TRIM28 maintains genome imprints and regulates development of porcine SCNT embryos

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Abstract

Pre-implantation embryos undergo genome-wide DNA demethylation, however certain regions, like imprinted loci remain methylated. Further, the mechanisms ensuring demethylation resistance by TRIM28 in epigenetic reprogramming remain poorly understood. Here, TRIM28 was knocked down in oocytes, and its effects on porcine somatic cell nuclear transfer (SCNT) embryo development was examined. Our results showed that SCNT embryos constructed from TRIM28 knockdown oocytes had significantly lower cleavage (53.9 ± 3.4% vs 64.8 ± 2.7%) and blastocyst rates (12.1 ± 4.3% vs 19.8 ± 1.9%) than control-SCNT embryos. The DNA methylation levels at the promoter regions of the imprinting gene IGF2 and H19 were significantly decreased in the 4-cell stage, and the transcript abundance of other imprinting gene was substantially increased. We also identified an aberrant two-fold decrease in the expression of CXXC1 and H3K4me3 methyltransferase (ASH2L and MLL2), and the signal intensity of H3K4me3 had a transient drop in SCNT 2-cell embryos. Our results indicated that maternal TRIM28 knockdown disrupted the genome imprints and caused epigenetic variability in H3K4me3 levels, which blocked the transcription activity of zygote genes and affected the normal developmental progression of porcine SCNT embryos.

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Introduction

Following SCNT, differentiated somatic cell genomes undergo global epigenetic reprogramming to achieve a totipotent state, and DNA demethylation is an important marker (Zlotorynski 2017). Some genomic loci are not reprogrammed and certain specific genomic regions must maintain DNA methylation to inherit important epigenetic characteristics of germ cells to somatic cells (Monk 2015). Sampath Kumar et al. reported that loss of maternal Trim28 causes depression of a Y chromosome-linked gene and results in male lethality during early embryogenesis in mice (Sampath Kumar et al. 2017). TRIM28 is a nuclear protein believed to be a coinhibitory of zinc finger proteins associated with the KRAB domain (Friedman et al. 1996). In mammals, TRIM28 and multiple KRAB domains inhibit transcriptional activity by recruiting remodeling factors that directly bind to DNA to participate in the formation of repressive chromatin modification (Kim et al. 1996, Cammas et al. 2000). Zlotorynski et al. found that TRIM28 mediates the establishment and maintenance of DNA methylation, when the Trim28 gene is deleted in oocytes, DNA methylation at imprinted genes is lost in preimplantation embryos (Zlotorynski 2017). Messerschmidt et al. found that the absence of maternal Trim28 results in the loss of DNA methylation and lack of paternal TRIM28 binding at later embryo development stages (Messerschmidt et al. 2012). It has been reported that TRIM28 can recruit ZFP57 and DNMT1 to form a complex, that can protect ICРs from active demethylation in mouse ES cells (Takikawa et al. 2013).

It has been reported that significant histone variant exchanges occur in SCNT embryos (Nashun et al. 2011, Wen et al. 2014). The authors found that H3K9me3 demethylation is generally required for successful SCNT reprogramming and that incomplete reprogramming of H3K27me3 is a barrier in SCNT embryos (Liu et al. 2016, Liu et al. 2018, Matoba et al. 2018). In addition, H3K4me3 might affect transcriptional reprogramming and efficient removal of a donor cell-specific H3K4me3 mark might contribute to SCNT reprogramming (Liu et al. 2016). Marta Klimczak et al. observed that the effect of TRIM28 in reprogramming is accompanied by differential enrichment of proteins involved in the cell cycle, adhesion and stemness (Klimczak et al. 2017). TRIM28 plays a crucial role in induced pluripotent stem cell reprogramming process.

However, the mechanism by which TRIM28 identifies imprint gene loci and confers resistance to demethylation...
reprogramming in SCNT embryo development is still unclear. Therefore, in this study, we used specific TRIM28-deleted oocytes to construct SCNT embryos to detect the effect of maternal TRIM28 on pig embryo development and epigenetic modification, and explored the mechanism of TRIM28 maintenance of DNA methylation and regulated SCNT embryo development, to provide a theoretical basis for obtaining higher SCNT efficiency.

Materials and methods

Chemicals
All chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise noted.

Isolation and cultivation of fetal fibroblasts (FFs)
FFs were isolated according to previously described protocols (Zhai et al. 2018). FFs were isolated from 30- to 35-day-old fetal pigs (approximately 35 mm in length).

Collection and in vitro maturation of porcine oocytes
The collection and maturation of porcine oocytes were conducted as previously described protocols (Zhai et al. 2018). Porcine ovaries were collected from a local abattoir. More than 600 COCs with at least three layers of cumulus cells were selected and cultured in in vitro maturation (IVM) media until 42–44 h.

Somatic cell nuclear transfer
SCNT was conducted according to previously described protocols (Zhai et al. 2018). More than 300 MII oocytes were operated and to construct about 150 embryos each time. The first polar body of MII oocytes was removed by using the blind-suction method. Then, porcine FFs were subsequently injected into the perivitelline space of the enucleated-oocytes to reconstruct the cloned embryo. The reconstructed cloned embryos were then activated by two successive direct-current pulses at 1.45 kV/cm for 100 μs using a CF-150B electro-fusion instrument (CF-150B, BLS, Hungary). The activated cloned embryos were then continuously cultured in PZM-3 medium at 38.5°C, 5% CO₂ and 100% humidity for 7 days.

In vitro fertilization (IVF)
Frozen porcine semen was purchased from the Hebei Mingge Animal Husbandry Co. Ltd. The frozen semen was quickly thawed. Groups of 20 oocytes were transferred to 50 μL of mTBDM covered with paraffin oil. The final sperm concentration was 2 × 10⁶ sperm/ml. The oocytes were cocultured with sperm for 6 h at 38.5°C with 5% CO₂, and then the oocytes were transferred to porcine zygote medium 3 (PZM3) for continued culture. More than 200 embryos were constructed each time.

siRNA synthesis and microinjection
Three groups of siRNAs were designed and synthesized by Sangon Biotech (Table 1). All microinjections were performed using an Eppendorf Femtojet 4i microinjector (Eppendorf, Hamburg, Germany). Approximately 10 pL synthetic siRNA was microinjected into the cytoplasm of denuded oocytes. The concentration of all microinjected siRNAs was adjusted to 20 μM. After microinjection, oocytes were washed and cultured in maturation medium at 38.5°C with 5% CO₂ for 24 h.

Western blotting (WB)
WB was conducted according to previously described protocols (Zhai et al. 2018). Protein samples of 200 embryos were separated by Biofuraw™ Precast Gel (Tanon, Shanghai, China). We repeated this experiment three times. Densitometry analysis of WB was performed using ImageJ software (Rasband, WS, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014).

Immunofluorescence (IF) staining
IF was conducted according to previously described protocols (Zhai et al. 2018). There are more than 10 embryos in each group for IF staining and repeated no less than three times. Embryos were incubated overnight at 4°C with primary antibodies (Table 2) and then stained at 37°C for 2 h with Alexa Fluor 488 goat anti-mouse (1:500 dilutions, A-11001) (Invitrogen) or Alexa Fluor 594 goat anti-rabbit (1:500 dilutions, A-11037) (Invitrogen). The DNA was stained for 10 min with 10 μg/mL DAPI prior to mounting and observation under a fluorescence microscope (Nikon).

Table 1  siRNA synthesis report form.

<table>
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<th>Sample</th>
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<th>Modification</th>
<th>Purification</th>
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<tr>
<td>Negative control</td>
<td>UUC UCC GAA CGU GUC ACG UTT</td>
<td>ACG UGA CAC GUU CGG AGA ATT</td>
<td>5’ FAM</td>
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</table>
**Microscopy and image analysis**

Microscopy and image analysis were conducted according to previously described protocols (Zhai et al. 2018). Fluorescence was examined with a Nikon Eclipse Ti-U microscope equipped with appropriate filters (Nikon). Images of the control and experimental groups were captured using the same microscope settings and exposure times. Evaluation of total fluorescence intensity of individual images was performed using ImageJ software (NIH), based on procedures described elsewhere (Amouroux et al. 2016). Background fluorescence intensity was measured as average intensity within the cytoplasmic area, and subtracted from the nuclear staining intensity for correction; labeling intensity in nuclei of porcine embryos were calculated accordingly. Semi-quantitative fluorescence intensity measurement was obtained by setting the average DAPI intensity from nucleus of embryos to 100%, and comparing the labeling intensity of specific signal in each nucleus to that value. Data shown are representative for at least three independent experiments.

**RNA isolation and quantitative polymerase chain reaction (PCR)**

RNA isolation and quantitative PCR were conducted according to previously described protocols (Zhai et al. 2018). The REPLI-g® WTA single cell kit (Qiagen, Hilden, Germany) was used to extract the total RNA and synthesize cDNA from 200 of porcine embryos and repeated three times. The primers used are listed in Table 3. Additionally, we defined the gene expression as cut-off when the Ct mean reached 35. GAPDH was used as the reference gene.

**Sodium bisulfite genomic sequencing**

Genomic DNA samples were achieved from 200 embryos. Genomic DNA was subjected to bisulfite transformation, followed by PCR using the primers listed in Table 4. PCR products were gel recovered using an ordinary Axy Prep DNA Gel Extraction Kit (Axygen, Beijing, China) and then ligated into the T-Vector pMD19 (TaKaRa). Recombinant plasmids were transformed into DH5α competent cells (Tiangen, Beijing, China) and 20 positive clones were selected and sequenced (Sangon Biotech, Changchun, China).

**RT² profiler PCR array and differential expression profiling**

RT² Profiler PCR Array were conducted according to previously described protocols (Zhai et al. 2018). The Pig Epigenetic Chromatin Modification Enzymes RT² Profiler PCR Array (Qiagen, PASS-085Z) was performed according to the manufacturer’s instructions. The detected genes can be found on the official website of Qiagen.

Differential expression profiling was analyzed according to previously described protocols (Zhang et al. 2018). Degust (http://vicbioinformatics.com/de gust/), an interactive web tool for visualizing differential gene expression data, was used to generate the parallel coordinate plot based on the data from the PCR array (Ritchie et al. 2015). The relative expression values of each tested gene were provided as a $\Delta\Delta CT$ value to evaluate the transcript level. The significantly differential expression genes were assessed using the LIMMA package integrated in the Degust public server. The heat maps showing the genic relationship matrices were created in R studio Version 1.1.383 with the Heat map package (Perez-Llamas & Lopez-Bigas 2011). The complete linkage hierarchical clustering was performed by the Euclidean distance measure.

**Statistical analysis**

All experiments were replicated at least three times. The statistical analysis was carried out by two-tailed Student’s $t$-test using SPSS (Statistics Production for Service Solution) v19.0 software. $P$-value $<0.05$ was considered statistically significant, and $P < 0.01$ was considered extremely significant.

**Results**

**Screening the most suitable siRNA**

TRIM28 expression was detected using IF staining and we found that TRIM28 is highly expressed in MII oocytes (Fig. 1A). Next, the siRNAs were used to target different areas of TRIM28 to knockdown its expression, and the efficacy was confirmed by qPCR and Western blotting. Three groups of siRNAs were designed and synthesized by Sangon Bioengineering Co., Ltd., and then were injected into oocytes (Fig. 1B). The siRNA targeting the TRIM28 CDS 629 site (siRNA-629) and TRIM28 CDS

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Table 2  Antibodies used for Immunofluorescence staining.

<table>
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<th>Antibodies</th>
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2444 site (siRNA-2444) could significantly knockdown TRIM28, reducing its expression in MII oocytes by 85% ($P < 0.01$) compared with the siRNA negative control. The siRNA targeting the TRIM28 CDS 1303 site (siRNA-1303) could also significantly knockdown TRIM28, reducing its expression in MII oocytes by 50% ($P < 0.01$) (Fig. 1C). These results were further verified at the protein level by Western blot analysis. The abundance of TRIM28 protein in the transfected siRNA-629 was 2 times lower than that in the control group (Fig. 1D) of MII oocytes, while the remaining two siRNAs had no significant influence on TRIM28 expression. Based on the above results, we chose siRNA-629 as the most suitable interfering RNA and applied it in subsequent experiments.

To further determine the optimal time for microinjection of siRNA, we performed cytoplasmic injections on oocytes matured in vitro for 20, 26, 32, and 42 h (Fig. 1E) and then, these oocytes were collected after 24 h. The qPCR results showed that the transcript abundance of TRIM28 in mature MII oocytes were significantly downregulated at the 20 and 26 h injections ($P < 0.01$) (Fig. 1E). Because cytoplasmic injection was easier at 26 h, and the expression level of TRIM28 at 26 h was significantly lower than that at 20 h, we selected 26 h as the optimal time for siRNA injection.

**TRIM28 knockdown inhibited the development of SCNT embryos**

The developmental capacity of SCNT embryos constructed with oocytes of interfering TRIM28, which was shortened to siRNA-SCNT, was evaluated. The cleavage and blastocyst rates of TRIM28-knockdown SCNT embryos (53.9 ± 3.4% vs 12.1 ± 4.3%) were significantly lower than those of the SCNT embryos (68.1 ± 1.9% vs 19.8 ± 1.9% (Table 5). Moreover, we used qPCR and IF staining to visualize the expression patterns of TRIM28 in different embryos. Our results also showed that TRIM28 had a higher expression level prior to the zygotic gene.
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activation (ZGA) period in the SCNT and IVF embryos (Fig. 2B, C, and D), while the expression of TRIM28 in the siRNA-SCNT embryos was significantly decreased compared with that of the control SCNT embryos until transcription from the paternal allele ensues after ZGA (Fig. 2A, B, and D). During embryonic development, TRIM28 expression was remarkably increased at the early 8-cell stage in the siRNA-SCNT embryos (Fig. 2A). The transcript abundance of TRIM28 had the same expression patterns (Fig. 2E).

Knocking down TRIM28 disturbed the dynamic patterns of DNA methylation during the early development of SCNT embryos

To determine whether TRIM28 is crucial for genome-reprogramming events, we assessed the global DNA modifications, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), in the siRNA-SCNT and control SCNT embryos using IF staining (Fig. 3A, B, C, and D). The total 5mC level of siRNA-SCNT embryos decreased significantly from the 2-cell to blastocyst stage ($P < 0.01$), but the 5mC level increased significantly at the 4-cell stage (Fig. 3A). The 5hmC level of siRNA-SCNT embryos was substantially lower than that of the control-SCNT embryos at the 2-cell and 4-cell stages, but significantly higher than that of the control-SCNT embryos at the blastocyst stage (Fig. 3B).

We also chose repeat elements (PRE-1 and SATELLITE) to assess the global genome-wide DNA methylation levels using sodium bisulfite genomic sequencing of the SCNT embryos. PRE-1 had an overall moderate methylation level and significantly increased the DNA methylation level at the MII stage and 4-cell stage, but was remarkably reduced at the 2-cell stage in the siRNA-SCNT embryos (Fig. 4A and C). SATELLITE had a remarkably lower methylation levels from MII stage to 8-cell stage in the siRNA-SCNT embryos than in the control-SCNT embryo (Fig. 4B and D). To determine whether knocking down TRIM28 affected the intergenic differentially methylated region (IG-DMR) located in $H19$-$IGF2$.
we then used bisulfite sequencing to measure the DNA methylation in DMRs of \( H19 \) and \( IGF2 \). \( H19 \) had an overall moderate methylation level in the siRNA-SCNT embryos and control-SCNT embryos, but had remarkably higher levels at the 2-cell and blastocyst stage in siRNA-SCNT embryos (Fig. 4E and F). The global DNA methylation levels at the promoter of \( IGF2 \) were apparently higher in the control-SCNT embryos than in the siRNA-SCNT embryos from the 2-cell to the blastocyst stage in (Fig. 4G and H).

**Figure 2** Global expression levels of TRIM28 in the SCNT and IVF embryos during early development. (A–C) Immunofluorescence staining of TRIM28 (red) in the siRNA-SCNT, SCNT and IVF embryos. Scale bars, 25 μm. (D) Semiquantitative fluorescence intensity analysis of TRIM28 staining. (E) Relative abundance of TRIM28 in the porcine IVF and SCNT embryos, quantities were normalized to GAPDH abundance. Data are presented as the mean ± s.d. *\( P < 0.05; \) **\( P < 0.01 \) between groups, as indicated. BLA, blastocyst. siRNA-TRIM28-SCNT, oocyte with TRIM28 depletion from somatic cell nuclear transfer.

**Figure 3** Abnormal methylation patterns of the global genome in the siRNA-TRIM28-SCNT embryos and control SCNT embryo. (A, B) Immunofluorescence staining of 5mC (green) and 5hmC (red) in the siRNA-SCNT and control-SCNT embryos. (C, D) Semiquantitative fluorescence intensity analysis of 5mC (C) and 5hmC (D) staining. Scale bars, 25 μm. *\( P < 0.05; \) **\( P < 0.01 \) between groups, as indicated.
Figure 4 Abnormal DNA methylation levels of PRE-1, SATELLITE, H19 and IGF2 in the siRNA-TRIM28-SCNT embryos and SCNT embryos. Measured using bisulfite sequencing, the DNA methylation level at the promoter region of PRE-1 (A and C), SATELLITE (B and D), H19 (E-F) and IGF2 (G-H); siRNA-TRIM28-SCNT, oocyte with TRIM28 depletion from somatic cell nuclear transfer.
Knockdown of TRIM28 interfered with the transcriptome of imprinted genes and TRIM28 complex related genes in the SCNT embryos

To explore whether TRIM28 is required for maintaining genomic imprinting, we evaluated the expression of maternal imprinting genes (IGF2, PLAGL1, DIO3 and DLK1) and paternal imprinting genes (H19 and MEG3) in siRNA-SCNT embryos by qPCR (Fig. 5). Most maternal and paternal imprinted genes were significantly upregulated from the 2-cell to the blastocyst stage in the siRNA-SCNT embryos, while H19 at the 2-cell and blastocyst stages was remarkably decreased (P < 0.05) (Fig. 5A, B, C, D, E, and F).

To understand the effect of maternal TRIM28 downregulation on mRNA expression of the TRIM28 complex during early embryonic development, we used qPCR to detect the expression changes of TRIM28-recruitment complex-associated genes, including DNA methyltransferases (DNMTs) (Fig. 5G, H, and I), histone acetyltransferase and histone deacetylase (HDAC1 and HAT1) (Fig. 5J and K), histone methyltransferases (SETDB1 and SUV39H1) (Fig. 5L and M), and the maternal effectors ZFP57 and PGC7 (Fig. 5N and O), in the siRNA-SCNT embryos. Our results showed that DNMT1 and DNMT3A were highly expressed at the siRNA-TRIM28-SCNT 4-cell to blastocyst stage (P < 0.05) (Fig. 5G and H), while the expression level of DNMT3B from the 2-cell to blastocyst stage was significantly lower than that in control-SCNT embryos (P < 0.01) (Fig. 5I). The expression level of SETDB1 in the 2-cell, 4-cell and blastocyst stages during embryonic development of siRNA-TRIM28-SCNT was significantly higher than that of control-SCNT embryo (P < 0.05), but was significantly lower in the 8-cell stage (Fig. 5M). Compared with that in the control-SCNT embryos, SUV39H1 was highly expressed from 2-cell to 8-cell stage during siRNA-SCNT embryonic development (P < 0.05) (Fig. 5N). The expression level of maternal effector factor PGC7 in siRNA-TRIM28-SCNT 2-cell to blastocyst stage was significantly lower than that of the control-SCNT embryo during the corresponding period (P < 0.01) (Fig. 5O), while ZFP57 was significantly higher in the siRNA-TRIM28-SCNT 4-cell to blastocyst stage (P < 0.05), and the 2-cell stage expression level was significantly lower than that of the control-SCNT embryo (P < 0.01) (Fig. 5P).

Figure 5 Abnormal expression of imprinted genes and TRIM28 complex-related genes in the siRNA-SCNT group. Relative abundance of the imprinting-related genes IGF2 (A), PLAGL1 (B), DIO3 (C) and DLK1 (D), H19 (E) and MEG3 (F) and the TRIM28 complex related genes DNMT1 (G), DNMT3A (H), DNMT3B (I), HDAC1 (J), HAT-1 (K), SETDB1 (L), SUV39H1 (M), ZFP57 (N) and PGC7 (O) in porcine siRNA-SCNT embryos and control-SCNT embryos. Quantities were normalized to GAPDH abundance. Data are presented as the mean ± s.d. *P < 0.05; **P < 0.01 between groups, as indicated.
Knocking down TRIM28 disturbed the dynamic expression of epigenetic chromatin modification enzymes in SCNT embryos

Epigenetic modifications play important roles in guiding and regulating transcriptional programs and reprogramming. To validate the effect of the maternal-mutation of TRIM28 on the transcriptional regulation of preimplantation embryos, we performed an epigenetic analysis of the 84 epigenetic chromatin modification enzymes expression patterns in the siRNA-SCNT embryos. Absolute (abs) value of log fold change (FC) value >1, false discovery rate (FDR) cut-off value <0.05. (C, D) Numbers of differentially expressed genes (DEGs) for all comparisons and heatmap analysis of DEGs in the siRNA-SCNT embryos. (E, F) The enrichment analysis for DEGs and clustered the upregulated and downregulated DEGs in gene ontology categories.

Figure 6 Abnormal expression of epigenetic chromatin modification enzymes in the siRNA-SCNT embryos. (A, B) Cluster analysis and Venn diagram analysis of the 84 epigenetic chromatin modification enzymes expression patterns in the siRNA-SCNT embryos. Absolute (abs) value of log fold change (FC) value >1, false discovery rate (FDR) cut-off value <0.05. (C, D) Numbers of differentially expressed genes (DEGs) for all comparisons and heatmap analysis of DEGs in the siRNA-SCNT embryos. (E, F) The enrichment analysis for DEGs and clustered the upregulated and downregulated DEGs in gene ontology categories.
The Venn diagram presents the numbers of differentially expressed genes (DEGs) for all comparisons between the siRNA-SCNT and control-SCNT embryos (Fig. 6B), and the details of the DEGs were shown in Fig. 6D. The heat maps showed that the number of downregulated transcripts was significantly higher than that of the upregulated transcripts during the 2-cell stage to 8-cell stage in the siRNA-SCNT embryos. Moreover, the expression level of 21 transcripts was at least two-fold higher at the blastocyst stage in the siRNA-SCNT embryos. We performed an enrichment analysis for DEGs and clustered the upregulated and downregulated DEGs in gene ontology categories (Fig. 6E and F). The downregulated DEGs were mainly enriched in covalent chromatin modification, chromosome organization, histone modification and macromolecule modification in the 2-cell, 4-cell and 8-cell embryos (Fig. 6E). Additionally, the upregulated DEGs in blastocyst stage were mainly enriched in covalent chromatin modification, chromosome organization, histone modification and macromolecule modification (Fig. 6F). Then, enrichment analysis of transcription factor (TF) binding motifs in the upstream 300 bp promoters of the DEGs were carried out. There was significant enrichment of TFs in the upregulated DEGs in the 2-cell and 8-cell embryos. There was significant enrichment of TF in downregulation of DEGs of the 2-cell embryo. The downregulated TF at the 2-cell stage was CXXC1 (Fig. 7A). We compared the downregulated of DEGs at the 2-cell stage of the siRNA-SCNT embryos with the control embryos (Fig. 7B). We classified the 25 enzymes and found that they included histone methyltransferases (H3K4 specific) (ASH2L, MLL2, SET6 and SETD7), the histone methyltransferases (H3K9 specific) (STEDB1, histone acetyltransferases (KAT2A, KAT2B, KAT6B and ESCO1), histone deacetylase (HDAC2, HDAC7, HDAC8, HDAC10, and HDAC11), and other modification enzymes. The PPI network of downregulated of DEGs at the 2-cell stage was constructed by STRING (Fig. 7C). There are direct interactions between CXXC1 with ASH2L, KMT2B(MLL2), SETD7, KAT2B and SETDB1(Fig. 7C).

Knocking down TRIM28 caused defects in H3K4me3 in the SCNT embryos

By analyzing the expression of DEGs, we found that H3K4-specific histone methyltransferases and H3K9 specific histone methyltransferases significantly decreased in the 2-cell stage, which might affect the expression levels of H3K4me3 and H3k9me3. To verify this assumption, we assessed the expression levels of TF binding motifs in upstream 300 bp promoters...
H3K4me3 and H3K9me3 using immunofluorescent staining (Fig. 8A, B, C, and D). The signal intensity of H3K4me3 in the siRNA-SCNT embryos was significantly lower than that in its control SCNT counterparts from the 2-cell to the 4-cell stage (Fig. 8B). The signal intensity of H3K9me3 in the siRNA-SCNT embryos was significantly higher than that in its control SCNT counterparts at the 4-cell and blastocyst stages (Fig. 8D).

**Discussion**

TRIM28 is located in the nucleus and can interact with specific regions of chromatin. It is a corepressor of the zinc-finger proteins associated with the Kruppel-associated box (KRAB) domain (Friedman et al. 1996). We now know that TRIM28 is essential for proper and successful epigenetic reprogramming and has also been
identified as a factor required for pluripotency and self-renewal of embryonic stem cells (ESCs) (Leseva et al. 2015, Hu et al. 2009). It has also been reported that maternal TRIM28 not only maintains DNA methylation at the germ imprinting site during the early stage of genome-wide reprogramming, but also regulates DNA methylation at the promoter of imprinted genes after genome-wide reprogramming (Alexander et al. 2015). However, the fundamental functions of TRIM28 in porcine SCNT embryo development and epigenetic reprogramming is still unclear. In this study, we identified TRIM28 as a crucial maternal-effect gene that required for maintaining genomic imprint and epigenetic stability during maternal-to-zygotic transition, and loss of maternal TRIM28 alone resulted in abnormalities of embryonic development and epigenetic reprogramming.

It has been reported that proper levels of site methylation of imprinted genes are essential to embryonic development, or are involved in adult cell and tissue homeostasis (Ferguson-Smith & Bourc’his 2018). Lorthongpanich et al. showed that loss of maternal Trim28 alone can result in dramatically aberrant expression of imprinted genes in midgestation mouse embryos, which resulted in a loss-of-ICR methylation and especially severe ICR demethylation, predominantly at the paternally imprinted H19/IGF2 locus (Messerschmidt et al. 2012, Lorthongpanich et al. 2013). Researchers found that TRIM28 controlled H19/IGF2 and Dlk1/Cgt2 expression by differential DMR methylation and ultimately influenced imprinted gene expression in sheep fibroblast proliferation (Luo et al. 2017). However, the molecular mechanism involved in imprinting maintenance of TRIM28 in porcine SCNT embryos is still poorly understood. In this study, we analyzed the DNA methylation levels of the paternally imprinted gene H19/IGF2. Our results showed that H19 DMR methylation loci were remarkable demethylation at the 4-cell and 8-cell stages. Severe ICR demethylation of IGF2 was observed during the progress of embryo development. We also analyzed the DNA methylation level of euchromatin repeat sequence PRE-1 and the heterochromatin repeat sequence SATELLITE, which are representative repeat elements overlapping the majority of the genome. The DNA methylation level of PRE-1 fluctuated in the 2-cell to 4-cell stage which was consistent with the methylation level of 5mC. The DNA methylation level of the SATELLITE was significantly decreased at the 2-cell and 8-cell stage, which is likely to cause embryonic death. All of these changes would be detrimental to chromatin stability. Our results indicated that TRIM28 is important for the maintenance of genomic imprints at the imprinting gene and the genome gene, which is conducive to the development of porcine SCNT embryos.

Before the zygote gene plays its role, the transcription level of SCNT embryonic genome will remain static, mainly due to the role of maternal factors in oocytes (Schulz & Harrison 2019). However, some mature oocytes cannot initiate normal embryonic development after SCNT probably due to defects in the genome reprogramming process (Svoboda 2018). TRIM28 mainly participates in the development of SCNT embryos by recruiting transcription factors to form transcription complexes (Liu et al. 2017), but the regulatory mechanism is rarely studied in pigs. The structure of TRIM28 in mice and human, with its amino-terminal RING domain, B-box, and coiled-coil (RBCC) domain, central HP1 binding domain and carboxy-terminal PHD-bromodomains, provides it with the means to interact with multiple transcription factors (TFs) and a variety of chromatin remodelers and epigenetic modifiers (Iyengar & Farnham 2011). Reports have shown that TRIM28 recruits ZFP57, HP1, and DNMT1 to form a molecular complex to maintain DNA methylation in proximity to their target sites in embryonic stem cells (Zuo et al. 2012). TRIM28 could induce repressive histone modifications by recruiting multiprotein complexes including SETDB1, the histone deacetylase-containing NuRD complex, and heterochromatin protein 1 (HP1) (Sripathy et al. 2006). We then analyzed the expression of epigenetic chromatin modification enzymes to investigate the effect of TRIM28 knockdown on the scripts of modification enzymes and transcriptional activation in SCNT embryos. Our results showed that the expression levels of these enzymes in TRIM28-deficient SCNT embryos were significantly downregulated before the blastocyst stage. The chromatin modification enzymes had a minor decrease before ZGA, of which 25 enzymes were downregulated at the 2-cell stage and 10 enzymes were downregulated at the 4-cell stage and had a major decrease in the 8-cell stage, of which 48 enzymes were downregulated. These results indicate that maternal TRIM28 knockdown has a stronger transcriptional inhibitory effect on SCNT embryos, and even the paternal Trim28 expression cannot rescue the transcriptional defect at the 8-cell stage. The deletion of maternal TRIM28 blocked the transcript activation of zygotic genes and had an adverse effect on epigenetic reprogramming.

Many enzymes are responsible for adding and removing epigenetic modifications associated with transcriptional active euchromatin or inhibited heterochromatin (Eckersley-Maslin et al. 2018). Schultz et al. found that TRIM28 acts as a scaffolding protein associated with the H3K9me3-writer SETDB1/ ESET, the H3K9me3-reader HP1, and the nucleosome remodeling and histone deacetylation complex NuRD to form the multisubunit, heterochromatin-inducing molecular complex (Schultz et al. 2001, Schultz et al. 2002). The methyltransferase SETDB1 was reported to establish and maintain the repressive epigenetic mark H3K9me2/3 in mouse embryos (Cho et al. 2012). Mouse blastocyst lacking the CXXC finger protein 1(CFP1) gene failed to gastrulate and CXXC1 is required for early development.
mammalian embryogenesis (Carlone and Skalnik 2001). Researchers found that defective H3K4 trimethylation due to Cxxc1 knockout in mice impaired the exchange of histone variants, reduced the genome accessibility, and decreased global transcription activity in oocytes and preimplantation embryos (Yu et al. 2017). Insufficient H3K4 trimethylation by Mll knockout in mouse embryos resulted in arrest at the 2-cell stage (Andreu-Vieyra et al. 2010). Our results showed that a group of chromatin-modifying enzymes were significantly decreased in 2-cell embryos including the H3K4 methyltransferases ASH2L, MLL2, SETD7 and SETD6 and the H3K9me3 methyltransferases STEDB1. However, the TF CXXC1 was identified by enrichment analysis of upstream 300 bp promoters of downregulated DEGs only at the 2-cell stage and other stages did not produce TFs. Moreover, H3K4me3 levels were markedly decreased in the TRIM28-deleted 2-cell stage. However, there were no changes in H3K9me3 levels in the 2-cell stage. These results showed that transient decrease in H3K4me3 levels coincided with the downregulation of CXXC1. These results indicated that TRIM28 knockdown affected the expression of transcription factor CXXC1, which decreased the transcripts of the H3K4 methyltransferases ASH2L and MLL2 and dropped the H3K4me3 levels. Insufficient H3K4me3 accumulation blocked zygote gene activation and impaired imprinting embryonic development. Further work to verify the direct interaction of TRIM28 and CXXC1 is required.

In conclusion, the results indicate that maternal accumulation of TRIM28 is required for maintaining genomic imprints and chromatin stability. TRIM28 participates in maintaining stability of the epigenetic state and the of H3K4me3 level in the process of maternal-to-zygotic transition. Our studies also reveal the long-range effects of maternal TRIM28 on epigenetic memory and highlight the importance of maternal TRIM28 in the developmental efficiency of porcine SCNT embryos.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Ziyi Li conceived the project, supervised the experiments and revised the manuscript. Yanhui Zhai performed most of the experiments, analyzed data and wrote the manuscript. Meng Zhang performed the bioinformatics analyses. Xinglan An, Sheng Zhang, Xiangjie Kong, Qi Li, Hao Yu, Xiangpeng Dai participated in the part of experiments and data analysis. All authors read and approved the final manuscript.

References


Ferguson-Smith AC & Bourc’his D 2018 The discovery and importance of genomic imprinting. eLife 7 e42368. (https://doi.org/10.7554/eLife.42368)


Huang XP, Friedman JR & Farnham PJ 2001 CpG binding protein is crucial for early mouse embryonic development. Gene Expression Patterns 1 286–297. (https://doi.org/10.1016/S1567-1335(01)00018-5)


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