriptional activity of *Nanog* is also subject to mono-allelic bursting and that this is crucial for maintenance of pluripotency, embryonic development, and reprogramming (Miyanari and Torres-Padilla, 2012). However, little is known about the allelic expression pattern of pluripotency-associated genes in other mammalian embryos. A better understanding of allelic expression patterns, and the location and repositioning of such important genes, would be potentially useful for embryonic development and stem cell research in pigs. In the present study, I examined the allelic expression pattern and repositioning of the pluripotency-associated genes, *SOX2* and *OCT4*, in 4- and 8-cell embryos during EGA in pigs.

3.3 MATERIALS AND METHODS

3.3.1 Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal cross-bred gilts (Landrace × Large White × Duroc) at a local slaughterhouse and carried to the laboratory in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 35–37ºC within 1 hr. Cumulus-oocyte complexes (COCs) were collected from follicles 3–6 mm in diameter in collection medium consisting of Medium 199 (with Hanks' salts; Sigma-

Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), and antibiotics (100 units/ml penicillin G potassium (Sigma-Aldrich) and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich)). IVM of oocytes was carried out as reported previously (Kikuchi et al., 2002). In brief, about 50 COCs were cultured in 500 μl of maturation medium, which was a modified form of NCSU-37 solution (Petters and Wells, 1993) containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich), 50 mM β-mercaptoethanol (Axon Medchem, Groningen, Netherlands), 1 mM dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP; Sigma-Aldrich), 10 IU/ml equine chorionic gonadotropin (Setrotropin; ASKA Pharmaceutical Co. Ltd., Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (Gonatropin; ASKA) in four-well dishes (Nunclon Multidishes, Nunc, Thermo Fisher Scientific) for 22 hr in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5ºC. The COCs were subsequently cultured in maturation medium without dbcAMP and hormones for an additional 24 hr.

3.3.2 In vitro fertilization and in vitro culture

Oocytes were in vitro fertilized according to the 2-step IVF method of Grupen and Nottle (2000) with modifications. The medium used for IVF was a modified Pig-FM medium (Suzuki et al., 2002) containing 10 mM HEPES, 2 mM caffeine and 5 mg/ml BSA. The oocytes were washed 3 times in IVF medium, then transferred into 90-μl IVF droplets (each containing approximately 20 oocytes) covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan). Frozen-thawed epididymal spermatozoa from a Meishan boar were preincubated at 38.5°C in Medium 199 (with Earle's salts, Gibco, Thermo Fisher Scientific, pH adjusted to 7.8) for 15 min (Kikuchi et al., 1998). To obtain

the final sperm concentration (1×10^5 sperms/ml), 10 μl of the sperm suspension was introduced into the IVF medium containing oocytes and co-incubated for 30 min at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2. The oocytes with the zona-bound sperms were then transferred to other fresh droplets of the IVF medium and subsequently incubated for 2.5 hr. At the end of IVF, spermatozoa were removed from the surface of the zona pellucida by gentle pipetting with a fine glass pipette. The day of IVF was defined as Day 0.

Presumptive IVF zygotes were then transferred to IVC media. Two types of IVC media were prepared (Kikuchi et al., 2002). The basic IVC medium was NCSU-37 modified by addition of 0.4% (w/v) BSA and 50 μM β-mercaptoethanol. Embryos were cultured at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2 in IVC-PyrLac (basic IVC medium with addition of 0.17 mM sodium pyruvate and 2.73 mM sodium lactate) from Day 0 to Day 2, and then in IVC-Glu (basic medium supplemented instead with 5.55 mM glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan)) from Day 2 until collected for RNA and DNA FISH.

3.3.3 RNA and DNA FISH

Four- and 8-cell embryos were collected at 48 and 79 hr after IVF, when developmental ability is known to be high (Nguyen et al., 2020). The zona pellucida was removed by incubation with 1% pronase (protease, Sigma-Aldrich) for approximately 1 min following by gentle pipetting until the zona pellucida had mostly dissolved, and then the embryos were immediately placed in IVC medium for 5 min. The embryos were washed twice with PBS before being used for RNA and DNA FISH, which were performed according to Miyanari and Torres-Padilla (2012) with modifications for pig

embryos. Briefly, for RNA FISH, selected 4- and 8-cell embryos were incubated in fixative containing 4% paraformaldehyde (Nacalai Tesque) in PBS for 20 min at room temperature. The embryos were permeabilized in fixative for 10 min at room temperature. After washing with PBS three times, the embryos were pre-hybridized in a 4-μl drop of hybridization buffer covered with mineral oil for 30 min at 50°C. The hybridization buffer consisted of 50% formamide (Wako), 10% dextran sulfate (Wako), 2x SSC, 1 μg/μl Hybloc DNA (Applied Genetics Laboratories), 1 mM vanadyl ribonucleotide complex (Sigma-Aldrich), 1 mg/ml polyvinyl pyrrolidone (PVP), 0.05% Triton X-100 and 0.5 mg/ml BSA. The embryos were then transferred to a 4-μl drop of hybridization buffer containing 10 ng/μl ChromaTide Alexa Fluor 488 fluorescent probe and incubated at 50°C overnight. After three washes with 2x SSC solution supplemented with 0.1% Triton X-100 and 1 mg/ml PVP at 50°C for 10 min, the embryos were mounted on glass slides and stained with Vectashield containing DAPI. Images were acquired on a LSM700 laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with a 488-oil immersion objective lens.

For DNA FISH, selected 4- and 8-cell embryos were incubated in fixative containing 4% paraformaldehyde in PBS for 15 min at room temperature, then permeabilized in fixative for 1 hr at 37°C. RNase was also added to the permeabilization buffer to digest the RNA. After briefly washing with PBS, the embryos were briefly treated with 0.3% HCl solution at room temperature to remove histones. After two washes with PBS, the embryos were equilibrated in a 4-μl drop of hybridization buffer covered with mineral oil for 3 hr at 55°C. The hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 2xSSC, 1 μg/μl Hybloc DNA, 5 mM EDTA, 1 mg/ml polyvinyl pyrrolidone (PVP), 0.1% Triton X-100 and 1 mg/ml BSA. Denaturation was performed

by incubation at 83°C for 10 min. The embryos were then pre-hybridized for 1 hr at 37°C before being transferred to a 4-µl drop of hybridization buffer containing 10 ng/µl fluorescent probe and incubated at 37°C overnight. After a brief wash with 2x SSC and twice with 0.2x SSC solution supplemented with 0.1% Triton X-100 and 1 mg/ml PVP at 52°C for 15 min, the embryos were mounted on glass slides and stained with Vectashield containing DAPI. Images were acquired on a laser scanning microscope with a 488-oil immersion objective lens.

Coding regions of *SOX2, OCT4, TUBA* and *ACTB* were amplified using ExTaq DNA Polymerase (TaKaRa) with the specific primers (Table 1). The PCR products were confirmed by DNA sequencing using an Applied Biosystems 3130xl Genetic Analyzer and then used as probes for RNA and DNA FISH. Probes were labelled with ChromaTide Alexa Fluor 488-dATP using Degenerate Oligonucleotide Primed (DOP) PCR according to Backx et al. (2008) and purified with a QIAquick PCR Purification Kit (QIAGEN).

3.3.4 Statistical analysis

Only interphase nuclei were selected for image analysis, which was performed using MATLAB version 2018 and ImageJ. Blastomere nuclear territory and positions of RNA/DNA signals in the nucleus were calculated in three dimensions by a MATLAB script using the fit_ellipse.m function written by Gal (2019) [https://www.mathworks.com/matlabcentral/fileexchange/3215-fit_ellipse]. Detected fluorescent spots located inside nuclear territory that were distinguishable from the background were considered real signals. Blastomeres with no, one or two well-separated RNA FISH signals were scored as no expression, mono-allelic or bi-allelic expression (Fig. 1A-C, Fig. 2A). Only nuclei with two DNA FISH signals were used for location

calculation (Fig. 2B). Two fluorescent spots in very close proximity, which might have resulted from DNA replication, were recorded as one signal. The position of a signal was the nearest distance from its center of mass to the nuclear membrane. Embryos in which all blastomeres showed no fluorescent signals were excluded from further image analysis.

All data were expressed as mean ± SD values. The data were analyzed by one-way ANOVA followed by Bonferroni correction by using the Stata/SE 15.0 software package (StataCorp., College Station, TX, USA). Differences at $p < 0.05$ were considered to be statistically significant.

3.4 RESULTS

3.4.1 Allelic gene expression during early development

The allelic expression patterns of two pluripotency-associated genes, *OCT4* and *SOX2*, and two housekeeping genes, *ACTB* and *TUBA*, were examined in 4- and 8-cell embryos. The proportions of blastomeres with mono-, bi-allelic or no expression for *OCT4* in 4-cell embryos were similar to those in 8-cell embryos (Fig. 4D). Likewise, no significant changes in *TUBA* and *ACTB* allelic expression patterns were recorded during 4- to 8-cell stage transition. Although it is not significant, the proportion of blastomeres bi-allelically expressing *SOX2* increased from 45% in 4-cell embryos to 60% in 8-cell embryos (Fig. 4D).

The ratios of mono-, bi-allelic and no expression for all four investigated genes were similar at the 4-cell stage. However, in 8-cell embryos, the percentage of blastomeres that expressed *SOX2* bi-allelically was significantly higher than was the case for *OCT4* (Fig. 4D).

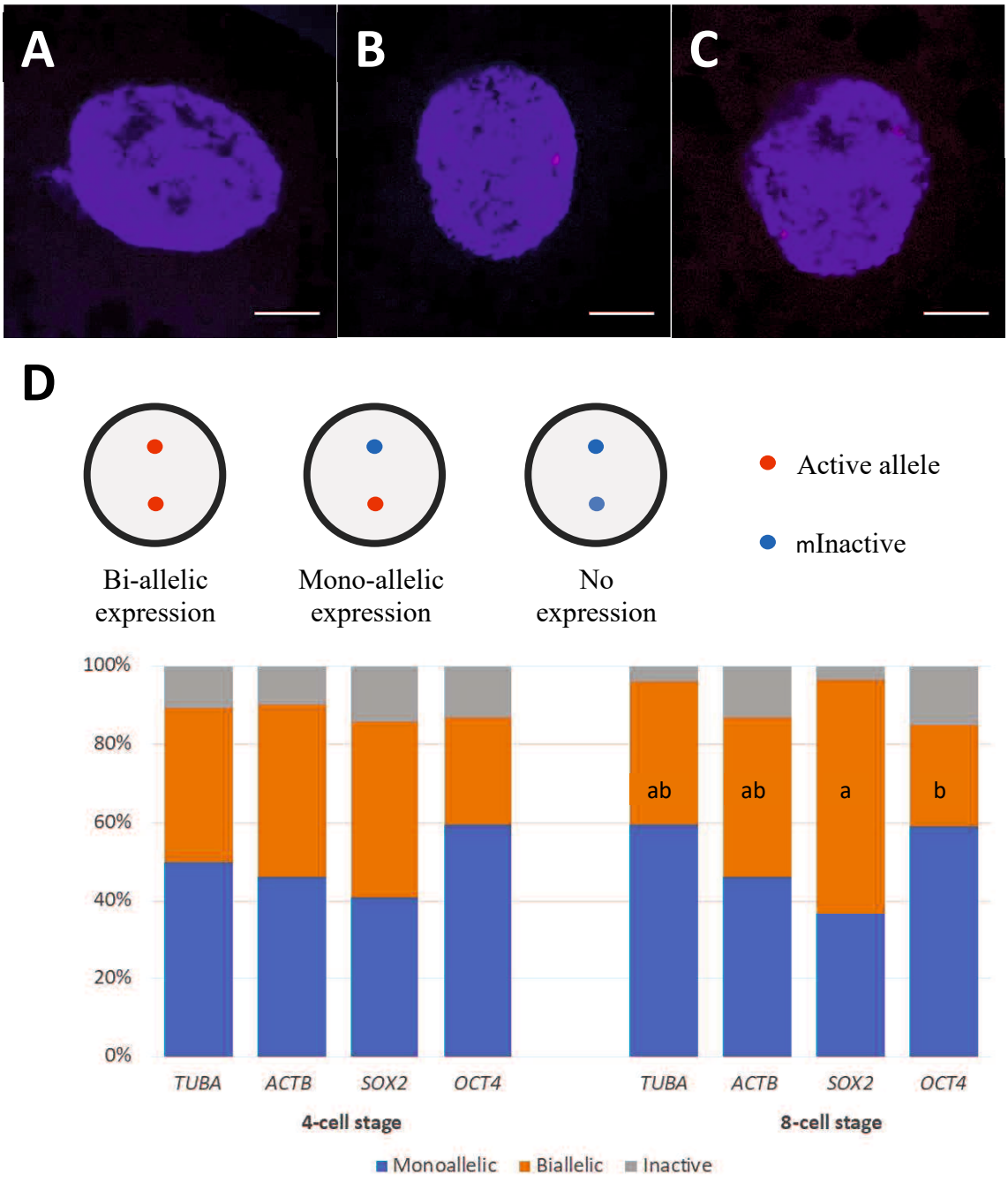
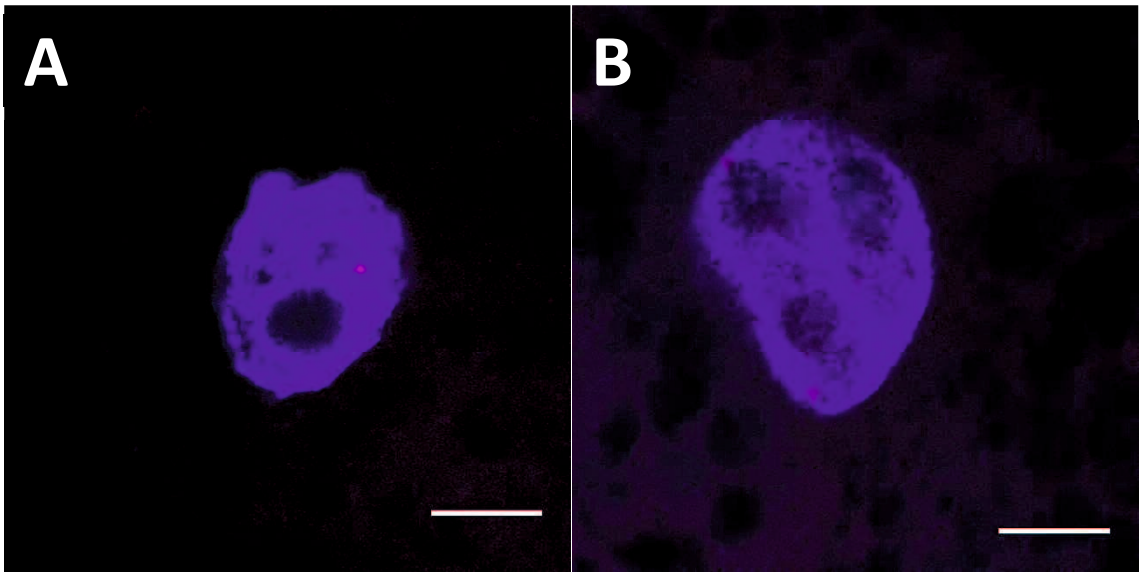


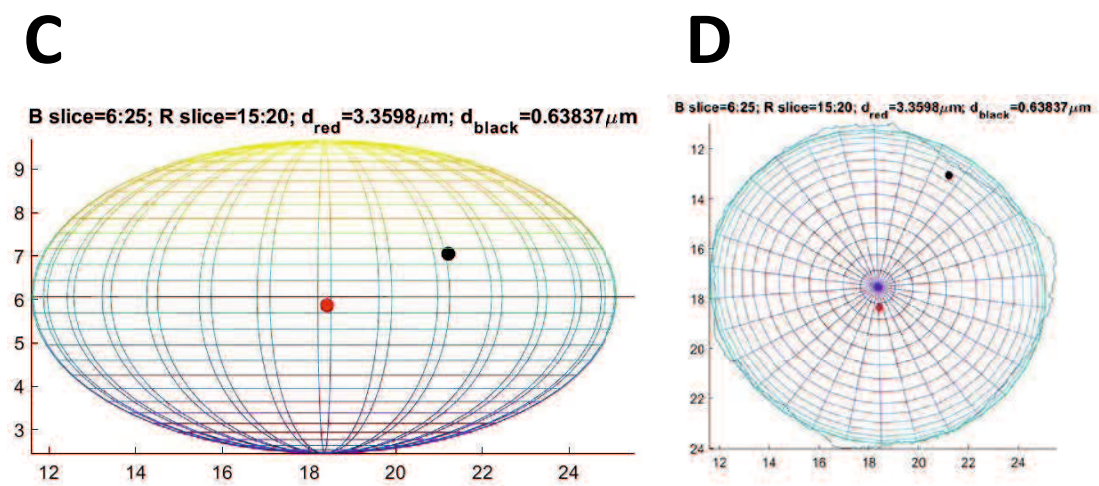
Figure 3.4. Allelic expression pattern of selected gene in porcine embryos. A 4-cell stage blastomere with no (A), mono-allelic (B), and bi-allelic expression (C) for *SOX2*. Allelic expression pattern for selected pluripotency-associated and house-keeping genes in 4- and 8-cell stage in porcine embryos (D). Number of blastomeres assessed for a specific gene at a specific stage ranged from 30 to 101. Scale bar presents 5 µm. Superscript letters denote significant difference ($P < 0.05$).

3.4.2 Gene repositioning during early development

The radial location of all *OCT4* alleles and active *OCT4* alleles in the nucleus, determined on the basis of DNA and RNA FISH signals, did not change significantly during 4- to 8-cell transition (Fig. 5E). Similarly, the distance from active *SOX2* alleles to the nuclear membrane was comparable at these developmental stages. However, *SOX2* alleles tended to move toward the nuclear interior during 4- to 8-cell transition (P=0.089) (Fig. 5F).

Based on DNA and RNA FISH signals, the locations of active and inactive alleles were calculated (Fig. 5G, H). The distance to the nuclear membrane from active and inactive *SOX2* alleles did not differ significantly in both 4- and 8-cell blastomeres. Inactive *OCT4* alleles were located in very close proximity to the nuclear membrane, whereas active *OCT4* alleles were more centrally disposed in the nucleus during 4- to 8-cell transition (Fig. 5G, H)


A
B


C
D
B slice=6:25; R slice=15:20; d red =3.3598μm; d black =0.63837μm
B slice=6:25; R slice=15:20; d red =3.3598μm; d black =0.63837μm

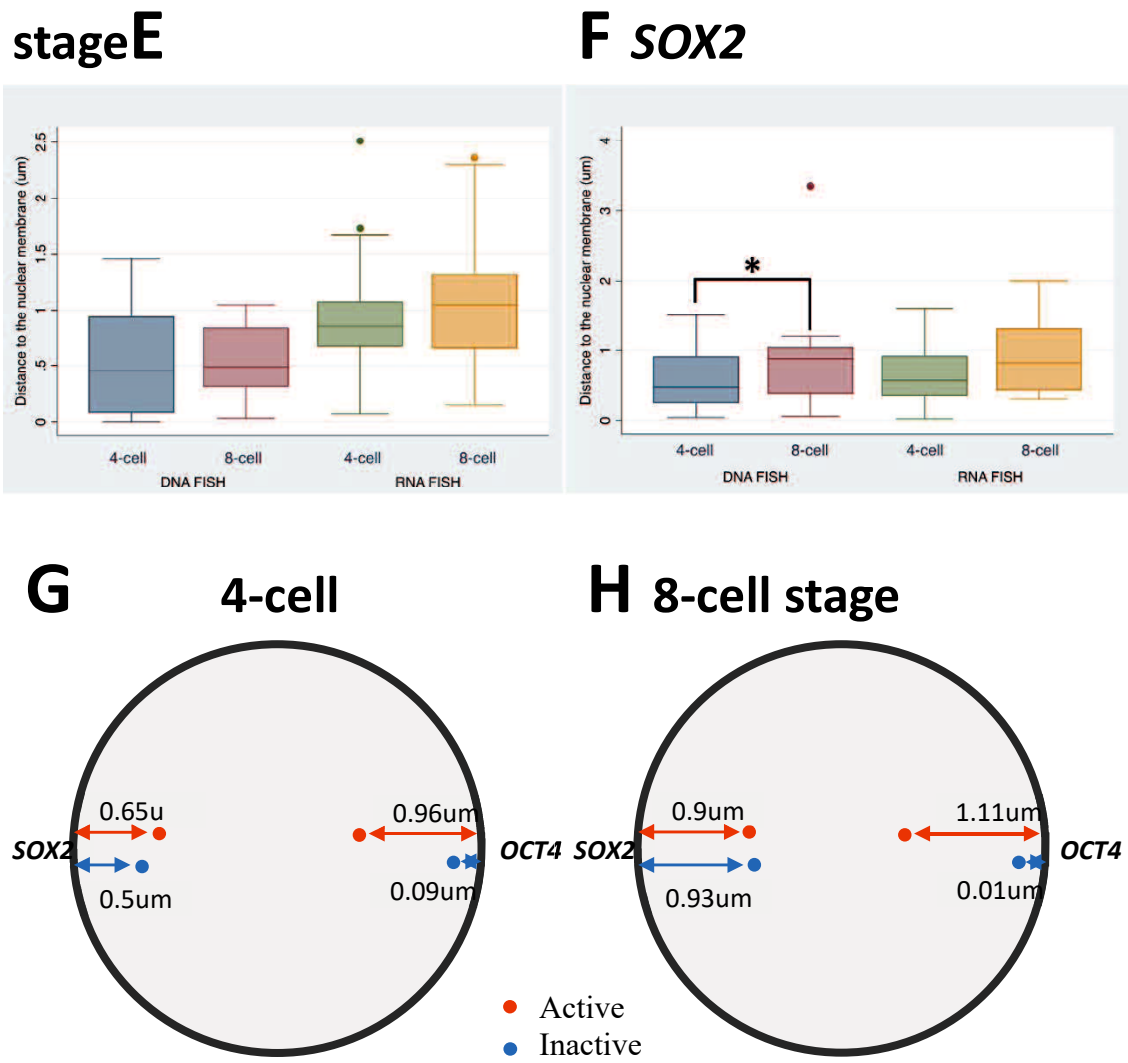


Figure 3.5. Nuclear location of pluripotency-associated genes in 4- and 8-cell stages in porcine embryos**.** An RNA FISH image of an 8-cell stage blastomere (A) and a DNA image of a 4-cell stage blastomere (B). Construction of nuclear territory and identification of RNA/DNA FISH signal in z (C) and in xy (D) using MATLAB. Location of *OCT4* (E) and *SOX2* (F) alleles, determined by distance of the detected FISH signals to the nuclear membrane, in 4- and 8-cell embryos by RNA and DNA FISH techniques. Nuclear location of active and inactive *OCT4* and *SOX2* alleles in 4- (G) and 8-cell embryos (H) calculated based on RNA (active alleles) and DNA FISH signals (both active and inactive alleles). Number of blastomeres assessed for a specific gene at a specific stage ranged from 16 to 56. Scale bar presents 5 µm. * P=0.089.

3.5 DISCUSSION

The allelic expression pattern and repositioning of several genes, including pluripotency-associated genes, has been reported in human somatic and stem cells (Croft et al., 1999; Wiblin, 2005), mouse somatic cells, stem cells and embryonic cells during differentiation and early embryonic development (Kosak et al., 2002; Chambeyron and Bickmore, 2004; Ragoczy et al., 2006; Miyanari and Torres-Padilla, 2012; Borsos et al., 2019). However, such information for any animal other than mice has not been published so far. In the present study, I examined the allelic expression patterns and nuclear location of two pluripotency-associated genes, *OCT4* and *SOX2*, during EGA in pigs. The location of these genes in the nucleus was determined in terms of the radial distance from the gene alleles to the nuclear membrane. I also have tried to examine allelic expression of *NANOG* which is another pluripotency-associated gene. However, *NANOG* expression could not be detected by FISH according to technical problems.

I did not detect any striking differences in the allelic expression patterns of *OCT4* and *SOX2*, or the two housekeeping genes, *ACTB* and *TUBA*, in both 4- and 8-cell embryos. The proportions of blastomeres with mono-, bi-allelic or no expression for all four of these genes were similar at both the 4- and 8-cell developmental stages (Fig. 4D). Notably, the percentages of mono-allelic expression were comparable to those of bi-allelic expression for all four examined genes and in both stages. Likewise, no significant changes in the allelic expression pattern of *SOX2, OCT4*, *TUBA* and *ACTB* were recorded during 4- to 8-cell stage transition. In mouse embryos, it has been reported that – except for *Nanog* – the majority of blastomeres show bi-allelic expression for all of the examined genes, including *Sox2, Oct4* and *Actb* (Miyanari and Torres-Padilla, 2012). These data suggest that there are differences in allelic expression profiles between pig and mouse

embryos during early embryonic development. However, it should be noted that in the study by Miyanari and Torres-Padilla (2012), cells with two, three or four separate RNA-FISH spots were scored as bi-allelism, whereas in my study only blastomeres with two RNA-FISH spots were defined as showing bi-allelic expression. This difference in the definition of bi-allelism might have partly contributed to the contrasting results between the two studies. Another factor that might also contribute to the difference between mouse and pig embryos is the synchronization of blastomere division. Using time lapse recording, it has been observed that, in pigs, the timing of division of some blastomeres may lag hours behind others (Anderson et al., 1999; Mateusen et al., 2005). In study of chapter 2 to optimize the timing of collection of 4- and 8-cell embryos (Nguyen et al., 2020), I usually found higher numbers of 5- to 7-cell embryos than 4- and 8-cell embryos at any time point of assessment. Furthermore, many 3-cell embryos were also found. In contrast, blastomeres in mouse embryos show somewhat synchronized division, with differences of only a few dozen minutes. These findings suggest that some of the blastomeres in the same embryos used in my study were arrested in different phases of the cell cycle. It has been reported that the gene expression profile of cells depends on the cell cycle phases (Grant et al., 2013; Liu et al., 2017). Although no report has documented this issue, cell cycle phase might also affect the allelic expression of certain genes. Utilization of inhibitors to synchronize the cell cycle transition in blastomeres is one potential solution to this problem. However, since most inhibitors interfere with embryonic development, I opted for natural development. Another point to note is that the use of RNA FISH only yields allelic expression data at the point of fixation, and is unable to indicate whether there are transcriptional bursts, where a gene allele switches between active and inactive states, or random allelic fluctuations, where the two alleles of a gene show ordered

activation. In order to catch such events, live imaging is required. However, for porcine embryos, live imaging is rather challenging due to the large amount of lipid droplets stored in the blastomeres.

Interestingly, I found that – although not significant – the proportion of blastomeres bi-allelically expressing *SOX2* increased from 45% in 4-cell embryos to 60% in 8-cell embryos, and that *SOX2* was expressed bi-allelically in a significantly greater number of blastomeres than was the case for *OCT4* in 8-cell embryos (Fig. 4D). This corresponded to the finding in my study that *SOX2* alleles tended to move toward the nuclear interior during 4- to 8-cell transition (Fig. 5F), whereas the location of *OCT4* alleles in the nucleus did not change significantly (Fig. 5E). Taken together, these observations may indicate a correlation between a change in the allelic expression pattern of *SOX2* and its repositioning during early embryonic development in pigs. However, it is noteworthy that one of the examined *SOX2* alleles was located 3.36 μm away from the nuclear membrane, which was considerably further than the remaining one, for which the average distance was 0.92 μm (Fig 5E).

I also calculated the radial locations of active and inactive alleles based on their DNA and RNA FISH signals. This revealed that inactive *OCT4* alleles were located in very close proximity to the nuclear membrane, whereas active *OCT4* alleles occupied the more inner area of the nucleus (Fig. 5G, H). Such behavior of *OCT4* alleles can be considered evidence for a correlation between gene expression and gene positioning. This is in agreement with a report by Stachecka et al. (2019), who demonstrated that active *PPARG* alleles are centrally positioned when inactive. Nevertheless, the data in my study also demonstrated that the distance of active and inactive *SOX2* alleles from the nuclear membrane did not differ significantly in both 4- and 8-cell blastomeres. Taken together,

these data suggest that although there is evidence to support a correlation between gene expression and gene positioning, this might not apply to all genes during EGA in pigs. Therefore, to obtain a better perspective on the correlation between gene expression and gene positioning, more genes should be studied. It should also be noted that, in my study, the nuclear locations of inactive alleles were computed indirectly based on the locations of active alleles and all alleles, as determined by RNA and DNA FISH, respectively. Sequential RNA-DNA FISH would allow direct measurement, and therefore yield more accurate results. Furthermore, artifacts might also lead to errors in the determination of allele locations using RNA and DNA FISH. One such artifact is the more oval rather than spherical shape of the nucleus, as shown in Fig. 5C, despite the efforts in my study to maintain the nuclear shape.

It is well documented that porcine embryos are characterized by nuclear and ploidy abnormalities (McCauley et al., 2003; Ulloa Ulloa et al., 2008; Dang-Nguyen et al., 2010), which would affect the results of FISH. In order to eliminate such aberrations, I carefully optimized methods for minimizing these abnormalities in the embryos utilizing for the FISH assays, which included strict selection of oocytes for IVF and embryos following IVF based on their morphology and the timing of early cleavages. This approach for optimizing the production and selection of good embryos has been proven to significantly reduce the incidence of nuclear and ploidy abnormalities (Nguyen et al., 2020).

In summary, the data in my study obtained using RNA and DNA FISH have yielded novel information on allelic expression patterns and positioning of two pluripotency-associated genes, *OCT4* and *SOX2*, during EGA in pigs. The results have shown that the repositioning of *SOX2* alleles coincided with an increase in the percentage

of blastomeres with bi-allelic expression during these stages of embryonic development, and that there was a correlation between the expression and nuclear location of *OCT4*. This information should be useful for improvement of embryonic development and stem cell research.

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CHAPTER 4 GENERAL DISCUSSION

Pluripotency-associated genes have important roles in maintenance of pluripotency in early embryos and stem cells (Miyanari and Torres-Padilla, 2012). Mono-allelic gene expression is considered as one of possible mechanisms for maintenance of pluripotency as well as for differentiation (Miyanari and Torres-Padilla, 2012, Eckersley-Maslin et al., 2014). Nevertheless, the allelic expression patterns, location and repositioning of pluripotency-associated genes in porcine embryo is not reported yet.

In mouse, the in vivo embryos are normally utilized for study. However, the use of in vivo embryos as starting material for study in pigs is laborious and expensive (Hou et al., 2016). The embryos produced by IVP, especially by IVF, with advantages of low cost and feasible availability, could be an alternative option for study in pigs. However, IVF in pigs is associated with high frequency of polyspermy which causes anomalies in embryos (Abeydeera & Day, 1997; McCauley et al., 2003; Koo et al., 2005). Since polyspermy in pigs remains as a major obstacle (reviewed by Nagai et al., 2006; Grupen, 2014), the establishment of selection system for high quality embryos under polyspermy condition is necessary for reproduction studies in pigs. The results of the first study revealed that although the frequency of anomalies in nucleus and number of chromosome in porcine embryos were affected by polyspermy, the subsequent selection based on morphological features of 4- and 8-cell embryos as single- and double-selection could be an alternative option for selecting porcine embryos with high developmental competence under moderate and high polyspermy conditions. Interestingly, it was found that only very low incidences of haploidy and triploidy in single-selected embryos and no haploid or triploid embryos among the double-selected embryos under moderate polyspermy condition. Therefore, those selected embryos produced under moderate polyspermy

condition would be helpful for improving the efficiency of DNA/RNA FISH assays which is useful tool for evaluation of allelic gene expression in pigs.

Although DNA/RNA FISH have been established successfully in mouse embryos, these techniques have several difficulties to apply on porcine embryos. For example, unlike human and mouse embryos, porcine embryos are characterized by a large amount of lipid droplets in cytoplasm (Kikuchi et al., 2002b). Although there are no reports or evidences, in my experience, lipid droplets seem to hamper the penetration of probes into the nucleus of the embryos thus decrease the efficiency of reaction in DNA/RNA FISH assays. In this study, I have tried several ways to improve the efficiency of DNA/RNA FISH techniques and succeeded to a certain extent, the techniques are still not perfect.

With my efforts, in the second study (Chapter 3), DNA and RNA FISH assays have been established in single blastomeres of 4- and 8-cell porcine embryos and results on allelic expression and position of two pluripotency-associated genes *OCT4* and *SOX2* and two housekeeping genes *ACTB* and *TUBA*. I did not detect any striking differences in the allelic expression patterns of *SOX2, OCT4*, *TUBA* and *ACTB* during 4- to 8-cell stage transition. These results suggested differences in allelic expression profiles between pig and mouse embryos during early embryonic development.

Interestingly, I found that the proportion of blastomeres expressed *SOX2* in bi-allelically increased from 45% at the 4-cell stage to 60% at the 8-cell stage which coincide with the finding in my study that *SOX2* alleles tended to move toward the nuclear interior during 4- to 8-cell transition. These observations may suggest a correlation between a change in the allelic expression pattern of *SOX2* and its repositioning during early embryonic development in pigs. Meanwhile, *OCT4* alleles did not change the location significantly during 4- to 8-cell transition. However, the location of inactive *OCT4* alleles

was very close proximity to the nuclear membrane, whereas the location of active *OCT4* alleles was more inner area of the nucleus. Such behavior of *OCT4* alleles can be considered evidence for a correlation between gene expression and gene positioning. Nevertheless, the distance from the nuclear membrane of active and inactive *SOX2* alleles was not significantly different in both 4- and 8-cell blastomeres. Taken together, these data suggest that although there is a correlation between gene expression and gene positioning, this might not apply to all genes during EGA in pigs. Therefore, to obtain a better perspective on the correlation between gene expression and gene positioning, more genes should be studied.

In conclusion, in the first study, the selection system for good quality embryos based on morphological features and timing of early cleavage under polyspermy conditions has been established. It would be useful for not only reproduction studies but also gene expression on embryos in pigs. The second study revealed novel information on allelic expression patterns and positioning of two key pluripotency-associated genes, *OCT4* and *SOX2*, during EGA in pigs for the first time. The information would be useful for further advances in embryonic development and stem cell research.

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