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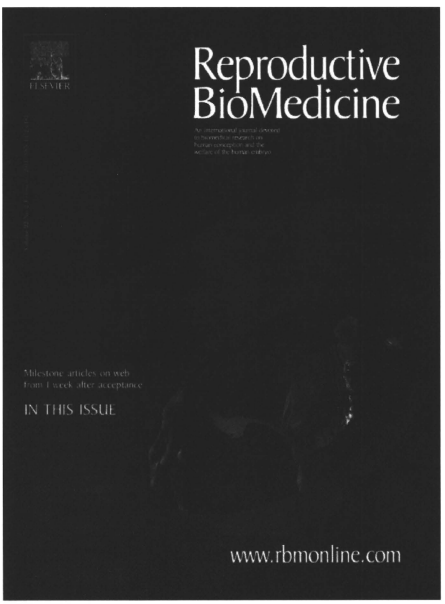

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COMMENTARY

Spermatozoal RNA profiling towards a clinical evaluation of sperm quality

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Abstract Human spermatozoal RNAs were recently profiled using microarrays and explored as clinical markers of male infertility. An appropriate study design with a considerable number of biological replicates (sperm samples) is necessary to validate the accuracy and reproducibility of these microarray data. If the genes identified as sperm quality markers by microarray studies are successfully attributed to the pathogenesis of male infertility, then the microarray strategy may be used as a clinical diagnostic tool for male infertility. On the other hand, spermatozoal RNAs may contain not only remnant RNAs after spermatogenesis, but also RNAs that may contribute extragenomically to early embryonic development. Therefore, spermatozoal RNA profiling may enable a better understanding of what is contributed to the oocyte by sperm, in addition to their genome, to facilitate early embryonic development.

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KEYWORDS: ICSI, male fertility, RNA profiling, sperm, spermatozoa, transcriptome

Assessments of male reproductive fitness have typically relied upon microscopic evaluation using semen parameters including morphology, motility, sperm concentration, presence of any cell types other than mature spermatozoa, and semen volume. The observation of normal semen features using these parameters does not necessarily guarantee male fertility. Recently, morphological real-time observation at high magnification (> ×6000) has been used to select sperm for intracytoplasmic sperm injection (ICSI). The morphological normalcy of the sperm nucleus is defined by both its shape (smooth, symmetric and oval) and its chromatin content (homogeneity of the chromatin mass containing no extrusion or invagination and no more than one vacuole involving less than 4% of the nuclear area) (Bartoov et al., 2002). Most publications have reported better rates of implantation and clinical pregnancy as well as a reduction in the rate of abortion where sperm cells were strictly morphologically selected at high magnification (Souza Setti et al., 2010). Prospective randomized clinical studies are still needed to confirm the preliminary findings on the efficacy of intracytoplasmic morphologically selected sperm injection (IMSI) over conventional ICSI. The further improvement of diagnosis and treatment of male infertility will need a new method to evaluate sperm quality based on molecular analysis, rather than on morphological observation.

Mature spermatozoa have little cytoplasm and a highly condensed chromatin architecture that is enriched in protamines. These structural features led to the long held view that mature spermatozoa are inert cells but both transcription and translation occur in the mitochondria, and not in the cytoplasm, of mature spermatozoa (Miller and Ostermeier, 2006). Spermatozoal nuclei, containing RNA polymerase and abundant transcription factors, are capable of transcribing RNA from endogenous templates (Hecht and Williams, 1978). Although mature spermatozoa do not contain some of the essential components of the 80S cytoplasmic ribosomes such as 28S and 18S rRNAs; 55S mitochondrial ribosomes are present in spermatozoal polysomal fractions (Gur and Breitbart, 2006). The incorporation of labeled amino acids into polypeptides occurs during sperm

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capacitation, and is completely inhibited by mitochondrial translation inhibitors but not by a cytoplasmic translation inhibitor (Gur and Breitbart, 2006). Therefore, it is apparent that mitochondrial ribosomes are actively involved in protein translation in spermatozoa.

The first mRNA that was identified in human mature spermatozoa was the c-Myc mRNA (Kumar et al., 1993). The existence of a complex population of mRNAs in ejaculated human mature spermatozoa was shown by expression profiling using oligo DNA microarrays (Ostermeier et al., 2002). Although these mRNAs were previously thought to be non-functional remnants of stored mRNAs that are synthesized at earlier stages of spermatogenesis, Ostermeier et al. proposed that a specific set of functional RNAs may be delivered into oocytes and support early embryonic development (Ostermeier et al., 2004). Although the specific functional significance of these mRNAs in mature ejaculate spermatozoa remains poorly investigated; they have been demonstrated to influence the phenotypic traits of offspring (Miller and Ostermeier, 2006). The poor developmental ratios relative to normal of both parthenogenetic embryos and cloned embryos obtained from somatic-cell nuclear transfer, are consistent with a developmental role for spermatozoal mRNAs.

Garcia-Herrero et al. used microarrays to investigate spermatozoal RNAs (this issue; Garcia-Herrero et al., 2011). They compared the profile gene of expression in spermatozoa that achieved pregnancy (group P) through an ICSI cycle in an oocyte donation program with the profile of those that did not achieve pregnancy (group NP) (Garcia-Herrero et al., 2011). In order to reduce female infertility as a bias factor, all of the oocytes originated from young female donors. Furthermore, the coupled pairs of women (pregnant and non pregnant) received the oocytes from the same donor. The total number of expressed transcripts detected in fresh sperm samples was 19,229. Of those transcripts, 16,035 (83.4%) were expressed in both groups, P and NP. Among these commonly expressed transcripts, only 44 sequences were overexpressed in group P versus NP and five in group NP versus P. Notably, the 44 differentially-expressed genes in group P included four cathepsins and six metallothioneins. Cathepsins are a family of cysteine proteases and are likely to prevent atrophy of seminiferous tubules and support spermatogenesis to pachytene spermatocytes (Gye and Kim, 2004; Wright et al., 2003). Metallothioneins function as detoxicants to prevent damage of the testes by heavy metals (Kusakabe et al., 2008). An ontology analysis by DAVID (http://david.abcc.ncifcrf.gov/home.jsp) of 1358 exclusively-expressed transcripts in group P, found the term 'embryo development ending in birth or egg hatching' as one of the highest-ranked gene ontology (GO) terms. The exclusively-expressed genes corresponding to this GO term include adducin 1 (*ADD1*), activin A receptor type-II like 1 (*ACVRL1*), androgen receptor (*AR*), and aryl-hydrocarbon receptor nuclear translocator (*ARNT*). All of those genes are potential pregnancy success markers rather than potential fertilization makers. In fact, because ICSI removes the physiological process of sperm entry, spermatozoal fertilization factors may not be necessary for pregnancy after ICSI. These highly expressed RNAs in group P may partially represent spermatozoal extra-genomic components that are required for successful pregnancies. In addition, DAVID also analyzed frozen spermatozoa used in ICSI in the same way, and demonstrated that the RNA profile of frozen spermatozoa was considerably changed by the sperm cryopreservation procedure.

Several other studies have profiled spermatozoal RNAs in clinical samples with the aim of finding a marker RNA or a distinctive expression pattern to represent sperm quality. A cross-platform microarray strategy was used to assess the profile of human spermatozoal transcripts from 13 fertile males who had fathered at least one child compared to those from eight teratozoospermic individuals (Platts et al., 2007). This analysis successfully distinguished between the normal and teratozoospermic groups using unsupervised hierarchical clustering. The teratozoospermic group lacked the RNAs of genes related to the ubiquitin-proteasome pathway and those genes transcribed at late stages of spermatogenesis including; an egg-activating sperm factor, *PLCZ1*; acrosomal proteins, *ACRV1* and *SPAM1*; and non-tubulin components of sperm tails, *ODF1-4*. These changes in gene expression are indicative of the failure of late-stage spermatogenesis in teratozoospermia. In a study by Lalancette et al., the spermatozoal RNAs of 24 healthy donors were expression profiled and a series of invariable transcripts were consistently present in all of the donor samples (Lalancette et al., 2009). Based on the expression of these consistently-expressed genes, only a single donor sample was not well correlated with the other 23 samples, suggesting that spermatozoal RNA profiling could be clinically applied to mark outliers. Furthermore, Garcia-Herrero et al. compared the transcriptomic profiles of sperm samples that achieved pregnancy after the first IUI to those that did not (Garcia-Herrero et al., 2009). They identified 756 genes that were significantly preferentially expressed in the pregnant group, and 194 genes that were significantly preferentially expressed in the non-pregnant group (Garcia-Herrero et al., 2009). Interestingly, these 756 genes include 20 of the 44 genes that were overexpressed in group P (pregnant after an ICSI in oocyte donation program) in the current study by the same group described in this volume of *Reproductive BioMedicine Online*. These 20 genes out of the 756 genes could be considered as potential pregnancy success markers rather than potential fertilization makers.

Thus far, microarray technologies have been used to assess the profiles of human spermatozoal RNAs and the utility of spermatozoal RNAs as clinical markers of male infertility. An appropriate study design with a considerable number of biological replicates (sperm samples) is necessary to validate the accuracy and reproducibility of these microarray data. If the genes identified as sperm quality markers by microarray studies are successfully attributed to the pathogenesis of male infertility, then the microarray strategy may be used as a clinical diagnostic tool for male infertility. On the other hand, spermatozoal RNAs may contain not only RNAs left over from failed or abnormal spermatogenesis, but also RNAs that may contribute extragenomically to early embryonic development. Therefore, spermatozoal RNA profiling may enable a better understanding of what is contributed to the oocyte by sperm, in addition to their genome, to facilitate early embryonic development.

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Induction of Oocyte Maturation by Hyaluronan-CD44 Interaction in Pigs

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Abstract. In most mammals, the oocyte is surrounded with compact multilayers of cumulus cells; these form cumulus-oocyte complexes (COCs). During oocyte maturation, the COCs dramatically expand and this is termed "cumulus expansion". We have previously demonstrated that cumulus expansion is the result of hyaluronan synthesis and accumulation in the extracellular space between cumulus cells in the COCs and that hyaluronan accumulation within the COCs affects oocyte maturation. We have also demonstrated that CD44, the principal hyaluronan receptor, is expressed in the COCs during cumulus expansion and that the interaction between hyaluronan and CD44 appears to be closely related to gap junctional communication of the COCs during the process of meiotic resumption. Based on our previous studies, we review herein that the physiological significance and the molecular mechanism of cumulus expansion for porcine oocyte maturation.

Key words: CD44, Cumulus expansion, Gap junction, Hyaluronan, Oocyte maturation

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In nearly all mammals, the oocyte is surrounded by compact multilayers of cumulus cells that form cumulus-oocyte complexes (COCs). During oocyte maturation, the COCs expand dramatically; this phenomenon is termed "cumulus expansion" and occurs after the pre-ovulatory surge of gonadotropins. It has been reported that cumulus expansion supports dissociation from the follicle wall and the expulsion of the oocyte through the ruptured follicle wall during ovulation. In addition to these effects, it has been reported that the expansion of COCs is essential for fertilization and the developmental potential of early embryos [1, 2]. Therefore, the degree of cumulus expansion is often cited as a major indicator in the selection of oocytes for *in vitro* fertilization (IVF) protocols [3–5]. Considering these observations, it is clear that an understanding of the physiological significance of cumulus expansion is important to the study of the developmental competence of mammalian oocytes that are matured and fertilized *in vitro*. Therefore, based on our previous studies, we describe herein the physiological significance and the molecular mechanism of cumulus expansion in porcine oocyte maturation.

Effects of Cumulus Expansion on Oocyte Maturation

Many researchers have documented that the formation of the COC matrix during cumulus expansion is characterized by the intercellular deposition of hyaluronan secreted from cumulus cells [6–8]. We first investigated whether the induction of cumulus expansion was due to the synthesis of hyaluronan during porcine oocyte maturation. COCs were cultured in maturation medium with or without 6-diazo-5-oxo-1-norleucine (DON; an inhibitor of hyaluronan synthesis) for 48 h. The degree of cumulus expansion increased gradually until 48 h in culture in the control medium. When the COCs were cultured with DON, they showed no evidence of cumulus expansion during the culture period. In addition, a remarkable accumulation of hyaluronan was confirmed in porcine expanded COCs by the immunostaining method, but hyaluronan accumulation was completely inhibited by treatment with DON (Fig. 1). These results indicate that the expanded cumulus mass of porcine COCs consists mainly of hyaluronan [9, 10].

Generally, serum and follicular fluid contain a factor(s) that stabilizes the hyaluronan-rich matrix. Previous studies have demonstrated that the inter-α-trypsin inhibitor (ITI) family plays an important role in the formation of the extracellular matrix of which hyaluronan is the predominant component [11]. Proteins of the ITI family are composed of a common light chain called "bikunin" and either one or two heavy polypeptide chains (HCs). In addition, serum-derived hyaluronan-associated proteins (SHAPs) have been isolated from the hyaluronan-rich extracellular matrix of mouse dermal fibroblasts cultured in the presence of serum [12], and these proteins were identical to the HCs of ITI [13]. It has been reported that the formation of a bond between SHAP and hyaluronan is also important for the stabilization of the hyaluronan-rich matrix [14, 15]. In our previous study, we documented that SHAPs were also present in porcine follicular fluid and serum as a single protein band at 70 kDa (Yokoo *et al.* unpublished data). In pigs, it has been reported that there is no apparent barrier to the transfer of SHAPs from the blood to the follicle [16]. Therefore, it is believed that SHAPs present in porcine follicular fluid are derived from serum.

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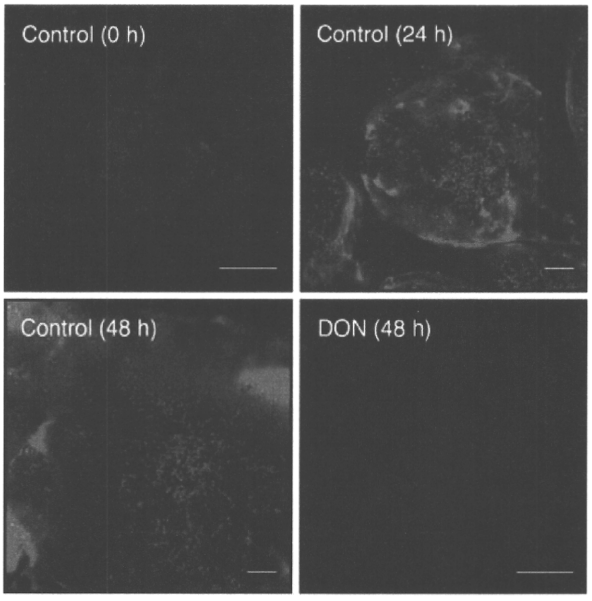

Fig. 1. Localization of hyaluronan in porcine cumulus-oocyte complexes (COCs) as detected by immunofluorescence using biotinylated hyaluronan-binding protein. Green=hyaluronan; red=nuclear; bars=100 μm.

Moreover, we demonstrated that the immunodepletion of SHAPs from follicular fluid produced not only incomplete cumulus expansion but also a decline in the oocyte maturation rates in a manner dependent on the antibody concentration against SHAPs. These results suggest that the retention and stabilization of hyaluronan within the COCs by the formation of the hyaluronan-SHAP complex during cumulus expansion is necessary for porcine oocyte maturation.

Expression of Hyaluronan-CD44 Interaction in COCs

Hyaluronan, which is the main component of cumulus expansion, is a linear glycosaminoglycan that is a high-molecular-weight polymer with repeating disaccharides linked by β 1–3 and β 1–4 glycosidic bonds. Despite its structural simplicity, hyaluronan is a biologically important biopolymer that is widely distributed in the extracellular matrix of connective tissues in the body. It plays important roles in diverse processes such as wound repair, cell motility, and cancer metastasis. Unlike others of the glycosaminoglycan family, hyaluronan is neither sulfated nor linked to a core protein. Hence, hyaluronan needs hyaluronan binding protein(s) for its biological functions. To obtain information regarding hyaluronan binding protein(s) during cumulus expansion, we investigated their expression in COCs during oocyte maturation by ligand blotting analysis with fluorescein isothiocyanate (FITC)-labeled hyaluronan. Interestingly, an 85-kDa hyaluronan binding protein was detected only in expanded COCs after maturation. Using immunoprecipitation assay, we showed that this protein was identical to CD44 [17].

CD44 is the principal cell-surface receptor for extracellular matrix hyaluronan and exists in a number of isoforms with different molecular sizes (approximately 80–250 kDa) on a wide variety of cell types [18–21]. It has been reported that the function of hyaluronan via CD44 is responsible for cell-to-cell and cell-to-extracellular matrix interactions [22], inhibition of apoptosis [23], augmentation of tumor cell motility and metastasis [24], and stimulation of lymphocytes [25]. Additionally, previous studies have indicated that the hyaluronan-CD44 interaction may influence fertility and the quality of oocytes [26, 27]. However, the role of CD44 in oocyte maturation remains poorly understood. To elucidate the role of hyaluronan-CD44 interaction in oocyte maturation, we examined the maturation-promoting factor (MPF) activity and the germinal vesicle breakdown (GVBD) rates in the COCs cultured in maturation medium containing with anti-CD44 antibody, which has been used for inhibition of hyaluronan binding [28, 29]. A low level of MPF activity was noted in oocytes from the COCs immediately after collection from follicles. After 24 h in culture, the MPF activity and the GVBD rates in oocytes cultured in the drug-free medium significantly increased compared to those of cumulus-oocyte complexes before culture. However, exposure of the COCs to anti-CD44 antibody during 24 h of culture significantly suppressed MPF activity and GVBD rates compared to those of the COCs cultured in the drug-free medium for 24 h (Table 1 and Table 2). Thus, these results clearly show that the hyaluronan-CD44 interaction is required for meiotic resumption in the oocyte maturation process.

We next examined the difference between the molecular size of CD44 expressed in the COCs matured *in vitro* and those matured *in vivo* [30]. The COCs matured *in vitro* showed bands of CD44 ranging from 81 to 88 kDa. However, the CD44 band in the *in vivo*-matured COCs was 73–83 kDa in size; thus, the size of the CD44 in the COCs matured *in vivo* was clearly smaller than the band of CD44 in the COCs matured *in vitro*. The amino acid sequence of CD44 predicts a polypeptide of <40 kDa, which contrasts with its apparent size on gel electrophoresis (approximately 80–250 kDa). This difference appears to be the result of extensive glycosylation of the extracellular domain [31]. It has been reported that CD44 glycosylation has been implicated in the regulation and function of CD44-mediated cell binding for hyaluronan [19]. Notably, Katoh *et al.* [32] reported that the terminal sialic acids on CD44 have an inhibitory effect on hyaluronan binding ability of CD44. Our results clearly demonstrated that the treatment with sialidase reduced the size of CD44 in the COCs matured *in vitro*, the size of which was not significantly different from that of the COCs matured *in vivo*. This evidence indicates the possibility that interaction between hyaluronan and CD44 during *in vitro* maturation may not be sufficient for oocyte maturation compared to that *in vivo*. In general, oocytes matured *in vitro* have a reduced capacity to be fertilized and a higher rate of abnormal fertilization and development as compared to their *in vivo* counterparts. In pigs, although oocytes matured *in vitro* can be penetrated by spermatozoa under appropriate conditions, *in vitro* maturation is associated with low rates of pronuclear formation and a high incidence of polyspermy [33]. Sun *et al.* [34] demonstrated that the rates of embryo development rates of *in vitro*-matured and fertilized COCs

Table 1. Effects of 6-diazo-5-oxo-norleucine (DON) and anti-CD44 antibody on maturation-promoting factor (MPF) activity in porcine oocytes

	Relative MPF activity (0 h=1)			
	0 h	6 h	12 h	24 h
Control	1[a]	1.09 ± 0.04[a]	1.94 ± 0.10[c]	5.87 ± 0.44[d]
DON	1[a]	1.32 ± 0.12[a]	0.99 ± 0.08[a]	2.51 ± 0.43[b]
Anti-CD-44	1[a]	1.01 ± 0.08[a]	1.00 ± 0.01[a]	2.16 ± 0.15[b]

Cumulus-oocyte complexes were cultured for 0, 6, 12 or 24 h with 1.0 mM DON or 5.0 mg/ml anti-CD44 antibody. Data are expressed as fold increases of MPF activity in oocytes just after collection from follicles, defined as 1. Experiments were replicated four times at least. Data represent mean ± standard deviation (SD). Different superscripts denote significant differences ($P<0.05$).

Table 2. Effects of 6-diazo-5-oxo-norleucine (DON) and anti-CD44 antibody on germinal vesicle breakdown (GVBD)

	Rate of GVBD (%)			
	0 h	6 h	12 h	24 h
Control	0[a]	2.5 ± 2.5[a]	4.8 ± 4.8[a]	63.8 ± 2.4[c]
DON	0[a]	2.4 ± 2.4[a]	4.5 ± 4.5[a]	19.0 ± 2.1[b]
Anti-CD44	0[a]	2.1 ± 2.1[a]	6.8 ± 6.8[a]	13.3 ± 7.0[b]

Cumulus-oocyte complexes were cultured for 0, 6, 12 or 24 h with 1.0 mM DON or 5.0 mg/ml anti-CD44 antibody. Experiments were replicated three times at least. Data represent mean ± SD. Different superscripts denote significant differences ($P<0.05$).

is significantly lower than that observed *in vivo*. Based on these observations, we speculate that the insufficient interaction of hyaluronan-CD44 during cumulus expansion *in vitro* may cause inferior fertilization and developmental capacities in oocytes compared to those matured *in vivo*.

Molecular Mechanism of Hyaluronan-CD44 Interaction for Oocyte Maturation

Since our findings suggested that the hyaluronan-CD44 interaction is involved in the induction of meiotic resumption, it was thought that CD44 was expressed in/on the oocytes. However, CD44 has been shown to be localized in cumulus cells, not in the oocyte, of the COCs by using reverse-transcription polymerase chain reaction (RT-PCR), western blotting, and immnunohistological staining [17, 35, 36]. Considering these results, we conclude that the hualuronan-CD44 interaction might function to promote the meiotic resumption of porcine oocytes through the cumulus cells.

Generally, the coordination of function between the oocyte and cumulus cells is mediated by cell-cell communication via gap junctions [37]. Early studies have shown that oocyte growth and development are strictly dependent upon the supply of nutrients transmitted from the follicle cells [38, 39]. Later studies have demonstrated that the meiotic maturation of oocytes is also subject to regulation by the somatic compartment of the ovarian follicle. MPF activation at the onset of meiotic resumption is inhibited by intra-oocyte cAMP, which is transferred from cumulus cells via gap junctional communication within COCs [37]. Interruption of gap junctions in the COCs, which occurs in response to the preovulatory surge of gonadotropins, leads to a drop in the intra-oocyte concentration of cAMP, followed by MPF activation and meiotic resumption [40–42]. We demonstrated that the reduction of the intra-oocyte cAMP concentration was suppressed by the inhibition of the interaction between hyaluronan and CD44 (Yokoo *et al.* unpublished data). This result supports the concept that hyaluronan-CD44 interaction is involved in the regulation of gap junctional communication and the termination of the flux of cAMP flux from cumulus cells to oocytes.

Gap junctions are specialized regions in closely opposed membranes of neighboring cells that allow cells to exchange small molecules, thus coordinating their activities. Each gap junction channel comprises two symmetrical hemispheres (termed "connexons") derived from two neighboring cells. The connexon comprises a hexagonal arrangement of six subunits of a protein named "connexin" (Cx). Connexins are encoded by members of a multigene family; they are defined by their molecular weight and share high homology. At present, at least 15 connexin genes have been reported in mammals and 7 genes (*Cx26, Cx30, Cx32, Cx37, Cx43, Cx45 and Cx60*) have been identified in the ovary. We examined the effects of the hyaluronan-CD44 interaction on the expression of Cx43, which is the most abundant Cx found in the ovarian follicle and in the COCs. Exposure of COCs to DON and anti-CD44 antibody had no effect on the expression of total Cx43 in the COCs. Conversely, these treatments significantly inhibited the tyrosine-phosphorylation of Cx43 in the COCs (Fig. 2). Previous studies have shown that a tyrosine kinase, such as $pp60^{Src}$, induces the tyrosine phosphorylation of Cx43 and inhibits intercellular junctional communication [43–47]. Therefore, it is suggested that hyaluronan-CD44 interaction controls the inhibition of Cx43 gap junctional communication in COCs. These findings strongly suggest that the hyaluronan-CD44 interaction during cumulus

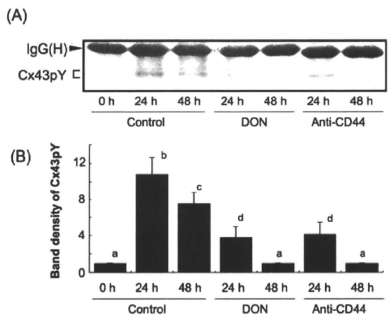

Fig. 2. Effects of 6-diazo-5-oxo-norleucine (DON) and anti-CD44 antibody on expression of Cx43. The cumulus-oocyte complexes (COCs) were cultured with 1.0 mM DON or 5.0 μg/ml anti-CD44 antibody. (A) Detection of tyrosine-phosphorylated Cx43 (Cx43pY). The extracts immunoprecipitated with anti-Cx43 antibody were probed with anti-phosphotyrosine antibody. Arrowhead means the band of heavy chain of immunoglobulin (IgG (H)). (B) Densitometric analysis of (A). Different superscripts denote significant differences ($P<0.05$). Data represent mean ± SD.

expansion induces disruption of the Cx43 gap junction in the COCs, inhibits the transport of cAMP from cumulus cells into oocytes, and leads to activation of MPF and meiotic resumption of oocytes (Fig. 3).

Concluding Remarks

Oocyte maturation is roughly divided into two types: nuclear maturation and cytoplasmic maturation. We have described herein the hyaluronan-CD44 interaction during cumulus expansion that concurrently controls the occurrence of meiotic resumption through the disruption of gap junctions in COCs. It has been demonstrated that volumetric expansion of COCs actively correlates, at least in the pig, with the progress of nuclear maturation. Although details of the mechanism controlling cytoplasmic maturation in mammals are still unclear, we believe that our findings as described here shed some light on the understanding of the cytoplasmic maturation process.

Recently, pigs have become increasingly important in the field of biomedical research and interest has grown in the use of transgenic pigs as potential xenograph donors. As most attempts to produce transgenic pigs by nuclear transfer/cloning techniques or pronuclear microinjection have used matured oocytes and early embryos, respectively, it is becoming more important to produce large numbers of developmentally competent oocytes and embryos. The developmental competence of porcine oocytes that are matured and fertilized *in vitro* has been enhanced by mimicking the active communication between the oocyte and follicular cells. Therefore,

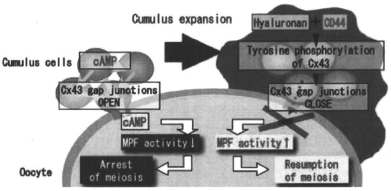

Fig. 3. Schematic representation of the oocyte maturation mechanism in the pig.

elucidation of the molecular mechanisms of oocyte maturation will enable substantial improvement of the quality of oocytes and embryos cultured *in vitro*.

During the past decade, new reproduction biotechnology based on molecular genetics, molecular biology, and embryology has rapidly developed. These techniques should enable us to increase the production of domestic animals. We now need to define issues and further develop the study of animal reproduction. The objective of our ongoing studies is to investigate in more detail the molecular mechanism of mammalian oocyte maturation and to develop an artificial technique for oocyte maturation *in vitro*.

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ORIGINAL ARTICLE

Early metaphase II oocytes treated with dibutyryl cyclic adenosine monophosphate provide suitable recipient cytoplasm for the production of miniature pig somatic cell nuclear transfer embryos

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ABSTRACT

We investigated the effects of *in vitro* maturation duration and treatment with dibutyryl cyclic adenosine monophosphate (dbcAMP) on the blind enucleation efficiency and developmental competence of miniature pig somatic cell nuclear transfer (SCNT) embryos. Oocytes were cultured for 22 h in NCSU-23 medium with or without 1 mM dbcAMP and then additionally cultured in dbcAMP-free NCSU-23 for 14, 18, or 22 h. Regardless of dbcAMP treatment, the rate of nuclear maturation reached a plateau at 36 and 40 h. However, mitochondrial distribution, a marker for cytoplasmic maturation, differed between the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h. The metaphase II chromosomes were adjacent to the first polar body in 68.8% and 63.5% of the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h, respectively. Furthermore, the blind enucleation efficiency by removing a small volume of cytoplasm was significantly higher in the dbcAMP-untreated oocytes at 36 h (82.9%) and dbcAMP-treated oocytes at 40 h (89.9%) than other groups. The rate of blastocyst formation was highest in the dbcAMP-treated oocytes at 40 h. Hence, this study demonstrated that dbcAMP-treated early metaphase II oocytes are suitable for the production of miniature pig SCNT embryos.

Key words: *enucleation, miniature pig, nuclear transfer, oocyte maturation.*

INTRODUCTION

Miniature pig cloning by somatic cell nuclear transfer (SCNT) has many potential biomedical applications such as xenotransplantation and the development of animal models for human diseases (Dai *et al.* 2002; Lai *et al.* 2002) because characteristics such as anatomy, physiology, and body size of the miniature pigs are similar to humans (Tucker *et al.* 2002). Despite recent successes in the miniature pig cloning technology, the efficiency of the procedure remains low (Lai & Prather 2003). One of the probable reasons for the low efficiency is the poor understanding of the factors determining the developmental ability of miniature pig SCNT embryos. Recently, we demonstrated that the factors determining *in vitro* developmental competence included the culture conditions for oocytes (Wakai *et al.* 2008), activation protocol (Yamanaka *et al.* 2007), and the presence of cytoskeletal inhibitors (Sugimura *et al.* 2008).

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For miniature pig cloning in particular, improving the *in vitro* maturation (IVM) of domestic pig oocytes is essential for ensuring a high production of SCNT embryos. Thus, the culture condition for oocytes that function as recipient cytoplasts is the main factor for a successful miniature pig cloning (Wakai *et al.* 2008). The enucleation of the IVM oocyte is a crucial step for subsequent development in cloned embryos (Liu *et al.* 2002; Simerly *et al.* 2004; Lee & Campbell 2006; Byrne *et al.* 2007). During enucleation, the position of the chromosomes is either indirectly determined by determining the location of the first polar body (PB) or directly determined by staining the oocytes with a DNA-specific dye (e.g., Hoechst 33342) and observing them under UV light (Li *et al.* 2004). However, the first PB often migrates from its place of origin and does not always remain in proximity to the chromosomes. This is because of oocyte aging that occurs as IVM progresses and/or the removal of cumulus cells by pipetting (Li *et al.* 2004; Miao *et al.* 2004). Recently, it has been speculated that Hoechst staining and the exposure of oocytes to UV light may disrupt functions in the cytoplast, especially the function of mitochondria, thus affecting subsequent development (Li *et al.* 2004; Byrne *et al.* 2007). Hence, a simple, facile and highly efficient enucleation technique, which does not require the visualization of DNA by exposure to UV light, is important for the high production of SCNT embryos exhibiting high developmental competence. A previous study revealed that the culture of activated oocytes in demecolcine resulted in efficient enucleation without the visualization of DNA by exposure to UV light. However, further studies on the reagent toxicity and further development are necessary for this method (Yin *et al.* 2002). Additionally, other previous studies have shown that the efficiency of 'blind' enucleation (without the visualization of DNA by exposure to UV light) of anaphase I (AI)-telophase I (TI) and AII-TII oocytes is very high because the chromosomes of these oocytes are located near the PB (Bordignon & Smith 1998; Nour & Takahashi 1999; Lee & Campbell 2006). However, these oocytes have low levels of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK), which may be the main factors involved in remodeling events, including nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) of G_0/G_1 phase donor somatic cells (Bordignon & Smith 1998; Lee & Campbell 2006). On the other hand, the distance between oocyte chromosome and first PB increases as the oocyte ages (Miao *et al.* 2004). Hence, it is hypothesized that metaphase II (MII) oocytes from which the first PB has just been extruded, which are termed as early MII oocytes, may exhibit high enucleation efficiency and high occurrence of NEBD and PCC after SCNT.

Other than enucleation, the homogeneity in the oocyte quality with respect to the cytoplasmic maturation status is important for the high production of SCNT embryos exhibiting high developmental competence. The heterogeneity of porcine oocytes is caused by spontaneous maturation, which is associated with a decrease in the intracellular concentration of cyclic adenosine monophosphate (cAMP) and subsequent inactivation of protein kinase A (PKA), may adversely affect the coordination between nuclear and cytoplasmic maturation, resulting in decreased developmental potential (Kim & Menino 1995; Bagg *et al.* 2006).

Previously, the treatment of prepubertal porcine oocytes for 20–22 h with dibutyryl cAMP (dbcAMP), a membrane permeable analog of cAMP, has been shown to inhibit GV breakdown (GVBD) and increase the incidence of blastocyst formation after IVF (Funahashi *et al.* 1997; Somfai *et al.* 2003). Thus, treatment with dbcAMP is considered helpful because it may improve the synchronization between nuclear and cytoplasmic maturation. However, little information is available on the beneficial effects of dbcAMP on cytoplasmic maturation.

In the present study, to gain information on the optimal IVM conditions of oocytes, we examined the effect of IVM duration and dbcAMP treatment for the first 22 h of IVM on the efficiency of blind enucleation, developmental competence after SCNT and mitochondrial distribution.

MATERIALS AND METHODS

Donor cells

Miniature pig fetuses (CSK, Suwa, Japan) were collected from sows at day 56 of pregnancy. Each fetus was decapitated and eviscerated. The remaining tissues were washed in Dulbecco's phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO, USA) and then digested with 0.1% (w/v) trypsin-EDTA (Sigma) for 45 min at 38.5°C. After digestion, fetal fibroblast cells were collected and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma). The culture medium was changed every 2 days until confluence was attained. Then the cells were harvested by treatment with 0.1% (w/v) trypsin in PBS containing 0.5 mmol/L EDTA for 5 min at 38.5°C, frozen with a cryoprotectant (Cellbanker; Zenyaku, Tokyo, Japan), and stored in liquid nitrogen (passage 0). Before starting the experiments, the cells derived

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from a single fetus were thawed and cultured in DMEM supplemented with 10% (v/v) FBS and used between passages 4 and 9.

IVM of oocytes

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory within 2 h in a container containing warm saline. Porcine oocytes were aspirated from antral follicles of diameter 3–6 mm using a 5-mL disposable syringe attached to an 18-gauge needle. Cumulus-oocyte complexes (COCs) with uniform ooplasm and compact cumulus cell mass were selected in PB1 (Quinn *et al.* 1982). After washing in PB1, the COCs were cultured in bovine serum albumin (BSA)-free NCSU-23 medium containing 10 IU/mL pregnant mares serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan), 0.1 mg/mL cysteine (Sigma), 1 mmol/L dbcAMP (Sigma) and 10% (v/v) porcine follicular fluid (Miyoshi *et al.* 2000) supplemented with or without 1 mmol/L dbcAMP (Sigma) (Funahashi *et al.* 1997) for 22 h at 38.5°C in a highly humidified atmosphere of 5% CO_2 in air and then transferred into the same medium, but without hormonal supplements and dbcAMP, for an additional 10, 14, 18 or 22 h of culture. After culturing, expanded cumulus cells of the COCs were removed by vortexing in PB1 containing 1 mg/mL hyaluronidase (Sigma) and oocytes showing the first PB under a stereomicroscope were selected as mature oocytes. The mature oocytes were transferred to PB1 and used for the experiments.

Oocyte enucleation

The cumulus-free oocytes were transferred into PB1 containing 1 mmol/L sucrose and 2.5 μg/mL cytochalasin D (Sigma). Enucleation of oocytes was performed by the blind method (without the visualization of DNA by exposure to UV light). Oocyte enucleation was performed by aspirating the first PB and the adjacent cytoplasm with a pipette using a piezo-drive unit (Primetech, Tokyo, Japan). The enucleated oocytes were washed thrice and transferred to a droplet of NCSU-23 medium for the subsequent microinjection of the donor nucleus.

Nuclear transfer

The cells were thawed and cultured for 1 week after reaching confluence, and a single-cell suspension was prepared prior to SCNT. Most of these cells were identified as being the G0/G1 phase of the cell cycle by analysis with a flow cytometry (Sugimura *et al.* 2008). The prepared donor cells were transferred to a 50-μL droplet of PB1, and their plasma membranes were damaged by repeated gentle aspirations using an injection pipette of 10 μm diameter. The denuded nucleus was microinjected into the cytoplasm of the enucleated oocyte.

Activation and culture of SCNT embryos

The embryos were activated using the protocol reported by Yamanaka *et al.* (2007). At 3 h after microinjection, SCNT embryos were activated by ionomycin (Sigma) and cycloheximide (Sigma). In order to activate the SCNT embryos with ionomycin, they were treated with 15 μmol/L ionomycin in NCSU-23 medium for 20 min at 38.5°C in a humidified atmosphere of 5% CO_2 in air and then washed 5 times with NCSU-23 medium. After the treatment, the SCNT embryos were cultured in NCSU-23 medium containing 5 μg/mL cycloheximide and 2.5 μg/mL cytochalasin D for 5 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air and then washed 5 times with cycloheximide and cytochalasin D-free NCSU-23 medium. Finally, the embryos were transferred to NCSU-23 medium and cultured at 38.5°C in an atmosphere of 5% CO_2 in air.

Experimental design

In **Experiment 1**, we studied the effect of dbcAMP treatment during IVM on the time-dependent changes in the nuclear maturation rate of oocytes. Oocytes were cultured in NCSU-23 medium with or without dbcAMP for 22 h and then without dbcAMP for an additional period of 10, 14, 18, or 22 h. In order to evaluate the nuclear maturation rate, after the culture for each period, the oocytes were mounted, fixed for 48 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and observed under a phase-contrast microscope.

In **Experiment 2**, we determined the location of MII chromosomes with respect to the location of the first polar body in untreated oocytes and oocytes treated with dbcAMP. Oocytes were examined at 36, 40 or 44 h of IVM. After staining the samples with 0.1 mg/mL Hoechst 33342 for 5–10 min, the location of first polar body relative to that of the MII chromosome was determined.

In **Experiment 3**, we determined the efficiency of blind enucleation in porcine oocytes treated with dbcAMP. The success rates for enucleation were assessed at 36, 40, and 44 h of IVM. Either 10% or 30% of the cytoplasm surrounding the first PB was removed by an enucleation pipette. Then, cytoplasts were stained with 0.1 mg/mL Hoechst 33342 for 5–10 min, and the enucleation efficiency was determined.

In **Experiment 4**, we studied the effect of the maturation period and treatment of dbcAMP on the *in vitro* development of SCNT embryos. Oocytes from which 10% cytoplasm was removed along with the nuclear material at 36, 40, or 44 h of IVM were used as recipient cytoplasts. PCC and pseudo pronucleus (pPN) formation were examined by orcein staining as described in Experiment 1 at 3 h after SCNT and 6 h of *in vitro* cultivation, respectively. The number of cleaved SCNT embryos was determined on day 2 of *in vitro* cultivation, and the number of SCNT embryos that developed to the blastocyst stage was determined on days 7 of *in vitro* cultivation. The total cells number of blastocysts stained with 0.1 mg/mL Hoechst 33342 for 5–10 min was determined on day 7.

In **Experiment 5**, we studied the mitochondrial distribution in untreated and dbcAMP-treated porcine oocytes. Oocytes were examined at 36, 40, or 44 h of IVM. First, the oocytes were stained with 10 μg/mL rhodamine 123 (Molecular Probes, Eugene, OR, USA) in PB1. Then, the oocytes were mounted on glass slides and immediately observed under a confocal scanning laser microscope (Hara *et al.* 2005). The fluorescence intensity depended on the mitochondrial membrane potential.

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Statistical analysis

There were at least three replicates for each experiment. The data for the rate of pronuclear formation, cleaved and blastocyst formed embryos, and mitochondrial distribution were analyzed by the chi-square test. Other data were analyzed by the analysis of variance (ANOVA); post hoc analysis was performed by the Bonferroni/Dunn test ($P < 0.05$).

RESULTS

Experiment 1. The time-dependant changes in the nuclear maturation rate in untreated and dbcAMP-treated oocytes

We examined the time-dependent changes in the nuclear maturation rate. The rates of nuclear maturation were examined at 32 (22 + 10), 36 (22 + 14), 40 (22 + 18), and 44 (22 + 22) h (Fig. 1). In the dbcAMP-untreated group, the rate of nuclear maturation was significantly lower (47.3 ± 4.6%) at 32 h IVM than other maturation periods and reached a plateau at 36 h (73.3 ± 8.1%). On the other hand, in the dbcAMP-treated group, the rate was significantly lower at 32 h (3.8 ± 0.8%) and 36 h (24.2 ± 3.1%) than other periods and reached a plateau at 40 h (88.8 ± 3.2%). The rate of MII did not differ significantly between the dbcAMP-untreated and dbcAMP-treated groups at 40 and 44 h. These results indicate that dbcAMP treatment does not affect the proportion of oocytes that attain nuclear maturation, but it affects the time required to attain maturity.

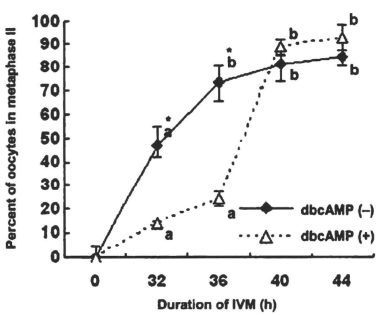

Figure 1 Incidence of meiotic progression to the MII stage during IVM in untreated and dbcAMP-treated porcine oocytes. The number of oocytes examined for each IVM period ranged from 79 to 150 in 3 replicate experiments. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups. Values are mean ± S.E. Values marked with different alphabets are significantly different ($P < 0.05$).

Experiment 2. Chromosome location of MII oocytes relative to that of the first PB in untreated and dbcAMP-treated oocytes

We determined the location of the MII chromosome relative to the first PB (Fig. 2 and 3). As shown in Figure 3, the chromosome location of MII oocytes was classified into 1 of 3 groups according to the angle formed by 2 lines: 1 line from the oocyte center to the first PB and 1 line from the center to the MII chromosome (0°–5°, 6°–30°, and 31°–180°). In both the dbcAMP-untreated and dbcAMP-treated groups, the percent of oocytes with angles of 0°–5° decreased as the IVM duration increased, and the percent was significantly higher in the dbcAMP-untreated oocytes at 36 h (69.0 ± 4.9%) and dbcAMP-treated oocytes at 40 h (63.5 ± 9.8%) than other groups ($P < 0.05$). In contrast, the percent of oocytes with angles of 6°–30° and 31°–180° increased as the IVM duration increased and these percents were significantly lower in the dbcAMP-untreated oocytes at 36 h (6°–30°, 21.3 ± 5.4% and 31°–180°, 9.7 ± 0.5%) and dbcAMP-treated oocytes at 40 h (6°–30°, 28.7 ± 6.0% and 31°–180°, 5.3 ± 1.0%) than other groups.

Experiment 3. Efficiency of blind enucleation in untreated and dbcAMP-treated oocytes

We determined the efficiency of blind enucleation (Fig. 4). In both the untreated and dbcAMP-treated groups, the enucleation efficiency decreased as the IVM duration increased. On removing 10% cytoplasm with MII chromosomes, the enucleation efficiency was significantly higher in the dbcAMP-untreated oocytes at 36 h (82.9 ± 8.2%) and dbcAMP-treated oocytes 40 h (89.9 ± 3.3%) than in the other groups (dbcAMP-untreated oocytes at 40 h: 58.0 ± 10.5%; dbcAMP-untreated oocytes at 44 h : 45.1 ± 2.2%; dbcAMP-treated oocytes at 44 h: 64.3 ± 3.4%). Moreover, the efficiency did not differ significantly with difference in the volume of the cytoplasm removed for the dbcAMP-unteated oocytes at 36 h, dbcAMP-treated oocytes at 40 h and dbcAMP-treated oocytes at 44 h.

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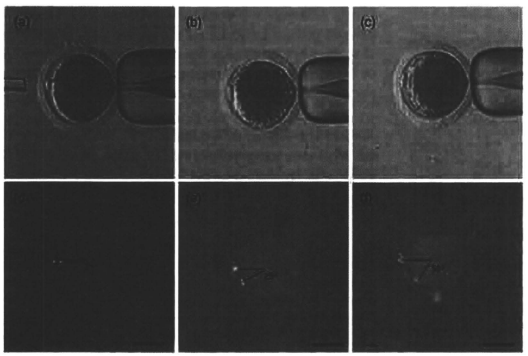

Figure 2 Time-dependent changes in the location of the MII chromosome relative to that of the first PB in untreated and dbcAMP-treated porcine oocytes. The number of oocytes examined for each IVM period ranged from 106 to 145 in 3 replicate experiments. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups. Values are mean ± S.E. Values marked with different alphabets (a and b) are significantly different in the same group of chromosome location ($P < 0.05$).

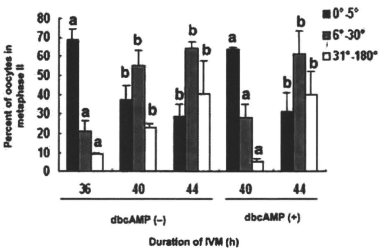

Figure 3 Representative photographs of porcine MII oocytes with different locations of the meiotic chromosome relative to that of the first PB. The chromosomes were visualized by staining with Hoechst 33342. These locations, determined by measuring the angle between a line connecting the oocyte centre with the meiotic nucleus and a line connecting the oocyte centre with the first PB, are 2° in (A) and (a), 25° in (B) and (b), and 50° in (C) and (c). These images represent the 3 oocyte groups with respect to the chromosome location (0–5°, 6–30°, and 31–180°, respectively). Bar = 60 μm.

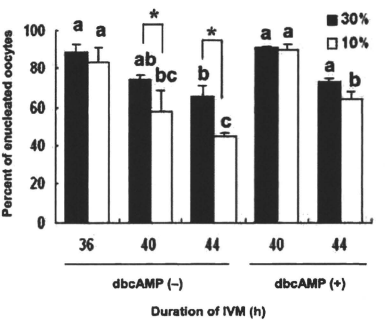

Figure 4 The efficiency of bind enucleation in untreated and dbcAMP-treated porcine oocytes. The number of oocytes examined for each IVM period ranged from 116 to 143 in 3 replicate experiments. In oocytes of the 30% and 10% group, 30% and 10% of the cytoplasm beneath the first PB was removed using an enucleation pipette of 20-μm diameter, respectively. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups. Values marked with different alphabets (a–c) are significantly different in the same groups (10% or 30% groups) and those marked with an asterisk (*) are different between the 2 groups ($P < 0.05$).

Experiment 4. Effect of maturation period and dbcAMP treatment on the in vitro development of SCNT embryos

First, we examined the occurrence of PCC at 3 h after SCNT (Fig. 5). The occurrence of PCC did not differ significantly in the dbcAMP-untreated oocytes at 36 h, dbcAMP-untreated oocytes at 40 h, dbcAMP-treated oocytes at 40 h and dbcAMP-treated oocytes at 44 h, but it was significantly lower in the dbcAMP-treated oocytes at 44 h (50.2 ± 5.4%) than other treatment groups (dbcAMP-untreated oocytes at 36 h and

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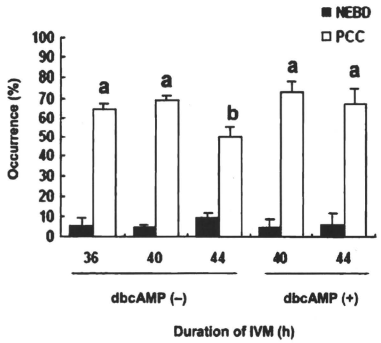

Figure 5 The occurrence of PCC and NEBD in SCNT embryos. At 3 h after nuclear injection, PCC and NEBD were observed. The number of SCNT embryos examined for each IVM period ranged from 60 to 116 in 3 replicate experiments. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups.Values are shown as the mean ± S.E. Values marked with different alphabets (a and b) are significantly different ($P < 0.05$).

dbcAMP-untreated oocytes at 40 h: 64.4 ± 2.6% and 69.2 ± 2.3%, respectively; dbcAMP-treated oocytes at 40 h and dbcAMP-treated 44 h: 73.5 ± 5.3% and 67.5 ± 6.9%, respectively).

Next, pPN and pseudo polar body (pPB) was observed at 6 h of *in vitro* culture (Table 1). The rate of activated oocytes did not differ significantly between the groups. However, 2pPN0pPB formation rate was significantly higher in the dbcAMP-untreated oocytes at 36 h (46.3%) and dbcAMP-treated oocytes at 40 h (47.2%) than in the dbcAMP-untreated oocytes at 44 h (22.9%).

Finally, we examined the *in vitro* developmental competence (Table 2). The rate of cleaved embryos did not differ significantly among all groups. However, the rate of blastocyst formation was significantly higher in the dbcAMP-treated oocytes at 40 h (26.4%) than in the dbcAMP-untreated oocytes at 36 h (10.3%) and dbcAMP-untreated oocytes at 44 h (11.2%).

Experiment 5. Mitochondrial distribution in untreated and dbcAMP-treated oocytes

We examined the mitochondrial distribution as a marker of cytoplasm maturation (Table 3). The pattern of mitochondrial distribution was categorized into 1 of the 4 types shown in Figure 6: peripheral (type I), semiperipheral (type II), diffused (type III), and weak (type IV). The distribution was more homogenous in oocytes of the dbcAMP-treated groups than in oocytes of the dbcAMP-untreated groups. The proportion of MII oocytes exhibiting the type II distribution pattern was significantly higher in the dbcAMP-untreated oocytes at 36 h (34.0%) than in the dbcAMP-untreated oocytes at 44 h (13.2%), dbcAMP-treated oocytes at 40 h (14.3%) and dbcAMP-treated oocytes at 44 h (12.9%). The proportion of MII oocytes exhibiting the type III distribution pattern was significantly higher in the dbcAMP-treated oocytes at 40 h (82.9%) and dbcAMP-treated oocytes 44 h (87.1%) than in the untreated oocytes at 36 h (54.0%) and untreated oocytes at 44 h (65.8%).

DISCUSSION

In the present study, in order to produce homogenous and high quality recipient cytoplasts, we evaluated the effects of IVM duration and dbcAMP treatment for the first 22 h of IVM on the following factors: the rate of nuclear maturation, mitochondrial distribution, chromosome location of MII oocytes relative to that of the first PB, efficiency of blind enucleation, and developmental competence of miniature pig SCNT embryos.

Enucleation is the key step in the preparation of a large number of high quality recipient cytoplasts. A highly efficient enucleation method, which does not involve the use of UV radiation and DNA-specific dyes, has been in demand. In the present study, we demonstrated that dbcAMP-untreated oocytes at 36 h of IVM and dbcAMP-treated oocytes at 40 h of IVM exhibit a high efficiency of blind enucleation. On the other hand, previous studies have indicated that the efficiency of blind enucleation is high in the AI-TI (Lee & Campbell 2006) and AII-TII (Bordignon & Smith 1998) oocytes compared to MII oocytes. However, it has been reported that the MPF and MAPK levels in these oocytes are lower than the levels in MII oocytes (Lee & Campbell 2006). It has been suggested that MPF and MAPK are important for the successful occurrence of nuclear remodeling events such as NEBD and PCC, which may be essential for the development of SCNT embryos with donor cells in the G_0/G_1 phase (Tani *et al.* 2003). Therefore, AI-TI or AII-TII stage oocytes were considered unsuitable for the induction of proper nuclear remodeling events, including NEBD and PCC, in the G_0/G_1 donor cells. In

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Table 1 Effect of IVM duration and dbcAMP treatment on pseudo-pronucleus formation of miniature pig SCNT embryos

dbcAMP	IVM duration	No. of oocytes used	No. (%) of activated oocytes	No. (%) of SCNT embryos having		
				1 pPN† and 1 pPB‡	1 pPN and 0 pPB	2 pPN and 0 pPB
Untreated	36	60	41 (63.3)	1 (2.4)	21 (51.2)	19 (46.3)[a]
	40	52	38 (73.1)	1 (2.6)	21 (55.3)	16 (42.1)[ab]
	44	50	35 (74.0)	2 (5.7)	24 (68.6)	8 (22.9)[b]
Treated	40	55	36 (65.5)	0 (0)	19 (52.8)	17 (47.2)[a]
	44	46	31 (67.4)	1 (3.2)	18 (58.1)	12 (38.7)[ab]

[a,b]Values with different superscripts are significantly different ($P < 0.05$). †Pseudo pronucleus. ‡Pseudo polar body.

Table 2 Effect of IVM duration and dbcAMP treatment on the development of miniature pig SCNT embryos

dbcAMP	IVM duration (h)	No. (%) of cultured oocytes	No. (%) of cleaved oocytes	No. (%) of blastocysts formed	Total cell number (mean ± SD)
Untreated	36	117	54 (46.2)	12 (10.3)[a]	26.6 ± 4.9
	40	93	63 (67.7)	17 (18.3)[ab]	30.1 ± 8.3
	44	98	50 (51.0)	11 (11.2)[a]	26.5 ± 4.8
Treated	40	110	72 (65.5)	29 (26.4)[b]	30.5 ± 3.5
	44	92	59 (64.1)	17 (18.5)[ab]	27.3 ± 5.0

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

Table 3 Mitochondrial distribution in untreated and dbcAMP-treated porcine oocytes

dbcAMP	IVM duration (h)	No. of oocytes used	No. (%) of oocytes having a distribution pattern of			
			Type I	Type II	Type III	Type IV
Untreated	36	50	5 (10.0)	17 (34.0)[a]	27 (54.0)[a]	1 (2.0)
	40	38	1 (2.6)	8 (21.1)[ab]	29 (76.3)[ab]	1 (2.6)
	44	38	4 (10.5)	5 (13.2)[b]	25 (65.8)[a]	4 (10.5)
Treated	40	35	1 (2.9)	5 (14.3)[b]	29 (82.9)[b]	0 (0)
	44	31	0 (0)	4 (12.9)[b]	27 (87.1)[b]	0 (0)

[a,b]Values with different superscripts are significantly different in same type of mitochondrial distribution ($P < 0.05$).

contrast, we used the dbcAMP-untreated oocytes at 36 h of IVM and dbcAMP-treated oocytes at 40 h of IVM and found that the frequency of oocytes undergoing PCC was high, which may be induced by high MPF and MAPK activities in the cytoplasts (Kawahara *et al.* 2005; Lee & Campbell 2006; Wakai *et al.* 2008). Additionally, it has been suggested that oocytes having high MPF activity may effectively induce the formation of 2 pPN from PCC with normal bipolar spindle, and SCNT embryos having 2 pPN induce proper mitotic division, resulting in high developmental potential (Ng *et al.* 2004; Wakai *et al.* 2008). In the present study, several SCNT embryos having 2 pPN were observed when the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM were used, indicating that the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM may be suitable for the induction of proper nuclear remodeling events, including NEBD, PCC, and pPN formation.

Moreover, we found that the use of the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM as recipients enabled blind enucleation by removing a smaller volume of cytoplasm. It has been suggested that appropriate cytoplasm volume is crucial for further normal development after reconstruction (Lee & Campbell 2006). Additionally, the removal of a smaller volume of cytoplasm may result in the loss of a smaller percentage of oocyte proteins that are associated with the meiotic spindle (Lee & Campbell 2006). A previous study on non-human primates suggested that proteins associated with the chromatin and spindle may vary between oocytes arrested in the early MII phase (pre-MII oocytes) and

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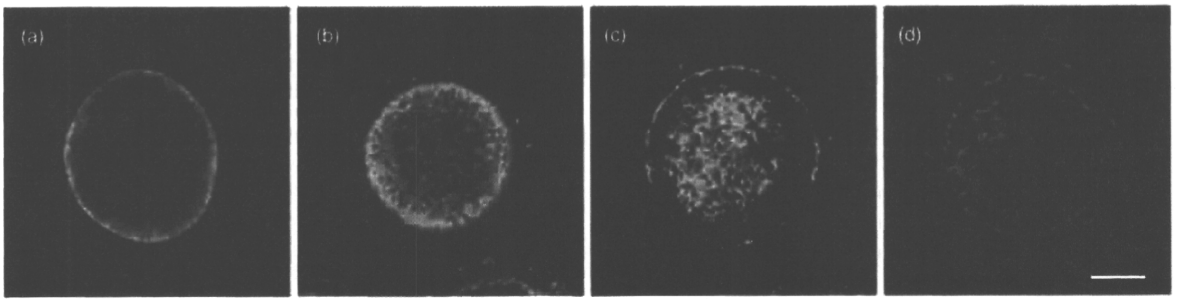

Figure 6 Representative confocal microscopy images of equatorial sections of porcine MII oocytes illustrating the different types of mitochondrial distributions: (A) type I (peripheral), (B) type II (semiperipheral), (C) type III (diffused), and (D) type IV (weak). In type I, the mitochondria were distributed in the peripheral region of cytoplasm; in type II, in the semiperipheral region of the cytoplasm resulting in small spots of florescence; in type III, in the inner region of the cytoplasm resulting in bigger aggregated spots of florescence; and in type IV, in the inner region of the cytoplasm resulting in weaker spots of florescence. Bar = 60 μm.

late MII oocytes from which the PB has been extruded (Simerly *et al.* 2004). In that study, Simerly *et al.* (2004) indicated that pre-MII meiotic spindle-chromosome complex (SCC), leaving some nuclear mitotic apparatus (NuMA), Eg5 and human spleen embryonic tissue and testis (HSET) mitotic molecular motor protein, which are responsible for spindle pole assembly, remaining which in the ooplasm. The lack of these proteins results in the failure of normal mitotic division in SCNT embryos of non-human primates (Simerly *et al.* 2003, 2004). Additionally, in mouse SCNT embryos, the removal of SCC along with several proteins such as polo-like kinase 1 (PLK1), translationally controlled tumor protein (TCTP), and calmodulin (CaM) has been suggested to increase the risk of mitotic errors (Miyara *et al.* 2006). Hence, the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM, in which blind enucleation is possible by the removal of a smaller volume of cytoplasm and a lower amount of the main proteins involved in mitotic progression, may be beneficial for the developmental competence of the SCNT embryos (Lee & Campbell 2006).

Interestingly, although both the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM have high competence of PCC and 2 pPN formation following SCNT, a significant difference was observed in the blastocyst formation rate between SCNT embryos formed using oocytes of these 2 types: the rate was higher for the latter group. Moreover, the time taken for the maturation rate to reach a plateau differed in the dbcAMP-untreated and dbcAMP-treated groups: the blastocyst formation rate was highest when oocytes at 40 h of IVM were used from both groups. Although more than 4 h had lapsed from the time of MII arrest in the dbcAMP-untreated oocytes at 40 h of IVM, MII arrest had just occurred in the dbcAMP-treated oocytes at 40 h of IVM. Therefore, these observations suggest that the synchronization between nuclear and cytoplasmic maturation may be better in the dbcAMP-treated oocytes than in the dbcAMP-untreated oocytes, and such synchronization is necessary for blastocyst formation in SCNT embryos.

In order to gain further understanding of the synchronization between nuclear and cytoplasmic maturation, we evaluated the mitochondrial distribution as a marker for cytoplasmic maturation. In a previous study, Brevini *et al.* (2005) indicated that most oocytes with high developmental competence exhibited a diffused pattern of mitochondrial distribution and suggested that diffused mitochondria are a marker of cytoplasmic maturation and are strongly associated with high developmental ability. In the present study, we also observed that 82.9% of the oocytes in the dbcAMP-treated oocytes at 40 h of IVM with a high developmental ability exhibited a diffused pattern of mitochondrial distribution. On the other hand, only 54.0% of the oocytes in the dbcAMP-untreated oocytes at 36 h of IVM exhibited a diffused mitochondrial distribution. This observation indicated that the dbcAMP-treated oocytes at 40 h of IVM have homogenous cytoplasm, which may indicate that the cytoplasm is mature and can efficiently function as recipient cytoplast during production of miniature pig SCNT embryos, whereas the dbcAMP-untreated oocytes at 36 h of IVM have heterogeneous cytoplasm,

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which may indicate that the cytoplasm is immature for functioning as a recipient cytoplast. Although the reasons for the improvement in the mitochondrial distribution by dbcAMP treatment are unclear, it is known that cAMP, A-kinase anchoring proteins (AKAPs), and the proteasome pathway play important roles in the regulatory mechanism of mitochondrial dynamics (Carlucci *et al.* 2008). Moreover, Kim *et al.* (2008) showed that the mitochondrial membrane potential is higher in SCNT blastocyst embryos derived from oocytes treated with dbcAMP than in SCNT embryos derived from the dbcAMP-untreated oocytes. Thus, these reports confirm that dbcAMP treatment during IVM may be beneficial to cytoplasmic maturation for supporting development of miniature pig SCNT embryos including mitochondrial distribution.

In conclusion, the present study demonstrated that the cytoplasm of dbcAMP-treated oocytes at 40 h of IVM, which are defended as early MII oocytes (Cheong *et al.* 2000; Ikeda & Takahashi 2001), is suitable for functioning as the recipient cytoplast during the production of miniature pig SCNT embryos, because of the homogeneity of cytoplasm, high efficiency of blind enucleation by the removal of a small volume of cytoplasm, and high developmental potential up to the blastocyst stage. Furthermore, these observations will help improve the miniature pig SCNT technique and cloning efficiency significantly.

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