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—Original Article—

The necessity of ZSCAN4 for preimplantation development and gene expression of bovine embryos

Kazuki TAKAHASHI[1)], Pablo J. ROSS[2)] and Ken SAWAI[1, 3)]

[1)]*The United Graduate School of Agricultural Sciences, Iwate University, Iwate 020-8550, Japan*
[2)]*Department of Animal Science, University of California Davis, CA 95616, U. S. A.*
[3)]*Faculty of Agriculture, Iwate University, Iwate 020-8550, Japan*

Abstract. Zinc finger and SCAN domain containing 4 (*Zscan4*) is a gene that is specifically expressed during zygotic genome activation (ZGA) in mouse preimplantation embryos, and a reduction of *Zscan4* transcripts leads to developmental failure. In mouse embryonic stem cells (ESCs), *Zscan4* is expressed transiently in as little as 1–5% of the cell population. *Zscan4* has also been shown to enhance the efficiency of mouse induced pluripotent stem cells (iPSCs) generation and their quality. Although ZSCAN4 plays important roles in murine embryos and stem cells, its expression and role in bovine embryos is unknown. This study examines *ZSCAN4* transcripts in bovine embryos at various developmental stages and attempts to elucidate the functions of ZSCAN4 during bovine preimplantation development. *ZSCAN4* transcripts were found to be upregulated at the 8- and 16-cell stages. We next attempted *ZSCAN4* downregulation in bovine early embryos by RNA interference and evaluated developmental competency and transcripts levels of genes involved in ZGA and iPSCs generation. Although the bovine embryos injected with *ZSCAN4*-siRNA could develop to the 8-cell stage, very few were developing beyond the 16-cell stage. *PIWIL2* expression was reduced in *ZSCAN4* downregulated embryos. It is possible that *ZSCAN4* downregulated embryos fail to regulate gene expression during ZGA. Our results indicate that *ZSCAN4* is an important factor for the preimplantation development of bovine embryos.

Key words: Bovine embryo, Early development, Gene expression, RNA interference, ZSCAN4

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Procedures for *in vitro* production (IVP) of embryos such as *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) have played an increasingly important role in domestic animal production. However, the efficiency remains low both for embryo development and offspring production after embryo transfer. Although the cause of such low efficiency is unknown, it may be due to the abnormal epigenetic status in bovine embryos obtained from IVF or SCNT procedures [1–3]. In mammalian embryos, zygotic genome activation (ZGA) is a critical event in preimplantation development [4]. ZGA occurs at the 2-cell stage in mouse embryos [5], at the 4- to 8-cell stage in human [6] and pig embryos [7], and at the 8- to 16-cell stage in bovine embryos [8, 9]. After fertilization, the developmental program controlled by maternally inherited transcripts in oocytes is replaced by a program controlled by embryonic transcripts [4, 10]. In case of SCNT, the epigenetic information of the donor nucleus needs to be reprogrammed in the early embryos reconstructed with donor cell and recipient oocyte. The epigenetic status in SCNT embryos is changed to an embryonic state by reprogramming of the donor nucleus [11]. Therefore, ZGA and reprogramming after fertilization or SCNT are essential for establishing the epigenetic status in IVP embryos. However, limited information is available regarding the mechanisms of ZGA and nuclear reprogramming in domestic animal embryos.

The Zinc finger and SCAN domain containing 4 (*Zscan4*) gene was originally identified as specifically expressed during ZGA in the late 2-cell stage of mouse preimplantation embryos [12]. Subsequently, *Zscan4* knockdown by small interfering RNA (siRNA) presented a progression delay from the 2- to 4-cell stage and, which resulted in implantation failure [12]. Furthermore, in mouse embryonic stem cells (ESCs), the expression of *Zscan4* is transient and reversible with infrequent transcriptional activation in only 1–5% of the cell population at a given time point [12, 13]. Additionally, *Zscan4* also enhances the efficiency of mouse induced pluripotent stem cells (iPSCs) generation and their quality [14, 15]. Within that period of *Zscan4* transcription, further biological events occur, including transient expression of other ZGA-specific genes [16, 17], rapid telomere extension [13], and blockage of global protein translation [18]. These data suggest that, in addition to ZGA in mouse early embryos, Zscan4 plays a role in reprogramming the somatic cell nucleus for iPSCs generation. However, the expression status and role of *ZSCAN4* in preimplantation development of bovine embryos is unclear.

The objectives of this study were to investigate the expression status of the *ZSCAN4* gene in bovine embryos at the preimplantation stage and to evaluate the role of *ZSCAN4* during the early development of bovine embryos using RNA interference targeting *ZSCAN4*.

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Correspondence: K Sawai (e-mail: kensawai@iwate-u.ac.jp)

Materials and Methods

In this study, we conducted three experiments, as follows. In experiment 1, we measured the transcript abundance of the *ZSCAN4* gene in bovine oocytes and preimplantation embryos (Figs. 1 and 2). In experiment 2, we evaluated the effect of siRNA injection on *ZSCAN4* expression in bovine embryos (Fig. 3) and the effect of *ZSCAN4* downregulation on the development of bovine embryos (Table 2 and Fig. 4). In experiment 3, we determined gene expressions in bovine embryos derived from ZSCAN4-siRNA injection (Figs. 5 and 6). Experiment 1 was performed in the University of California, Davis, and experiments 2 and 3 were performed in The United Graduate School of Agricultural Sciences, Iwate University. All experiments were approved by the Animal Ethics Committee in each university.

Chemicals

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

Oocyte collection and in vitro maturation

Cow ovaries were collected at a local slaughterhouse and maintained at room temperature during transportation to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2–8 mm. In experiment 1, 50 bovine COCs were matured in 500 µL modified M199 medium (Sigma M2154) supplemented with 0.1 mM ALA-glutamine, 0.2 mM sodium pyruvate, 5 mg/ml gentamicin, 50 ng/ml EGF, 50 ng/ml oFSH (National Hormone and Peptide Program, St. Torrance, CA, USA), 3 µg/ml bLH (National Hormone and Peptide Program), 0.1 mM cysteamine, and 10% FBS (Hyclone; South Logan, UT, USA). In experiment 2 and 3, 10 bovine COCs were matured in a 100 µl drop of IVMD-101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) [19]. *In vitro* maturation (IVM) was performed at 38.5°C in a humidified atmosphere containing 5% CO_2 in air for 22–24 h.

In vitro fertilization and in vitro culture

After IVM, COCs were fertilized in SOF-IVF medium containing 107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.49 mM $MgCl_2$, 1.17 mM $CaCl_2$, 5.3 mM sodium lactate, 25.07 mM $NaHCO_3$, 0.20 mM sodium pyruvate, 0.5 mM fructose, 1X non-essential fatty acid, 5 µg/ml gentamicin, 10 µg/ml heparin, and 6 mg/ml BSA (experiment 1) or IVF-100 medium (Research Institute for the Functional Peptides, Yamagata, Japan; experiments 2 and 3) [19]. Cryopreserved semen was thawed, and sperms were washed twice by centrifugation (at 1800 rpm for 5 min) in IVF medium. Sperm were resuspended in the IVF medium at a final concentration of 1.0×10^6/ml (experiment 1) or 5.0×10^6/ml (experiment 2 and 3). Between 15 and 25 COCs were placed into each sperm suspension drop. COCs and sperm were incubated for 6 h (experiments 2 and 3) or 18 h (experiment 1) at 38.5°C in a humidified atmosphere containing 5% CO_2 in air.

In experiment 1, embryos were cultured in KSOMaa Evolve Bovine media (ZEBV-100; Zenith Biotech, Guilford, CT, USA) supplemented with 4 mg/ml BSA at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. On day 3 (IVF = Day 0), 5% FBS (Gemini Bio Products, West Sacramento, CA, USA) was supplemented into the culture drops. In a group of embryos, 50 µg/ml α-amanitin, which is an inhibitor of RNA synthesis, was added in the *in vitro* culture (IVC) medium, 4- and 8-cell embryos were collected at 28 and 40 h after IVC, respectively. In experiments 2 and 3, following microinjection of siRNA, embryos were cultured in modified TALP (mTALP) medium [20], with 0.1% BSA (fraction V) at 39°C in 5% CO_2, 5% O_2, and 90% N_2. On Day 2, embryos were transferred to mTALP supplemented with 3% new-born calf serum (Invitrogen, Carlsbad, CA, USA) and subsequently cultured at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2 until Day 7. Rates of embryo development were assessed on Day 2 (2-cell ≤), Day 3 (8-cell ≤), Day 4 (16-cell ≤, 32-cell ≤), Day 5 (morula ≤) and Day 7 (blastocyst).

Design of siRNA and microinjection into embryos

The target sites of the *ZSCAN4* transcript were selected from bovine sequences (GenBank accession number: XM_005195522.1). Then, specific siRNA for *ZSCAN4* were developed using siRNA design software, BLOCK-iT RNAi Designer (Thermo Fisher Scientific, Tokyo, Japan). Both sense and antisense RNA sequences for siRNA were commercially synthesized (Table 1).

After insemination, cumulus cells and excess sperm were removed from presumptive zygotes by pipetting. These embryos were subsequently transferred to a 20 µl drop of mTALP containing 1 mg/ml BSA for microinjection. Using a Femtojet microinjector (Eppendorf, Hamburg, Germany), approximately 10 pl of 50 µM specific siRNA duplexes with 5 µM tetramethylrhodamine isothiocyanate (TRITC)-dextran (Dx; Invitrogen) were injected into the cytoplasm of a group of embryos (ZSCAN4-siRNA) during their 1-cell stage. Approximately 10 pl of 20 µM nonsilencing siRNA (AllStars Negative Control siRNA, Qiagen, Tokyo, Japan) with Dx was injected into another group of embryos (Control-siRNA) by the same method. Finally, some embryos were not injected with siRNA (Uninjected). Embryos were washed three times immediately after microinjection, and cultured as described above. Injection of siRNA into the cytoplasm of embryos was verified by the detection of red fluorescence from Dx on Day 2, and embryos devoid of red fluorescence were removed from further analysis.

Determination of the relative abundance of gene transcripts in bovine embryos

In experiment 1, total RNA was extracted from oocytes before or after IVM and from embryos developed to 1-cell (18 h after IVF), 2-cell (10–14 h after IVC), 4-cell (28 h after IVC), 8-cell (40 h after IVC), 16-cell (56 h after IVC), morula (96 h after IVC), and blastocyst (144 h after IVC) stage. The RNA was extracted from pools of 10 oocytes or embryos using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA, USA) and treated with RNase-free DNase I (Qiagen) to remove the genomic DNA according to the manufacturer's instructions. Before RNA isolation, each sample was spiked with 8 µl of 250 fg/µl *HcRed1* cRNA, used as an exogenous control [21]. cDNA synthesis was performed using Superscript II Reverse Transcriptase with random hexamer priming, following the manufacturer's instructions. In experiments 2 and 3, 8- to 16-cell (56 h after IVC) stage embryos were treated with 0.1% protease in 1% PVP-PBS for 5 min, and washed seven times in 1% PVP-PBS. Pools of five embryos were added to 5 µl lysis buffer [0.8% Igepal (ICN Biomedicals Inc., Aurora, OH, USA), 5 mM DTT (Invitrogen)

Table 1. Primers and siRNA sequences

Name	Nucleotide sequences (5'-3')	Annealing temperature (°C)	Fragment size	Genbank accession no.
ZSCAN4	F- GTCCCTGGAAACAGGACAAA	60	179	XM_005195522.1
	R- TTCTGGAGTTCCGTGGATTC			
EIF1AX	F- TTCAAAGAGGATGGGCAGGAGTATG	53	287	NM_001412.1
	R- GGACCAAATGTATCAGTTTCATTGA			
DPPA2	F- CAGACCACTCTTGGCAGACA	60	163	NM_001206470.1
	R- GCATTCAGGGCATAACAGGT			
PIWIL2	F- AGGCCCAGTGAGAGACAGAA	60	235	XM_010798716.1
	R- CCCAACGTGTCAGTTCATTG			
HcRed1	F- GCCCGGCTTCCACTTCA	60	79	N/A
	R- GGCCTCGTACAGCTCGAAGTA			N/A
Histone H2A	F- AGGACGACTAGCCATGGACGTGTG	60	208	NM_174809
	R- CCACCACCAGCAATTGTAGCCTTG			
ZSCAN4-siRNA	S- GCAUGUAUCCUGGCUCCAUTT	N/A	N/A	N/A
	AS- AUGGAGCCAGGAUACAUGCTT	N/A	N/A	N/A

F, forward; R, reverse; S, sense; AS, antisense strand.

and 1 U/µl of RNasin (Promega, Madison, WI, USA)], snap-frozen in liquid nitrogen and stored at −80°C. Extracted RNA samples were heated to 80°C for 5 min and subjected to reverse transcription (RT) using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

In experiment 1, quantitative real-time PCR was performed in a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) with a final reaction volume of 20 µl containing 10 µl Fast SYBR Green Master Mix (Qiagen), 0.5 µl forward primer, 0.5 µl reverse primer (Table 1), 7 µl water, and 2 µl cDNA sample. *HcRed1* (external control) abundance, which maintained a same expression level in across the different developmental stages, was determined in each sample and used to normalize for differences in RNA extraction and RT efficiency. Four samples were used for quantitative analysis, and each sample was run in duplicate for real-time PCR. In experiments 2 and 3, real-time PCR was performed using a StepOne™ system (Thermo Fisher Scientific) with a final reaction volume of 20 µl containing 10 µl QuantiTect SYBR Green Master Mix (Qiagen), 1.0 µl forward primer, 1.0 µl reverse primer (Table 1), 6 µl water, and 2 µl cDNA sample. A standard curve was generated for each amplicon based on the serial dilution amplification 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 NanoDrop (ND-1000; Thermo Fisher Scientific), and diluted as appropriate. 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 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 the StepOne™ quantification software (Thermo Fisher Scientific). Expression levels of the target gene in each run were normalized to the internal control *Histone H2A*, which is stably expressed in bovine embryos at each developmental stage [22]. Six samples were used for quantitative analysis, and each sample was run in duplicate for real-time PCR.

Statistical analysis

The expression levels of *ZSCAN4*, *EIF1AX*, *DPPA2* and *PIWIL2* mRNA were analyzed using the Kruskal-Wallis test, followed by multiple pairwise comparisons using Scheffé's method (Figs. 1, 3, 5 and 6). The levels of expression of *ZSCAN4* in α-amanitin-treated or untreated embryos were analyzed using the F-test, followed by Mann-Whitney's U test (Fig. 2). Percentage data for embryo development were subjected to an arcsine transformation. The transformed values were analyzed by Bartlett's test for the equality of multiple variances to analyze between-group differences in variance. The measures with equal variances were analyzed using one-way analysis of variance, and the measures with unequal variances were analyzed by the Kruskal-Wallis test, followed by multiple pairwise comparisons using the Scheffé method (Table 2). P value < 0.01 or 0.05 was considered statistically significant.

Results

Expression of ZSCAN4 mRNA in bovine oocytes and embryos at various stages

Figure 1 shows the *ZSCAN4* mRNA levels in oocytes before or after IVM and in embryos at various stages. *ZSCAN4* mRNA levels are low until the 4-cell stage. *ZSCAN4* expression increased from the 4- to 8-cell stage, and transcript levels in the 16-cell stage embryos were significantly ($P < 0.05$) higher than levels in oocytes and other embryos stages, with the exception of the 8-cell stage embryos. *ZSCAN4* expression was significantly ($P < 0.05$) decreased at the morula stage (Fig. 1).

At the 4-cell stage, there was no significant difference in the relative abundance of *ZSCAN4* mRNA between α-amanitin-treated and untreated embryos (Fig. 2A). However, at the 8-cell stage, the relative abundance of *ZSCAN4* in the α-amanitin-treated embryos was significantly ($P < 0.05$) lower than that in the α-amanitin-untreated

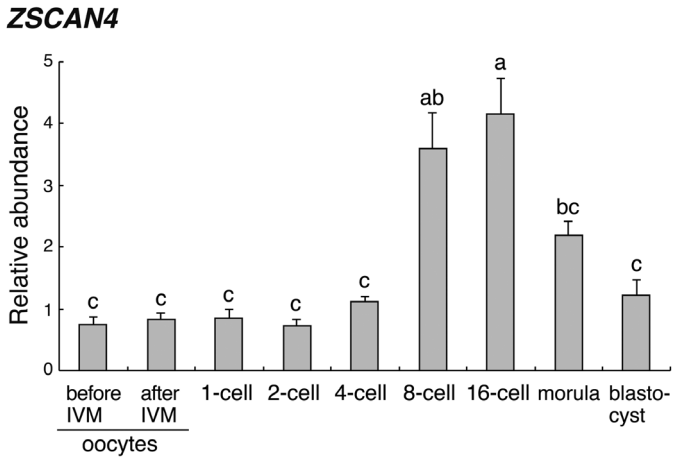

Fig. 1. Relative abundance (mean ± SEM) of *ZSCAN4* transcripts in bovine oocytes and preimplantation embryos (n = 4). The relative abundance represents the normalized quantity compared with exogenous control (*HcRed1*) RNA. [a, b, c] Different superscript letters indicate a significant difference ($P < 0.05$).

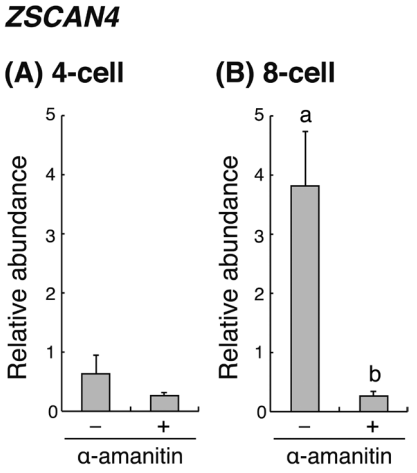

Fig. 2. Relative abundance (mean ± SEM) of *ZSCAN4* transcripts in bovine (A) 4-cell and (B) 8-cell embryos treated with or without α-amanitin (n = 4). The relative abundance represents the normalized quantity compared with exogenous control (*HcRed1*) RNA. [a, b] Different superscript letters indicate a significant difference ($P < 0.05$).

embryos (Fig. 2B). It is well known that α-amanitin is an inhibitor of RNA synthesis. This result indicated that the significant increase of *ZSCAN4* transcripts at the 8-cell stage results from the *de novo* synthesis of zygotic *ZSCAN4*.

Effect of siRNA injection on ZSCAN4 expression in bovine embryos

The expression levels of *ZSCAN4* mRNA in 8- to 16-cell stage embryos obtained from Uninjected, Control-siRNA-injected, or ZSCAN4-siRNA-injected were evaluated (Fig. 3). *ZSCAN4* gene expression was significantly ($P < 0.01$) lower in embryos injected with ZSCAN4-siRNA than in Control-siRNA injected or Uninjected embryos.

Effect of ZSCAN4 downregulation on the development of bovine embryos

In vitro developmental competence of ZSCAN4-siRNA-injected embryos was evaluated (Table 2). No difference in developmental rates for the 2-cell ≤ (Day 2) and 8-cell ≤ (Day 3) stages were observed between ZSCAN4-siRNA-injected and control (Uninjected or Control-siRNA-injected) embryos. However, the rate of ZSCAN4-

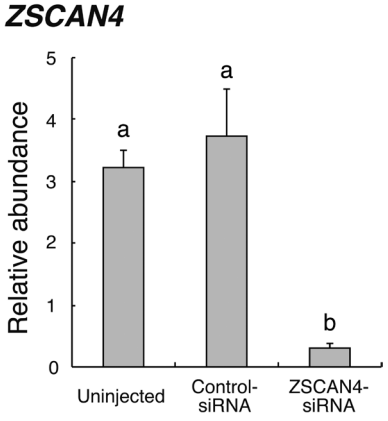

Fig. 3. Relative abundance (mean ± SEM) of *ZSCAN4* transcripts at the 8- to 16-cell stage in Uninjected, Control-siRNA-injected, or ZSCAN4-siRNA-injected embryos (n = 6). The relative abundance represents the normalized quantity compared with *Histone H2A*. [a, b] Different superscript letters indicate a significant difference ($P < 0.01$).

Table 2. Effect of ZSCAN4-siRNA injection on *in vitro* development of bovine embryos *

Treatment	Numbers of embryos cultured ††	No. (%) † of embryos develop to					
		Day 2	Day 3	Day 4		Day 5	Day 7
		2-cell ≤	8-cell ≤	16-cell ≤	32-cell ≤	morula ≤	blastocyst ≤
Uninjected	191	153 (80.1)	148 (77.5)	123 (64.4) [a]	101 (52.9) [a]	77 (40.3) [a]	86 (45.0) [a]
Control-siRNA	176	142 (80.7)	129 (73.3)	105 (59.7) [a]	80 (45.5) [a]	65 (36.9) [a]	81 (46.0) [a]
ZSCAN4-siRNA	148	122 (82.4)	107 (72.3)	60 (40.5) [b]	3 (2.0) [b]	5 (3.4) [b]	4 (2.7) [b]

* Experiments were replicated five times. † Percentages of the number of embryos cultured. †† Control-siRNA, ZSCAN4-siRNA; number of embryos with Dx signals. [a, b] Values with different superscripts within each column differ significantly ($P < 0.01$ or $P < 0.05$).

siRNA-injected embryos that developed to 16-cell ≤ stage (Day 4; 40.5%) was significantly ($P < 0.05$) reduced than that of control embryos (59.7–64.4%). Remarkable developmental arrest occurred in the ZSCAN4-siRNA-injected embryos at the 32-cell ≤ (Day 4), the morula ≤ (Day 5), and the blastocyst stage (Day 7). The developmental ratios of ZSCAN4-siRNA-injected embryos (2.0%, 3.4%, and 2.7%, respectively) were significantly ($P < 0.01$) lower than those of Uninjected (52.9%, 40.3%, and 45.0%, respectively) and Control-siRNA-injected embryos (45.5%, 36.9%, and 46.0%, respectively).

Representative photographs of embryos morphology are shown in Fig. 4. In the Uninjected and Control-siRNA groups, embryos had developed to the blastocyst stage at Day 7 and the expanded blastocysts are shown. However, the ZSCAN4-siRNA-injected embryos showed for the majority a developmental arrest at the 16-cell stage, with only a few embryos reaching the blastocyst stage (Fig. 4).

Gene expressions in bovine embryos derived from ZSCAN4-siRNA injection

The expression levels of the ZGA marker, eukaryotic translation initiation factor 1a X-linked (*EIF1AX*), were not significantly different between groups, as shown in Fig. 5. To elucidate the effect of *ZSCAN4* downregulation on gene transcripts related to reprogramming, we examined the mRNA levels of developmental pluripotency associated 2 (*DPPA2*) and piwi-like RNA-mediated gene silencing 2 (*PIWIL2*) in 16-cell stage embryos. As shown in Fig. 6A, the relative abundance of *DPPA2* did not differ between treatment groups. On the other hand, *PIWIL2* transcript level in ZSCAN4-siRNA-injected embryos was significantly ($P < 0.05$) lower than that in Uninjected and Control-siRNA-injected embryos (Fig. 6B).

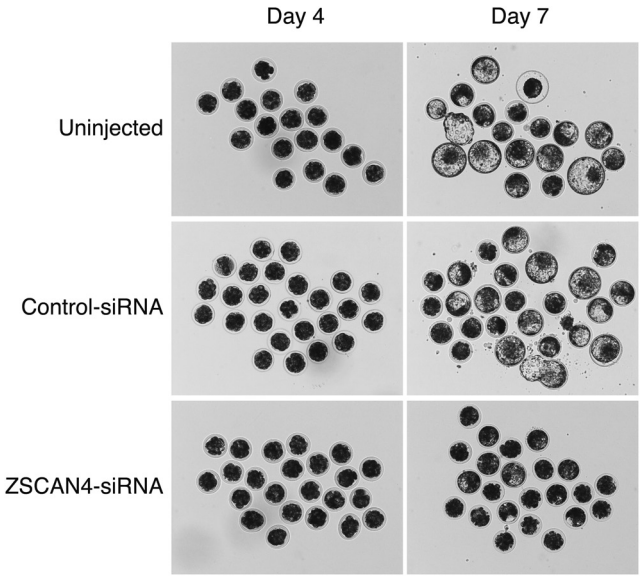

Fig. 4. Representative photographs showing the developmental morphology in late culture periods of bovine embryos obtained from Uninjected, Control-siRNA-injected, or ZSCAN4-siRNA-injected embryos. Embryos obtained from each treatment were cultured continuously until Day 7.

Discussion

Zscan4 has been reported as a reprogramming factor in the generation of murine iPSCs, and it may play a role in the development of mouse embryos after implantation [12, 14]. In the present study, we report the *ZSCAN4* expression status at different developmental stages

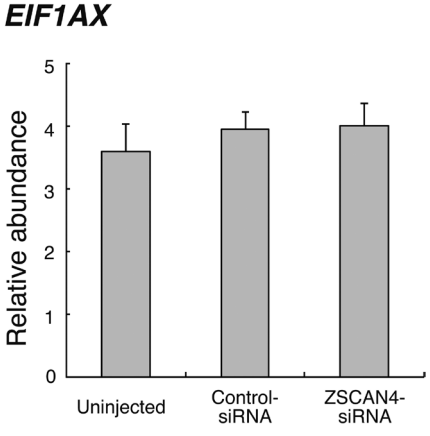

Fig. 5. Relative abundance (mean ± SEM) of *EIF1AX* transcripts at the 8- to 16-cell stage in Uninjected, Control-siRNA-injected, or ZSCAN4-siRNA-injected embryos (n = 6). The relative abundance represents the normalized quantity compared with *Histone H2A*.

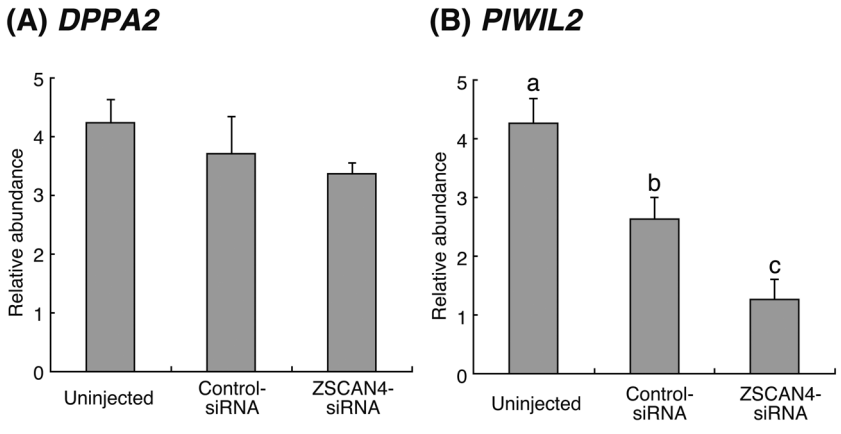

Fig. 6. Relative abundance (mean ± SEM) of (A) *DPPA2* and (B) *PIWIL2* transcripts at the 16-cell stage in Uninjected, Control-siRNA-injected, or ZSCAN4-siRNA-injected embryos (n = 6). The relative abundance represents the normalized quantity compared with *Histone H2A*. [a, b, c] Different superscript letters indicate a significant difference ($P < 0.05$).

and the necessity of *ZSCAN4* transcription for the preimplantation development of bovine embryos.

Zscan4 is a DNA-binding protein that is specifically expressed in 2-cell stage embryos during mouse development [12]. The expression of *Zscan4* in mouse ESCs is transient and reversible, resulting in a unique expression pattern with expression limited 1–5% of ESCs [12, 13, 23]. In human preimplantation embryos, *ZSCAN4* transcripts are significantly upregulated between the 4- and 8-cell stages of embryo development, with a mean expression level peaking at the 8-cell stage [24, 25]. In our study, the level of *ZSCAN4* mRNA in bovine preimplantation embryos was low in the oocyte through the 4-cell stage embryos and increased at the 8-cell stage. *ZSCAN4* transcript level was kept high in 16-cell stage embryos and decreased to low levels from the morula stage. Furthermore, in the present study, we indicated that the higher levels of *ZSCAN4* transcripts at 8- to 16-cell stages result from the *de novo* synthesis of *ZSCAN4*. In the present study, the *ZSCAN4* expression period in bovine embryos was in concordance with the timing of ZGA, like it is the case in murine and human embryos.

In the present study, due to the lack of bovine-specific ZSCAN4 antibodies and the low conservation of ZSCAN4 proteins across species, with only 35.3–42.1% between mouse and bovine and 56.5% between human and bovine according to HomoloGene [26], we could not assess the effect of ZSCAN4-siRNA injection on ZSCAN4 protein levels. However, we clearly demonstrated the downregulation efficiency of *ZSCAN4* mRNA by siRNA injection at the 8- and 16-cell stage. In mouse embryos, the reduction of *Zscan4* transcripts delayed the progression from the 2- to 4-cell stage and produced blastocysts that failed implantation or did not proliferate in blastocyst outgrowth cultures [12]. In the present study, downregulation of *ZSCAN4* expression in bovine embryos had no effects on development to the 8-cell stage. However, in the *ZSCAN4* downregulated embryos, developmental competence at the 16-cell stage was reduced, and embryo development to the 32-cell stage was clearly inhibited. The effects of *ZSCAN4* downregulation also appeared in the development to the morula and blastocyst formation, and developmental competences to both stages were very low.

As described above, *ZSCAN4* shows high expression during ZGA period in mouse [12], human [24, 25] and bovine embryos (present study). In our study, embryo development from the 16- to 32-cell stage was inhibited by *ZSCAN4* downregulation; i.e., developmental arrest was observed just after ZGA. Therefore, to test the hypothesis that bovine embryos obtained from ZSCAN4-siRNA injection are defective in terms of ZGA, we evaluated the expression level of ZGA related gene. *EIF1AX* is a transiently expressed endogenous marker of ZGA found in mouse and bovine embryos [27, 28]. Furthermore, *Eif1a* is found to be co-upregulated with *Zscan4* in mouse 2-cell embryos [29–31] and is one of the most upregulated genes in mouse ESCs, which are enriched for *Zscan4* [17]. Moreover, many preimplantation-specific genes, including *Eif1a*, were activated during the early phase of mouse iPSCs formation generated with *Zscan4* [14]. Therefore, we used the *EIF1AX* transcript level as a ZGA marker in *ZSCAN4* downregulated embryos. However, there was no significant difference in *EIF1AX* transcripts between *ZSCAN4* downregulated embryos and control embryos. These findings suggest that ZGA in *ZSCAN4* downregulated embryos occurs normally. Moreover, ZGA is not related to the developmental arrest of bovine embryos obtained from ZSCAN4-siRNA injection. Alternatively, *EIF1AX* expression might be not regulated by ZSCAN4 in bovine preimplantation embryos.

Zscan4 has also been shown to enhance the efficiency of generating mouse iPSCs and their quality [14, 15]. Thus, there was the possibility that ZSCAN4 has a role in regulating gene expression involved in reprogramming the somatic cell nuclei [14]. During early development, epigenetic reprogramming occurs in order to remove gamete-specific epigenetic patterns [32, 33]. Therefore, to examine the ability of ZSCAN4 to act as a regulator of gene transcription in bovine embryos, we focused on the genes which were highly expressed in iPSCs generated using *Zscan4*. Compared with iPSCs generated using conventional methods, *Dppa2* and *Piwil2* mRNA transcripts showed higher levels of expression in the mouse iPSCs generated using *Zscan4* [14]. Dppa2 is involved in the proliferation of mouse ESCs [34, 35] and has a role in maintaining their pluripotency and self-renewal *in vitro* [36]. Piwil2, a member of the Piwi gene family, promotes proliferation and inhibits apoptosis in tumor cells [37]. Notably, when *Piwil2* was knocked down, the proliferation and invasion of mouse cervical cancer cell lines were significantly inhibited [38]. In the present study, although the DPPA2 transcripts were not different among the experimental groups, *PIWIL2* transcript levels in *ZSCAN4* downregulated embryos were significantly lower than that in control embryos. Piwi proteins and their associated small RNAs, Piwi-interacting RNA (piRNAs), repress transcription of transposable elements in metazoan germ cells [39]. It is also reported that knock out of *Piwil2* led to significant overexpression of long terminal repeat retrotransposons in mouse male germline [40]. A large number of retrotransposons are expressed when the zygotic genome is first transcribed, including the endogenous retroviruses [41]. It is reported that both piRNA-like RNAs [42, 43] and retrotransposons [44] were expressed in bovine preimplantation embryos. Furthermore, recent *in silico* analysis showed that piRNA-like RNAs were markedly reduced in bovine 8-cell stage embryos as compared with oocytes, but appeared to partially rebound at the blastocyst stage [43]. From these observations, PIWI-piRNA associated regulation of retrotransposons expression may prove critical for successful ZGA and embryo development in mammals [43]. Additionally, piRNAs have been shown to be essential for the targeted elimination of mRNA transcripts during pachytene spermatogenesis [45]. It is worth noting that Zscan4 protein was highly detected in late pachytene or diplotene spermatogenesis [46]. We examined the expression levels of retrotransposons, *BERV-K1* and *BERV-K2* [44] in *ZSCAN4* downregulated bovine embryos (data not shown). In *ZSCAN4* downregulated embryos, aberrant transcript levels of both retrotransposons were not observed. However, it is well known that various retrotransposons express in mammalian preimplantation embryos [44]. It is possible that the developmental arrest after ZSCAN4-siRNA injection is due to failed gene transcript regulation including other retrotransposons in bovine embryos caused by *PIWIL2* downregulation. However, the interaction between ZSCAN4 and PIWI-piRNA pathway in bovine embryos remain to be clarified. Further studies are necessary to clarify the relationship between ZSCAN4 and the epigenetic status including retrotransposons expression in bovine embryos.

In conclusion, we found that *ZSCAN4* is essential for early development of bovine embryos. The present study is the first to demonstrate

the critical importance of *ZSCAN4* in bovine embryos and provide new insights for understanding the mechanisms of gene expression after fertilization.

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