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—Original Article—

Changes in the Expression Patterns of the Genes Involved in the Segregation and Function of Inner Cell Mass and Trophectoderm Lineages During Porcine Preimplantation Development

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Abstract. In mouse embryos, segregation of the inner cell mass (ICM) and trophectoderm (TE) lineages is regulated by genes, such as *OCT-4*, *CDX2* and *TEAD4*. However, the molecular mechanisms that regulate the segregation of the ICM and TE lineages in porcine embryos remain unknown. To obtain insights regarding the segregation of the ICM and TE lineages in porcine embryos, we examined the mRNA expression patterns of candidate genes, *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc* and *FGFR2-IIIc*, in blastocyst and elongated stage embryos. In blastocyst embryos, the expression levels of *OCT-4*, *FGF4* and *FGFR1-IIIc* were significantly higher in the ICM than in the TE, while the *CDX2*, *TEAD4* and *GATA3* levels did not differ between the ICM and TE. The expression ratio of *CDX2* to *OCT-4* (*CDX2*/*OCT-4*) also did not differ between the ICM and TE at the blastocyst stage. In elongated embryos, *OCT-4*, *NANOG*, *FGF4* and *FGFR1-IIIc* were abundantly expressed in the embryo disc (ED; ICM lineage), but their expression levels were very low in the TE. In contrast, the *CDX2*, *TEAD4* and *GATA3* levels were significantly higher in the TE than in the ED. In addition, the *CDX2*/*OCT-4* ratio was markedly higher in the TE than in the ED. We demonstrated that differences in the expression levels of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc* and *FGFR2-IIIc* genes between ICM and TE lineages cells become more clear during development from porcine blastocyst to elongated embryos, which indicates the possibility that in porcine embryos, functions of ICM and TE lineage cells depend on these gene expressions proceed as transition from blastocyst to elongated stage.

Key words: Elongated embryo, Gene expression, Inner cell mass, Porcine embryo, Trophectoderm

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The pig has attracted increasing attention as a suitable source for xenotransplantation, as a transgenic animal to produce specific proteins and as a biomedical model for the study of human physiology and pathology. Successful piglet production from *in vitro* produced embryos, such as those produced by *in vitro* fertilization (IVF) or somatic cell nuclear transfer [1, 2], has accelerated these processes. However, *in vitro* production (IVP) of porcine embryos is still inefficient compared with in other mammals, such as mice and cattle, because of the low rate of development to the blastocyst stage and the poor blastocyst quality [3]. One of the reasons for the developmental retardation of the porcine IVP system is limited knowledge of the molecular mechanisms for early embryonic development. Therefore, in order to improve the IVP system for porcine embryos, it is important to perform a further basic research on molecular mechanisms that regulate the early embryonic development.

Differentiation of unspecialized cells into other cell types is a crucial process of development. Thus, understanding the molecular mechanisms governing lineage segregation during early embryonic development is critical to dissect fundamental developmental pathways. In early mammalian development, the first lineage segregation occurs during the transition from the morula to blastocyst stage when blastomeres differentiate into the inner cell mass (ICM) and the trophectoderm (TE). The ICM is a group of pluripotent cells attached to the inside of the TE that gives rise to the embryonic tissue comprising the ectoderm, mesoderm and endoderm [4]. On the other hand, the TE is a single layer of polarized cells surrounding the blastocoel, which gives rise to the embryonic portion of the placenta [5, 6]. The segregation of the ICM and TE lineages is regulated by the interaction of various genes. In mouse embryos, the transcription factors, POU domain class 5 transcription factor 1 (OCT-4) and Caudal-related homeobox 2 (CDX2) play pivotal roles in the segregation of the ICM and TE [7–9]. Recently, interaction between NANOG and CDX2 was observed [10], indicating that NANOG is also involved in regulating the segregation of the ICM and TE. In addition, TEA domain family transcription factor 4 (TEAD4) and GATA binding protein 3 (GATA3) have been identified as important factors for TE development that act upstream of *CDX2* [11–13]. Furthermore, fibroblast growth factor 4 (FGF4) is required for functional ICM formation [14], and FGF4-FGF receptor (FGFR)-2 signaling plays an important role in proliferation and differentiation of TE cells [15, 16].

As just described, molecular mechanisms that regulate the seg-

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regation of the ICM and TE lineages have been well characterized in mouse embryos. However, little information is available for the segregation of the ICM and TE lineages in porcine embryos. Recently, some researchers reported that in contrast to the mouse, *OCT-4* expression does not appear to be restricted to the ICM, even in expanded blastocysts in pigs and cattle [17–20]. These findings led us to expect a difference in the molecular mechanisms that regulate the segregation of the ICM and TE lineages between species.

Patterns of preimplantation development and implantation differ remarkably between mammalian species. For instance, mouse embryos invasively implant at the blastocyst stage, and then form an egg cylinder. On the other hand, porcine blastocysts elongate before implantation, transforming from a spherical to ovoid shape and then subsequently into a long thin filament [21]. These developmental differences may influence the mechanisms that regulate the segregation of the ICM and TE lineages. Therefore, in order to obtain better understanding of the molecular mechanism responsible for the segregation of the ICM and TE lineages in porcine embryos, it is necessary to study changes in the expression of genes during preimplantation development including the elongated stage. In the present study, we comprehensively examined the mRNA expression patterns of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc* and *FGFR2-IIIc*, which are well known as principal factors responsible for segregation of the ICM and TE lineages of mouse embryos in porcine blastocyst and elongated stage embryos.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Oocyte collection and in vitro maturation

Ovaries from prepubertal gilts were obtained at a local slaughterhouse and transported to the laboratory at 37 C. Cumulus-oocyte complexes (COCs) were collected from follicles 2–6 mm in diameter in TCM 199 medium supplemented with 10% (v/v) fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA), 20 mM Hepes, 0.68 mM L-glutamine, 100 U/ml penicillin G potassium (Meiji Seika, Tokyo, Japan) and 100 mg/ml streptomycin sulfate (Meiji Seika). *In vitro* maturation of oocytes was essentially performed according to a previous study [2]. In brief, approximately 50 COCs with uniform ooplasm and a compact cumulus cell mass were cultured separately in 500 µl of maturation medium, a modified North Carolina State University (NCSU)-37 (mNCSU-37 [22]) solution containing 10% porcine follicular fluid, 0.6 mM cysteine, 0.05 mM β-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP), 10 IU/ml pregnant mare serum gonadotropin (PMS 1000, ZENOAQ, Nippon Zenyaku Kogyo, Koriyama, Japan), and 10 IU/ml human chorionic gonadotropin (hCG; Puberogen 1500, Novartis, Tokyo, Japan), in four-well dishes (Nunclon Multidishes; Nalge Nunc International, Denmark) for 20 h. The COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for 24 h. The maturation culture was carried out at 39 C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2.

In vitro fertilization

After *in vitro* maturation, COCs were washed three times with modified Pig-FM (mPig-FM) medium [2], and 15–20 COCs were transferred into a 90 µl volume of mPig-FM medium. Cryopreserved semen was thawed, and spermatozoa were washed twice by centrifugation (at 1800 rpm for 3 min) in sperm washing medium (TCM 199 medium supplemented with 20 mM Hepes, 0.68 mM L-glutamine, 100 U/ml penicillin G potassium, 100 mg/ml streptomycin sulfate, 0.91 mM sodium pyruvate, 4.12 mM calcium lactate, 3.0 mM glucose and 10% [v/v] FCS) adjusted to pH 7.8 [23]. The spermatozoa were resuspended in the sperm washing medium, and 10 µl of this suspension was added to 90 µl of mPig-FM containing matured COCs. The final sperm concentration was adjusted to 1.0×10^7/ml. COCs and sperm were incubated for 12 h at 39 C under a 5% CO_2, 5% O_2, and 90% N_2 atmosphere. Following incubation with sperm, presumptive zygotes were freed from the cumulus cells and attached spermatozoa.

In vitro culture of fertilized embryos

Following IVF, presumptive zygotes were cultured in mNCSU-37 supplemented with 2.7 mM sodium lactate, 0.17 mM sodium pyruvate, 0.05 mM β-mercaptoethanol and 4 mg/ml BSA (fraction V) at 39 C under a 5% CO_2, 5% O_2 and 90% N_2 atmosphere. On day 2 (the day of IVF was considered day 0), embryos were transferred to mNCSU-37 media supplemented with 5.56 mM glucose, 0.05 mM β-mercaptoethanol and 4 mg/ml BSA and cultured at 39 C under a 5% CO_2, 5% O_2 and 90% N_2 atmosphere until day 7.

Production of elongated embryos

All experiments using animals were approved by the Animal Ethics Committee, Hokkaido Animal Research Center, Shintoku, Japan. Prepubertal gilts approximately 205 days of age were superovulated and mated using artificial insemination (AI) for *in vivo* elongated embryo production. A dose of 1500 IU/gilt equine chorionic gonadotropin (eCG; Yell Pharmaceutical, Tokyo, Japan) was administered to donor gilts. Approximately 72 h after administration of eCG, 1000 IU/gilt hCG (Kyoritsu Seiyaku, Tokyo, Japan) was administered. Gilts were bred by AI at 18–36 h after administration of hCG. On day 11 (AI = day 0), two donor gilts were sacrificed, their reproductive tracts removed, and elongated embryos were collected by retrograde uterine flushing from the horns on both sides.

Determination of the relative abundances of gene transcripts in porcine embryos

Under an inverted microscope, blastocysts derived from IVF were divided into the following two parts using a microsurgical blade (Feather, Osaka, Japan): intact ICM with surrounding TE cells and a TE portion (Figs. 1A and 1B). ICM and TE samples obtained from 8–10 different blastocyst embryos were pooled to form single samples. ICM and TE portions were washed three times in PBS containing 1% polyvinyl pyrrolidone (PVP), added to 5 µl lysis buffer (0.8% Igepal [ICN Biomedical, Aurora, OH, USA], 5 mM DTT [Invitrogen], and 1 U/µl RNasin [Promega, Madison, WI, USA]), snap-frozen in liquid nitrogen, and stored at –80 C. Recovered elongated embryos were transferred to 1% PVP-PBS. Elongated embryos in which we could identify both the embryo

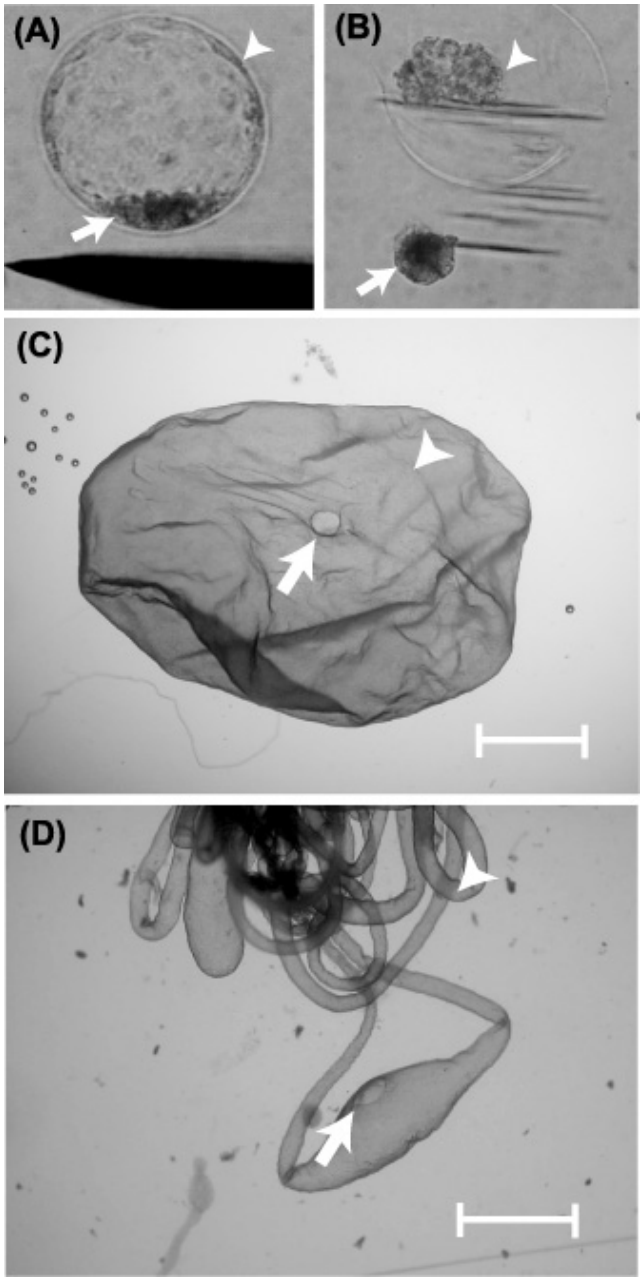

Fig. 1. Representative photographs of porcine blastocyst (A, B) and elongated stage embryos (C, ovoid; D, filamentous). Scale bars represent 2 mm (C) and 2.5 mm (D). Arrows indicate the ICM (A, B) or ED (C, D) in embryos. Arrowheads indicate the TE in embryos (A, B, C, D).

disc (ED) and TE were used for analysis (Figs. 1C and 1D). Under a stereomicroscope, elongated embryos were then divided into the ED and TE regions using a surgical knife. The TE region was divided into several pieces, approximately 3–5 mm wide. Pieces of ED and TE from single elongated embryos were washed three times in 1% PVP-PBS. Total RNA was isolated from pieces of ED and TE using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions and stored at –80 C.

RNA samples were heated to 80 C for 5 min and reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The reaction mixture of blastocyst and elongated stage samples was diluted with DEPC-treated water to final volumes of 24 µl and 40 µl, respectively. Real-time PCRs were performed using StepOne^{TM} (Applied Biosystems, Tokyo, Japan), and products were detected with SYBR Green included in QuantiTect SYBR Green PCR Master Mix (Qiagen). For each quantifications, 1.5 or 2 µl of the RT product was used. The amplification program was as follows: preincubation at 95 C for 15 min to activate HotStarTaq DNA Polymerase (Qiagen), followed by 45 cycles of denaturation at 94 C for 15 sec, annealing of primers at different temperatures (Table 1) for 30 sec, and elongation at 72 C for 30 sec. After the end of the last cycle, a melting curve was generated by starting fluorescence acquisition at 60 C and taking measurements in 0.3 C steps up to 95 C.

A standard curve was generated for each amplicon by amplifying serial dilutions of a known quantity. PCR products for each gene were purified using the a QIAquick PCR Purification Kit (Qiagen), quantified by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Kanagawa, Japan) and diluted as described. Serial 10-fold dilutions for creating the standard curve were amplified in every real-time PCR run. The standards and cDNA samples were then co-amplified in the same reaction prepared from a master mix. Fluorescence was acquired in each cycle to determine the threshold cycle or in the cycle during the log-linear phase of the reaction at which the fluorescence rose above the background for each sample. Final quantification was performed using the StepOne^{TM} quantification software. Expression of the target gene in each run was normalized to the internal standard, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Statistical analysis

Differences in mRNA expression levels in blastocyst stage embryos were analyzed by the Mann-Whitney U test. Data regarding mRNA expression levels in elongated stage embryos were analyzed by the Kruskal-Wallis test followed by multiple pairwise comparisons using the Scheffé's method. A P value < 0.05 denoted a statistically significant difference.

Results

Relative expression levels of mRNA transcripts in porcine blastocyst embryos

To describe the expression patterns of the genes involved in the segregation of the ICM and TE lineages in porcine blastocyst embryos, mRNA transcripts levels of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc* and *FGFR2-IIIc* in blastocyst embryos (Figs. 1A and 1B) were examined. As shown in Figs. 2A and 2B, *OCT-4* and *CDX2* transcripts were detected in both ICM and TE portions. *OCT-4* expression was significantly ($P<0.01$) higher in the ICM than in the TE, while the *CDX2* expression level did not differ between the ICM and TE. In addition, the expression ratio of *CDX2* to *OCT-4* (*CDX2*/*OCT-4*) did not differ between the ICM and TE (Fig. 2C). Like the *CDX2* expression, the *TEAD4* and *GATA3* expression levels

Table 1. Primers used for RT-PCR

Genes	Primer sequences (5′– 3′)[a]	Annealing temperature (C)	Fragment size (bp)	GenBank accession no.
OCT-4	F- GTTCTCTTTGGGAAGGTGTT	56	313	AJ251914
	R- ACACGCCGGACCACATCCTTC			
CDX2	F- GTCACCAGAGCTTCTCTGGG	53	144	EU137688
	R- AGACCAACAACCCAAACAGC			
TEAD4	F- AAGTTCTGGGCAGACCTCAA	60	157	XM_605145
	R- GTGCTTCAGCTTGTGGATGA			
GATA3	F- CATGTCCTCTCTCAGCCACA	60	206	NM_001044567
	R- TGCGAAAATGCACGTAGAAG			
NANOG	F- GTACCTCAGCCTCCAGCA	57	161	AJ877915
	R- CTGAGCCCTTCTGAATCAC			
FGF4	F- TTCTTCGTGGCCATGAGCAG	52	206	XM_003122418
	R- AGGAAGTGGGTGACCTTCAT			
FGFR1-IIIc	F- ACTGCTGGAGTTAATACCACCG	60	125	AJ577088
	R- GCAGAGTGATGGGAGAGTCC			
FGFR2-IIIc	F- GGTGTTAACACCACGGACAA	60	139	AJ439896
	R- CTGGCAGAACTGTCAACCAT			
GAPDH	F- TCGGAGTGAACGGATTTG	60	219	AF017079
	R- CCTGGAAGATGGTGATGG			

[a] Primer orientations: F, forward; R, reverse.

did not differ between the ICM and TE (Figs. 2D and 2E). The gene expressions of *FGF4* and *FGFR1-IIIc* were significantly (*FGF4*, P<0.01; *FGFR1-IIIc*, P<0.05) higher in the ICM than in the TE (Figs. 2F and 2G). At the blastocyst stage, *NANOG* and *FGFR2-IIIc* transcripts were not detected in the ICM and TE portions.

Relative expression levels of mRNA transcripts in porcine elongated embryos

To describe the expression patterns of the genes involved in the segregation of the ICM and TE lineages in porcine elongated embryos, mRNA transcripts levels of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc*, and *FGFR2-IIIc* in elongated embryos (ovoid, 6–10 mm, Fig. 1C; filamentous, ≥100 mm, Fig. 1D) were examined. As shown in Fig. 3A, *OCT-4* expression was observed in the ED, while the expression was very low in the TE. In contrast, *CDX2* expression in ovoid stage embryos was significantly (P<0.01) higher in the TE than in the ED (Fig. 3B). As embryonic development progressed from the ovoid to filamentous stage, the expression level of *CDX2* was significantly (P<0.01) reduced in TE lineages (Fig. 3B). The *CDX2*/*OCT-4* ratio in elongated embryos was markedly higher in the TE compared with the ED (Fig. 3C). The *TEAD4* expression level in filamentous embryos and *GATA3* expression levels in ovoid and filamentous embryos were significantly (P<0.01) higher in the TE than in the ED (Figs. 3D and 3E). On the other hand, *NANOG* expression was detected in the ED in elongated embryos, but not in the TE portion (Fig. 3F). The *NANOG* expression level was significantly (P<0.05) reduced as development progressed from the ovoid to filamentous stage (Fig. 3F). The *FGF4* and *FGFR1-IIIc* expression levels were significantly (P<0.01) higher in the ED than in the TE in both ovoid and filamentous stage embryos (Figs. 3G and 3H). In addition, *FGFR2-IIIc* expression was detected in elongated embryos, and, like the *FGF4* and *FGFR1-IIIc* expressions, was significantly (P<0.01) higher in ED than in TE (Fig. 3I).

Discussion

Several studies of mouse embryos have indicated clearly that the segregation of the ICM and TE lineages is regulated by the mutually antagonistic effect of OCT-4 and CDX2 [8]. At the morula stage, OCT-4 and CDX2 are expressed throughout the embryo, but after initiation of blastocyst formation, OCT-4 and CDX2 expressions gradually segregate to the ICM and TE, respectively [8]. The OCT-4 and CDX2 proteins form a transcription network by which they activate their own transcription, while reciprocally suppressing each other's expression. This transcription network contributes to the establishment of mutually exclusive OCT-4 and CDX2 expression, and thus establishment of the ICM and TE lineages [8]. On the other hand, in porcine and bovine blastocyst embryos, OCT-4 protein and mRNA are detectable in both the ICM and TE [17, 19, 20, 24, 25]. Thus, Kuijk *et al*. [20] suggested the possibility that OCT-4 is not involved in defining the pluripotent ICM population in porcine and bovine embryos. In the present study, *OCT-4* and *CDX2* transcripts were detected in both the ICM and TE at the blastocyst stage. On the other hand, at the elongated stage, *OCT-4* expression appeared to be largely restricted to the ED, consistent with previous studies of porcine embryos [18, 26, 27]. In addition, the *CDX2* level in ovoid embryos was significantly higher in the TE than in the ED. Thus, the difference in the *CDX2*/*OCT-4* ratio between ED and TE cells became more clear during development from the blastocyst to elongated stage. This pattern of *OCT-4* and *CDX2* expressions observed in porcine embryos is similar to that of bovine embryos [17, 28]. Our results present a persuasive argument that interplay

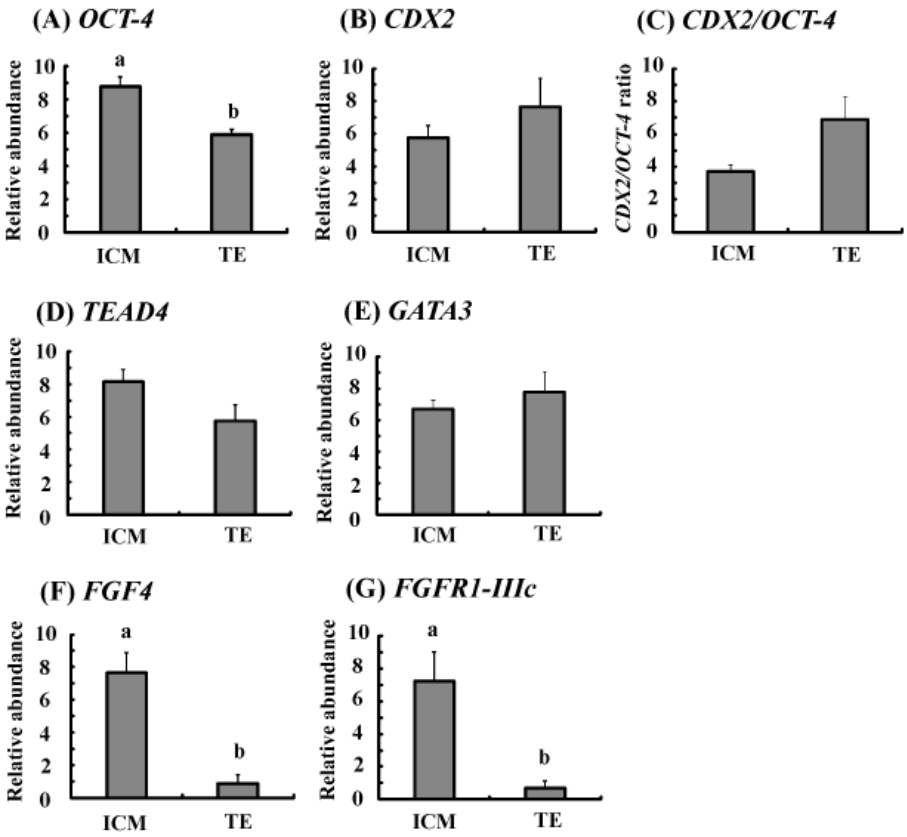

Fig. 2. Relative abundance (mean ± SE) of (A) *OCT-4*, (B) *CDX2*, (D) *TEAD4*, (E) *GATA3*, (F) *FGF4*, and (G) *FGFR1-IIIc* transcripts in porcine blastocyst embryos (n = 5). (C) The expression ratio of *CDX2* to *OCT-4* mRNAs (*CDX2*/*OCT-4*) in porcine blastocyst embryos (n = 5). Expression levels of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *FGF4* and *FGFR1-IIIc* mRNAs were normalized to *GAPDH* expression. [a,b] Different superscripts indicate a significant difference (P<0.01–0.05). *NANOG* and *FGFR2-IIIc* mRNA were not detected in porcine blastocyst embryos.

of OCT-4 and CDX2 could be important for the segregation and functionalization of the ICM and TE lineages in porcine embryos. Furthermore, our results indicate the possibility that in porcine embryos, functionalization of ICM and TE lineage cells caused by interplay of OCT-4 and CDX2 may progress with development from the blastocyst to elongated stage.

In the present study, we used different methods to produce the blastocyst and elongated stage embryos; blastocyst embryos were obtained from IVF, while elongated embryos were obtained *in vivo*. We have acknowledged that *in vitro* procedures may affect the expression of several genes in porcine and bovine embryos [29, 30]. However, we previously reported that in bovine blastocyst embryos, the expression patterns of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG* and *FGF4* genes in ICM and TE cells did not differ between IVF and *in vivo* embryos [17]. Thus, in the present study, we considered that it is possible to discuss the changes in the expression pattern of eight genes in the ICM and TE lineages during development from blastocysts to elongated embryos.

Recently, it has been revealed that TEAD4 and GATA3 play important roles in specification and development of the TE lineages in mouse embryos [11–13]. *TEAD4*-deficient mouse embryos did not express CDX2 gene after the morula stage, and these embryos exhibited defects in the specification of the TE lineage [12, 13]. Knockdown of *GATA3* by RNA interference also reduced *CDX2* expression and inhibited the morula to blastocyst transformation [11]. These findings suggest that TEAD4 and GATA3 regulate TE development through a pathway that requires the activation of CDX2 expression. In mouse blastocysts, TEAD4 expression was observed in both the ICM and TE [12], while GATA3 was selectively expressed in the TE portion [11]. In this study, *TEAD4* and *GATA3* mRNA were found to be expressed in both the ICM and TE portions, and their expression levels did not differ between the ICM and TE. These patterns of *TEAD4* and *GATA3* expression in blastocyst embryos are similar to those we described previously in bovine embryos [17]. On the other hand, in elongated embryos, *TEAD4* and *GATA3* expression levels were higher in the TE than in the ED. These results may be linked to the expression pattern of *CDX2* in the ED and TE at the ovoid stage. However, although the *TEAD4* expression level was maintained from the ovoid to filamentous stage, *CDX2* expression was remarkably downregulated. Recently, Home *et al*. [31] reported that subcellular localization of TEAD4 is important for regulation of expression for target genes, such as *CDX2* in mammalian species. Thus, further

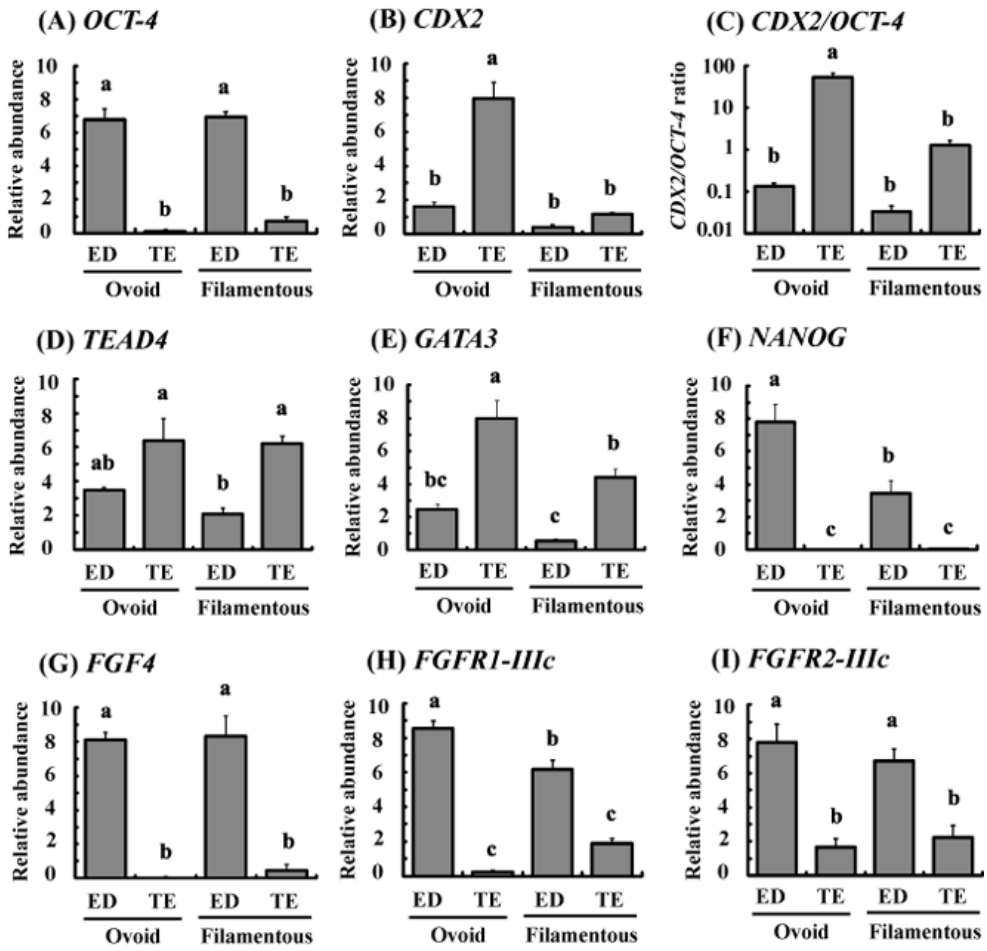

Fig. 3. Relative abundance (mean ± SE) of (A) *OCT-4*, (B) *CDX2*, (D) *TEAD4*, (E) *GATA3*, (F) *NANOG*, (G) *FGF4*, (H) *FGFR1-IIIc* and (I) *FGFR2-IIIc* transcripts in porcine elongated embryos (ovoid [n = 4] and filamentous [n = 5]). (C) The expression ratio of *CDX2* to *OCT-4* mRNAs (*CDX2*/*OCT-4*) in porcine elongated embryos (ovoid [n = 4] and filamentous [n = 5]). The bars for the *CDX2*/*OCT-4* ratio represent a log scale. Expression levels of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc* and *FGFR2-IIIc* mRNAs were normalized to *GAPDH* expression. [a,b,c] Different superscripts indicate a significant difference (P<0.01–0.05).

analyses such as immunohistochemistry were necessary to uncover the transcription network between *TEAD4*, *GATA3*, and *CDX2* in porcine embryos. The present study is the first to demonstrate the changes in the expression of *TEAD4* and *GATA3* mRNAs during porcine preimplantation development, and our findings suggest the possibility that TEAD4 and GATA3 participate in the regulation of TE development in porcine preimplantation embryos.

NANOG is an ICM-specific transcription factor under the control of OCT-4 [32] that is expressed in early mouse blastocysts. Like OCT-4 and CDX2, NANOG and CDX2 mutually repress the expression of the other, suggesting that NANOG is also involved in the regulation of the segregation of the ICM and TE lineages [10]. In the present study, the *NANOG* transcript was not detected at the blastocyst stage, but was exclusively expressed in the ED of elongated embryos, corroborating previous reports in porcine embryos [18, 26]. In addition, *NANOG* expression levels were reduced as the embryos transitioned from the ovoid to filamentous stage. Recently, Wolf *et al*. [27] demonstrated that NANOG is downregulated in the epiblast as the primitive streak develops. Although the timing of *NANOG* expression between mouse and porcine embryos is different, these findings indicate that *NANOG* expression may be involved in the maintenance of pluripotency in ICM lineage (the ED) cells in porcine embryos.

Fibroblast growth factors bind to a group of FGF receptors (FGFRs) with tyrosine kinase activity. The FGF4 isoform interacts primarily with FGFR1-IIIc and FGFR2-IIIc [33, 34]. In the mouse embryos, it is established that the FGF4 signaling pathway is required for maintaining the proliferation of TE cells [16, 35, 36]. FGF4 is highly expressed in the ICM and epiblast, and activates the membrane-associated FGFR2 expressed by the TE lineage [37, 38]. Embryos with targeted disruption of *FGF4* or *FGFR2* show peri-implantation lethality, caused by defects in functional ICM formation as well as a

placental defect [14, 15], which together indicate the importance of the FGF4 signaling pathway for proliferation and differentiation of both the ICM and TE cells. In the present study, we demonstrated that *FGF4* is expressed in porcine preimplantation embryos. In blastocyst embryos, expression of *FGF4* and *FGFR1-IIIc* was significantly higher in the ICM than in the TE. Furthermore, these gene expression patterns were maintained in the elongated embryos. These findings suggest that FGF4-FGFR1-IIIc signaling may be important for segregation and proliferation of the ICM lineage in porcine embryos. On the other hand, *FGFR2-IIIc* mRNA was not detected in porcine blastocysts. However, *FGFR2-IIIc* expression was detected in both the ED and TE portions of the elongated embryos, which indicates the possibility that FGF4 from the ED portion activates the FGFR2-IIIc, and this signaling functions in the differentiation and proliferation of the ED and TE in porcine elongated embryos as seen in the mouse embryos. In addition, Powers *et al*. [31] reported that FGFR1-IIIc and FGFR2-IIIc are also activated by FGF1, 2, 6 and 9. Thus, it is possible that these other FGF isoforms also activate FGFR1-IIIc and FGFR2-IIIc in porcine elongated embryos, and thereby regulate the differentiation and proliferation of the ED and TE.

In conclusion, we demonstrated dramatic changes in the expression patterns of *OCT-4*, *CDX2*, *TEAD4*, *GATA3*, *NANOG*, *FGF4*, *FGFR1-IIIc* and *FGFR2-IIIc* genes in ICM and TE lineage cells during porcine preimplantation development. Changes in the expression patterns concomitant with embryonic stage transition suggests that these genes may help guide the segregation and functionalization of the ICM and TE lineages in porcine preimplantation embryos. In the mouse embryos, the expressions of most genes involved in the segregation of the ICM and TE were restricted in ICM or TE cells at the blastocyst stage, and regulate the ICM and TE cell functions. Thus, in mouse embryos, morphological and functional segregation of the ICM and TE lineages occurred at the blastocyst stage. On the other hand, our findings suggest that in porcine embryo, the functions of ICM and TE lineages cells depend on these gene expressions might proceed as transition from blastocyst to elongated stage, which indicates the possibility of differences in the molecular mechanism that regulates early lineage segregation between species. Further studies, such as immnohistochemical analysis and knockdown of target genes by siRNA, are necessary to obtain better understanding for the mechanism of early lineage segregation in porcine embryos.

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