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Maternal *DDB1* regulates apoptosis and lineage differentiation in porcine preimplantation embryos

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ABSTRACT

Context. Maternal-effect genes (MEGs) play a critical role in modulating both cellular and molecular biology events in preimplantation embryonic development. Damage-specific DNA binding protein 1 (DDB1) is a gene that participates in meiotic resumption, ovulation, and embryonic stem cell maintenance. Its function in preimplantation development is not well-studied. **Aims.** We aimed to explore the expression pattern, genomic heritage, and potential molecular mechanisms of *DDB1* in preimplantation embryos in porcine. **Methods.** In this study, RNA interference, microinjection, RT-qPCR, immunofluorescence staining and single-cell RNA sequencing were used to explore the molecular function of *DDB1* in porcine preimplantation embryos. **Key results.** *DDB1* was found to be expressed in germinal vesicle (GV) and Meiosis II (MII) oocytes and in preimplantation embryos. We confirmed it is a MEG. *DDB1*-deficient blastocysts had a significantly reduced number of trophectoderm cells, an increased apoptotic cell number and increased apoptosis index. According to a next-generation sequencing (NGS) analysis, 236 genes (131 upregulated and 105 downregulated) significantly changed in the *DDB1*-deficient morula. The myeloid leukaemia factor 1 (*MLF1*) and yes-associated protein 1 (*YAP1*) expressions were significantly upregulated and downregulated respectively, in the *DDB1*-deficient morula. In combination with the decreased expression of *TEAD4*, *CDX2*, *GATA3*, *OCT4*, and *NANOG* and the increased expression of *SOX2* in the blastocyst, *DDB1* may play a role in determining lineage differentiation and pluripotency maintenance. **Conclusions.** *DDB1* is a MEG and it plays a crucial role in porcine preimplantation embryonic development. **Implications.** This study provides a theoretical basis for further understanding the molecular mechanisms of preimplantation embryo development.

Keywords: blastocyst, cellular apoptosis, *DDB1*, embryo, lineage differentiation, maternal-effect gene, porcine, preimplantation development.

Introduction

Mammalian early embryonic development is a precise and complex biological process that includes a series of morphological (e.g. cleavage or compaction), cytological (e.g. polarisation or lineage differentiation), and molecular biological (e.g. maternal regulation or epigenetic modification) events before implantation. These events are controlled by the accurate temporal and spatial expression of genes (Hamatani *et al.* 2004; Wang *et al.* 2021). Maternal-effect genes (MEGs) are maternal origin genes that play a critical role in modulating early embryonic development such as embryonic genome, cleavage, and embryonic cell lineage establishment (Li *et al.* 2010; Zhang and Smith 2015). An impairment in the expression of MEGs can lead to abnormal embryo development, impairing cleavage or compaction (Wu *et al.* 2003; de Vries *et al.* 2004; Xu *et al.* 2015), blastocyst formation (Kim *et al.* 2016; Cao *et al.* 2019), and trophectoderm (TE) and inner cell mass (ICM) differentiation (Wu *et al.* 2010; Cockburn *et al.* 2013).

TE and ICM are the major components of a blastocyst and they are both formed in the blastocyst after the first lineage differentiation. TE is a type of squamous epithelium derived from the polar cells produced by an asymmetric division during the 8-cell to the morula stage

(Saini and Yamanaka 2018). It is located on the outer edge of the embryo and mediates implantation and placentation (Maître 2017). Several MEGs play a critical role in the formation and differentiation of TE cells. For example, maternal *E-cadherin*-deficient embryos can have a delayed cell division (de Vries *et al.* 2004) and an *E-cadherin* null mutant embryo failed to form an intact TE cell (Larue *et al.* 1994). ICM takes an inner position within the embryo, which develops the fetus and the extra-embryonic yolk sac (Saini and Yamanaka 2018). During mammalian embryonic development, it is well-known that the Hippo/YAP signalling cascade plays a central role in the TE/ICM lineage specification (Chazaud and Yamanaka 2016).

Damage-specific DNA binding protein 1 (DDB1) is one of the subunits of the damaged DNA binding protein complex that is involved in the nucleotide excision repair pathway (the other component is DDB2) (Chu and Yang 2008). DDB1 is an adapter protein between Cullin 4A (CUL4A) and CUL4-associated factors (DCAFs) that acts as a component of the CUL4A-RING ubiquitin E3 ligases (CRL4) complex to target substrates for ubiquitination (Iovine *et al.* 2011). DDB1 is required for meiotic resumption and meiosis I progression in mice oocytes (Yu *et al.* 2015*a*). Oocyte-specific *Ddb1* knockout mice demonstrated oocyte loss and female infertility due to increased apoptosis and poor responses to ovulation signals of the granulosa cells (Yu *et al.* 2013, 2015*b*). The deletion of maternal DDB1 in oocytes contributes to embryonic deaths before the 8-cell stage (Yu *et al.* 2013). Although *Ddb1* has been reported to be a MEG in mice, with a role in oocyte meiosis maturation, whether it is a conservative MEG among species – as well as its function in preimplantation embryonic development of other species – is still unknown.

In this study, we identified *DDB1* as a MEG during porcine preimplantation embryonic development. We further explored the dynamic expression pattern of *DDB1* in porcine oocytes and preimplantation embryos. In addition to this novel finding, we also assessed the molecular function of *DDB1* using RNA interference and RNA-seq analysis methods. Our work provides evidence supporting the critical role of *DDB1* in cellular apoptosis, lineage differentiation, and the pluripotency maintenance of blastocyst formations in porcine models.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (Shanghai, China) unless specific statement.

Porcine oocytes collection and *in vitro* maturation (IVM)

Porcine ovaries were collected from a local slaughterhouse and transported into the lab for processing within 2 h of collection. The cumulus-oocyte complexes (COCs) with 3–6 mm diameter were used for maturation culture. Briefly, the COCs were randomly placed in four-well Petri dishes. Eighty oocytes were cultured in 400 μL IVM medium [Medium-199 supplemented with 10% fetal bovine serum, 10% porcine follicular fluid, 10 ng/mL epidermal growth factors, 10 IU/mL equine chorionic gonadotropin, 10 IU/mL human chorionic gonadotropin, 0.1 mg/mL L-Cysteine (Ding *et al.* 2017)] per well, and covered with mineral oil. After 42–44 h culture (38.5°C, 5% CO_2, saturated humidity), the cumulus cells were removed using 1 mg/mL hyaluronidase. The oocytes with normal morphology and first polar body (pb1) were identified as mature oocytes and used for the subsequent experiments. The subsequent experiments were replicated at least three times.

Matured oocytes parthenogenetic activation (PA), *in vitro* fertilisation (IVF), and embryo culture

The matured oocytes PA, IVF and embryo culture were performed as previous described (Ding *et al.* 2017). Briefly, matured oocytes (metaphase II stage, MII) were activated with a single direct current pulse of 1.56 kV/cm for 80 μs in activation medium (0.3 M mannitol supplemented with 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.01% polyvinyl alcohol) using cell fusion apparatus (CF-150B, BLS, Hungary). Then, the oocytes were further incubated with assisting activation medium [porcine zygote medium 3 (PZM-3 was prepared as previous described (Yoshioka *et al.* 2002)) supplemented with 10 μg/mL cycloheximide and 10 μg/mL Cytochalasin B] for 4 h at 38.5°C with 5% CO_2 and saturated humidity.

For the IVF, fresh Duroc boar semen was collected from a local pig breeding farm (Hefei Antai, China) and transported into the lab for processing within 2 h of collection. A fertilisation medium droplet (55 μL) was made by adding 5.5 μL of resuspended fresh boar sperm solution (1×10^6 cells/mL) into 49.5 μL fertilisation medium (modified tris-buffered medium (components are listed in Supplementary Table S1) plus 2 mg/mL BSA and 2 mM caffeine). A total of 15 MII oocytes were co-incubated with sperm in the fertilisation droplet for 6 h in the incubator (38.5°C, 5% CO_2, saturated humidity). After washing out the surrounding sperm, the presumptive zygotes were processed into embryo culture. A pool of 15 zygotes were cultured in one 50 μL PZM-3 droplet, covered with mineral oil and placed in the incubator (38.5°C with 5% CO_2 and saturated humidity). The cultured embryos were assessed and collected at the following hours: pronucleus (PN, 16 h); 2-cell (2C, 32 h); 4-cell (4C, 44 h); 8-cell (8C, 82 h); morula (MO, 104 h); blastocyst-day 5 (BL, 120 h); blastocyst-day 6 (BL, 144 h); blastocyst-day 7 (BL, 168 h).

Inhibition of zygote transcripts synthesis

The embryos were treated with 25 μg/mL α-amanitin (RNA polymerase II inhibitor) for 30 min at the 2C, 4C, and 8C stages. The embryos in the control groups were treated with

solvent (dimethylsulfoxide). 2C embryos from treatment and control groups were washed with PZM-3 three times and cultured and then were collected at 4C for RT-qPCR. 4C embryos from treatment and control groups were washed with PZM-3 three times and cultured and then collected at 8C for RT-qPCR. 8C embryos from treatment and control groups were washed with PZM-3 three times and cultured and then collected at MO for RT-qPCR. All experiments were replicated three times.

Knockdown of the target gene

DDB1 expression was knocked down in MII oocytes by cytoplasm microinjecting two small interfering RNA species (siRNA sequences are listed in Table S2). A non-specific siRNA was used as a negative control (GenePharma, Shanghai, China). Briefly, MII oocytes were placed in an operating medium (TCM199 with 2% fetal bovine serum and 7.5 μg/mL Cytochalasin B), and ~10 pL of the siRNA solution (25 μM) was cytoplasm microinjected per cell with a micromanipulator system (Olympus, Japan). After 30 min of recovery in PZM-3 media, the oocytes were processed into PA or IVF. FAM-siRNA (green fluorescence) was used to validate injection efficiency. Recovered oocytes with tightness, margin smooth and good refraction of cytoplasm were considered survival (Fig. S1*a*). The non-treated (control), RNase-free water injection (sham water), and non-specific siRNA injection (NC-siRNA) groups were set as controls. There were more than 20 oocytes per group and the experiment was replicated three times.

Real-time quantitative PCR (q-PCR)

More than 20 oocytes and 10 embryos were used for total RNA extraction (74034, Qiagen, Germany). After that, total RNA was used for reverse transcription (205311, Qiagen, Germany). Quantitative real time PCR (StepOnePlus, Applied Biosystems, USA) was used to measure target gene expression. The amplification reaction consisted of template denaturation and polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s. and 72°C extension for 30 s. The genes expression level was normalised against H2AFZ (Lee *et al.* 2017) and presented as relative fold change to the controls (n = 3). Three independent experiments and qPCR reactions were performed. All primers are listed in Table S3.

Terminal deoxynucleotidyl transferase-mediated nick-end labelling (TUNEL) staining

Blastocyst apoptotic cells were stained using an *In Situ* Cell Death Detection Kit (12156792910, Roche, USA). Briefly, blastocyst (day 7) embryos were fixed with 4% paraformaldehyde for 15 min and permeabilised with 1% Triton X-100 for 30 min. After that, the blastocyst embryos were incubated with TUNEL reaction solution for 1 h at 37°C, then with 10 μg/mL Hoechst 33342 for 15 min. Finally, the apoptotic cells were imaged using an inverted fluorescence microscope (Olympus, Japan) and quantified with Image J software (ImageJ 1.52a, NIH, USA). Five blastocysts were quantified in each group, and the experiment was replicated three times.

Immunofluorescence staining

CDX2 protein was stained for immunofluorescence as previously described (Ding *et al.* 2017; Gao *et al.* 2020). Briefly, blastocyst (day 7) embryos were fixed (4% paraformaldehyde, 15 min) and permeabilised (1% Triton X-100, 30 min), then blocked (2% BSA, 2 h, RT). The embryos were then followed by incubating with anti-CDX2 antibody (CDX2, AM392, Biogenex, USA) overnight at 4°C. After washing, the embryos were incubated with secondary antibody (1:200, goat anti-mouse IgG, Alexa Fluor 488, A11029, Invitrogen, USA) for 2 h at 37°C. After being counterstained with propidium iodide or 4,6-diamidino-2-phenylindole dihydrochloride for 10 min, the stained embryos were imaged using an inverted fluorescence microscope (Olympus, Japan) and quantified using Image J software (ImageJ 1.52a, NIH, USA).

RNA sequencing and bioinformatic analyses

The non-specific siRNA injected (NC) and *DDB1*-siRNA injected (*DDB1*-KD) porcine morulae were collected for RNA sequencing (five morulae per group, n = 3). Briefly, the morulae RNA was extracted (TRK1001, LC Science, Houston, TX) and the cDNA library was constructed (TruSeq Nano DNA LT Library Preparation Kit, FC-121-4001, Illumina, USA). Then the cDNA was fragmented by dsDNA Fragmentase (NEB, M0348S, USA) and paired-end sequencing was performed on an Illumina Novaseq™ 6000 (LC-bio, China) using Illumina paired-end RNA-seq approach. A total of 57 G bp of cleaned, paired-end reads were produced and mapped to the *Sus scrofa* reference genome. StringTie was used to determine mRNA expression levels by calculating the fragments per kilobase of exon model per million mapped fragments (FPKM). The differentially expressed genes were screened with $\log_2$ fold change > 1 or $\log_2$ fold change < −1 and with a statistical significance (P-value < 0.05) by the R package. Gene ontology (GO) analysis was performed using http://geneontology.org.

Statistical analysis

At least three replicates were performed for all experiments. The statistical analyses were performed using a student's t-test or a one-way ANOVA (SPSS 17.0, USA). The graphs were constructed by GraphPad Prism (GraphPad software 5.01, USA). A P value of < 0.05 was considered to be statistically significant.

Results

Expression pattern of DDB1 in porcine oocytes and preimplantation embryos

The mRNA expression levels of *DDB1* in oocytes and preimplantation embryos were measured by RT-qPCR. The expression level of the *DDB1* transcript level was highest in oocyte at the GV stage and was significantly higher than the MII stage and embryos in different developmental stage (Fig. 1*a*). After fertilisation, the *DDB1* mRNA abundance peaked at the stage of 2C, followed by 4C, PN, MO, BL, and was lowest in 8C (Fig. 1*a*).

Verification of the heritage of the transcripts and the RNA interference efficiency

Porcine zygotic genome activation occurs at the 4-cell stage (Cao *et al.* 2014). α-amanitin, an inhibitor of RNA polymerase II, has been commonly used to block zygote mRNA synthesis during preimplantation stage (Uh and Lee 2017). We treated the 2C, 4C, and 8C embryos with α-amanitin to inhibit zygote mRNA produce. The *DDB1* mRNA expression level did not change between the treatment and the control groups (Fig. 1*b*), suggesting that *DDB1* is one of the maternal origin transcripts in porcine early embryos.

We designed two siRNA species (*DDB1*-siRNA1, D1; *DDB1*-siRNA2, D2) to target the porcine *DDB1* sequence for the *DDB1* knockdown. 25 μM siRNA was determined to be the most appropriate concentration (Fig. S1*d*). As shown in Fig. 1*c*, the endogenous *DDB1* mRNA was significantly reduced in the 4C embryos (D1, 78.21%; D2, 79.65%; D1 + D2, 84.49%) and the 8C embryos (D1, 80.43%; D2, 81.58%; D1 + D2, 91.46%). A *DDB1*-siRNA cocktail was used for the subsequent knockdown experiments. The injection efficiency of FAM-siRNA was 97.75% and survival rate of MII oocytes after injection was 95.75% (Fig. S1*b*, *c*).

DDB1-deficient porcine embryo has an impaired developmental competence

We knocked down *DDB1* in IVF embryos to explore the effect of DDB1 on preimplantation development. As can be seen in Fig. 2*a*, *b*, the *DDB1*-deficient IVF embryos had an impaired development ability as the ratios of 8C, MO, and BL were all significantly lower than the controls. The *DDB1*-deficient blastocysts had a significantly decreased total cell number and TE cell number (Fig. 2*c*, *d*). Although the *DDB1*-deficient embryos had no change in ICM cell population, the ICM/TE significantly increased compared with the controls (Fig. 2*d*). Similar results were detected in the *DDB1*-deficient PA embryos (Fig. S2).

DDB1 ablation induces apoptosis in the blastocysts

Due to the decreased number of TE cells in the *DDB1*-deficient blastocysts, TUNEL staining was applied to establish whether *DDB1* ablation caused cell apoptosis. As shown in Fig. 3, there was a greater number of apoptotic cells and a higher apoptotic index in the *DDB1*-deficient blastocysts relative to the control group.

DDB1-deficient morula embryos have a different gene expression profile

An RNA-seq analysis was used for exploring the molecular difference in the *DBB1*-deficient embryos (Fig. 4*a*). All five replicates in the same group had a high correlation (*DDB1*-KD groups, $R = 0.81 \sim 0.96$; NC groups, $R = 0.88 \sim 0.97$) (Fig. S3*a*, *b*).

Our result demonstrated that 236 DEGs accounted for the transcriptome changes between the *DDB1*-deficient and control groups. There were 131 upregulated and 105 downregulated genes in the *DDB1*-deficient morula (Fig. 4*b*, *d*).

A total of 19 differentially expressed genes (DEGs) (FC > 2 or <0.5, $P < 0.05$) were validated by RT-qPCR (UP: *EZH1*, *JAM3*, *PHF1*, *PPP1CB*, *BMP15*, *KPNA7*, *MAPK9*, *CD3E*, *PIWIL2*, *FKBP6*; DOWN: *YAP1* (also known as *YAP*), *SH2B3*, *ANXA1*, *CSF1R*, *IL1B*, *ERBB4*, *FRMD4B*, *DAB2*, *TCIRG1*) (Fig. 4*c*) and their expression patterns were consistent with the RNA-seq result.

A gene ontology (GO) analysis was performed on the DEGs and three function categories (the biological process, cellular component, and molecular function) were used for the classification (Fig. 4*e*). Several blastocyst genesis key genes were found to be enriched in the biological process such as *YAP1* (Cao *et al.* 2019), *JAM3* (Su *et al.* 2012), *KPNA7* (Tejomurtula *et al.* 2009), and *PIWIL2* (Roovers *et al.* 2015).

Knockdown of DDB1 affects the Hippo signalling pathway

The Hippo signalling pathway plays an essential role in the establishment of TE-specific features (Saini and Yamanaka 2018). Our results demonstrated a significantly downregulated *YAP1* in the *DDB1*-deficient morula (Fig. 4*c*). The expression level of *TEAD4*, *CDX2*, and *GATA3* – the downstream effectors of *YAP* – were all significantly decreased in the *DDB1*-deficient blastocysts compared to the control groups (Fig. 5*a*). Immunofluorescent staining confirmed the reduced protein CDX2 level in the *DDB1*-deficient blastocysts (Fig. 5*b*, *c*). The expression of the ICM-specific genes *OCT4* and *NANOG* decreased, while *SOX2* increased in the *DDB1*-deficient blastocysts (Fig. 5*a*).

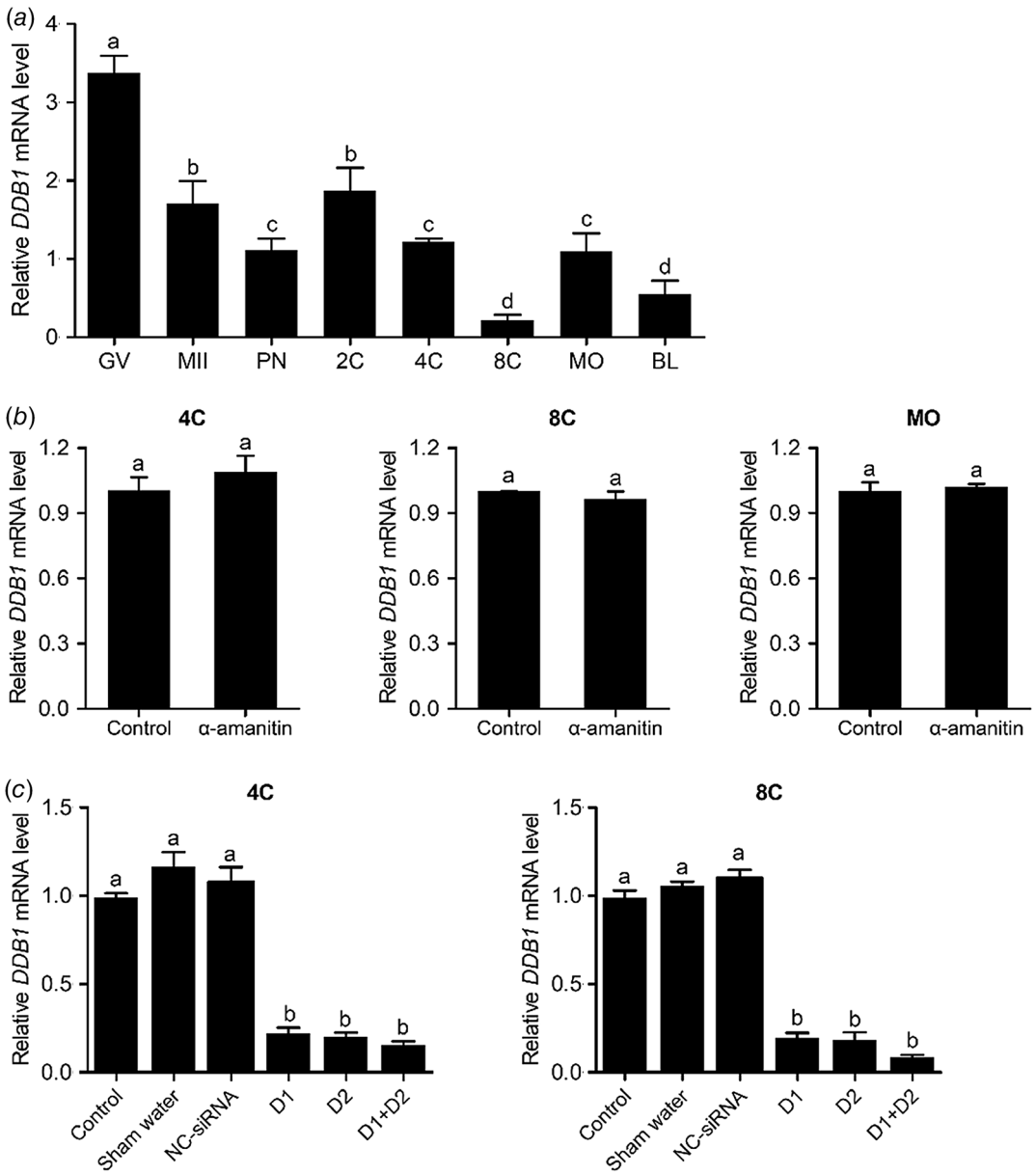

Fig. 1. Characterisation of *DDB1* in porcine oocytes and preimplantation embryos. (*a*) *DDB1* mRNA was analysed in oocytes and preimplantation embryos by RT-qPCR at different developmental stages (at least 20 oocytes and 10 embryos per pool, $n = 3$). (*b*) Relative abundance of *DDB1* mRNA in 4C, 8C, and MO embryos derived from α-amanitin treated 2C, 4C, and 8C embryos, respectively, determined by RT-qPCR (10 embryos per pool, $n = 3$). (*c*) RT-qPCR analysis of *DDB1* mRNA levels in non-injected (control), RNase-free water injection (sham water), negative control siRNA (NC-siRNA), and *DDB1*-siRNA injected 4C or 8C embryos (10 embryos per pool, $n = 3$). D1 and D2 correspond to two different *DDB1* siRNA sequences; D1 + D2, cocktail of D1 and D2 (1:1). All data are presented as mean ± s.e.m. and different letters on the column indicate significant differences ($P < 0.05$).

Discussion

DDB1 is a multifunctional gene and plays a role in embryonic stem cell differentiation, oocyte maturation, and early embryonic development (Yu *et al.* 2013, 2015*a*; Gao *et al.* 2015). In this study, we demonstrated that *DDB1* was a transcript of maternal origin in the porcine model and was consistently expressed in the oocytes and embryos. Our generated *DDB1*-deficient embryos showed an impaired 8C, MO, and BL ratio as well as a significantly decreased TE

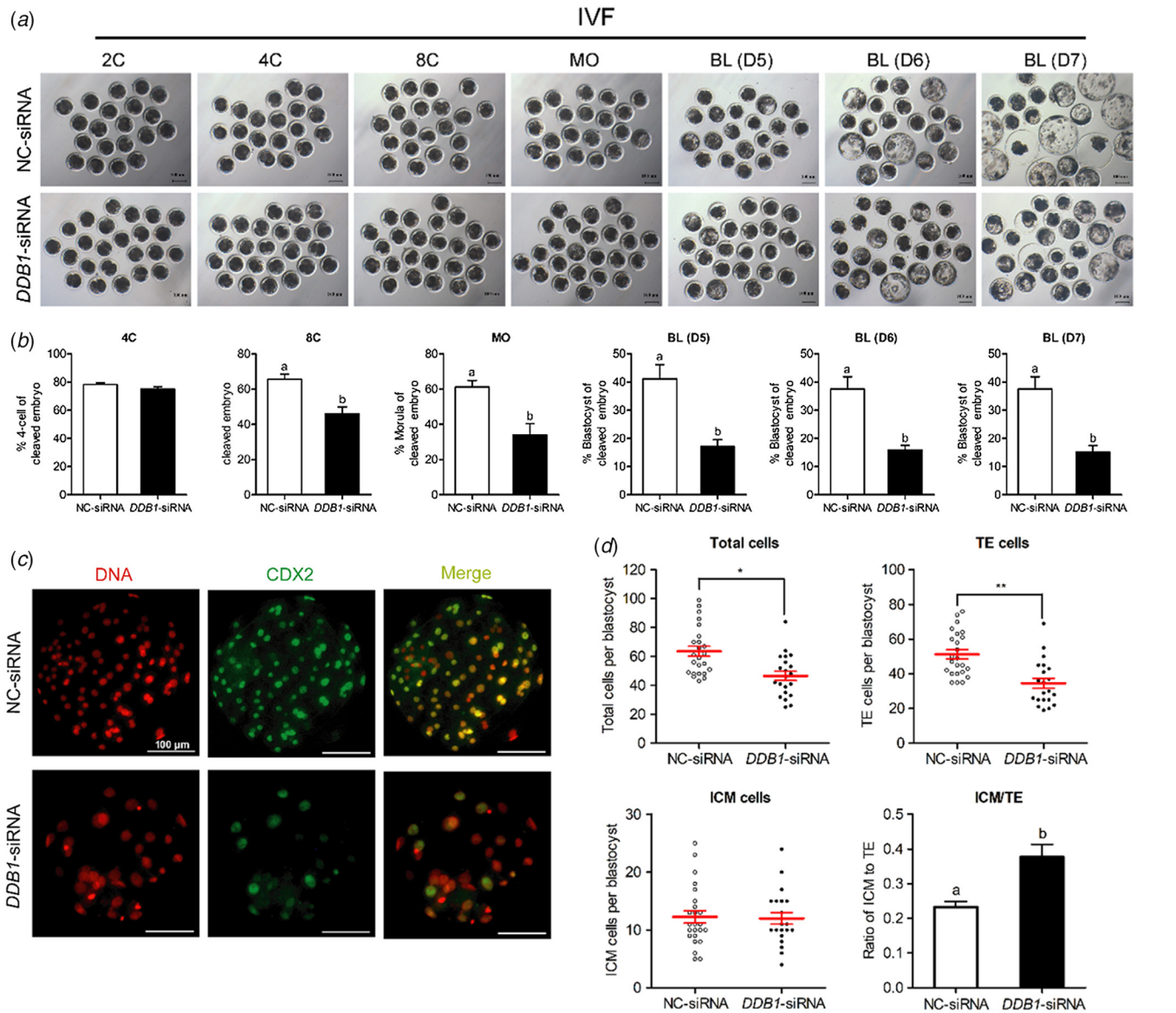

Fig. 2. Effect of *DDB1* knockdown on *in vitro* development of embryos derived from IVF. (*a*) Representative brightfield images of embryos at different developmental stages. Scale bar: 100 μm. (*b*) Developmental rate of embryos at different stages. The number of 2C embryos was regarded as the initial data and the embryos of all other stages were compared to it (at least 20 oocytes per treatment, $n = 4$). Data are shown as mean ± s.e.m. and different letters indicate significant difference ($P < 0.05$). (*c*) Representative fluorescence staining images of *DDB1* knockdown and the control. Nuclei (left panel, red), CDX2 (middle panel, green), and merged images between the DNA and CDX2 (right panel). Scale bar: 100 μm. (*d*) Detection of the quality of blastocysts by an analysis of the number of total cells, TE cells, ICM cells, and the ratio of ICM to TE (at least eight blastocysts per group, $n = 3$). Data are presented as mean ± s.e.m. and different letters indicate significant difference ($P < 0.05$).

population associated with increased apoptotic cells in blastocysts. The RNA-seq analysis showed a changed gene expression profile in the *DDB1*-deficient embryos indicating that *DDB1* could regulate porcine embryo development by modulating the differentiation of the embryonic cell lineages and pluripotency. Our work highlights the critical function of *DDB1* in porcine embryonic development, which is of benefit to embryonic development-related research.

MEGs are not all highly conserved among mammal species. For example, Linker histone *H1FOO* as a maternal gene is essential for the morula–blastocyst transition in bovine early embryonic development (Li *et al.* 2021); however, the knockdown of *H1foo* did not affect the preimplantation development to the blastocyst stage for mice (Funaya *et al.* 2018). Ddb1 was first reported in mice, which is a maternal factor required for zygotic genome activation, and a *Ddb1*-deficient embryo died before the 8-cell stage (Yu *et al.* 2013).

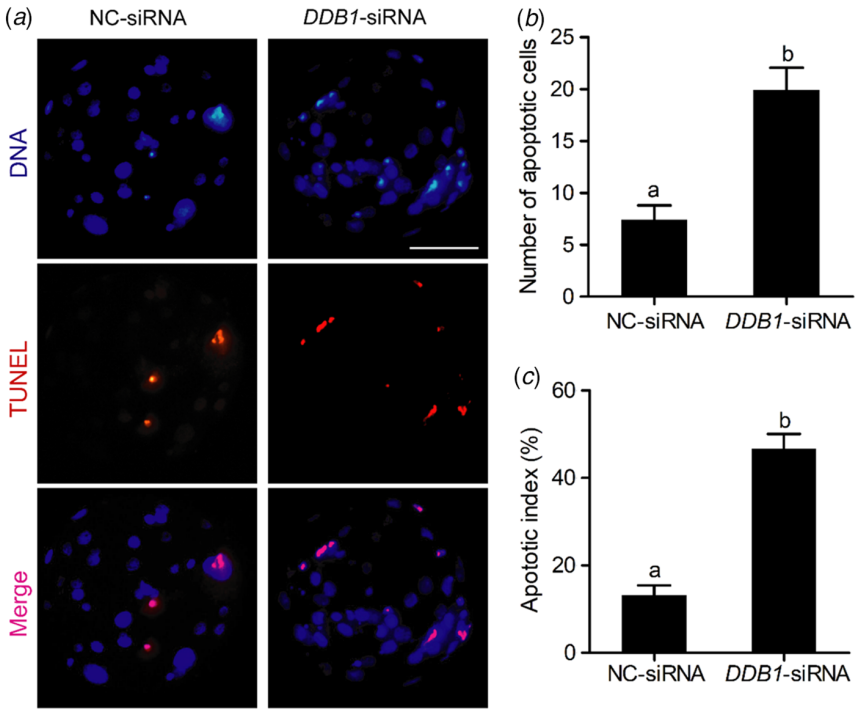

Fig. 3. Effect of *DDB1* deficiency on cell apoptosis in blastocysts. (*a*) Apoptosis of blastocysts was detected by TUNEL staining and representative images of the *DDB1*-KD groups and the control groups are shown. DNA (top panel, blue), TUNEL (middle panel, red), merged images between the DNA and TUNEL (bottom panel). Scale bar: 100 μm. (*b*) The number of TUNEL positive cells was statistically analysed in the *DDB1*-KD and control groups, respectively (at least 10 blastocysts per group, $n = 3$). (*c*) The apoptotic index (ratio of apoptotic cells to total cells) was statistically analysed in the *DDB1*-KD and control groups. Data are presented as the mean ± s.e.m. and the different letters indicate significant difference ($P < 0.05$).

The expression pattern of *Ddb1* in mice oocytes and embryos was unclear. In this study, we reported the expression pattern of *DDB1* in porcine oocytes and embryos. A *DDB1*-deficient porcine embryo could develop to the blastocyst stage but the quality of the resultant blastocyst was poor. These results suggested that the function of maternal *DDB1* was less conserved in preimplantation embryonic development between the mouse and porcine models.

Apoptosis is a critical factor that correlated with embryo developmental arrest in bovine and porcine studies (Mateusen *et al.* 2005; Antunes *et al.* 2010). In our study, we found that *DDB1* depletion stimulated apoptosis in the developing porcine embryos. DDB1 takes part in the nucleotide excision repair pathway (Chu and Yang 2008). The loss of DDB1 led to transcriptional p53 pathway activation, cell cycle deregulation, and an increase in apoptosis in zebrafish embryos (Hu *et al.* 2015). The deletion of *DDB1* in hematopoietic stem cells has been shown to induce p53 pathway activation leading to DNA damage and rapid apoptosis (Gao *et al.* 2015). Our RNA-seq result showed that myeloid leukaemia factor 1 (*MLF1*) was upregulated in the *DDB1*-KD morula (Fig. 4*d*). MLF1 is a negative regulator of cell cycle progression functioning upstream of the p53, which induced p53-dependent cell cycle arrest in mice embryonic fibroblasts (Yoneda-Kato *et al.* 2005). Overexpressed *MLF1* has been shown to increase apoptosis in neonatal rat cardiomyocytes (Rangrez *et al.* 2017). Therefore, MLF1 may be a potential regulator of increased cellular apoptosis in *DDB1*-deficient porcine embryos, however further work is required.

The morula is a pivotal period for blastocyst formation, controlled by a significant amount of differentially expressed genes between the morula and blastocyst stage (Hsu *et al.* 2012). In this study, the most changed genes in the *DDB1*-deficient morula were found to be associated with signal transduction, cell adhesion, and stem cell differentiation including *YAP1* (also known as *YAP*). The Hippo/YAP signalling cascade is central to cell fate specification processes indicating a TE/ICM lineage specification (Sasaki 2015; Chazaud and Yamanaka 2016). In mice, the polarisation of the outer cells promoted YAP nuclear localisation through the regulation of the Hippo signalling pathway (Nishioka *et al.* 2009; Saini and Yamanaka 2018). Non-phosphorylated YAP entered the nucleus and interacted with TEAD4 to promote the expression of downstream genes such as *CDX2* and *GATA3*, which are related to trophoblast differentiation (Ralston *et al.* 2010). The formation of the TE lineage depends on the transcription factor CDX2. The deletion of CDX2 has been shown to result in a failure to form TE and impair the TE function (Strumpf *et al.* 2005; Goissis and Cibelli 2014; Bou *et al.* 2017; Liu *et al.* 2018). Our result showed downregulated gene and protein levels of CDX2 and *GATA3* and significantly reduced TE population, indicating that CDX2-mediated TE was reduced in the *DDB1*-deficient porcine embryos.

Pluripotency is one property for ICM cells that generates all somatic lineages and germlines (Nichols and Smith 2012). OCT4, NANOG, and SOX2 are key transcription factors that maintain the pluripotency of ICM cells (Boyer *et al.* 2005). OCT4 and NANOG are highly expressed in embryonic stem

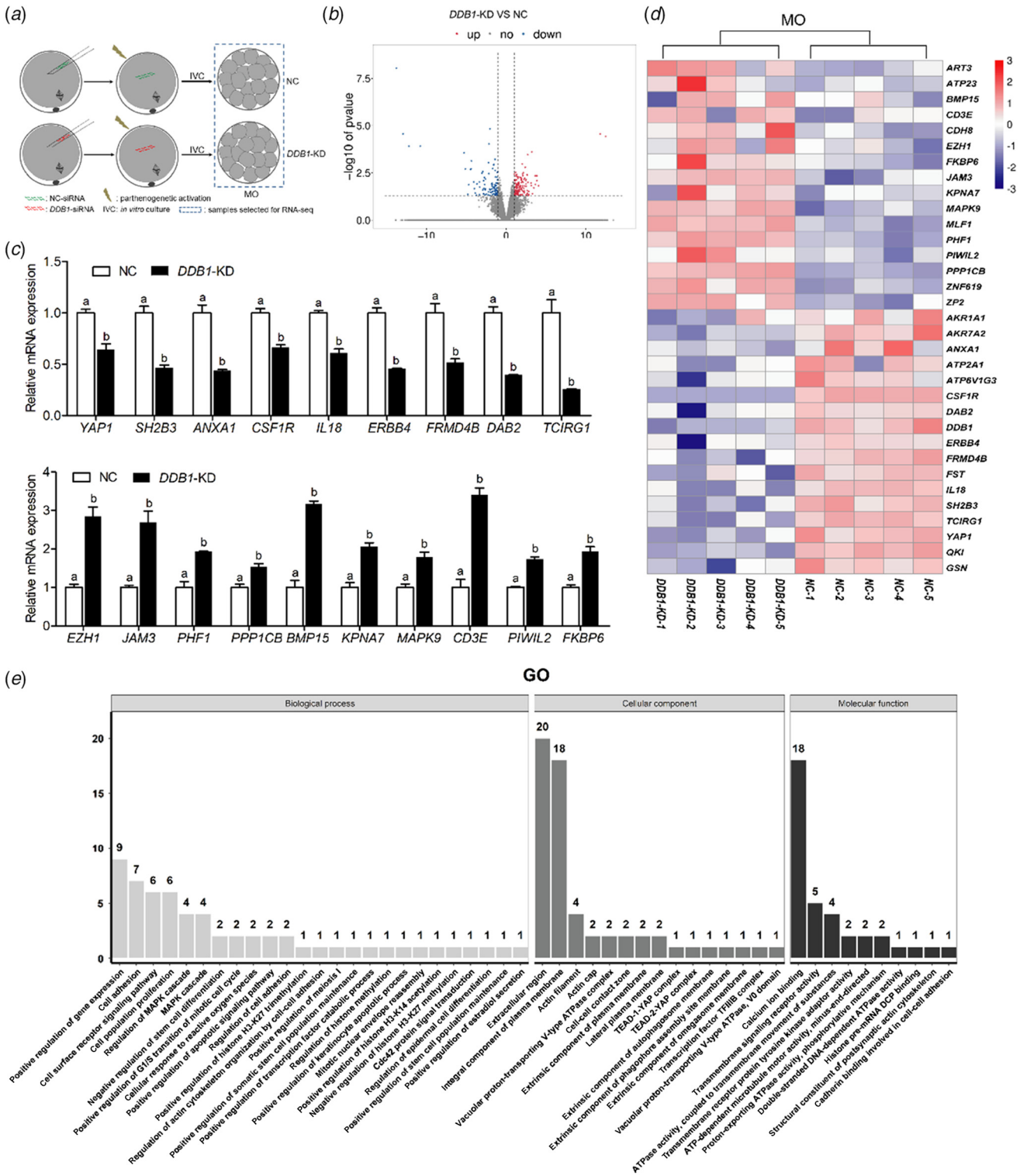

Fig. 4. Transcriptome analysis of the morula embryos deficient of *DDB1*. (*a*) Schematic design of the morula selected for RNA-seq (three morulae per group, $n = 5$). (*b*) Volcano plot showing all DEGs between the *DDB1*-KD and NC groups. (*c*) Validation of selected downregulated (top panel) and upregulated genes (bottom panel) in the *DDB1*-KD and the control groups by RT-qPCR (10 morulae per pool, $n = 3$). Data are shown as mean ± s.e.m. and the different letters indicate significant difference ($P < 0.05$). (*d*) Heatmaps showing partial DEGs at the morula between the *DDB1*-KD and NC groups ($P < 0.05$, $\log_2$ fold change > 0.8 or < −0.8). (*e*) GO analysis exhibiting the most enriched functional categories by the DEGs.

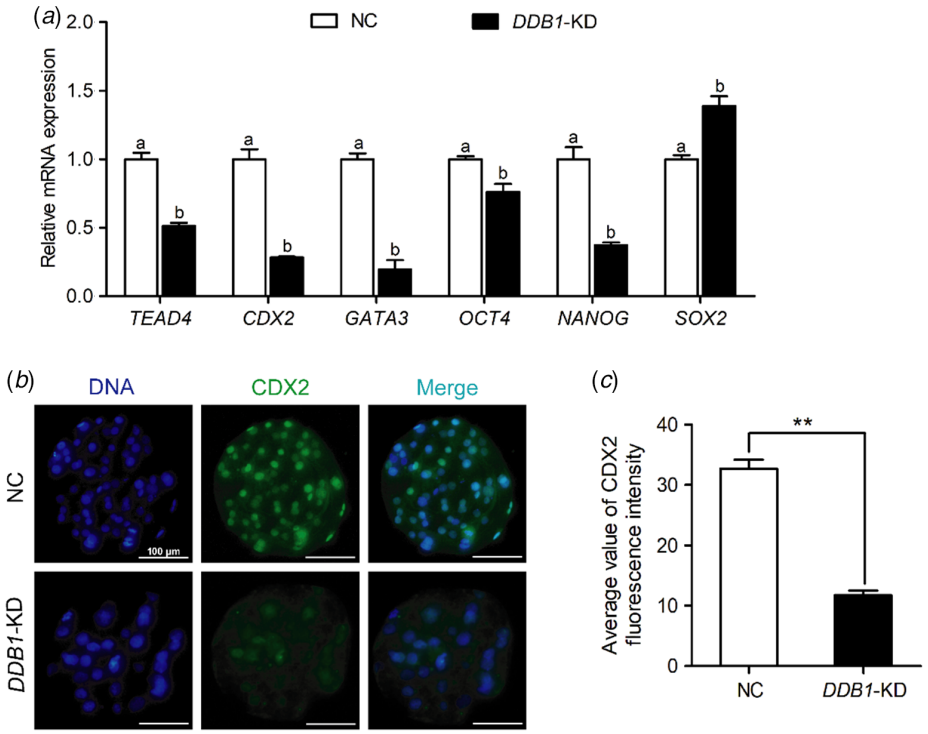

Fig. 5. *DDB1* deficiency disturbs the expression of pluripotency and the differentiation of associated genes in the blastocysts. (*a*) RT-qPCR analysis of the pluripotency and differentiation-associated gene mRNA abundance in the NC and *DDB1*-KD groups (five blastocysts per pool, $n = 3$). Data are shown as mean ± s.e.m. and the different letters indicate significant difference ($P < 0.05$). (*b*) Representative fluorescence staining images of the nuclei and CDX2 protein. Nuclei (left panel, blue), CDX2 (middle panel, green), and merged images between the DNA and CDX2 (right panel). Scale bar: 100 μm. (*c*) The average values of the fluorescence intensities of CDX2 were assessed in the NC and *DDB1*-KD groups by densitometry (at least five blastocysts per treatment; $n = 3$ replicates). $^{**}P < 0.01$.

cells (ESCs) and have been shown to trigger differentiation when repressed (Pan and Thomson 2007; Shi and Jin 2010). In contrast, an increase in SOX2 can induce the differentiation of ESCs (Kopp *et al.* 2008). Our results showed a decreased *OCT4* and *NANOG* and an increased *SOX2* expression in the *DDB1*-deficient blastocysts (Fig. 5*a*), suggesting an essential role of *DDB1* in ICM pluripotency maintenance.

In conclusion, our study demonstrated the maternal origin of *DDB1* in a porcine model. A lack of *DDB1* enhanced cellular apoptosis and impaired TE formation and pluripotency maintenance in porcine blastocysts. The decreased expression of CDX2 was the potential molecular mechanism of the decreased TE population in *DDB1*-deficient porcine blastocysts. Further studies are required to explore the function of DDB1 in embryonic development.

Supplementary material

Supplementary material is available online.

References

Antunes G, Chaveiro A, Santos P, Marques A, Jin HS, Moreira da Silva F (2010) Influence of apoptosis in bovine embryo's development. *Reproduction in Domestic Animals* **45**(1), 26–32. doi:10.1111/j.1439-0531.2008.01131.x

Bou G, Liu S, Sun M, Zhu J, Xue B, Guo J, Zhao Y, Qu B, Weng X, Wei Y, Lei L, Liu Z (2017) CDX2 is essential for cell proliferation and polarity in porcine blastocysts. *Development* **144**(7), 1296–1306. doi:10.1242/dev.141085

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**(6), 947–956. doi:10.1016/j.cell.2005.08.020

Cao S, Han J, Wu J, Li Q, Liu S, Zhang W, Pei Y, Ruan X, Liu Z, Wang X, Lim B, Li N (2014) Specific gene-regulation networks during the pre-implantation development of the pig embryo as revealed by deep sequencing. *BMC Genomics* **15**, 4. doi:10.1186/1471-2164-15-4

Cao Z, Xu T, Tong X, Wang Y, Zhang D, Gao D, Zhang L, Ning W, Qi X, Ma Y, Yu T, Knott JG, Zhang Y (2019) Maternal yes-associated protein participates in porcine blastocyst development *via* modulation of trophectoderm epithelium barrier function. *Cells* **8**(12), 1606. doi:10.3390/cells8121606

Chazaud C, Yamanaka Y (2016) Lineage specification in the mouse preimplantation embryo. *Development* **143**(7), 1063–1074. doi:10.1242/dev.128314

Chu G, Yang W (2008) Here comes the sun: recognition of UV-damaged DNA. *Cell* **135**(7), 1172–1174. doi:10.1016/j.cell.2008.12.015

Cockburn K, Biechele S, Garner J, Rossant J (2013) The Hippo pathway member Nf2 is required for inner cell mass specification. *Current Biology* **23**(13), 1195–1201. doi:10.1016/j.cub.2013.05.044

de Vries WN, Evsikov AV, Haac BE, Fancher KS, Holbrook AE, Kemler R, Solter D, Knowles BB (2004) Maternal β-catenin and E-cadherin in mouse development. *Development* **131**(18), 4435–4445. doi:10.1242/dev.01316

Ding B, Cao Z, Hong R, Li H, Zuo X, Luo L, Li Y, Huang W, Li W, Zhang K, Zhang Y (2017) WDR5 in porcine preimplantation embryos: expression, regulation of epigenetic modifications and requirement for early development. *Biology of Reproduction* **96**(4), 758–771. doi:10.1093/biolre/iox020

Funaya S, Ooga M, Suzuki MG, Aoki F (2018) Linker histone H1FOO regulates the chromatin structure in mouse zygotes. *FEBS Letters* **592**(14), 2414–2424. doi:10.1002/1873-3468.13175

Gao J, Buckley SM, Cimmino L, Guillamot M, Strikoudis A, Cang Y, Goff SP, Aifantis I (2015) The CUL4-DDB1 ubiquitin ligase complex controls adult and embryonic stem cell differentiation and homeostasis. *eLife* **4**, e07539. doi:10.7554/eLife.07539

Gao D, Xu T, Qi X, Ning W, Ren S, Ru Z, Ji K, Ma Y, Yu T, Li Y, Cao Z, Zhang Y (2020) CLAUDIN7 modulates trophectoderm barrier function to maintain blastocyst development in pigs. *Theriogenology* **158**, 346–357. doi:10.1016/j.theriogenology.2020.09.038

Goissis MD, Cibelli JB (2014) Functional characterization of CDX2 during bovine preimplantation development *in vitro*. *Molecular Reproduction and Development* **81**(10), 962–970. doi:10.1002/mrd.22415

Hamatani T, Carter MG, Sharov AA, Ko MSH (2004) Dynamics of global gene expression changes during mouse preimplantation development. *Developmental Cell* **6**(1), 117–131. doi:10.1016/S1534-5807(03)00373-3

Hsu CC, Lin EC, Chen SC, Huang SC, Liu BH, Yu YH, Chen CC, Yang CC, Lien CY, Wang YH, Liu CW, Mersmann HJ, Cheng WTK, Ding ST (2012) Differential gene expression between the porcine morula and blastocyst. *Reproduction in Domestic Animals* **47**(1), 69–81. doi:10.1111/j.1439-0531.2011.01803.x

Hu Z, Holzschuh J, Driever W (2015) Loss of DDB1 leads to transcriptional p53 pathway activation in proliferating cells, cell cycle deregulation, and apoptosis in zebrafish embryos. *PLoS ONE* **10**(7), e0134299. doi:10.1371/journal.pone.0134299

Iovine B, Iannella ML, Bevilacqua MA (2011) Damage-specific DNA binding protein 1 (DDB1): a protein with a wide range of functions. *The International Journal of Biochemistry & Cell Biology* **43**(12), 1664–1667. doi:10.1016/j.biocel.2011.09.001

Kim J, Zhao H, Dan J, Kim S, Hardikar S, Hollowell D, Lin K, Lu Y, Takata Y, Shen J, Chen T (2016) Maternal Setdb1 is required for meiotic progression and preimplantation development in mouse. *PLoS Genetics* **12**(4), e1005970. doi:10.1371/journal.pgen.1005970

Kopp JL, Ormsbee BD, Desler M, Rizzino A (2008) Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. *Stem Cells* **26**(4), 903–911. doi:10.1634/stemcells.2007-0951

Larue L, Ohsugi M, Hirchenhain J, Kemler R (1994) E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proceedings of the National Academy of Sciences of the United States of America* **91**(17), 8263–8267. doi:10.1073/pnas.91.17.8263

Lee W-J, Jang S-J, Lee S-C, Park J-S, Jeon R-H, Subbarao RB, Bharti D, Shin J-K, Park B-W, Rho G-J (2017) Selection of reference genes for quantitative real-time polymerase chain reaction in porcine embryos. *Reproduction, Fertility and Development* **29**(2), 357–367. doi:10.1071/RD14393

Li L, Zheng P, Dean J (2010) Maternal control of early mouse development. *Development* **137**(6), 859–870. doi:10.1242/dev.039487

Li S, Shi Y, Dang Y, Luo L, Hu B, Wang S, Wang H, Zhang K (2021) NOTCH signaling pathway is required for bovine early embryonic development. *Biology of Reproduction* **105**(2), 332–344. doi:10.1093/biolre/ioab056

Liu S, Bou G, Zhao J, Guo S, Guo J, Weng X, Yin Z, Liu Z (2018) Asynchronous CDX2 expression and polarization of porcine trophoblast cells reflects a species-specific trophoderm lineage determination progress model. *Molecular Reproduction and Development* **85**(7), 590–598. doi:10.1002/mrd.22994

Maître J-L (2017) Mechanics of blastocyst morphogenesis. *Biology of the Cell* **109**(9), 323–338. doi:10.1111/boc.201700029

Mateusen B, Van Soom A, Maes DGD, Donnay I, Duchateau L, Lequarre A-S (2005) Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. *Reproduction* **129**(4), 443–452. doi:10.1530/rep.1.00533

Nichols J, Smith A (2012) Pluripotency in the embryo and in culture. *Cold Spring Harbor Perspectives in Biology* **4**(8), a008128. doi:10.1101/cshperspect.a008128

Nishioka N, Inoue K-i, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N, Makita R, Kurihara H, Morin-Kensicki EM, Nojima H, Rossant J, Nakao K, Niwa H, Sasaki H (2009) The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Developmental Cell* **16**(3), 398–410. doi:10.1016/j.devcel.2009.02.003

Pan G, Thomson JA (2007) Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Research* **17**(1), 42–49. doi:10.1038/sj.cr.7310125

Ralston A, Cox BJ, Nishioka N, Sasaki H, Chea E, Rugg-Gunn P, Guo G, Robson P, Draper JS, Rossant J (2010) Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development* **137**(3), 395–403. doi:10.1242/dev.038828

Rangrez AY, Pott J, Kluge A, Frauen R, Stiebeling K, Hoppe P, Sossalla S, Frey N, Frank D (2017) Myeloid leukemia factor-1 is a novel modulator of neonatal rat cardiomyocyte proliferation. *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research* **1864**(4), 634–644. doi:10.1016/j.bbamcr.2017.01.004

Roovers EF, Rosenkranz D, Mahdipour M, Han CT, He NN, Chuva de Sousa Lopes SM, van der Westerlaken LAJ, Zischler H, Butter F, Roelen BAJ, Ketting RF (2015) Piwi proteins and piRNAs in mammalian oocytes and early embryos. *Cell Report* **10**, 2069–2082. doi:10.1016/j.celrep.2015.02.062

Saini D, Yamanaka Y (2018) Cell polarity-dependent regulation of cell allocation and the first lineage specification in the preimplantation mouse embryo. *Current Topics in Developmental Biology* **128**, 11–35. doi:10.1016/bs.ctdb.2017.10.008

Sasaki H (2015) Position- and polarity-dependent Hippo signaling regulates cell fates in preimplantation mouse embryos. *Seminars in Cell & Developmental Biology* **47–48**, 80–87. doi:10.1016/j.semcdb.2015.05.003

Shi G, Jin Y (2010) Role of Oct4 in maintaining and regaining stem cell pluripotency. *Stem Cell Research & Therapy* **1**(5), 39. doi:10.1186/scrt39

Strumpf D, Mao C-A, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* **132**(9), 2093–2102. doi:10.1242/dev.01801

Tejomurtula J, Lee KB, Tripurani SK, Smith GW, Yao J (2009) Role of importin alpha8, a new member of the importin alpha family of nuclear transport proteins, in early embryonic development in cattle. *Biology of Reproduction* **81**, 333–342. doi:10.1095/biolreprod.109.077396

Su RW, Jia B, Ni H, Lei W, Yue SL, Feng XH, Deng WB, Liu JL, Zhao ZA, Wang TS, Yang ZM (2012) Junctional adhesion molecule 2 mediates the interaction between hatched blastocyst and luminal epithelium: induction by progesterone and LIF. *PLoS One* **7**, e34325. doi:10.1371/journal.pone.0034325

Uh K, Lee K (2017) Use of chemicals to inhibit DNA replication, transcription, and protein synthesis to study zygotic genome activation. In 'Methods in molecular biology. Vol. 1605'. (Ed. K Lee) pp. 191–205. (Humana Press: New York, NY) doi:10.1007/978-1-4939-6988-3_13

Wang Y, Yuan P, Yan Z, Yang M, Huo Y, Nie Y, Zhu X, Qiao J, Yan L (2021) Single-cell multiomics sequencing reveals the functional regulatory landscape of early embryos. *Nature Communications* **12**(1), 1247. doi:10.1038/s41467-021-21409-8

Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick SL, Matzuk MM (2003) Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for

the oocyte-to-embryo transition. *Nature Genetics* **33**(2), 187–191. doi:10.1038/ng1079

Wu G, Gentile L, Fuchikami T, Sutter J, Psathaki K, Esteves TC, Araúzo-Bravo MJ, Ortmeier C, Verberk G, Abe K, Schöler HR (2010) Initiation of trophectoderm lineage specification in mouse embryos is independent of Cdx2. *Development* **137**(24), 4159–4169. doi:10.1242/dev.056630

Xu Q, Wang F, Xiang Y, Zhang X, Zhao Z-A, Gao Z, Liu W, Lu X, Liu Y, Yu X-J, Wang H, Huang J, Yi Z, Gao S, Li L (2015) Maternal BCAS2 protects genomic integrity in mouse early embryonic development. *Development* **142**(22), 3943–3953. doi:10.1242/dev.129841

Yoneda-Kato N, Tomoda K, Umehara M, Arata Y, Kato J-Y (2005) Myeloid leukemia factor 1 regulates p53 by suppressing COP1 *via* COP9 signalosome subunit 3. *The EMBO Journal* **24**(9), 1739–1749. doi:10.1038/sj.emboj.7600656

Yoshioka K, Suzuki C, Tanaka A, Anas IM-K, Iwamura S (2002) Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biology of Reproduction* **66**(1), 112–119. doi:10.1095/biolreprod66.1.112

Yu C, Zhang Y-L, Pan W-W, Li X-M, Wang Z-W, Ge Z-J, Zhou J-J, Cang Y, Tong C, Sun Q-Y, Fan H-Y (2013) CRL4 complex regulates mammalian oocyte survival and reprogramming by activation of TET proteins. *Science* **342**(6165), 1518–1521. doi:10.1126/science.1244587

Yu C, Ji S-Y, Sha Q-Q, Sun Q-Y, Fan H-Y (2015*a*) CRL4–DCAF1 ubiquitin E3 ligase directs protein phosphatase 2A degradation to control oocyte meiotic maturation. *Nature Communications* **6**, 8017. doi:10.1038/ncomms9017

Yu C, Xu Y-W, Sha Q-Q, Fan H-Y (2015*b*) $CRL4^{DCAF1}$ is required in activated oocytes for follicle maintenance and ovulation. *Molecular Human Reproduction* **21**(2), 195–205. doi:10.1093/molehr/gau103

Zhang K, Smith GW (2015) Maternal control of early embryogenesis in mammals. *Reproduction, Fertility and Development* **27**(6), 880–896. doi:10.1071/RD14441

Data availability. The data that support the findings of this study are openly available in Gene Expression Omnibus (GEO) database (accession number GSE189720).

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