

Junction-mediating and regulatory protein (JMY) is essential for early porcine embryonic development

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Abstract. Junction-mediating and regulatory protein (JMY) is a regulator of both transcription and actin filament assembly. JMY is a critical nucleation-promoting factor (NPF); however, its role in the development of mammalian embryos is poorly understood. In the current study, we investigated the functional roles of the NPF JMY in porcine embryos. Porcine embryos expressed JMY mRNA and protein, and JMY protein moved from the cytoplasm to the nucleus at later embryonic developmental stages. Knockdown of JMY by RNA interference markedly decreased the rate of blastocyst development, validating its role in the development of porcine embryos. Furthermore, injection of JMY dsRNA also impaired actin and Arp2 expression, and co-injection of actin and Arp2 mRNA partially rescued blastocyst development. Taken together, our results show that the NPF JMY is involved in the development of porcine embryos by regulating the NPF-Arp2-actin pathway.

Key words: Junction-mediating and regulatory protein (JMY), Nucleation-promoting factor (NPF), Pig embryo
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Actin nucleators, which are proteins that promote rapid actin polymerization in cells [1], including the Arp2/3 complex [2], formins [3] and spire [4], play essential roles in the dynamic reorganization of the actin cytoskeleton. These proteins also control many different cellular processes, including cell motility [5], cytokinesis [6] and oocyte maturation [7, 8]. In addition to these actin nucleators, various nucleation-promoting factors (NPFs), including N-WASP [9], WAVE2 [10] and junction-mediating and regulatory protein (JMY) [11], which bind to and activate the Arp2/3 complex in response to Rho family GTPase signaling, are essential components in dynamic actin reorganization during oocyte maturation.

Among the NPFs, JMY is unique because it has dual roles as a NPF and transcriptional regulator [12–14]. JMY was initially identified as a transcriptional regulator that binds p300 and facilitates p53-mediated transcriptional responses [12, 13]. JMY can bind and activate the Arp2/3 complex, and it is involved in cell migration [14, 15]. The region of JMY that is responsible for its actin nucleation activity and interaction with the Arp2/3 complex is located in the C-terminal WWCA region, which is composed of three tandem actin monomer-binding sequences (Wiskott-Aldrich syndrome protein homology 2 [WH2] domains) and an Arp2/3-binding central and acidic (CA) region [14, 16]. JMY has been reported to be one of the essential components for asymmetric divisions during oocyte maturation in mice [11] and pigs [17].

After fertilization, the mammalian zygote divides into two cells

under maternal regulation as embryonic development begins [18]. In contrast to the asymmetric cell divisions that occur during meiotic maturation in the oocyte [8], early embryos undergo symmetric cell divisions, and precise divisions at each developmental stage are crucial for proper embryonic development [18]. After the 8-cell stage, each cell in the embryo undergoes compaction and inner cell mass polarization, followed by formation of the blastocyst cavity. Since cytokinesis and cell movement in other somatic cells are mainly driven by dynamic actin polymerization [1], actin nucleators and/or NPF, which is the main regulator of the actin cytoskeleton, play important roles in early embryogenesis. For example, the Arp2/3 complex [19] has been reported to be important for early mouse embryogenesis, and various NPFs, including WAVE2 and JMY, are involved in this process [20]. However, the exact roles and function of JMY in early porcine embryonic development are not yet well understood.

In this study, we investigated the roles of JMY in porcine preimplantation development. By knocking down JMY expression, we found that JMY is essential for preimplantation development of porcine embryos.

Materials and Methods

In vitro maturation (IVM) and parthenogenetic activation of porcine oocytes

Porcine cumulus oocyte complexes (COCs) were collected as described previously [21]. COCs with intact and unexpanded cumulus cells were isolated and cultured in tissue culture medium (TCM)-199 containing 0.1% polyvinyl alcohol (PVA, w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO, USA), 10 IU/ml pregnant mare serum gonadotropin (PMSG), 10 IU/ml human chorionic gonadotropin (hCG), 75 µg/ml penicillin G and 50 µg/ml streptomycin sulfate under mineral oil for 44 h at 38.5 C in a humidified

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atmosphere containing 5% CO₂ (v/v) in air. Parthenogenetic embryos were used in this study to avoid sperm penetration. Oocytes were subsequently exposed to 5 mg/l ionomycin diluted in North Carolina State University (NCSU)-23 medium for 5 min and then cultured in NCSU-23 medium supplemented with 7.5 µg/ml cytochalasin B (Sigma) for 3 h. The porcine embryos were washed three times in NCSU-23 medium containing 0.4% BSA (w/v) and cultured (activation = day 0) in the same medium at 39 C in an atmosphere containing 5% CO₂.

Preparation of mRNA and double-stranded RNA (dsRNA)

Total RNA was extracted from 50 GV oocytes with an RNeasy Micro Kit (QIAGEN, Duesseldorf, Germany). Then, total cDNA was synthesized with a cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The full length Arp2 and actin CDSs were cloned by PCR using the primer pairs listed in Table 1. After sequencing and performing BLAST searches, the full-length CDSs were subcloned into the *EcoRI/NotI* (NEB, Ipswich, MA, USA) restriction sites of the PRN3 vector. The plasmid was linearized by digestion with *SfiI* (TaKaRa, Tokyo, Japan) and purified with a Gel Extraction Kit (Promega, Madison, WI, USA). A MEGAscript T7 transcription Kit (Ambion, Austin, TX, USA) was used to produce the mRNA, which was purified with an RNeasy MinElute Cleanup Kit (QIAGEN). The mRNA concentration was determined with a Beckman DU 530 Spectrophotometer (Fullerton, CA, USA) and then diluted to 0.4 mg/ml for injection.

To knockdown JMY expression in porcine embryos, JMY dsRNA was generated as described previously [17]. Using two primers (shown in Table 1), a fragment (from bp 2266 to bp 2811) of the porcine JMY gene (XM_003123744.1) was amplified as a 570-bp fragment containing the T7 promoter from the cDNA. The PCR product was gel purified and then used as a template for *in vitro* transcription using an mMessage mMachine T7 Transcription Kit (Ambion). T7 promoters located at both ends of the PCR product initiated transcription in both directions to produce sense and antisense JMY transcripts. After the *in vitro* transcription reaction, the template DNA was digested with TURBO DNase (Life Technologies, Foster City, CA, USA), and the transcripts were purified by phenol-chloroform extraction and isopropanol precipitation. The dsRNA sample was stored at -80 C until use.

Microinjection of oocytes with dsRNA or mRNA

Microinjections were performed as described previously [17] using an Eppendorf microinjector (Hamburg, Germany) and a Nikon Diaphot ECLIPSE TE300 inverted microscope (Nikon UK, Kingston upon Thames, Surrey, UK) equipped with a Narishige MM0-202N hydraulic three-dimensional micromanipulator (Narishige, Sea Cliff, NY, USA) and were completed within 1 h. The dsRNA or mRNA was injected in a volume of approximately 10 pl (1 µg/µl), and then the zygotes were cultured under paraffin oil at 38.5 C. All microinjection experiments were performed at least five times, and approximately 80 oocytes in each group were injected.

Immunofluorescence and confocal microscopy

Porcine embryos were fixed with 3.7% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) containing 0.1% PVA (w/v) for

30 min at room temperature. Embryos were washed three times with PBS-0.1% PVA and permeabilized with 1% Triton X-100 (v/v) for 30 min at 37 C and then blocked with 1% BSA (w/v) for 1 h. To determine the cellular distribution of proteins, embryos were incubated overnight at 4 C with anti-JMY, anti-Arp2 and anti-actin antibodies (Santa Cruz, CA, USA) diluted 1:100 in blocking buffer. Alexa Fluor 568 Goat anti-rabbit IgG (Invitrogen) was used as the secondary antibody. The nuclear status of the embryos was determined by staining with 10 µg/ml propidium iodide (PI) for 20 min. Following extensive washing, oocytes were mounted between a coverslip and a glass slide. For the negative control in each group, the primary antibodies were omitted, and the oocytes were incubated with only the secondary antibody and stained with DAPI. Embryos were examined under a Zeiss LSM 710 META confocal laser-scanning microscope (Zeiss, Jena, Germany).

Real-time quantitative PCR

Total RNA was isolated from frozen porcine embryos with a Dynabeads mRNA DIRECT Kit (DynaL Biotech ASA, Oslo, Norway) and reverse transcribed into cDNAs with oligo(dT)₁₂₋₁₈ and SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA). RT-qPCR was performed with a DyNamo HS SYBR Green qPCR Kit (Finnzymes, Helsinki, Finland) and a CFX96 real-time qPCR system (Bio-Rad, Hercules, CA, USA) under the following conditions: 94 C for 30 sec, followed by 40 cycles of 94 C for 30 sec, 60 C for 30 sec and 72 C for 25 sec. A final extension at 72 C for 5 min was included at the end of the run. Relative gene expression was quantified by normalization to GAPDH mRNA levels using the $\Delta\Delta$ CT method [22]. Briefly, for each independent experiment, mRNA was extracted from 20 embryos of each stage. The primers used for RT-qPCR are listed in Table 1.

Statistical analysis

All percentage data were subjected to arcsine transformation before statistical analysis. The general linear models (GLM) procedure in the SAS software (SAS Institute, Cary, NC, USA) was used to analyze the data. Differences with *p* values less than 0.05 were considered significant. For fluorescence intensity data, 10 × 10 pixels in different areas of 10 oocytes were analyzed using the ZEN 2009 software.

Results

Dynamic localization of JMY during early porcine embryonic development

We investigated the expression of JMY mRNA and the subcellular localization of JMY protein in porcine parthenogenetic embryos. As shown in Fig. 1, JMY mRNA was detected at the 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst stages. JMY mRNA expression increased significantly after the 2-cell stage and was maintained at a similar level until the blastocyst stage. Next, we examined the subcellular localization of JMY protein using immunostaining (Fig. 1B). JMY was predominantly located in the cytoplasm of 1-, 2- and 4-cell stage embryos. Interestingly, JMY protein was distributed in the plasma surrounding the nucleus at the 8-cell stage and was selectively enriched in the nucleus at the morula and blastocyst stages. These results suggested that JMY is present during all stages of porcine

Table 1. Primers used in this study

	GenBank accession number	Primer sequence (5'-3')	Length (bp)
<i>JMY</i> ^a	XM_003123744.1	F: ATTAATACGACTCACTATAGGGAGAACTGCCTCCCACTGTATCG R: ATTAATACGACTCACTATAGGGAGATGCTCCGTGTAGAGGGTCT	570
<i>JMY</i>	XM_003123744.1	F: TTCCGAGACATGCGAGAACT R: TGCTGCCCATGATGCTTTAC	467
<i>Arp2</i>	XM_005662508.1	F: GGCAGTCTGTTAGGTGGC R: CCTGGCGTTGAAGGAGTT	1477
<i>Actin</i>	XM_003357928.2	F: CGTGTCTTCTCGCCGCTCC R: CTCCGTCTCAGATTCTCAACC	1569
<i>Nanog</i>	NM_001129971	F: AGCGAATCTTCACCAATGCC R: TGCTTCTTGACTGGGACCTT	230
<i>Oct4</i>	JN633978.1	F: AGGGCAAACGATCAAGCAGT R: CTTCACCTCCCTCCAACCA	408
<i>Sox2</i>	EU519824.1	F: AACAGCCAGACCGAGTTAA R: CATGAGCGTCTTGGTTTTCC	150
<i>Foxp3</i>	NM_001128438.1	F: GGCATCATCTGACAAAGGGTT R: AGGCTCAAGTTGTGGCGAAT	357
<i>Stat3</i>	HM462247.1	F: GTGGAACCGTACACCAAGCA R: TAGAGGTAGACCAGCGGAGACA	113
<i>GAPDH</i>	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	230

^a T7 promoter sequences used to generate the JMY dsRNA for knockdown are indicated in bold.

preimplantation embryo development; however, its localization changes drastically during the course of development.

Knockdown of JMY impairs cell division in porcine embryos

To determine the role of JMY in porcine embryonic development, we performed knockdown experiments by injecting porcine JMY dsRNA. After knockdown, the JMY mRNA level in 4-cell stage embryos decreased to $27.1 \pm 6.1\%$ of that in the control embryos (Fig. 2A). Using immunofluorescence staining (Fig. 2B), we confirmed that the JMY protein level also decreased following dsRNA injection. Next, we injected JMY dsRNA into porcine zygotes and assessed its effect on the development of early porcine embryos. There was no significant difference in the percentage of 2-cell embryos between those injected with JMY dsRNA and those injected with the control RNA. However, at 30 h, only $36.6 \pm 5.9\%$ of the JMY-knockdown embryos developed to the 4-cell stage compared with $72.7 \pm 6.7\%$ of the embryos in the control group. At 144 h, only a few embryos developed to the blastocyst stage in the JMY-knockdown group ($3.9 \pm 0.9\%$), with more than half of the embryos in the control group did so ($53.3 \pm 2.0\%$). The embryos in the JMY-knockdown group were irregular in both shape and blastomere distribution (Fig. 2C).

Knockdown of JMY alters the level of actin and Arp2 in porcine embryos

Because few embryos developed to the blastocyst stage in the JMY-knockdown group, we analyzed the mechanism underlying aberrant embryo cleavage. The localization of actin and Arp2 was investigated after JMY depletion. As shown in Fig. 3, JMY knockdown decreased both actin and Arp2 fluorescence intensity in 4-cell arrested and blastocyst embryos compared with the levels

in control embryos, suggesting possible failure of cell division in the JMY-knockdown group.

The JMY knockdown-mediated disturbance in blastocyst development was not completely rescued by actin and Arp2 mRNA injection

In addition to its roles as a nucleation-promoting factor, JMY can also function as a transcriptional coactivator [12, 13]. Previous studies in mouse [23] and porcine [17] oocytes showed that knockdown of JMY affects Arp2 and actin mRNA and/or protein levels. We tested whether decreased actin or Arp2 mRNA levels are the main cause of the developmental arrest induced by JMY knockdown. We attempted to rescue the JMY knockdown phenotypes with actin or Arp2 mRNA injections. As shown in Fig. 4A, injection of neither actin nor Arp2 mRNA alone affected the rate of blastocyst development, whereas simultaneous injection of actin and Arp2 mRNA partially rescued the phenotypes caused by JMY knockdown. These results suggested that the phenotype caused by JMY knockdown is due in part to decreased levels of Arp2 and actin mRNA.

JMY knockdown disturbed the levels of embryo development-related mRNA expression

Because Arp2 or actin mRNA was not sufficient to rescue the developmental defects caused by JMY knockdown, we hypothesized that JMY may affect the expression of other development-related transcripts, including Nanog, Oct4, Sox2, Stat3, and Foxp3. We analyzed the level of these mRNAs following parthenogenetic activation in the control and JMY-knockdown groups at 72 h after activation using real-time qPCR. As shown in Fig. 4B, significantly lower mRNA levels of all the developmental-related genes examined

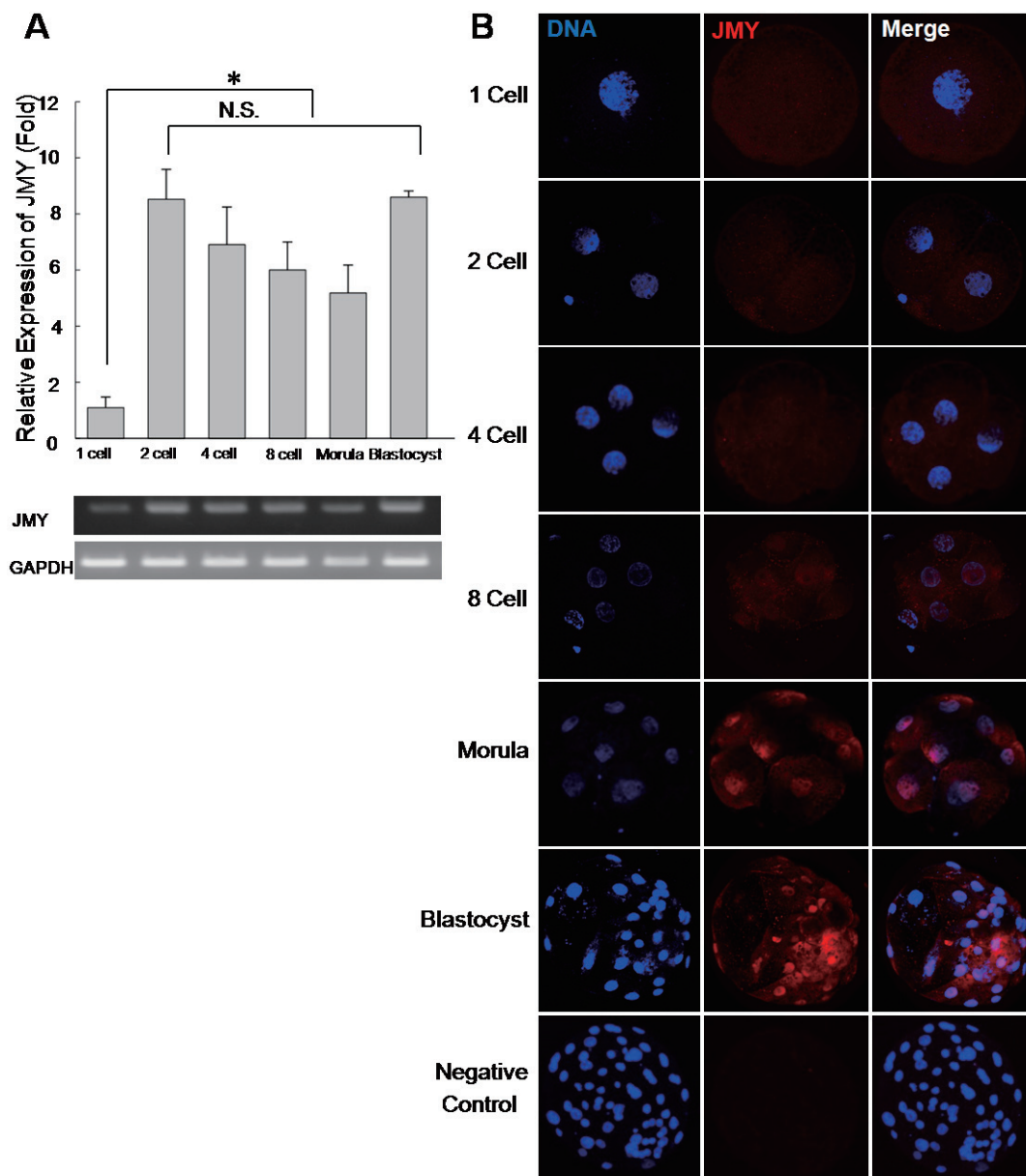


Fig. 1. Expression of JMY during early parthenogenetic embryo development in pigs. **A:** The relative expression of JMY in porcine embryos was measured by RT-qPCR, and mRNA levels were normalized to the 1-cell stage. Values represent the mean \pm SEM. * $P < 0.05$. **B:** Subcellular localization of JMY protein during porcine embryonic development as determined by immunofluorescence staining. Red, JMY; blue, chromatin.

were observed in the JMY-silenced embryos at 72 h compared with the corresponding levels in the control embryos.

Discussion

It has been reported that JMY plays crucial roles in mammalian oocyte maturation in mice [11] and pigs [17]. In addition to its role as an actin nucleator [14, 16, 24], JMY was first identified as a transcriptional coactivator involved in the p53 response [12, 13, 15]; however, its roles in preimplantation development are not yet

known. Based on its roles in oocyte maturation and transcriptional control, we hypothesized that JMY may contribute to early embryonic development.

In this study, we initially found that the subcellular localization of JMY changed dramatically during development. Although JMY was mainly dispersed in the cytoplasm and plasma membrane up until the 4-cell stage, nuclear localization was evident in 8-cell and later stage embryos. Considering the dual functions of JMY as an actin nucleation factor and transcriptional coactivator, these results imply that in preimplantation embryo development, the role of JMY

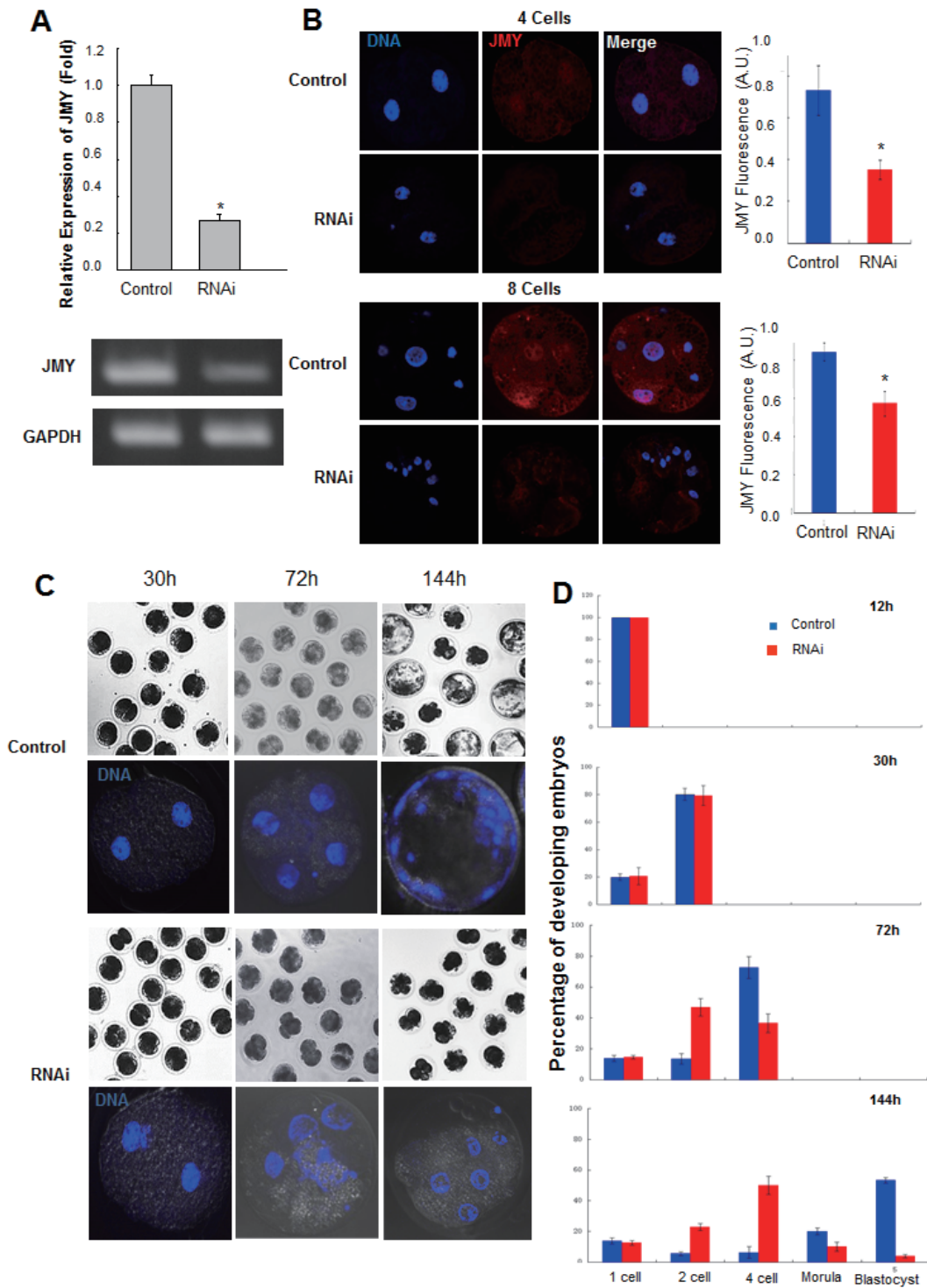


Fig. 2. Effect of JMY dsRNA on the early cleavage of porcine parthenogenetic embryos. A: Knockdown of JMY was performed by dsRNA injection. The mRNA levels of JMY after dsRNA microinjection are shown. mRNA levels in dsRNA-injected parthenogenetic 1-cell embryos (n = 20) are expressed relative to those in negative control siRNA-injected embryos. Data are shown as the mean \pm SEM (three independent experiments). B: Immunostaining and Fluorescence intensity for JMY in negative control siRNA-injected (control) and dsRNA-injected (RNAi_{JMY}) 4-cell and 8-cell stage embryos. Red, JMY; blue, DNA. C, D: Few embryos developed to the blastocyst stage after JMY RNAi. Data are shown as the mean \pm SEM. * P < 0.05 (four independent experiments).

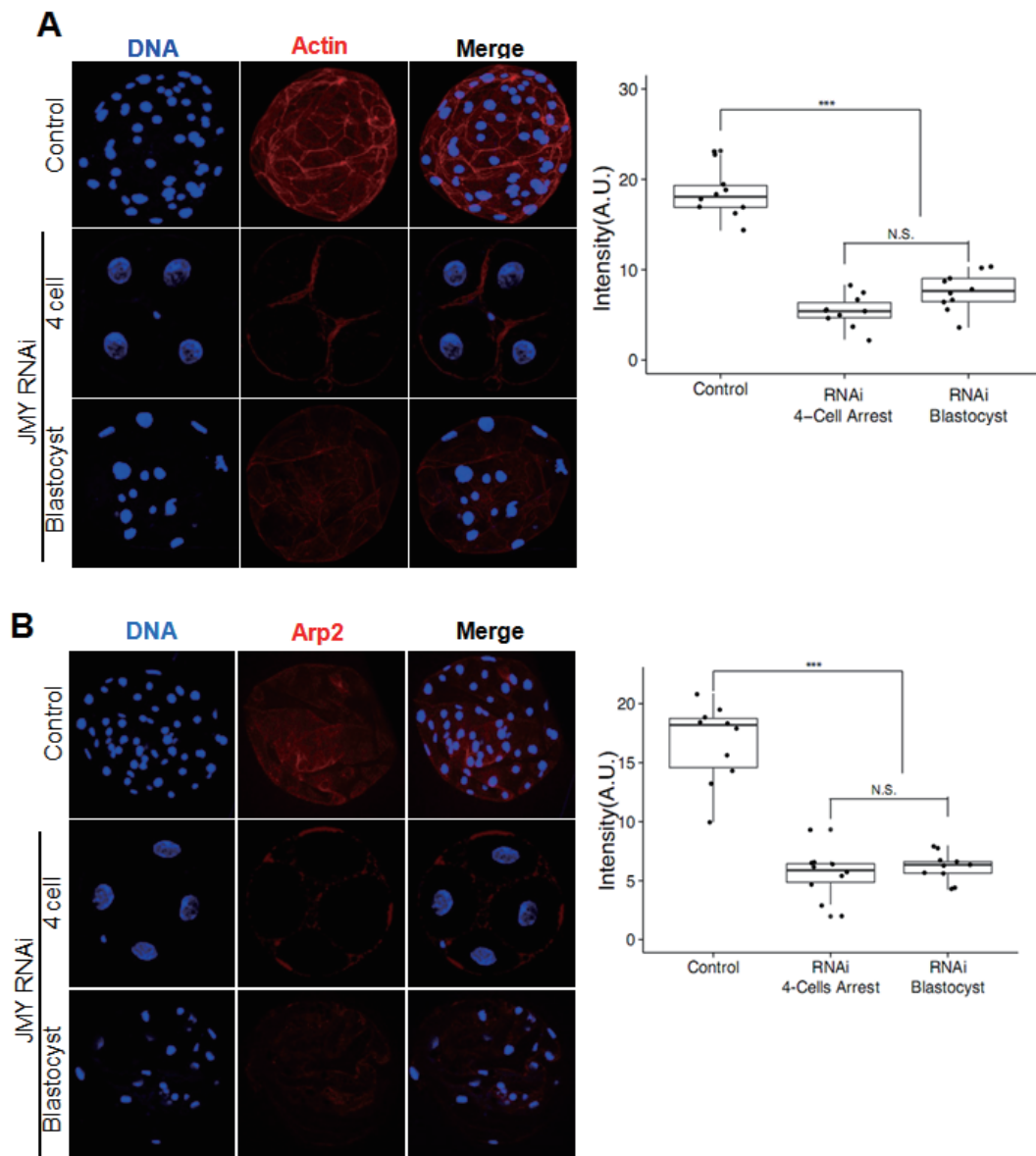


Fig. 3. Effect of JMY RNAi on actin and Arp2 expression in porcine embryos. Actin (A) and Arp2 (B) expression was lower in the JMY RNAi group at both the 4-cell and blastocyst stages. $n = 20$ (three independent experiments). Red, actin or Arp2; blue, chromatin. The histogram shows the relative fluorescence intensity of actin and Arp2 in the control and JMY RNAi groups. *** $P < 0.001$. N.S., not statistically significant ($P > 0.05$).

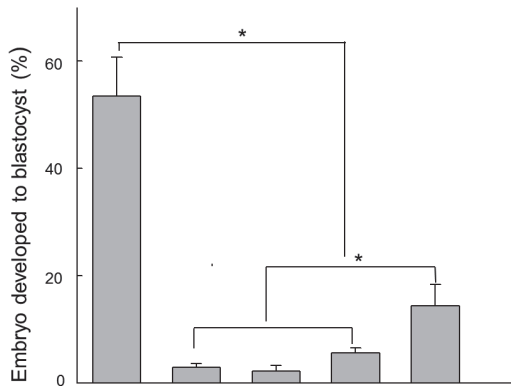
may be related to both functions.

Knockdown of JMY in porcine embryos caused developmental abnormalities comparable to those observed in mouse embryos [20]. Interestingly, the development rate of JMY-knockdown embryos did not differ from that of the control up until the 2-cell stage; however, development beyond the 4-cell stage differed significantly from that observed in the control embryos. These results, along with the translocation of JMY into the nucleus after the 8-cell stage, suggested the possibility that JMY may be associated with the embryonic genome activation (EGA) process, which is initiated at the 4-cell stage in porcine embryos [25]. This idea is supported by the significant decrease in the mRNA levels of embryogenesis-related genes in

JMY-knockdown embryos. However, the exact mechanism underlying the function of JMY in the activation of development-related genes requires further investigation.

We observed that the amount of filamentous actin and Arp2 was significantly lower in JMY-knockdown embryos than in control embryos (Fig. 3B) and that introduction of Arp2 or actin mRNA only partially rescued embryonic development, indicating that the effect of JMY knockdown may be caused by decreased expression of actin-related transcripts. Because JMY can bind to actin, which regulates its translocation into the nucleus [17], JMY translocation may affect the transcription of actin or actin-related proteins. However, its exact effect on preimplantation development remains

A



Control dsRNA	+				
JMY-dsRNA		+	+	+	+
Arp2 mRNA			+		+
Actin mRNA				+	+

B

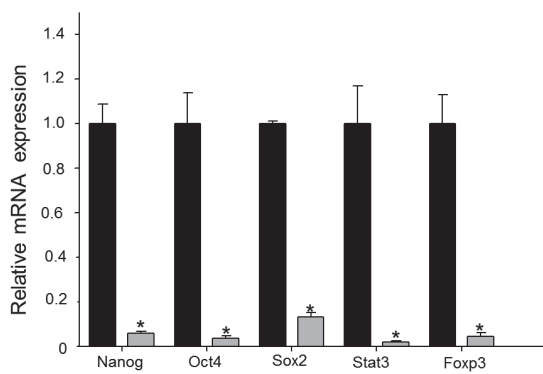


Fig. 4. JMY mediates porcine embryonic development and the levels of transcription-related mRNA expression. A: The percentage of porcine embryos that developed to the blastocyst stage was affected by JMY dsRNA and actin or Arp2 mRNA microinjection. B: JMY RNAi affected the expression of development-related transcripts, including Nanog, Oct4, Sox2, Stat3, and Foxp3. n = 20 (three independent experiments). * P < 0.05.

to be determined.

Here, we present experimental evidence that JMY is an essential factor in porcine embryonic development. Further investigation of the underlying mechanism, especially the dual roles of JMY, this is as an actin nucleation-promoting factor and a transcriptional coactivator, will shed light on the molecular mechanism of JMY function in embryogenesis.

Acknowledgments

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