

Effects of downregulating GLIS1 transcript on preimplantation development and gene expression of bovine embryos

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Abstract. Krüppel-like protein Gli-similar 1 (GLIS1) is known as a direct reprogramming factor for the generation of induced pluripotent stem cells. The objective of this study was to investigate the role of *GLIS1* in the preimplantation development of bovine embryos. *GLIS1* transcripts in *in vitro*-matured oocytes and 1-cell to 4-cell stage embryos were detected, but they were either absent or at trace levels at the 8-cell to blastocyst stages. We attempted *GLIS1* downregulation of bovine early embryos by RNA interference and evaluated developmental competency and gene transcripts, which are involved in zygotic gene activation (ZGA) in *GLIS1*-downregulated embryos. Injection of specific siRNA resulted in a distinct decrease in *GLIS1* transcript in bovine embryos at the 4-cell stage. Although the bovine embryos injected with *GLIS1*-siRNA could develop to the 16-cell stage, these embryos had difficulty in developing beyond the 32-cell stage. Gene transcripts of *PDHA1* and *HSPA8*, which are transcribed after ZGA, showed lower level in *GLIS1* downregulated embryos. It is possible that *GLIS1*-downregulated embryos fail to initiate ZGA. Our results indicated that *GLIS1* is an important factor for the preimplantation development of bovine embryos.

Key words: Bovine embryo, Early development, Gene expression, GLIS1, RNA interference

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Although the procedures for *in vitro* production (IVP) of domestic animal embryos such as *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) have improved, the efficiencies of embryo development and offspring production after embryo transfer are not high. In particular, neonatal and postnatal aberrations have been observed at varying incidence levels after the use of bovine IVF and SCNT procedures [1–4]. Although the cause of such abnormalities is unknown, the phenomenon may be correlated with abnormal epigenetic status [5–7].

During early development, the epigenetic status of embryos, such as DNA methylation levels, changes considerably [8]. This process of epigenetic reprogramming in early embryos removes gamete-specific epigenetic patterns inherited from the parents [8–10]. Similarly, SCNT requires the epigenetic information of the donor nucleus to be reprogrammed to an embryonic state [11]. It is a persuasive argument that aberrant epigenetic reprogramming of IVP embryos, such as those produced by IVF and SCNT is responsible for the developmental failure of these embryos. Oocytes can remarkably induce transcription in sperm after fertilization and in somatic nuclei after SCNT. Therefore, oocytes can efficiently reprogram transplanted somatic nuclei to an embryonic state [12, 13]. However,

the reprogramming factor(s) in oocytes have not yet been determined, and limited information concerning the mechanism of nuclear reprogramming in oocytes is available.

The Krüppel-like protein Gli-similar 1 (GLIS1) constitutes a subfamily of Krüppel-like zinc finger transcription factors that are closely related to the Gli family [14–16]. Recently, GLIS1 has been shown to be a direct reprogramming factor for somatic cell nuclei, in that, *GLIS1* markedly enhances the generation of induced pluripotent stem cells (iPSCs) from both mouse and human fibroblasts when it is expressed along with *Pou5f1* (*OCT3/4*), *SOX2* and *KLF4* [17]. *GLIS1* is able to replace oncogenic *MYC*, resulting in decreased tumorigenicity as well as improved safety and efficiency of iPSCs production [17]. Notably, *Glis1* transcript is enriched in unfertilized oocytes and 1-cell stage embryos in mice [17]. Furthermore, *Glis1* was reported to be both temporally and spatially regulated, suggesting that it may play a role in the regulation of embryonic development programs at specific stages [15, 18]. However, the role of *GLIS1* in preimplantation development of bovine embryos is unclear.

The objectives of this study were to investigate the expression status of the *GLIS1* gene in bovine embryos at preimplantation stages and to investigate the role of *GLIS1* during the early development of bovine embryos using RNA interference targeted to *GLIS1*.

Materials and Methods

Chemicals

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

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Table 1. Primers and siRNA sequences

Name	Nucleotide sequences (5'–3')	Annealing temperature (C)	Fragment size (bp)	GenBank accession no.
<i>GLIS1</i>	F- ACTGCTCCCAGGCATGTATC R- CCCTTCAGTGGACTGACCAT	60	216	XM_002686397.1
<i>PGK1</i>	F- CTGCTGTTCCAAGCATCAAA R- GCACAAGCCTTCTCCACTTC	60	202	NM_001034299
<i>PDHA1</i>	F- TGGTCAGGAAGCTTGTGTG R- TGCATTGATCCTCCTTTTCC	60	177	NM_001101046
<i>HSPA8</i>	F- CTGCTGTGAGCGTGCTAAG R- GAGCCACCAACCAAGACAAT	60	226	NM_174345
<i>XIST</i>	F- AATAATGCGACAGGCAAAGG R- TCCCCTCATTTCATTAG	58	168	AF104906
<i>Histone H2A</i>	F- AGGACGACTAGCCATGGACGTGTG R- CCACCACCAGCAATTGTAGCCTTG	60	208	NM_174809
GLIS1-siRNA	S- GCAUGUAUCCUGGCUCAUTT AS- AUGGAGCCAGGAUCAUGCTT	N/A N/A	N/A N/A	N/A N/A

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

Oocyte collection and *in vitro* maturation

Cow ovaries were collected at a local slaughterhouse and maintained at room temperature during transport to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2–8 mm. Ten bovine COCs were matured in a 100 µl drop of IVMD-101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) [19] at 39 C in a humidified atmosphere containing 5% CO₂ in air for 22 h.

In vitro fertilization and *in vitro* culture

After *in vitro* maturation, COCs were washed with IVF-100 medium (Research Institute for the Functional Peptides, Yamagata, Japan) [19]. Cryopreserved semen was thawed, and sperm were washed twice by centrifugation (at 1800 rpm for 5 min) in IVF-100 medium. The sperm were resuspended in the IVF-100, and 50 µl of this suspension was added to 50 µl drops of IVF-100. The final concentration of sperm was adjusted to 5.0×10^6 /ml. COCs (15–20 COCs/drop) were placed into each sperm suspension drop. COCs and sperm were incubated for 6 h at 39 C in a humidified atmosphere containing 5% CO₂ in air.

Following microinjection of siRNA, embryos were cultured in modified TALP (mTALP) medium [20], with 0.1% BSA at 39 C in 5% CO₂, 5% O₂, and 90% N₂. On day 2 (IVF = day 0), embryos were transferred to mTALP supplemented with 3% newborn calf serum (Invitrogen, Carlsbad, CA, USA) and subsequently cultured at 39 C in 5% CO₂, 5% O₂, and 90% N₂ until day 7. Rates of embryo development were assessed on day 2 (2-cell ≤), day 3 (8-cell ≤), day 4 (16-cell ≤), day 5 (32-cell ≤), day 6 (morula ≤), and day 7 (blastocyst).

Design of siRNA and microinjection into embryos

The target sites of the *GLIS1* transcript were selected from bovine sequences (GenBank accession number: XM_002686397.1). The specific siRNA (GLIS1-siRNA) was designed using siRNA design software, BLOCK-iT RNAi Designer (<http://rnaidesigner.invitrogen.com/rnaiexpress/>). 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 (fraction V) for microinjection. Approximately 10 pl of 50 µM specific siRNA duplexes were injected into the cytoplasm of each embryo using a Transjector 5246 (Eppendorf, Hamburg, Germany). Approximately 10 pl of 20 µM nonsilencing siRNA (AllStars Negative Control siRNA, Qiagen, Tokyo, Japan) was injected as control siRNA by the same method. The embryos were washed three times immediately after microinjection and cultured as described above.

Determination of the relative abundance of gene transcripts in bovine embryos

In vitro matured (IVM) oocytes and embryos developed to the 1-cell [12 h after *in vitro* culture (IVC)], 2-cell (18–24 h after IVC), 4-cell (24–48 h after IVC), 8-cell (24–48 h after IVC), 16-cell (48–72 h after IVC), morula (96–120 h after IVC), and blastocyst (144–168 h after IVC) stages were treated with 0.1% protease in 1% PVP-PBS for 5 min, and washed seven times in 1% PVP-PBS. Pools of 20 IVM oocytes, 10 (1-cell to 16-cell stages) or five (morula and blastocyst stages) embryos were added to 5 µl of lysis buffer [0.8% Igepal (ICN Biomedicals, Aurora, OH, USA), 5 mM DTT (Invitrogen) and 1 U/µl of RNasin (Promega, Madison, WI, USA)], snap-frozen in liquid nitrogen and stored at –80 C. RNA samples were heated to 80 C for 5 min and treated for reverse transcription (RT) using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Real-time PCRs were performed using a StepOne™ system (Applied Biosystems, Tokyo, Japan), and the products were detected with SYBR Green included in the QuantiTect SYBR Green PCR Master Mix (Qiagen). A 2 µl aliquot of the RT product was used for each quantification. The amplification program was as follows: preincubation at 95 C for 15 min to activate HotStarTaq DNA Polymerase (Qiagen), followed by 45 cycles of denaturation at 94 C for 15 sec, annealing of primers at different temperatures (Table 1) for 30 sec, and elongation at 72 C for 30 sec. After the

end of the last cycle, a melting curve was generated by starting fluorescence acquisition at 60 C and 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 NanoDrop (ND-1000; Thermo Fisher Scientific, Kanagawa, Japan), and diluted. 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 increased above the background for each sample. Final quantification was performed using the StepOne™ quantification software. Expression of the target gene in each run was normalized to the internal standard, *Histone H2A*. Five or six RT samples were used for quantitative analysis, and each sample was run in duplicate for real-time PCR.

Statistical analysis

The percentage data for embryo development were subjected to arcsine transformation. The transformed values were analyzed by one-way analysis of variance, followed by multiple pairwise comparisons using the Tukey-Kramer method. *GLIS1* mRNA expression levels in IVM oocytes and embryos at various stages were analyzed by the Kruskal-Wallis test, followed by multiple pairwise comparisons using the Scheffé method. Differences in all mRNA expression levels between control siRNA-injected embryos and *GLIS1*-siRNA injected embryos were analyzed by the F-test, followed by the Mann-Whitney's U test or the Student's *t*-test. A P value less than 0.05 denoted a statistically significant difference.

Results

Expression of *GLIS1* mRNA in IVM oocytes and embryos at various stages

As shown in Fig. 1, *GLIS1* mRNA levels in IVM oocytes and embryos at various stages are indicated. *GLIS1* transcripts in the 1-cell stage embryos were significantly higher than that in IVM oocytes and 4-cell to blastocyst stage embryos ($P < 0.05$). *GLIS1* transcript levels were decreased at the 8-cell stages, and it was difficult to detect in the embryos following the 8-cell stage (Fig. 1).

Effect of siRNA injection on *GLIS1* expression in bovine embryos

The expression levels of *GLIS1* mRNA in 1-, 2- and 4-cell stage embryos obtained from control siRNA or *GLIS1*-siRNA injection were evaluated (Fig. 2). The relative abundance of *GLIS1* in the 4-cell stage embryos injected with *GLIS1*-siRNA was significantly lower than that in control siRNA-injected embryos ($P < 0.05$, Fig. 2C). The *GLIS1* transcript levels in both 1- and 2-cell embryos did not differ between treatment groups (Fig. 2A and B).

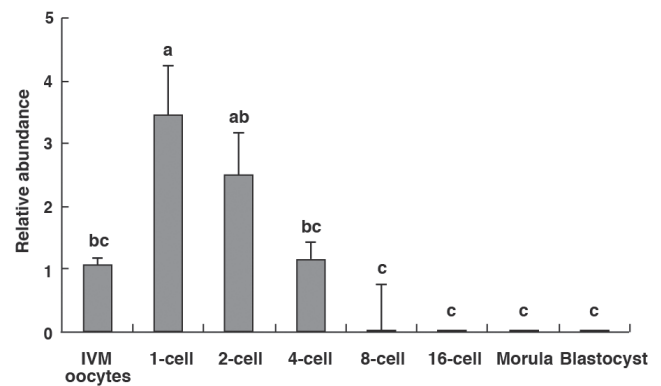


Fig. 1. Relative abundance (mean ± SEM) of *GLIS1* transcripts in bovine *in vitro* matured (IVM) oocytes and 1-cell to blastocyst stage embryos (n = 5 or 6). The relative abundance represents the normalized quantity compared with *Histone H2A*. ^{a, b, c} Different superscripts indicate a significant difference ($P < 0.05$).

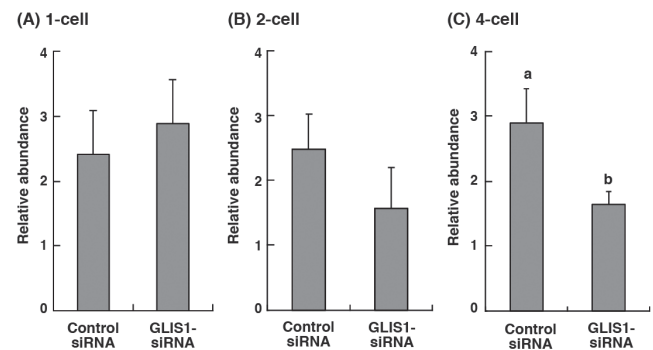


Fig. 2. Relative abundance (mean ± SEM) of *GLIS1* transcripts in bovine (A) 1-cell, (B) 2-cell, and (C) 4-cell stage embryos obtained from control siRNA or *GLIS1*-siRNA injection (n = 5 or 6). The relative abundance represents the normalized quantity compared with *Histone H2A*. ^{a, b} Different superscripts indicate a significant difference ($P < 0.05$).

Effect of *GLIS1* downregulation on the development of bovine embryos

In vitro developmental competence of *GLIS1*-siRNA-injected embryos was evaluated (Table 2). No differences in developmental rates for the 2-cell ≤ (day 2) to 16-cell ≤ (day 4) stages were observed between *GLIS1*-siRNA-injected and control (uninjected and control siRNA injected) embryos. However, the rate of *GLIS1*-siRNA-injected embryos that developed to 32-cell ≤ stage (day 5) was significantly lower than that of uninjected embryos (22.6 and 45.6%, respectively, $P < 0.05$). Both morula ≤ (day 6) and blastocyst (day 7) rates of *GLIS1*-siRNA-injected embryos (9.0 and 6.5%, respectively) were significantly ($P < 0.05$) lower than those of the uninjected (45.0 and 43.9%, respectively) and control siRNA-injected (36.9 and 34.5%, respectively) embryos.

Table 2. Effect of *GLIS1* siRNA injection on *in vitro* development of bovine embryos*

Treatment	Number of embryos cultured	No. (%) [†] of embryos develop to					
		Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
		2-cell ≤	8-cell ≤	16-cell ≤	32-cell ≤	Morula ≤	Blastocyst
Uninjected	171	141 (82.5)	114 (66.7)	83 (48.5)	78 (45.6) ^a	77 (45.0) ^a	75 (43.9) ^a
Control siRNA	168	142 (84.5)	107 (63.7)	62 (36.9)	63 (37.5) ^{ab}	62 (36.9) ^a	58 (34.5) ^a
<i>GLIS1</i> -siRNA	155	139 (89.7)	94 (60.6)	49 (31.6)	35 (22.6) ^b	14 (9.0) ^b	10 (6.5) ^b

* Experiments were replicated five times. [†] Percentages of the number of embryos cultured. ^{a, b} Values with different superscripts within each column differ significantly ($P < 0.05$).

Gene expressions in bovine embryos derived from *GLIS1*-siRNA injection

To clarify the effect of *GLIS1* downregulation on gene transcripts, phosphoglycerate kinase 1 (*PGK1*), pyruvate dehydrogenase 1 α (*PDH1*), heat shock cognate protein 70 (*HSPA8*), and X inactive specific transcript (*XIST*) mRNA expression in 8- to 16-cell stage (48–72 h after IVC) embryos were examined. As shown in Fig. 3B and 3C, *PDH1* and *HSPA8* transcript levels in *GLIS1*-siRNA-injected embryos were significantly lower than those in control siRNA-injected embryos ($P < 0.05$). On the other hand, the relative abundance of *PGK1* and *XIST* did not differ between treatment groups (Fig. 3A and 3D).

Discussion

GLIS1 has been reported as a reprogramming factor in generation of murine and human iPSCs, and it may have a role in the development of mouse embryos after implantation [18]. In the present study, we indicated the *GLIS1* expression status and the necessity of *GLIS1* transcription for the preimplantation development of bovine embryos.

Glis1 is expressed in a temporal and spatial manner during mouse development; *Glis1* expression is most prominent in several defined structures of the mesodermal lineage of the fetus, including craniofacial regions or limb buds [18]. On the other hand, *Glis1* mRNA is enriched in metaphase II oocytes and 1-cell stage murine embryos [17]. *Glis1* expression levels in mouse embryos decreased at the 2-cell stage, and it was difficult to detect them from the 4-cell to blastocyst stages [17]. In our study, bovine *GLIS1* transcripts were detected clearly in IVM oocytes and 1-cell to 4-cell stage embryos, and the *GLIS1* transcript level in the 1-cell stage embryos was higher than that in the IVM oocytes. A rise in *GLIS1* expression after fertilization has been observed in murine embryos [17]. Thus, it is possible that *GLIS1* expression in mammalian embryos increases transiently just after fertilization. On the other hand, the *GLIS1* expression in bovine embryos was either absent or at trace levels from the 8-cell to blastocyst stage. These findings indicated that in mammalian embryos, *GLIS1* expression is limited to the early stage after fertilization and that *GLIS1* transcription resumes after implantation. In the present study, downregulation of *GLIS1* mRNA by siRNA injection was detected at the 4-cell stage, but there was no difference in both 1-cell and 2-cell stage embryos. It is well known that siRNA-based RNAi in mammalian cells varies considerably depending on the target sequences selected [21, 22]

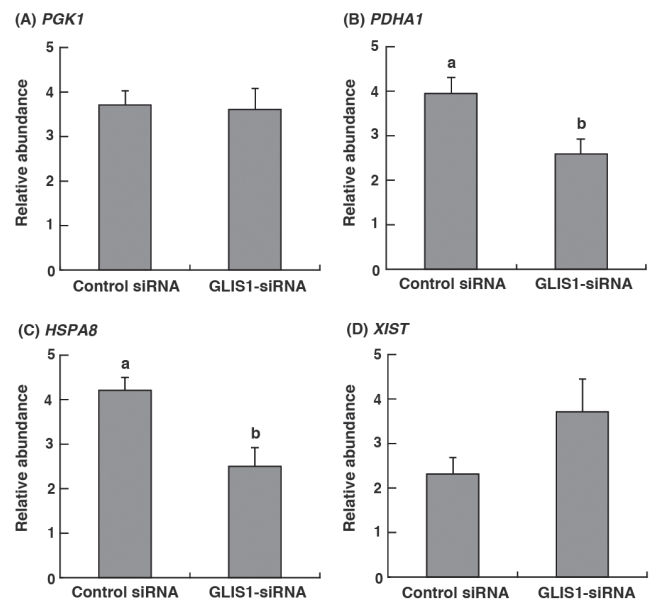


Fig. 3. Relative abundance (mean \pm SEM) of (A) *PGK1*, (B) *PDH1*, (C) *HSPA8* and (D) *XIST* transcripts in bovine 8- to 16-cell stage embryos obtained from Control siRNA or *GLIS1*-siRNA injection ($n = 6$). The relative abundance represents the normalized quantity compared with *Histone H2A*. ^{a, b} Different superscripts indicate a significant difference ($P < 0.05$).

but that the time required for gene silencing after siRNA injection is unclear. It is possible that a certain amount of time is required to silence genes in early embryos.

In the present study, downregulation of *GLIS1* expression in bovine embryos had no effects on development to the 16-cell stage, but embryo development to the 32-cell stage was inhibited. The markedly effects of *GLIS1* downregulation also appeared in both development to the morula and blastocyst formation, and developmental competences to both stages were very low. In the mouse, *Glis1* mutants were found to be viable and to have no obvious phenotype [23]. The cause of developmental arrest in bovine embryos with *GLIS1* suppression was not clear; thus we focused on the developmental stage in which developmental arrest occurred in *GLIS1* knockdown embryos. In bovine embryos, zygotic gene activation (*ZGA*) is well known as an important event that occurs around the 16-cell stage. After

fertilization, the developmental program controlled by maternally inherited transcripts in oocytes is replaced by a program controlled by embryonic transcripts [24, 25]. In mammalian embryos, the transition from maternal to embryonic control is an essential event during early development and is termed ZGA [24]. ZGA occurs at the 2-cell stage in mouse embryos [26], at the 4- to 8-cell stage in human [27] and pig embryos [28] and at the 8- to 16-cell stage in bovine embryos [29, 30]. In our study, embryo development from the 16-cell to 32-cell stage was inhibited by *GLIS1* downregulation, i.e., developmental arrest was observed at just after ZGA. Therefore, to test the hypothesis that bovine embryos obtained from *GLIS1*-siRNA injection are defective in terms of ZGA, we evaluated the transcript levels of four genes that start to be transcribed after ZGA in bovine embryos [31]. Consequently, the transcripts of two genes (*PDHAI* and *HSPA8*) in *GLIS1* downregulated-embryos were found to have lower levels than control embryos.

PGK1, *PDHAI* and *HSPA8* are essential housekeeping genes that regulate various physiological functions [31–33]. *PGK1* and *PDHAI* are involved in glucose metabolism, and play an important role in bovine and murine embryo development [34, 35]. *HSPA8* encodes heat shock cognate protein 70 (HSC70) [33]. HSC70 has been reported to be involved in numerous of housekeeping and chaperoning functions, such as folding of nascent polypeptides, protein translocation across membranes and chaperone-mediated autophagy [36]. RNAi-based knockdown of HSC70 results in massive cell death in various cell types [37]. *XIST* is well known as a key factor for X chromosome inactivation [38]. Song *et al.* reported that in bovine IVF embryos, transcription levels of these four genes markedly increased after ZGA [31]. It has also been reported that *PGK1*, *PDHAI* and HSC70-encoding genes began to express in nonhuman primate embryos after ZGA and that these transcriptions were inhibited by α -amanitin (RNA polymerase inhibitor) treatment [39]. It was suggested that aberrant expression of nuclear proteins causes abnormal nucleogenesis in bovine SCNT embryos [40], which leads to failed ZGA and developmental arrest [41]. In bovine SCNT embryos, after ZGA, the transcript levels of *PGK1* and *XIST* increased, but the *HSPA8* and *PDHAI* levels did not increase [31]. In the present study, transcripts of *HSPA8* and *PDHAI* were downregulated in the *GLIS1*-siRNA-injected embryos, but *PGK1* and *XIST* expressions did not differ from those of control embryos. Although it is possible that the *XIST* expression level in our results was affected by the sex ratio of the embryos used for gene expression analysis, at least three other genes in the *GLIS1*-siRNA-injected embryos showed expression patterns similar to SCNT embryos. Together with these results, it is possible that the developmental arrest manifested after *GLIS1*-siRNA injection is due to failed ZGA in embryos caused by *GLIS1* downregulation.

ZGA is a nuclear reprogramming event that changes the transcriptionally inactive embryonic genome to an active one after fertilization [25]. Although little is known regarding the molecular mechanisms of ZGA, oocyte-derived mRNAs and proteins probably play an important role in this reprogramming [25]. As described in the Introduction, *GLIS1* is a direct reprogramming factor for the generation of iPSCs from both mouse and human fibroblasts [17]. However, it is not clear whether *GLIS1* is involved in nuclear reprogramming after fertilization. Further studies, such as those related

to the relationship between *GLIS1* and the epigenetic status of the nucleus after fertilization, are necessary. In addition, investigation of *GLIS1* has been performed using a limited number of species. Further studies using various species are essential to elucidate the function of *GLIS1* in embryo development.

In conclusion, we found that *GLIS1* is essential for early development of bovine embryos. The present study is the first to demonstrate the critical presence and importance of *GLIS1* for bovine oocytes and embryos and may also provide the basis for understanding the role of mRNAs accumulated in oocytes for embryo development after fertilization.

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