Effect of Downregulating the Hippo Pathway Members YAP1 and LATS2 Transcripts on Early Development and Gene Expression Involved in Differentiation in Porcine Embryos

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Abstract

In mouse development, differentiation of the inner cell mass (ICM) and trophectoderm (TE) during the transition from the morula to blastocyst stage is regulated by the Hippo pathway; however, the functions of the Hippo pathway in porcine embryogenesis have not been investigated. In the present study, we examined the gene expression patterns of the Hippo pathway members yes-associated protein 1 (YAP1) and large tumor suppressor 2 (LATS2) and the functions of these genes during porcine preimplantation development using RNA interference. Both *YAP1* and *LATS2* mRNA levels were shown high in the *in vitro* matured oocytes and 1-cell stage embryos and fell progressively with development. YAP1 nuclear localization was detected at the morula and blastocyst stages. Downregulation of either *YAP1* or *LATS2* inhibited porcine preimplantation development and affected the expression levels of POU class 5 homeobox 1 (*OCT-4*) and SRY-related HMG-box gene 2 (*SOX2*), transcription factors necessary for the ICM/TE differentiation. Taken together, YAP1 and LATS2 are essential for porcine preimplantation development, and it is possible that the Hippo pathway has important roles in porcine ICM/TE segregation.

Keywords: embryo, early development, porcine, IVF, Hippo pathway

Introduction

THE FIRST VISIBLE DIFFERENTIATION EVENT during mammalian embryonic development is the segregation of the trophectoderm (TE) and the inner cell mass (ICM) from the morula to blastocyst stage. The TE is an epithelial outer layer that develops into the placenta, whereas the ICM is a group of cells attached to the inside of the TE that gives rise to the fetus and extraembryonic tissues (Pedersen et al., 1986). In mice, ICM and TE can be isolated and maintained *in vitro* as embryonic stem cells (ESCs) and trophoblast stem cells (TSCs), respectively (Evans and Kaufman, 1981; Martin, 1981; Tanaka et al., 1998). Thus, it is important to elucidate the molecular mechanism of the ICM/TE segregation, which is the starting point of cell differentiation, and it is known that the differentiation requires the precisely timed expression of multiple transcription factors (Yamanaka et al., 2006).

The Hippo pathway controls a number of cellular events such as cell proliferation, cell death, and cell differentiation (Yu et al., 2013; Zhao et al., 2007) and is one of the main regulators of the ICM/TE lineages divergence in murine embryos (Hirate et al., 2013; Nishioka et al., 2009). Activation of the Hippo pathway depends on the cell position in early em-

bryos. In the inner cells of murine morulae, the Hippo pathway is activated, allowing the activation of large tumor suppressor 1/2 (Lats1/2) kinases to phosphorylate yes-associated protein 1 (Yap1) for preventing its nuclear accumulation (Hirate et al., 2013; Nishioka et al., 2009).

As a result, Yap1 transition into nucleus is inhibited, and Yap 1 cannot bind to its partner protein TEA domain transcription factor 4 (Tead4) to drive expression of caudal-related homeobox 2 (*Cdx2*), which is essential for TE segregation (Home et al., 2012; Nishioka et al., 2008, 2009; Rayon et al., 2014; Yagi et al., 2007). And thus, in the absence of Tead4–Yap1 activity, ICM-induced genes such as POU class 5 homeobox 1 (*Oct-4*) and SRY-related HMG-box gene 2 (*Sox2*) are expressed in the inner cells (Cockburn et al., 2013; Frum et al., 2018; Lorthongpanich et al., 2013; Wicklow et al., 2014; Yu et al., 2016). In the outer cells, the Hippo pathway is repressed and Lats1/2 is not activated. Thus, Yap1 is not phosphorylated and can shuttle into the nucleus. Consequently, Yap1 binds to Tead4 and drives *Cdx2* expression for TE segregation (Nishioka et al., 2009).

We have demonstrated previously that development from the morula to blastocyst stage is inhibited when *TEAD4*

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expression is downregulated by RNA interference in porcine embryos (Emura et al., 2019). In addition, *SOX2* transcript levels were increased in these *TEAD4*-downregulated embryos (Emura et al., 2019). These findings suggest that *TEAD4* is needed for TE segregation and that the Hippo pathway controls this function in porcine embryos as in mice. However, roles of the Hippo pathway in porcine embryos have not been investigated in detail.

Moreover, there are evidences for differences in molecular mechanisms of the ICM/TE segregation between mice and pigs. For example, our previous report has demonstrated that OCT-4 is essential for both ICM and TE segregation in porcine embryos, while it is needed only for ICM formation in mice (Emura et al., 2016; Nichols et al., 1998; Sakurai et al., 2013). In addition, *CDX2* is dispensable for blastocyst formation in porcine embryos, but not in murine embryos (Bou et al., 2017). Therefore, the molecular mechanisms of the ICM/TE segregation may differ among animal species, and further investigation of YAP1 and large tumor suppressor 2 (*LATS2*) functions in preimplantation development of porcine embryos is necessitated.

In the present study, we report the functions of the Hippo pathway related genes YAP1 and *LATS2* in porcine preimplantation development. First, we characterized expression patterns of YAP1 and *LATS2* in porcine oocytes and embryos during progression to the blastocyst stage. We then conducted independent YAP1 and *LATS2* knockdown to assess their functions in preimplantation development and gene expression involved in differentiation in porcine embryos.

Materials and Methods

Chemicals

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

Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and maintained at 37°C during transport to the laboratory. Cumulus-oocyte complexes (COCs) were harvested from 2 to 6 mm diameter follicles in TCM-199 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Kanagawa, Japan), 20 mM Hepes, 0.68 mM L-glutamine, 100 IU/mL penicillin G potassium (Meiji Seika, Tokyo, Japan), and 0.1 mg/mL streptomycin sulfate (Meiji Seika).

Approximately 50 COCs with uniform ooplasm and a cumulus cell mass were cultured separately in four-well dishes (Thermo Fisher) for 20 hours in 500 μ L of maturation medium, composed of a modified North Carolina State University (NCSU)-37 (mNCSU-37) (Petters and Wells, 1993) solution supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 0.05 mM β -mercaptoethanol, 1 mM dibutyl cAMP (dbcAMP), 10 IU/mL pregnant mare serum gonadotropin (Serotropin, Aska Animal Health, Tokyo, Japan), and 10 IU/mL human chorionic gonadotropin (hCG; Gestron 1500, Kyoritsu Seiyaku, Tokyo, Japan). The COCs were subsequently cultured in maturation medium without dbcAMP and hormones for 24 hours. The maturation culture was performed at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂.

In vitro fertilization and embryo culture

After *in vitro* maturation, COCs were washed with modified Pig-FM (mPig-FM) (Kikuchi et al., 2002), and 15–20 COCs were suspended in a 90 μ L drop of mPig-FM. Cryopreserved semen was thawed, and spermatozoa were washed twice by centrifugation at 1800 rpm for 3 minutes in sperm washing medium (TCM 199 medium supplemented with 20 mM Hepes, 0.68 mM L-glutamine, 100 U/mL penicillin G potassium, 0.1 mg/mL streptomycin sulfate, 0.91 mM sodium pyruvate, 4.12 mM calcium lactate, 3.0 mM glucose, and 10% [v/v] FBS) adjusted to pH 7.8 (Nagai et al., 1988). The spermatozoa were resuspended in sperm washing medium, and 10 μ L of this suspension was added to 90 μ L of mPig-FM containing matured COCs. The final concentration of sperm was adjusted to 1.0×10^6 /mL. COCs and sperm were incubated for 6 hours at 39°C under a 5% CO₂, 5% O₂, and 90% N₂ atmosphere.

Following microinjection of siRNA, the embryos were washed and cultured in PZM-5 (Suzuki and Yoshioka, 2006) at 39°C under a 5% CO₂, 5% O₂, and 90% N₂ atmosphere until day 5 (*in vitro* fertilization = day 0). Rates of embryo development were assessed on day 2 (2-cell $\leq$ stage), day 3 (8-cell stage), day 4 (16-cell $\leq$ and morula stage), and day 5 (blastocyst stage).

Design of siRNA and microinjection into embryos

The siRNA target sights of the YAP1 and *LATS2* transcript were selected from porcine sequences in GenBank (accession nos.: YAP1, XM_021062706.1; *LATS2*, NM_001177919.1), and specific siRNAs were designed using siRNA design software BLOCK-iTTM RNAi Designer (<http://rnaidesigner.invitrogen.com/rnaiexpress/>). Both sense and antisense RNA sequences for siRNAs were commercially synthesized (Table 1).

After insemination, cumulus cells and excess spermatozoa were removed from presumptive zygotes by pipetting. Embryos were subsequently transferred to a 20 μ L drop of PZM-5. Approximately 10 pL of 50 μ M specific siRNA duplexes were injected into the cytoplasm of each embryo using a FemtoJet (Eppendorf, Hamburg, Germany). Approximately 10 pL of 20 μ M nonsilencing siRNA (AllStars Negative Control siRNA; Qiagen, Tokyo, Japan) was injected as a control by the same method. After microinjection, the embryos were cultured as described above.

Determination of gene transcript levels in porcine embryos

Oocytes and embryos at the indicated developmental stages were treated with 0.1% protease in 1% polyvinylpyrrolidone (PVP)-phosphate buffered saline (PBS) for 5 minutes and washed seven times in 1% PVP-PBS. Pools of 10 (*in vitro* matured oocytes, 1-cell, 2–4-cell, and 8–16-cell stage embryos) or 5 (morula and blastocyst stage embryos) oocytes or embryos were added to 5 μ L lysis buffer (0.8% Igepal [ICN Biomedicals, Inc., Aurora, OH], 5 mM dithiothreitol [Thermo Fisher], and 1 U/ μ L of RNasin [Promega, Madison, WI]) and snap-frozen in liquid nitrogen and stored at –80°C.

RNA samples were heated to 80°C for 5 minutes and treated for reverse transcription (RT) using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The reaction mixture was diluted

TABLE 1. PRIMERS AND siRNA SEQUENCES

Name	Nucleotide sequences (5'-3')	Annealing temperature (C)	Fragment size (bp)	GenBank accession no.
<i>YAP1</i>	F- ATCAGTCAAAGCGCTCCAGT R- TTGGAGAATTTGCTGTGCTG	60	208	XM_021062706.1
<i>LATS2</i>	F- TACCAGAAAGGGAGCCACAC R- AAGAGAATCACGCCGACACT	60	239	NM_001177919.1
<i>SOX2</i>	F- GCCCTGCAGTACAACTCCAT R- GCTGATCATGTCCCGTAGGT	60	216	EU503117.1
<i>OCT-4</i>	F- GTTCTCTTTGGGAAGGTGTT R- ACACGCGGACCACATCCTTC	55.4	313	NM_001113060
<i>GAPDH</i>	F- TCGGAGTGAACGGATTTG R- CCTGGAAGATGGTGATGG	52	219	AF017079
YAP1 siRNA	S- GCUCAUCCUCUCCAGCUUTT AS- AAGCUGGAGAGGAAUGAGCTT	N/A	N/A	N/A
LATS2 siRNA	S- GGAAGAUCUCCUACCAGAATT AS- UUCUGGUAGAGGAUCUUCCTT	N/A	N/A	N/A

AS, antisense strand; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LATS2, large tumor suppressor 2; N/A, not applicable; OCT-4, POU class 5 homeobox 1; R, reverse; S, sense strand; SOX2, SRY-related HMG-box gene 2; YAP1, yes-associated protein 1.

with DEPC-treated water to obtain a final volume of 21 μ L. Real-time PCRs were performed using a StepOne™ system (Applied Biosystems, Tokyo, Japan), and products were detected with SYBR Green included in the QuantiTect SYBR Green PCR master mix (Qiagen).

A 2 μ L aliquot of the RT product was used for each quantification. The amplification program was as follows: pre-incubation at 95°C for 15 minutes to activate HotStarTaq DNA Polymerase (Qiagen), followed by 45 cycles of denaturation at 94°C for 15 seconds, annealing of primers at different temperatures (Table 1) for 30 seconds, and elongation at 72°C for 30 seconds. At the end of the last cycle, a melting curve was generated by starting fluorescence acquisition at 60°C and recording measurements at 0.3°C increments 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 a QIAquick PCR Purification Kit (Qiagen), quantified by measuring absorbance at 260 nm using a NanoDrop (ND-1000; Thermo Fisher Scientific), and diluted as described. Serial 10-fold dilutions for creating the standard curve were amplified in all real-time PCR runs. The standards and cDNA samples were then coamplified in the same reaction prepared from a master mix. Fluorescence was acquired at each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction, at which fluorescence rose above the background for each sample. Final quantification was performed using StepOne™ quantification software. Expression of the target gene in each run was normalized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Immunofluorescence staining

Oocytes or embryos at the indicated developmental stage were fixed in PBS containing 4% (w/v) paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 20 minutes and then washed twice in PBS containing 0.1% (v/v) Triton X-100 (TXPBS) for 10 minutes each time. Samples were subsequently permeabilized in 0.2% (v/v) Triton X-100 in PBS for 30 minutes, incubated in Image-iT FX Signal Enhancer (Thermo Fisher Scientific) for 30 minutes, and washed twice in TXPBS for 10 minutes each time. Samples were blocked by incubation

in 0.5% (w/v) bovine serum albumin (BSA) and 1% (w/v) skimmed milk in TXPBS for 1.5 hours and then washed in TXPBS for 5 minutes. The oocytes or embryos were incubated with an anti-YAP1 primary antibody (1:200, H00010413-MO1; Abnova, Taipei, Taiwan) in PBS supplemented with 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100 at 4°C for 1 hour.

Immunolabeled oocytes or embryos were washed four times in TXPBS for 15 minutes each time, and incubated at room temperature with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:400, A11029; Thermo Fisher Scientific) for 1 hour in PBS containing 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100. Oocytes or embryos were then washed four times in TXPBS for 20 minutes each time and mounted onto slides in a drop of VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindol (DAPI; Vector Laboratories, Burlingame, CA) for nuclear counterstaining. Fluorescent images were obtained using an inverted fluorescence microscope and digital camera systems (ECLIPSE Ti-U and DS-Fi2-L3; Nikon, Tokyo, Japan).

Statistical analysis

Percentage data for embryonic development was subjected to an arcsine transformation. The transformed values were analyzed using the one-way analysis of variance (ANOVA) or Kruskal–Wallis test, followed by multiple pairwise comparisons using the Tukey–Kramer or Scheffé method. The mRNA expression levels of *YAP1* and *LATS2* were analyzed using the Kruskal–Wallis test, followed by multiple pairwise comparisons using the Scheffé method. *OCT-4* and *SOX2* mRNA expression levels were analyzed by one-way ANOVA, following by multiple pairwise comparisons using the Fisher's PLSD or Tukey–Kramer method. A difference with $p < 0.05$ was regarded as statistically significant for all tests.

Results

Temporal expression of YAP1 and LATS2 mRNA and protein in early embryos

YAP1 and *LATS2* mRNA levels in the *in vitro* matured oocytes and embryos at various developmental stages are shown in Figure 1. *YAP1* mRNA level in the 1-cell stage

FIG. 1. Relative abundance (mean ± SEM) of (A) *YAP1* and (B) *LATS2* transcripts in porcine *in vitro* matured oocytes and 1-cell to blastocyst stage embryos ($n=5$). ^{a-c}Different superscripts indicate significant differences ($p<0.05$). *LATS2*, large tumor suppressor 2; SEM, standard error of the mean; *YAP1*, yes-associated protein 1.

embryos was significantly ($p<0.05$) higher than that in the 2-cell to blastocyst stage embryos (Fig. 1A). *YAP1* transcript levels in the following 2–4-cell stage were decreased (Fig. 1A). *LATS2* mRNA level in the *in vitro* matured oocytes was significantly ($p<0.05$) higher than that in the 1-cell to blastocyst stage embryos, and which the expression level was beginning to decline at the following 1-cell stage (Fig. 1B).

Temporal expression status of YAP1 protein was evaluated by immunofluorescence labeling (Fig. 2). Nuclear localization of the YAP1 protein was detected at the morula and blastocyst stage. In the blastocyst stage embryos, nuclear YAP1 signals were clearly observed in TE at least.

Effect of siRNA injection on YAP1 and LATS2 expression

To evaluate the efficacy of *YAP1* and *LATS2* downregulation using RNA interference, *YAP1* and *LATS2* mRNA levels in

uninjected, control siRNA-injected, and YAP1 or LATS2 siRNA-injected embryos at the 16-cell stage were investigated (Fig. 3). The relative abundance of *YAP1* mRNA in YAP1 siRNA-injected embryos was significantly ($p<0.05$) lower than that in uninjected and control siRNA-injected embryos (Fig. 3A). Similarly, *LATS2* mRNA level was significantly ($p<0.05$) reduced in LATS2 siRNA-injected embryos compared to embryos subjected to control siRNA injection (Fig. 3B). *LATS2* mRNA level in LATS2 siRNA-injected embryos was slightly ($p=0.07$) lower than that in uninjected embryos (Fig. 3B).

Effect of YAP1 or LATS2 downregulation on the development of porcine embryos

We first evaluated the *in vitro* developmental competence of YAP1 siRNA-injected embryos (Table 2). No significant

FIG. 2. Representative photographs of YAP1 protein expression in porcine *in vitro* matured oocytes and 1-cell to blastocyst stage embryos. The oocytes and embryos are labeled with DAPI (blue) and YAP1 (green). DAPI, 4',6-diamidino-2-phenylindol. Color images are available online.

differences in the rate of development to the 2-cell $\leq$ embryos on day 2 were observed among the experimental groups. On day 3, however, the rate of development to the 8-cell stage was significantly ($p < 0.05$) higher in uninjected embryos than in control siRNA-injected embryos. On day 4, the rate of development to the 16-cell $\leq$ embryos was significantly ($p < 0.05$) higher in the uninjected group than in the YAP1 siRNA-injected and control siRNA-injected groups.

The rate of the morula stage development was significantly ($p < 0.05$) lower in YAP1 siRNA-injected embryos than in uninjected embryos, while there was no difference between uninjected and control siRNA-injected embryos. On day 5, the rate of the blastocyst stage development was significantly ($p < 0.05$) lower in YAP1 siRNA-injected embryos (0.5%) than in uninjected (20.4%) and control siRNA-injected embryos (15.9%).

The developmental competence of LATS2 siRNA-injected embryos is summarized in Table 3. No differences among experimental groups were observed until the 8-cell stage on day 3. On day 4, the rates of the 16-cell $\leq$ and morula stage development were significantly ($p < 0.05$) lower in LATS2 siRNA-injected embryos than in uninjected and control siRNA-injected embryos. The number of embryos reaching the blastocyst stage (day 5) was also significantly ($p < 0.05$) lower in the LATS2 siRNA-injected group (1.0%) than in the uninjected (34.6%) and control siRNA-injected groups (24.2%).

Relative expression levels of the ICM/TE segregation-related mRNA transcripts in porcine embryos derived from YAP1 or LATS2 siRNA injection

OCT-4 and *SOX2* transcript levels were analyzed at the 16-cell stage to clarify the effects of YAP1 and LATS2 down-

regulation on expression of genes involved in the ICM/TE segregation (Fig. 4). YAP1 siRNA injection significantly ($p < 0.05$) increased both *OCT-4* and *SOX2* expression levels (Fig. 4A). As shown in Figure 4B, although there was no difference in *OCT-4* mRNA levels between uninjected and LATS2 siRNA-injected embryos, *OCT-4* transcript level in LATS2 siRNA-injected embryos was significantly ($p < 0.05$) lower than that in control siRNA-injected embryos. Similarly, the expression level of *SOX2* in LATS2-injected embryos was significantly ($p < 0.05$) lower than that in uninjected and control siRNA-injected embryos (Fig. 4B).

Discussion

It is well known that the Hippo pathway has important roles of the ICM/TE segregation in mice (Hirate et al., 2013; Nishioka et al., 2009). Lats1/2 kinases and the downstream effector Yap1 are the core components of the Hippo pathway. At the murine morula stage, the Hippo pathway is quiescent in the outer cells, and Yap1 is not phosphorylated by Lats1/2 kinases. Thus, Yap1 can localize to the nucleus and combine with Tead4 to induce expression of the TE-related genes such as *Cdx2* (Home et al., 2012; Nishioka et al., 2008, 2009; Rayon et al., 2014; Yagi et al., 2007).

Conversely, the Hippo pathway is activated in the inner cells, resulting in Yap1 phosphorylation by Lats1/2 in the cytoplasm and inhibition of Yap1 nuclear translocation (Hirate et al., 2013; Nishioka et al., 2009). In this condition, the TE-related gene expression does not take place, and the inner cells adopt the ICM phenotype. Taken together, Yap1 and Lats1/2 are necessary for the segregation of the TE and ICM, respectively, in mice. In this study, we evaluated the functions of YAP1 and LATS2 in preimplantation porcine embryos using RNA interference.

FIG. 3. Relative abundance (mean ± SEM) of (A) *YAP1* and (B) *LATS2* transcripts at the 16-cell stage in uninjected ($n=5$), control siRNA-injected ($n=5$), and *YAP1* (A) or *LATS2* (B) siRNA-injected embryos ($n=5$). ^{a,b}Different superscripts indicate a significant difference ($p < 0.05$).

In mice, *Yap1* transcription levels are high from the oocyte to 2-cell stage and then decrease with development to the blastocyst stage (Yu et al., 2016). In the present study, *YAP1* mRNA expression in porcine *in vitro* matured oocytes and embryos was detected at all developmental stages. Similar to the case with mice, *YAP1* mRNA levels in the oocytes and embryos just after fertilization were the highest in pigs. On the contrary, nuclear signals of YAP1 protein in porcine embryos were detected from the morula stage, and they were clearly observed in TE at the blastocyst stage. Our previous research has shown that TEAD4 protein was in nuclei of porcine embryos from the 16-cell to blastocyst

stage (Emura et al., 2019). These findings suggest that *YAP1* mRNA is transcribed at early stages, and is progressively translated and shuttled to the nucleus where it cooperates with TEAD4 to activate genes required for preimplantation development at late stages.

Although *LATS2* mRNA expression was detected at all developmental stages in porcine oocytes and embryos, *LATS2* mRNA expression was higher in the *in vitro* matured oocytes and 1-cell stage embryos than at the other developmental stages, similar to the expression pattern of *YAP1* mRNA. While *Lats2* mRNA expression and enzyme activity are well described in murine oocytes and embryos (Nishioka et al., 2009), expression status in porcine oocytes and preimplantation embryos was unknown. In the present study, we demonstrate that both *LATS2* and *YAP1* are expressed in porcine oocytes and preimplantation embryos, and that the Hippo pathway may play a role in functional regulation of gene expression involved in the ICM/TE segregation of porcine embryos.

Interestingly, our study showed that downregulation of *YAP1* transcript allowed porcine embryos to develop to the morula stage, but strongly inhibited blastocyst formation. In murine embryos, *Cdx2* expression by Tead4–Yap1 complex in the outer cells of morulae is important for TE segregation (Home et al., 2012; Nishioka et al., 2008, 2009; Rayon et al., 2014; Yagi et al., 2007).

Our previous study has found porcine embryos with *TEAD4* downregulated cannot form blastocysts, similar to the *YAP1* downregulation in this study (Emura et al., 2019). Therefore, it is possible that the TEAD4–YAP1 complex is essential for TE differentiation of porcine embryos. However, *TEAD4* downregulation does not affect *CDX2* expression (Emura et al., 2019), and *CDX2* is dispensable for TE segregation in porcine embryos (Bou et al., 2017). Further studies are necessary to identify the downstream factors of the TEAD4–YAP1 complex in porcine embryos. On the contrary, *LATS2* downregulation inhibited porcine embryo development before the morula stage. This result indicates that *LATS2* regulates the preimplantation development of porcine embryos in addition to segregating the ICM/TE lineages from the morula to blastocyst stage.

Oct-4 is a marker for pluripotency as possessed by ESCs (Niwa et al., 2000; Yeom et al., 1996). In murine embryos, Oct-4 is required for ICM formation, but is dispensable for TE segregation (Nichols et al., 1998). In porcine blastocyst stage embryos, OCT-4 is expressed in both ICM and TE (du Puy et al., 2011; Fujii et al., 2013; Kirchhof et al., 2000), and our previous research has indicated that OCT-4 expression

TABLE 2. EFFECT OF YES-ASSOCIATED PROTEIN 1 siRNA INJECTION ON *IN VITRO* DEVELOPMENT OF PORCINE EMBRYOS

Treatment	No. of embryos cultured	No. (%) ^a of embryos developed to				
		Day 2	Day 3	Day 4	Day 5	
		2-cell ≤	8-cell	16-cell ≤	Morula	Blastocyst
Uninjected	196	138 (70.4)	61 (31.1) ^a	104 (53.1) ^a	37 (18.9) ^a	40 (20.4) ^a
Control siRNA	195	119 (61.0)	40 (20.5) ^b	81 (41.5) ^b	27 (13.8) ^{ab}	31 (15.9) ^a
YAP1 siRNA	192	118 (61.5)	44 (22.9) ^{ab}	60 (31.3) ^b	20 (10.4) ^b	1 (0.5) ^b

Experiments were replicated five times.

^aPercentages of the number of embryos cultured.

^{a,b}Values with different superscripts within each column differ significantly ($p < 0.05$).

TABLE 3. EFFECT OF LARGE TUMOR SUPPRESSOR 2 siRNA INJECTION ON *IN VITRO* DEVELOPMENT OF PORCINE EMBRYOS

Treatment	No. of embryos cultured	No. (%) ^a of embryos developed to				
		Day 2	Day 3	Day 4	Day 5	
		2-cell ≤	8-cell	16-cell ≤	Morula	Blastocyst
Uninjected	211	152 (72.0)	85 (40.3)	115 (54.5) ^a	47 (22.3) ^a	73 (34.6) ^a
Control siRNA	207	138 (66.7)	69 (33.3)	87 (42.0) ^a	37 (17.9) ^a	50 (24.2) ^a
LATS2 siRNA	205	137 (66.8)	84 (41.0)	57 (27.8) ^b	4 (2.0) ^b	2 (1.0) ^b

Experiments were replicated five times.

^aPercentages of the number of embryos cultured.

^{a,b}Values with different superscripts within each column differ significantly ($p < 0.05$).

is essential for porcine TE segregation (Emura et al., 2016; Sakurai et al., 2013). Therefore, OCT-4 functions in the formation of both ICM and TE in porcine embryos.

Lacking maternal *Yap1* in murine embryos increased expression of ICM-related genes, such as Oct-4 and Nanog (Yu et al., 2016). The Tead4–Yap1 complex is not formed in the nucleus in cells with *Yap1* deletion, so it is possible that expression of the target gene *Cdx2* is decreased under such conditions. Moreover, *Cdx2* downregulation induces Oct-4 expression in blastomeres. In addition, *Lats1/2* downregulation in mice reduces phosphorylated Yap1, resulting in transition of Yap1 into the nucleus

(Lorthongpanich et al., 2013; Nishioka et al., 2009). As a result, *Cdx2* is expressed in every cell while Oct-4 expression is unchanged (Lorthongpanich et al., 2013; Nishioka et al., 2009).

In the present study, *YAP1* downregulation led to *OCT-4* upregulation as in murine embryos. Furthermore, *LATS2* downregulation decreased *OCT-4* expression in porcine embryos. These results imply that YAP1 and LATS2 regulate *OCT-4* expression in porcine preimplantation embryos. However, *TEAD4* downregulating in porcine embryos has no influence on *OCT-4* expression despite findings that *Tead4*-deficient murine embryos express high levels of Oct-

FIG. 4. (A) Relative abundance (mean $\pm$ SEM) of *OCT-4* and *SOX2* transcripts at the 16-cell stage in uninjected ($n = 5$), control siRNA-injected ($n = 5$), and YAP1 siRNA-injected embryos ($n = 5$). ^{a,b}Different superscripts indicate a significant difference ($p < 0.05$). (B) Relative abundance (mean $\pm$ SEM) of *OCT-4* and *SOX2* transcripts at the 16-cell stage in uninjected ($n = 5$), control siRNA-injected ($n = 5$) and LATS2 siRNA-injected embryos ($n = 5$). ^{a,b}Different superscripts indicate a significant difference ($p < 0.05$). OCT-4, POU class 5 homeobox 1; SOX2, SRY-related HMG-box gene 2.

4 (Emura et al., 2019; Yagi et al., 2007). Thus, in porcine embryos, YAP1 controls *OCT-4* expression independently from TEAD4.

Sox2 is also a marker for pluripotency and interacts with Oct-4 for forming the Oct–Sox enhancer in ESCs (Kuroda et al., 2005; Masui et al., 2007; Okumura-Nakanishi et al., 2005). In murine embryos, Sox2 is essential for ICM formation (Avilion et al., 2003), and Sox2 expression is absent in *Lats1/2*-downregulated embryos (Lorthongpanich et al., 2013). Conversely, lacking either *Yap1* or *Tead4* increases ectopic Sox2 expression (Frum et al., 2018; Wicklow et al., 2014). Taken together, Tead4 and Yap1 contribute to TE segregation in murine embryos through Sox2 repression.

In the present study, *SOX2* expression was upregulated by *YAP1* downregulation in porcine embryos. In contrast, *LATS2* downregulation impaired *SOX2* expression. Our recent study also has shown that TEAD4 negatively regulates *SOX2* expression in porcine embryos (Emura et al., 2019). These results suggest that the molecular mechanisms for regulation of *SOX2* expression in porcine embryos are similar to those in murine embryos.

In conclusion, we demonstrated that YAP1 and LATS2 are important for early porcine embryo development and expression of downstream genes involved in the segregation of ICM and TE lineages. *YAP1* downregulation inhibited blastocyst formation and increased expression of *OCT-4* and *SOX2*. In addition, *LATS2*-downregulated embryos could not develop to the morula stage and exhibited reduced *OCT-4* and *SOX2* expression. This is the first time to indicate the necessity of YAP1 and LATS2 and the findings about the mechanism of early lineage segregation in porcine embryos. This study showed the possibility that the Hippo pathway plays an important role in the ICM/TE segregation of porcine embryos; however, further studies are required to elucidate the functions of the Hippo pathway in porcine embryogenesis.

Author Disclosure Statement

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