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—Original Article—

Overexpression of *OCT4A* ortholog elevates endogenous *XIST* in porcine parthenogenic blastocysts

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Abstract. X-chromosome inactivation (XCI) is an epigenetic process that equalizes expression of X-borne genes between male and female eutherians. This process is observed in early eutherian embryo development in a species-specific manner. Until recently, various pluripotent factors have been suggested to regulate the process of XCI by repressing *XIST* expression, which is the master inducer for XCI. Recent insights into the process and its regulation have been restricted in mouse species despite the evolutionary diversity of the process and molecular mechanism among the species. *OCT4A* is one of the represented pluripotent factors, the gate-keeper for maintaining pluripotency, and an *XIST* repressor. Therefore, in here, we examined the relation between *OCT4A* and X-linked genes in porcine preimplantation embryos. Three X-linked genes, *XIST*, *LOC102165544*, and *RLIM*, were selected in present study because their orthologues have been known to regulate XCI in mice. Expression levels of *OCT4A* were positively correlated with *XIST* and *LOC102165544* in female blastocysts. Furthermore, overexpression of exogenous human *OCT4A* in cleaved parthenotes generated blastocysts with increased *XIST* expression levels. However, increased *XIST* expression was not observed when exogenous *OCT4A* was obtained from early blastocysts. These results suggest the possibility that *OCT4A* would be directly or indirectly involved in *XIST* expression in earlier stage porcine embryos rather than blastocysts.

Key words: Blastocyst, *OCT4A*, Transgenesis, *XIST*

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Inactivation of the X-chromosome in female eutherians is an epigenetic process essential to achieving normal embryo development by equalizing the expression of X-linked genes between male and female embryos. This process is known to be regulated by X-chromosome inactivation specific transcript (*XIST*). This non-coding RNA (ncRNA) gene is a key factor for initiating X-chromosome inactivation (XCI) [1], and overexpression of the gene in cloned mouse embryos revealed differential expression of X-linked genes compared to fertilized embryos [2]. This finding suggests that the delicate and accurate regulation of *XIST* expression is required for successful XCI in early embryos. The genes or enhancers located on the specific genomic region called X-chromosome inactivation center (XIC) have been known to regulate *Xist* expression positively (*Rlim*, *Ftx*, and *Jpx*) [3–5] or negatively (*Tsix*, *DXpas34*, and *Xite*) [6–8]. Additionally, pluripotent factors like *Oct4*, *Sox2*, *Nanog*, and *Rex1* have been reported as negative regulators of expression of *Xist* and its positive regulator, *Rlim*, and may prevent initiation of XCI in mice [9–11]. These reports reveal that female XCI in mice is a complex event controlled accurately with numerous regulators. However, the strategy for inducing XCI in early developing embryos has been considered to vary among species [12], and the roles of regulators of *XIST* expression in other species including pigs are unclear.

Porcine *XIST* gene was identified recently [13] and abnormal expression of *XIST* were observed in cloned porcine embryos [14, 15]. Although the epigenetic differences of *XIST* expression between fertilized and cloned embryos have been observed, the regulation of expression of XCI-regulators including *XIST* by pluripotent factors have not been focused until recently in pigs.

OCT4 is one of the most popular and important pluripotent genes among the various factors regulating XCI. The gene has been well-known as a gate-keeper maintaining pluripotency in the inner cell mass (ICM) and its derivate, embryonic stem cells (ESCs), in mice [16]. Interestingly, a recent report demonstrated that *OCT4,* a well-conserved gene, is shared as a pluripotent marker even in vertebrates [17]. However, it is unclear whether the gene also has conserved functions associated with XCI and *XIST* suppression among the species. Contrary to *OCT4*, ncRNAs in XIC, have been known to have low sequence homology caused by rapid evolution [18],

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and the mechanism of XCI was considered to vary among species [12] as referred above. Considering the differences in genetic and functional conservation between *OCT4* and X-linked XCI regulators, examining their association in non-mouse species is necessary. Although a few studies have tried to uncover the functions of *OCT4* in embryo development and reprogramming capacity during nuclear transfer (NT) [19, 20], the relationship between *OCT4* and X-linked XCI regulators is still unclear in pigs.

Therefore, in present study, we aimed to examine whether over-expression of *OCT4A*, which has been reported to be pluripotent gene and major variant of *OCT4A* gene in pigs [21], could affect expression of X-linked genes regulating XCI in female porcine preimplantation embryos.

Materials and Methods

Ethics statement

All experiments are conducted with approval of Institutional Animal Care and Use Committees, Seoul National University (SNU-140325-3).

In vitro embryo production

In vitro maturation: Prior to generating *in vitro* embryos, oocytes were maturated *in vitro* following the procedure from our previous report [22]. Ovaries of pre-pubertal gilt were gifted from Sooam Biotech Research Institute (Seoul, Korea). Cumulus-oocyte-complexes (COCs) were extracted from 3–6 mm follicles, and those with multiple layered cumulus cells and granulated cytoplasm were selected. Prepared COCs were washed using TL-Hepes-PVA [23] and cultured in tissue culture medium (TCM-199; Life Technology, Rockville, MD, USA) containing 10% follicular fluid, 10 ng/ml epidermal growth factor (EGF), and 1 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA) for 44 h at 39 C in 5% CO_2 conditions. Human and equine chorionic gonadotropins (4 IU/ml, Intervet, Boxmeer, Netherlands) were treated for the first 22 h. Forty-four h later, expanded cumulus cells were detached from the zona pellucida (ZP) by gentle pipetting with 0.1% of hyaluronidase (Sigma-Aldrich). The oocytes without cumulus cells were assessed for *in vitro* fertilization and parthenogenesis.

In vitro fertilization: Fertilized embryos were produced *in vitro* following the procedure from our previous report [22]. Commercial semen from ducoc breed (DARBI A.I center, Jochiwon, Korea) was assessed, and the sperm were washed with Dulbecco's phosphate buffered saline (DPBS; Welgene, Seoul, Korea) supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich) before use. Each 20 to 25 mature oocytes and prepared sperm (1×10^5 cells/ml) were co-incubated on the modified tris-buffered medium (mTBM) [24] for 6 h at 39 C in 5% CO_2 conditions. After incubation, the binding sperms were detached from oocytes by gentle pipetting, and oocytes were moved to porcine zygote medium 3 (PZM3) [25] and cultured at 39 C in 5% CO_2 and 5% O_2 conditions.

Parthenogenesis: To generate parthenotes, denuded oocytes were activated by electric pulse (1.0 kV/cm for 60 μsec) using BTX Electro-cell Manipulator (BTX, CA, USA) in activation medium (280 mM mannitol, 0.01 mM $CaCl_2$, and 0.05 mM $MgCl_2$). The oocytes were transferred to PZM3 containing 2 mM 6-dimethylainopurine (6-DMAP; Sigma-Aldrich) and incubated for 4 h at 39 C in 5% CO_2 and 5% O_2 conditions. After 4 h, the oocytes were moved to PZM3 without 6-DMAP and incubated in the same conditions with *in vitro* fertilized embryos.

RNA extraction and RT-PCR

RNA of individual blastocysts without ZP was extracted using Dynabeads® mRNA DIRECT™ Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacture's instructions. Extracted RNA from blastocysts were reverse-transcribed with High Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Synthesized cDNA was assessed by PCR, and the reaction was carried out using 0.5 μM of primers and 2x PCR master mix solution (i-MAX II) (iNtRON Bio Technology, Seongnam, Korea) with the following conditions: One cycle at 95 C for 7 min; 40 cycles at 95 C for 15 sec, 60 C for 20 sec, and 72 C for 30 sec; and one cycle at 72 C for 10 min.

Genomic DNA extraction

Genomic DNA (gDNA) from HEK293-LTV cells were extracted using G-DEX™ llc Genomic DNA Extraction Kit (iNtRON Bio Technology) following the manufacturer's instructions. Extracted gDNA was applied for titration of virus.

Quantitative RT-PCR

Quantitative RT-PCR was performed using 0.1 μM of primer sets (Supplementary Table 1: online only) and DyNAmo HS SYBR Green qPCR kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's guidance. The reaction was conducted under the following conditions: one cycle at 50 C for 5 min; one cycle at 95 C for 5 min; and 40 cycles at 95 C for 15 sec and 60˚C for 1 min. *ACTB* and *RN18S* were used as reference genes. Sexing of fertilized embryos was conducted by comparing the expression levels of *XIST* [15] prior to measuring expression levels of target genes.

Lentiviral vector plasmid cloning and virus preparation

Lentiviral vector plasmids coding *enhanced green fluorescent protein* (*EGFP*) and human *OCT4* (*hOCT4*) were prepared. *EGFP* and *hOCT4* inserts are obtained by PCR amplification from pLL3.7 (Invitrogen) and FU-tet-o-*hOCT4* (Addgene, Cambridge, MA, USA), respectively. Each insert was passed to the *LIN28* coding region of the pSIN-EF2-LIN28-PUR plasmid (Addgene). Preparation of lentivirus particles followed the previous report [26] with modification. Prepared self-inactivating lentiviral vector plasmids harboring transgenes, packaging plasmids (pLP1 and pLP2; Invitrogen), and envelop plasmids (pLP/VSVG; Invitrogen) were used for lentivirus particle production, and HEK293 LTV cells (Cell Biolabs, USA) were used for packaging lentivirus particles. The detailed procedure is described in the Supplementary Materials and Methods (online only).

Lentivirus titration

Virus titration was performed by calculating the integrated copy numbers in HEK293-LTV cells inoculated with the prepared virus. To calculate copy numbers of integrated vectors and genomes, a linear-regression plot was prepared following our previous report [21]. Human genomic region (*hGAPDH*) and internal ribosome entry

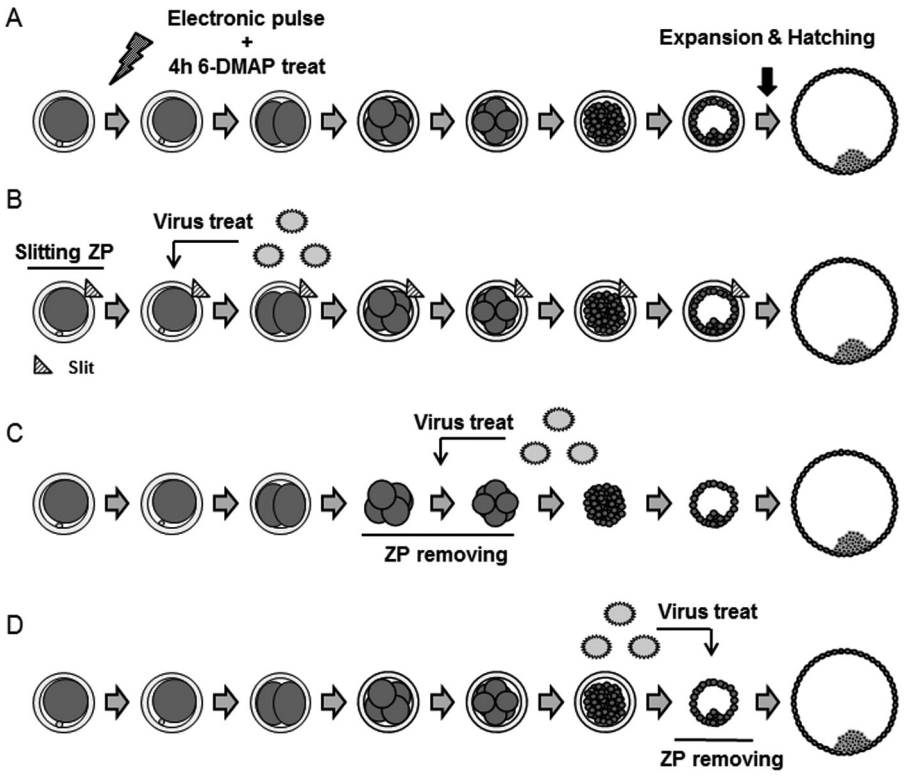

Fig. 1. Strategies for lentivirus transduction. To infect lentivirus into porcine cells developing into parthenotes, various strategies were applied. Parthenotes were generated by electronic activation and 6-DMAP treatment (A). The ZP of mature oocytes was slit before generating parthenotes, and the oocytes were co-cultured with virus particles after 4 h of treatment with 6-DMAP (B). To inoculate the virus at the cleaved embryos (C) and early blastocysts (D), ZP of the embryos was removed at embryonic day 3 and 5, respectively. Each embryo group was cultured with lentiviral particles for one day.

site (IRES) in lentiviral vector plasmids were assessed to calculate the number of integrated transgenes in the genome. Each region was amplified by PCR, and the Ct-value of the serially diluted amplicon was used for plotting linear-regression. Log of copy number and its Ct-value were plotted (Supplementary Fig. 1A and Supplementary Table 2: online only). Copy number of transgenes in one cell was calculated using the plot. To titrate stock of lentivirus particles, each virus stock was serially diluted and transduced to HEK293-LTV cells. The cells were harvested, and gDNA extracted from the cells was assessed by quantitative PCR to obtain Ct-values of *hGAPDH* and *IRES*. Copy number of transgenes in one cell was calculated using Ct-values, and log of copy number was plotted against the log of dilution rate of virus stock (Supplementary Fig. 1B). The detailed procedure for lentivirus titration is written in the supplementary materials and methods.

Lentivirus transduction into embryos

Lentivirus transduction was performed carried out using two previously reported methods with some modifications [27, 28]. Two types of embryos were assessed for transgenesis, zygotes with slit ZPs or embryos without ZPs. Lentivirus was co-cultured with zygotes with slit ZP (immediately after 6-DMAP treatment, embryonic day 0, D0), and ZP-removed cleaved embryos (embryonic day 3, D3) and early blastocysts (embryonic day 5, D5) (Fig. 1). Slit ZPs were produced by cutting the ZP of mature oocytes with glass needles before generating parthenotes. At each stage of embryo development, the ZP was removed by Tyrode's Acid (Sigma-Aldrich). These embryos were treated with lentivirus for one day followed by washing four times with DPBS containing 0.4% BSA. Washed embryos were transferred to PZM3. Transgenesis of embryos was confirmed by observing green fluorescence (*EGFP*-lentivirus inoculation) or analyzing expression of puromycine resistance gene (*PURO*) using RT-PCR.

Statistical analysis

Statistical analysis was carried out with the Graphpad Prism statistical program (Graphpad Software, San Diego, CA, USA). Comparisons of developmental competence among the embryos and expression levels of blastocysts inoculated with *hOCT4*- or *EGFP*-lentiviruses were performed using Student *t*-test. Analysis of correlative expression between endogenous *OCT4* and X-linked genes in individual blastocysts was conducted using Pearson correlation coefficient. All data were exhibited as mean ± standard error mean (SEM), and $P < 0.05$ was considered to be statistically significant.

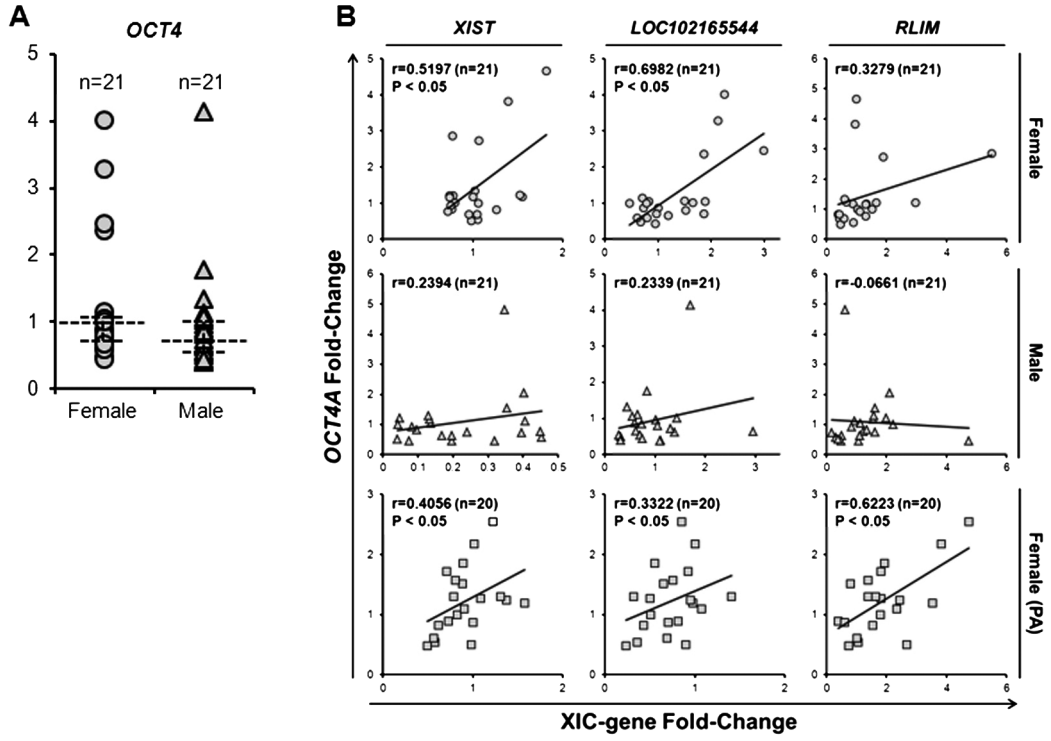

Fig. 2. Correlative analysis of *OCT4* expression and X-linked genes in blastocysts. (A) *OCT4* expression level comparison between male and female fertilized blastocysts. Each circle and triangle indicates *OCT4* expression levels of individual fertilized female and male blastocysts, respectively. Box-plot in each column represents quartile of expression level distribution. (B) Correlative comparison of expression levels between *OCT4* and X-linked genes. $P < 0.05$ was considered to be statistically significant. *ACTB* and *RN18S* were used for reference genes, and the median of each gene expression level in fertilized female blastocysts was set to one-fold.

Results

Correlative expression analysis of OCT4A and X-linked genes in male and female blastocysts

Expressions of the *OCT4* gene and X-linked genes in male and female blastocysts were compared. Three X-linked genes (*XIST*, *RLIM*, and *LOC102165544)* were selected because their orthologues have been suggested to mediate XCI in association with *OCT4* in mice. *LOC102165544* was considered to be an ortholog of mouse *Jpx* because of its coding location close to *XIST*. The sex of fertilized blastocysts was classified by *XIST* expression levels [15]. Expression levels of endogenous *OCT4A* were not different in male and female blastocysts (Fig. 2A). Correlative comparison of the expression levels between *OCT4A* and the X-linked genes revealed that *OCT4A* expression is significantly correlated with *XIST* and *LOC102165544* in female fertilized blastocysts but not in males (Fig. 2B). This result raised the possibility that *OCT4A* expression is related to the *XIST* and *LOC102165544* in porcine female blastocysts. Parthenotes also showed similar results as fertilized female blastocysts, and the following experiments were carried out with parthenotes to obtain female embryos only.

Lentivirus transduction in preimplantation embryos

Embryo transgenesis using lentiviruses was conducted by exposing the membrane of embryos to an external environment supplemented with lentivirus particles (Fig. 1). Embryos denuded at the zygote stage were not assessed in the following experiment because of severe and uncontrollable aggregation (data not shown). The ZP was slit or removed to expose the membrane to virus. The embryos normally developed into blastocysts (Fig. 3), and their developmental capacity was not affected by denuding or slitting of the ZP (Supplementary Tables 3 and 4: online only). Embryos with slit or removed ZP were cultured with EGFP-lentivirus particles for one day and were not influenced to proceed with further embryo development (Supplementary Tables 5 and 6: online only). Successful transgenesis was observed only in denuded embryos, and exogenous gene expression was not observed in blastocysts with slit ZP (Fig. 4). Therefore, denuded embryos were used to infect viral vectors in this study.

Effect of hOCT4 overexpression on X-linked genes in parthenogenic blastocysts

To evaluate the effect of *OCT4A* on the expressions of X-linked genes in blastocysts, its ortholog in humans was transduced to the denuded parthenotes (Fig. 5). Unexpectedly, transduction of *hOCT4A* to D3 embryos showed reduced blastocysts formation (Fig. 5A). Also, a five-fold increase in *XIST* expression was observed in the *hOCT4A*-overexpressing blastocysts (Fig. 5B). Similar results on expansion and maintenance of blastocysts or expression levels of *XIST*

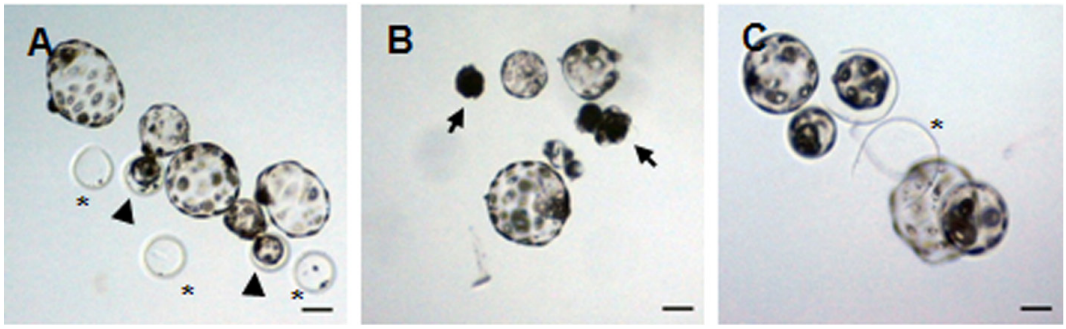

Fig. 3. Generation of parthenogenic blastocysts with slit or removed ZP. Blastocysts developing from parthenotes with slit ZP (A) or without ZP (B) were exhibited. Blastocysts originated from parthenotes with intact ZP were used for control (C). Asterisks and arrowheads in panel (A) indicate empty ZP and blastocysts, respectively, showing assisted hatching. Arrows in panel (B) mean that ZP embryos arrested development and failed to form blastocysts. Scale bars = 100 μm.

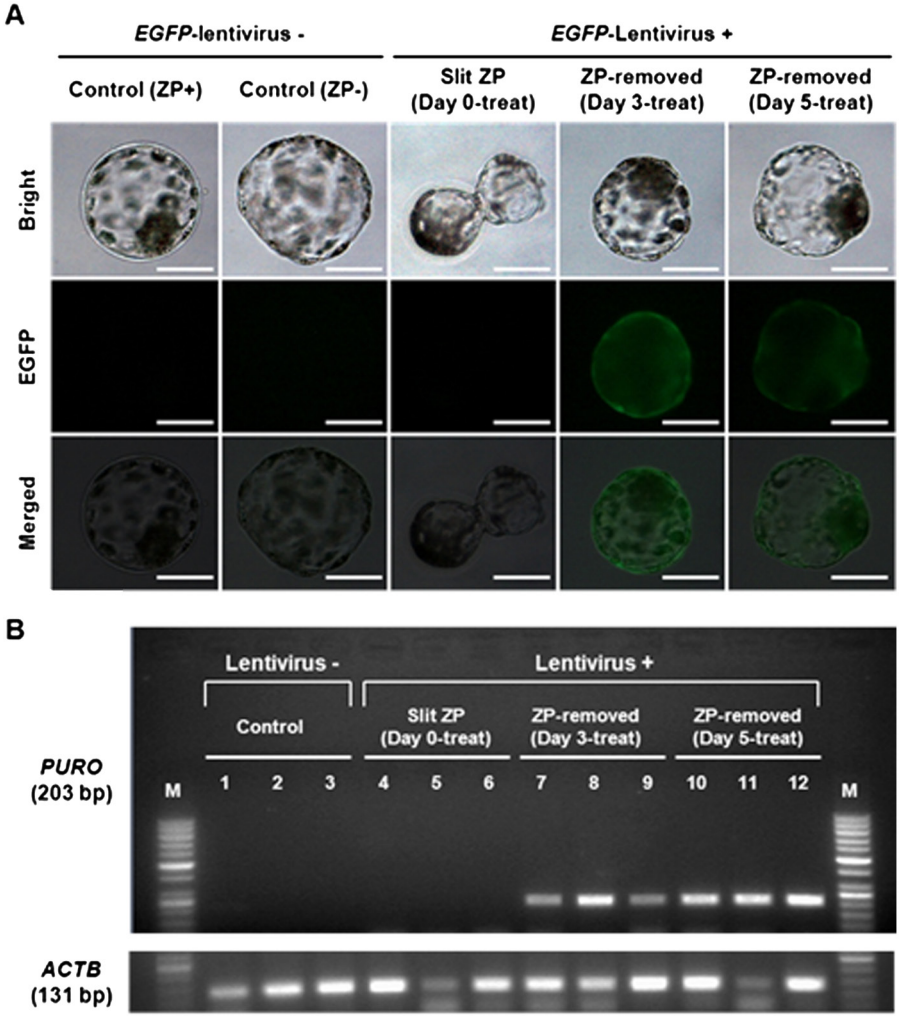

Fig. 4. Validation of transgenesis of porcine blastocysts. (A) *EGFP* expression in blastocysts. Successful transgenesis was observed in blastocysts that had been denuded at D3 and D5. However, blastocysts with slit ZP failed to successfully undergo transgenesis. No infected blastocysts with or without ZP were used for control. Scale bars = 100 μm. (B) Confirming transgene expression by amplifying transgene. Expression of transgene *PURO* (203 bp) was examined in blastocysts in which transgenesis had been attempted with differential strategies. Expression of *PURO* was not observed in blastocysts with intact or slit ZP (line 1–3 and 4–6, respectively). However, blastocysts from denuded parthenotes (line 7–12) showed transgene expression. M indicates 50 bp-DNA size markers.

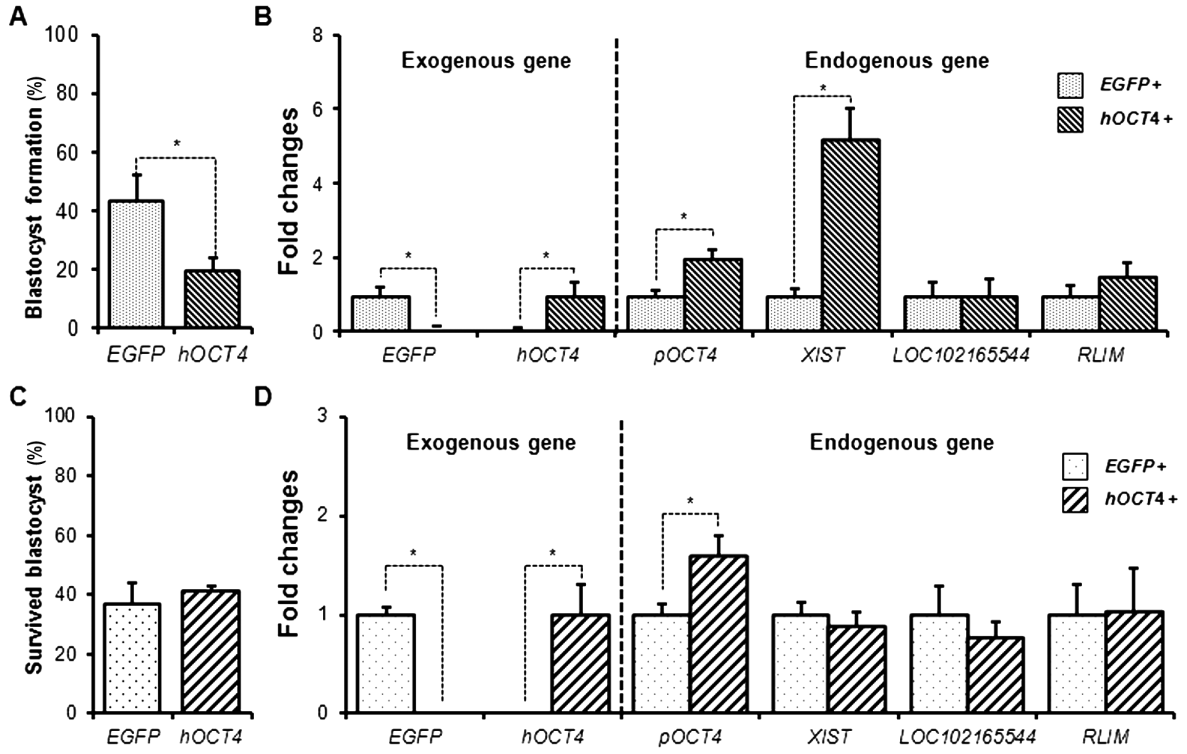

Fig. 5. Effect of *OCT4A* ortholog overexpression in parthenogenic blastocysts. Expression levels of target genes in each individual blastocyst were examined (n = 7 for blastocysts transfected at D3, A and B; n = 5 for blastocysts transfected at D5, C and D). Formation and maintenance of *hOCT4A* overexpressing blastocysts were significantly reduced in embryos denuded at D3 (A), but there was no change in embryos infected at D5 (C). The experiment was replicated three times. Relative expression levels of endogenous *OCT4A* and X-linked genes were compared between blastocysts transfected with *EGFP* and *hOCT4* (B and D). Median of each gene expression level in *EGFP*-expressing blastocysts was set to one-fold except for *hOCT4* expression. Expression levels of *hOCT4* were normalized by the median value of *hOCT4*-expressing blastocysts.. Asterisk indicates significant difference ($P < 0.05$).

were not observed in blastocysts when *hOCT4A* was transduced to D5 embryos (Fig. 5C, D). Increased level of endogenous *OCT4A* and non-effect to the other two X-linked genes, *RLIM* and *LOC102165544,* were observed in transgenic blastocysts that underwent transgenesis at differential embryonic days.

Discussion

XCI is a chromosome-wide gene silencing process occurring in the X-chromosome of early developing embryos to equalize the expression levels of X-linked genes between male and female individuals. Recently, several studies have demonstrated that the mechanism of XCI would be closely related to pluripotent factors. *Oct4A* in particular has been suggested as a regulator for preventing XCI initiation by suppressing or supporting expression of *Xist* [9] and *Tsix* [11], respectively, in mice. Although the functions of *OCT4A* orthologues in maintaining pluripotency and inducing reprogramming have been suggested to be well-conserved among vertebrates [17], it is unclear whether the genes have conserved roles and relations with XCI among different species. Since processes for XCI and its initiation timing in developing embryos vary among different species [12], examination of their relationship in various species is necessary. Although numerous studies were conducted to understand the mechanism of this complex epigenetic event and its associated regulators using mouse models, its extension to non-mouse species has rarely been studied. Therefore, the relationship between *OCT4A* and three X-linked genes whose orthologues (*XIST* and *RLIM*) or expected counterpart (*LOC10216554*) were reported to induce and support XCI in mice was studied to determine if the function of *OCT4A* on XCI is conserved in pigs.

As introduced above, *Oct4A* has been suggested to prevent *Xist* expression by binding to its intron 1 in mouse embryonic stem cells (ESCs) [9]. Dissociation of Oct4A from the intron and rapid elevation of *Xist* transcription (within 24 h) was observed in differentiating ESCs. Another report suggested that Oct4A suppresses *Xist* expression indirectly by forming complexes with Ctcf and promoting expression of *Tsix* [11], which is a ncRNA antisense to *Xist* and repress *Xist* and XCI in mice [6]. Even though the two reports suggested different mechanisms of *Xist* repression by *Oct4* in mouse ESCs, it looks clear that *Oct4A* negatively regulate *Xist* expression and its subsequent result, XCI, at least in mice. However, the results in this study had the opposite tendency compared to the studies in mice. Expression levels of *OCT4A* and *XIST* were positively correlated in female fertilized and parthenogenic blastocysts (Fig. 2).

However, it is unclear whether *OCT4A* and *XIST* are really positively interacted each other or just expressed parallel in porcine blastocysts. Overexpression of *hOCT4A* in cleaved embryos induced elevation of *XIST* expression in parthenogenic blastocysts in the present study (Fig. 5B). The result supports the possibilities that *OCT4A* could be related to *XIST* expression in porcine developing embryos. However, it is still unclear how *OCT4A* regulate *XIST* expression in porcine embryonic stages. Although it is possible that *hOCT4A* directly target and induce *XIST* expression in porcine embryos, overexpression of *OCT4A* ortholog would induce confusion of pluripotent molecular networking during early embryo development. Indeed, despite the importance of *Oct4* in maintenance of pluripotency, overdose of the gene induces differentiation into primitive endoderm and mesodermal cell lineage in mouse ESCs [29]. In our results, expression level of exogenous *hOCT4A* in blastocysts transfected at D3 embryos was about ten-fold higher than that of controls. This finding might lead to accelerated differentiation of cells in embryos during their growth. Considering that XCI is initiated in differentiating mouse ESCs and ICM, and that *Xist* accumulation is a key inducer for XCI, rapid differentiation which could be induced by *hOCT4*-overexpression would result in increased *XIST* expression in porcine blastocysts. Previously reported studies commonly addressed that overexpression of *OCT4A* could improve the developmental competence and qualities of cloned porcine embryos [19, 20]. However, contrary to the reports, our results showed reduced developmental competence of embryos by inducing *hOCT4A* transgenesis (Fig. 5A). This finding may originate from differences in the assessed embryos and expression levels of exo- and endogenous *OCT4A* orthologues in embryos. Endo- and exogenous expression levels of *OCT4A* orthologues in blastocysts transfected at D3 were about two-fold and ten-fold higher, respectively, compared to controls. Increased expression levels of endogenous *OCT4A* could be originated by localization of *hOCT4A* into OCT4A binding site, which is present in distal enhancer region of *OCT4* gene [30, 31].This level is higher than the previous reports and could exceed the threshold for affecting cell status of blastocysts. Furthermore, upregulated *XIST* expression in the blastocysts would induce malformation of blastocysts because *XIST* expression is closely linked to abnormal embryo development in various species [2, 15, 32].

The elevation of *XIST* expression was not observed in blastocysts transfected at early blastocyst stages (Fig. 5C, D). As shown in our previous study, *XIST* expression is observed after the morula stage [33]. This might indicate that XCI initiates at the blastocyst stage in pigs and that the cells in early blastocysts might already begin segregation into specific cell lineages because XCI and pluripotency are tightly linked in the embryonic stage and in ES cells [34, 35]. Therefore, even though almost a fifty-fold increase in expression of exogenous factors was observed, this overdose might not affect the molecular networking of blastocysts. On the other hand, a short period of exogenous gene expression in developing embryos could be another reason for a weak effect on *XIST* expression in the blastocysts.

Expression levels of other X-linked genes examined in this study did not show any changes following induction of *hOCT4* (Fig. 5B, D). Even though *Rlim* is likely regulated negatively by *Oct4* in mouse ES cells [10], expression of its orthologues was not influenced by the exogenous gene. This might be explained by the differential *OCT4A* roles in *Rlim* regulation in pigs. One remaining question is why the expression level of these X-linked genes was not affected by elevation of *XIST* expression level. This is especially curious when considering that *Rlim* is subjected to XCI [4] and that repression of chromosome-wide X-linked gene by ectopic *Xist* expression was observed in cloned mouse embryos [2]. This could be explained by insufficient *XIST* accumulation for chromosome-wide inactivation of the X-chromosome. Another possible interpretation of the results is that *LOC102165544* and *RLIM* might escape XCI in pigs.

In this study, we examined the effect of *OCT4A* overexpression on X-linked gene expressions during porcine preimplantation embryo development by transducing *hOCT4A*. It is expected that *OCT4A* influence *XIST* expression in early stage of porcine embryos. Our results were different compared to previous studies in mice [9, 11]. Even though the difference might originate from accelerated lineage segregation in cleaved embryos, there is still a possibility that the functions of porcine *OCT4* on *XIST* expression in preimplantation embryos would be different than those of mouse *Oct4*. Indeed, one study reported that RNAi-mediated knockdown of *OCT4* did not influence *CDX2* expression despite reducing the blastocyst formation rate [36]. This might mean that porcine *OCT4* can regulate its targets differently in pigs than in mice. And also, as present study was conducted mainly using parthenogenic embryos, it could be originated from differential expression patterns of parthenotes and *in vivo* fertilized embryos which showed differential expression patterns of certain genes during development. Therefore, relation between *OCT4* and X-linked genes including *XIST* should be examined further using *in vivo* embryos. To clearly understand how *OCT4* functions to control XCI and expression of *XIST* in pigs, epigenetic changes of various genes related to pluripotency, lineage segregation, and diverse X-linked genes should be examined in porcine embryos following the regulation of *OCT4A* expression.

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