XIST Derepression in Active X Chromosome Hinders Pig Somatic Cell Nuclear Transfer

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SUMMARY

Pig cloning by somatic cell nuclear transfer (SCNT) remains extremely inefficient, and many cloned embryos undergo abnormal development. Here, by profiling transcriptome expression, we observed dysregulated chromosome-wide gene expression in every chromosome and identified a considerable number of genes that are aberrantly expressed in the abnormal cloned embryos. In particular, XIST, a long non-coding RNA gene, showed high ectopic expression in abnormal embryos. We also proved that nullification of the XIST gene in donor cells can normalize aberrant gene expression in cloned embryos and enhance long-term development capacity of the embryo. Furthermore, the increased quality of XIST-deficient embryos was associated with the global H3K9me3 reduction. Injection of H3K9me demethylase Kdm4A into NT embryos could improve the development of pre-implantation stage embryos. However, Kdm4A addition also induced XIST derepression in the active X chromosome and thus was not able to enhance the in vivo long-term developmental capacity of porcine NT embryos.

INTRODUCTION

Cloned pigs have been produced by somatic cell nuclear transfer (SCNT) for many years (Lai et al., 2002; Park et al., 2001). However, the overall cloning efficiency in terms of blastocyst development and the birth of full-term pigs remains extremely low (<0.5%) (Mao et al., 2012; Yuan et al., 2014; Zeng et al., 2016). This low efficiency seriously hinders the application of genetically modified cloned pigs.

In pig cloning, many surrogates can progress to early pregnancy, but only a few pregnancies develop to term. Most implanted SCNT embryos suffer growth retardation and are eventually absorbed by surrogate sows during pregnancy (Huang et al., 2013). Comparison of the transcriptional differences among normal nuclear transfer (NT) fetuses, abnormal NT fetuses and fertilized ones would help provide insights into this phenomenon.

SCNT is a process of reprogramming differentiated cells into totipotent stem cells by oocytes (Meisner and Jaenisch, 2006; Ogura et al., 2013). The abnormal development of SCNT-derived embryos might be attributed to the aberrant reprogramming of the donor genome (Rodriguez-Osorio et al., 2012; Yuan et al., 2014). One SCNT-specific error identified in mouse is the large-scale downregulation of X chromosome-linked genes in the cloned embryos; this downregulation is mainly caused by the ectopic expression of XIST gene, a non-coding RNA and maternally expressed imprinted gene responsible for the X chromosome inactivation (XCI) at the pre-implantation stages of mouse development (Bao et al., 2005; Inoue et al., 2010; Matoba et al., 2011). By contrast, deletion of XIST or repression of XIST expression by specific short interfering RNA (siRNA) from the active X chromosome in the donor genome can elevate about 10-fold normal birth rate of mouse cloning (Inoue et al., 2010; Matoba et al., 2011).

In mouse, many cloned embryos also arrest before implantation stage (Liu et al., 2016). The residual status of repressive histone modifications on specific regions is a reprogramming error in these early-stage embryos (Inoue et al., 2010). The transformation of differentiated donor nuclei to a totipotent state in reconstructed embryos must overcome epigenetic barriers, such as the reduction of H3 lysine 9 methylation (H3K9me), which is the primary epigenetic determinant for the intermediate insufficient pluripotent stem cell state. The removal of such epigenetic...
barriers produces fully reprogrammed pluripotent stem cells (Chen et al., 2013; Chung et al., 2015; Liu et al., 2016; Matoba et al., 2014). In cloned mouse embryos, gene expression abnormalities begin at the two-cell stage, which corresponds to the major wave of zygotic genome activation (ZGA) in normal embryogenesis of the mouse (Matoba et al., 2014; Schultz, 2002). Abnormal gene reactivation in cloned mouse embryos can be partly rescued through H3K9me3 demethylation using histone H3 lysine 9 trimethylation demethylases, including Kdm4b (Liu et al., 2016) or Kdm4d (Matoba et al., 2014).

In the present study, through analysis of the global transcriptome of cloned embryos we found that pig SCNT-specific abnormalities are associated with aberrant $XIST$ expression and persistent H3K9me3 residues. Nullification of the $XIST$ gene could significantly impede $XIST$ expression, which leads to the significant reduction of global H3K9me3 level and improvement of the developmental capacity of NT embryos. We also found that injecting porcine H3K9me3 demethylase $Kdm4A$ could greatly reduce the global H3K9me3 level. However, the injection of $Kdm4A$ into SCNT embryos induced H3K9me3-enriched $XIST$ derepression and resulted in wide-scale gene downregulation, and thus failed to improve the developmental capacity of the reconstructed pig NT embryos.

RESULTS

Global Gene Expression Pattern of Cloned Fetuses

A total of 944 NT embryos were transferred into 6 surrogates. Four of these surrogates were found to be pregnant, as confirmed by ultrasound check 25 days after embryo transfer. The fetuses with gestational periods of 30 and 35 days were collected (Table S1). Many of the fetuses underwent developmental retardation (abnormal), only a few developed normally (Figures 1A and S1A).

To define the transcriptional differences between the normal and abnormal NT embryos, tissues derived from day 30 (n = 5) and day 35 fetuses (n = 5) from the same surrogate sows were used for RNA sequencing (RNA-seq) analysis. The tissue from the in vivo fertilized fetuses at the same day (day 30, n = 2; day 35, n = 2) were used as the controls. The collected fetuses were sex defined and their RNA integrity was validated by agarose gel electrophoresis (Figure S1B). Comparative RNA-seq analysis revealed that the naturally fertilized, normal cloned and abnormal cloned fetuses featured different global transcriptome patterns (Figures 1B and S1C). Compared with the fertilized fetuses, the normal cloned and abnormal cloned fetuses harbored different numbers of up- and downregulated genes (Figure S1D). These genes were distributed across all the 19 chromosomes (Figures 1C and S1E). The number of commonly downregulated genes (CDGs) solely found in day 30 and day 35 normal and abnormal male cloned fetuses were 120 and 805, respectively. For the upregulated genes, a subset of 475 commonly upregulated genes (CUGs) in the normal fetuses failed to be activated in the abnormal cloned fetuses. By contrast, a subset of 267 genes was found commonly to be activated in the abnormal cloned fetuses (Figure S1F). Chromosome distribution analysis revealed that these CDGs and CUGs in both day 30 and day 35 normal and abnormal male fetuses were located not only in X chromosome but also in the other autosomes (Figure S1G). Gene ontology (GO) analysis revealed that in the abnormal fetuses (in both day 30 and day 35 fetuses), these genes (475 CUGs) that failed to be activated were enriched in embryo development pathways, while the upregulated genes (267 CUGs) appeared to be involved in metabolism (Figure 1D).

To determine the master genes correlating with fetus abortion, we further analyzed all of the up- and downregulated genes (p < 0.05, fold change > 2) based on the transcriptomes in all the cloned and fertilized male fetuses on
A diagram of the XIST locus showing the targeting donor and targeted regions with TALEN-L and TALEN-R. The regions are labeled with P5F, P5R, P3F, and P3R. The figure also includes a bar graph showing XIST expression levels with control blastocysts, XIST-KO blastocysts, and pathenogenetic blastocysts.

B A box plot showing the cell number of the blastocyst with different treatments: WT female, WT male, XIST-KO #1, and XIST-KO #2.

C Images of control blastocysts, XIST-KO blastocysts, and pathenogenetic blastocysts.

D A scatter plot showing the log2 TPM of marker1798-1, 1798-2, 1798-3, 1798-4, 1798-5, 1798-6, and 1798-7 with positive and negative controls.

E Images of piglets labeled with markers and gel images showing the log2 TPM of P3F/P3R(2.4K).

F A scatter plot showing the log2 TPM and log2 TPM of P3F/P5R(3.6K) with Wildtype and XIST-KO.

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day 30 and day 35. We found one non-coding RNA XIST, which is required for full-term normal growth and plays important roles in X chromosome-wide gene inactivation in mouse, was expressed at a significantly higher level (among the top 10) in the abnormal cloned fetuses than in the normal and fertilized counterpart (Figure 1E; Table S2). qRT-PCR analysis further confirmed that upregulation of XIST expression only existed in abnormal fetuses (both male and female cloned fetuses) (Figure 1F). Imprinted genes DLK1 and IGF2 also differentially expressed between the groups in day 30 and day 35 fetuses (Figure S1H).

Previous studies demonstrated that XIST is the master regulator of XCI and provokes the stable silencing of the entire X chromosome. XIST would recruit polycomb repressive complex 2 (PRC2) by unknown mechanism and showed linear correlations of PRC2-mediated tri-methylation of lysine 27 of histone H3 (H3K27me3) (Brockdorff, 2017; Cerase et al., 2015; da Rocha and Heard, 2017). We next assessed the whole genome repressive pattern (PRC2 module) by performing principal-component analysis (PCA) and gene set enrichment analysis of microarray data for the expression of polycomb complex target genes. These analyses revealed that, in comparison with fertilized fetuses, the day 30 normal cloned fetuses had already attained high-fidelity transcription of the repressive patterns that were indistinguishable from the day 35 ones but were significantly different from the abnormal cloned fetuses (Figures 1G and S1I). The day 30 and day 35 abnormal cloned fetuses harbored a subset of up- and downregulated genes, which were down- and upregulated in the normal cloned and fertilized fetuses, respectively (Figure S1J). Collectively, the bioinformatics studies revealed that highly ectopic expression of XIST was closely linked to fetus abortion and would greatly hinder pig somatic NT.

**Development Capacity of Reconstructed Embryos Derived from XIST-Null Donor Cells**

The results above showed that the high ectopic expression of the XIST gene may cause developmental retardation in the cloned fetuses. To determine whether the impeded XIST expression in donor cells could improve the developmental capacity of the cloned fetuses, we nullified the XIST gene of both male and female porcine fetal fibroblasts using the TALEN gene-editing system before using them as donor cells. A pair of TALENs targeting the functional repeated region in the first exon of the pig XIST gene (Figures 2A and S2A) was designed. To disrupt XIST expression, an EGFP CDS and puromycin-resistance cassette were inserted downstream the XIST promoter (Figure 2A). In the process, EGFP was used as an indicator of XIST expression nullification in the reconstructed embryos. The correct targeted mutation of selected fibroblast colonies was confirmed by PCR and sequencing with two pairs of primers, which resulted in products with the length of 2,379 and 3,727 bp, respectively (Figures S2B and S2C). Fifteen cell lines (male and female) with correct mutations were achieved (Table S3). The wild-type allele of mutant female clones was untouched, which was confirmed by sequencing (Figure S2D). The NT embryos derived from the mutant cells emitted green fluorescence. This result further validated the vector and the correct mutation in the donor cells (Figure S2E).

Day 6 blastocysts generated from male XIST-deficient cells (n = 2 male clones) also showed barely detectable Xist expression, which was significantly lower than the wild-type male blastocysts (Figure 2B). When the mutant cells (male and female) were used as donors for SCNT, the rate of blastocysts derived from XIST-deficient cells (121/332, 36.4%) was significantly higher than that of blastocysts derived from the control cells (101/398, 25.4%) (p < 0.01,

**Figure 2. XIST Mutant Fibroblast Cells and Their Developmental Capacity**

(A) Schematic of the genomic region containing the pig XIST gene and the targeting homologous recombination vector used for electroporation. In the mutant locus, the genomic sequence was replaced with an EGFP and puromycin expression cassette. Exons are represented as black boxes.

(B) Relative expression of Xist in day 6 in vitro blastocysts (mean ± SD, two-tailed unpaired Student’s t test, n = 3 biological replicates from 3 separate embryo extracts *p < 0.01).

(C) Day 6 blastocyst phenotype from XIST-nullified and wild-type fibroblast cells. Parthenogenetic blastocysts served as control to demonstrate oocyst quality. Scale bar, 500 μm. Representative DAPI staining image of day 6 in vitro embryos from the XIST-nullified donor cell, wild-type cell, and parthenogenetic blastocyst. Scale bar, 500 μm.

(D) Statistical analysis of the total cell number per blastocyst from the XIST-null (n = 26) and wild-type fibroblasts (n = 18), with parthenogenetic blastocysts (n = 25) as the control. Mean ± SEM, two-tailed unpaired Student’s t test, n = 3 biological replicates to collect embryos. *p < 0.05.

(E and F) One of the surrogate sows, 1,798, which was transplanted with 100 male XIST-defective and 100 male wild-type reconstructed embryos, underwent caesarean section on day 40 of gestation. Seven normal fetuses showed XIST-defective mutation, whereas one normal fetus was wild-type. Primer sequences are listed in Table S5. PCR products are 2,379 bp.

(G) One of the surrogate sows, which was transplanted with 246 wild-type and 127 XIST-KO reconstructed NT embryos, gave birth to two piglets. PCR identification showed these piglets generated from XIST-defective embryos.

(H) Scatterplots comparing gene expression levels between XIST-KO and wild-type fetuses. Genes that expressed higher in XIST-KO (FC > 2.0, XIST-KO high) and higher in wild-type fetus (FC > 2.0, wild-type high) are colored red and blue, respectively.
the same 5 surrogates. All 5 surrogates were found pregnant confirmed by ultrasound confirmation 25 days after embryo transfer (Table S3). One of the surrogates transferred 100 male XIST-deficient embryos and 100 wild-type male embryos was killed 40 days after embryo transfer (Figure 2E). Eight male fetuses of normal sizes were retrieved. Genotyping results identified by PCR showed seven out of the 8 fetuses harboring the XIST mutation (Figure 2F). Two of the 4 other surrogates, which were transferred 193 male XIST-deficient embryos and 404 wild-type embryos in total, developed to full-term pregnancies. Six living piglets (male) were also delivered. Four of the piglets were XIST-knockout (XIST-KO) piglets, and two were wild-type piglets, as confirmed by PCR and sequencing (Figure S2G). Furthermore, the average cell number of XIST-deficient embryos (N = 26, mean = 45) was significantly higher than that of the control group embryos (N = 18, mean = 34), and even higher than that of the parthenogenetic (PA) embryos (N = 25, mean = 39), which served as an index of oocyte quality (Figures 2C and D).

To determine whether the cells containing a XIST-deficient Xa (male fibroblasts) hold long-term growth advantages over the control cells, a total of 530 embryos derived from male XIST-deficient embryos and 953 embryos derived from wild-type cells were transferred into the same 5 surrogates. All 5 surrogates were found pregnant confirmed by ultrasound confirmation 25 days after embryo transfer (Table S3). The DNA demethylation of some transcription factors and imprinted genes can reflect the degree of reprogramming completion (Huan et al., 2015; Mao et al., 2015; Zhao et al., 2013). Therefore, we analyzed the DNA methylation statuses of POU5F1, XIST, and IGF2 loci in the porcine female donor cells and a variety of day 6 in vitro culture embryos including NT, PA, and in-vitro-fertilized (IVF) (mix of male and female) embryos. The POU5F1 locus methylation level was relatively higher in the differentiated donor cells (30/140, 21.4%) than in the embryos. The POU5F1 locus DNA methylation level greatly decreased in the embryos derived from XIST-deficient cells (1/140 = 0.7%) compared with parthenogenetic embryos (PEs) (13/140 = 9.3%) and wild-type cell-derived embryos (10/140 = 7.1%). However, the former level was very close to that in the IVF embryos (0%). In the female donor cells, the DNA methylation level in the XIST loci showed completely unmethylated and completely methylated status in the inactive and active allele, respectively. In day 6 in vitro culture embryos, the IVF (mix) and PA embryos had only unmethylated alleles, while control embryos had mostly unmethylated but still had three to four amplicons with elevated methylation, while XIST-KO embryos showed only one significantly methylated amplicon. The IGF2 loci of the donor cells were highly methylated (122/170 = 71.7%). In the PA embryos, the IGF2 loci were also highly methylated (54.7%). The NT embryos exhibited a much lower methylation level than those in the donor cells and PEs. The methylation level of XIST-null cell-derived embryos (48/170 = 28.2%) was also moderately reduced relative to that of the wild-type cell-derived embryos (57/170 = 33.5%), and very close to that of the IVF embryos (48/170 = 28.2%) (Figure 3A).

To explore the potential mechanism of the nullification of XIST gene in improving the development of cloned embryos, the transcriptional differences between early XIST nullification embryos and control embryos in in vitro culture for 6 days were compared by RNA-seq analysis. Comparative RNA-seq analysis revealed that the IVF (n = 2), wild-type cell-derived (n = 2), and XIST-deficient cell-derived embryos (n = 2) also featured different global transcriptome patterns but XIST-deficient cell-derived embryos were closer to IVF embryos (Figures 3B and S3A). Compared with IVF embryos, the wild-type cell-derived embryos had 2,136 commonly differentially dysregulated genes, of which 1,286 were upregulated (CUGs) and 850 were downregulated (CDGs) (Figure S3C). These dysregulated genes were distributed across all the 19 chromosomes (Figure S3D). Comparing with the wild-type cell-derived embryos, XIST-deficient cell-derived embryos had fewer upregulated genes (536 versus 1,255) and downregulated genes (562 versus 1,497) (Figure S3E). And these genes were also distributed across all 19 chromosomes.
### Table

| Condition        | H3K9me3 | DAPI | Merge |
|------------------|---------|------|-------|
| WT-1             |         |      |       |
| WT-2             |         |      |       |
| IVF-1            |         |      |       |
| IVF-2            |         |      |       |
| XIST-KO-1        |         |      |       |
| XIST-KO-2        |         |      |       |

### Figure A

- **POUSF1** (Sus scrofa)
- **XIST** (Sus scrofa)
- **IGF2** (Sus scrofa)

### Figure B

- **WT-1**
- **WT-2**
- **IVF-1**
- **IVF-2**
- **XIST-KO-1**
- **XIST-KO-2**

### Figure C

- **Signal**
- **Disulfide bond**
- **Extracellular exosome**
- **Metabolic pathways**
- **Extracellular space**

### Figure D

- **GADD45G**
- **POUSF1**
- **DPPAS5**
- **CD9**
- **GATA6**
- **KLF4**
- **ZFP42**

### Figure E

- **Suv39h1 Expression**
- **Kdm4a Expression**
- **Kdm4b Expression**
- **SEFDB1 Expression**

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immunostaining of the IVF, PA, control, and by further examining the global H3K9me3 level through corresponding methyltransferases.

Two H3K9me3 demethylases (SUV39h1 and EDTB1) in the early stage of IVF embryos based on published RNA-seq data (Cao et al., 2014). For the two demethylases, Kdm4A expression was not detectable in one- and two-cell-stage embryos, but dramatically increased from four- to eight-cell stage (ZGA phage), and Kdm4B expression remained at a very low level and did not exhibit a large change from one-cell stage to blastocyst stage. For methyltransferases, SUV39h1 was undetectable during the whole pre-implantation stage, and EDTB1 showed a high-expression level in one- to two-cell-stage embryos, but became undetectable after the four-cell stage (Figure 4A). Based on these retrospective analysis data we postulated that demethylases might play a vital role in early pre-implantation of embryos. To verify the hypotheses, synthesized mRNAs of three porcine polyadenylated H3K9me3 demethylases, Kdm4A, Kdm4B, and Kdm4D were injected separately into the reconstructed NT embryos derived from wide-type fetal fibroblasts 5 hr after activation (Figure S4A).

In the initial comparison experiments, we found that global H3K9me3 level reduced by Kdm4A addition was much higher than that of Kdm4B and Kdm4D injection; Kdm4A addition also resulted in a higher blastocyst rate (83/224, 37%, n = 3) than Kdm4B (55/237, 23.2%, n = 3), Kdm4D (27/150, 18%, n = 3), and the control (31/146, 21.2%, n = 3) (Figures S4B and S4C).

We retrospectively analyzed the dynamic change of expression pattern of Kdm4A, Kdm4B, SUV39h1, and EDTB1 in the control and PA embryos, but was close to that in the fertilized embryos.

**Figure 3. Gene Expression Related to Pluripotency and Epigenetics Status in Reconstructed Embryos Derived from XIST-Null Donor Cells**

(A) Methylation statuses of the POU5F1, XIST, and IGF2 loci in the female donor cells and day 6 SCNT embryos. IVF embryos were mixture of female and male embryos. Solid-filled circles represent the methylated cytosine, whereas open circles represent the unmethylated cytosine in each CpG site. The horizontal line represents one individual clone. The left diagram in each figure denotes the genomic location of the target DNA methylation region.

(B) RNA-seq analysis (Spearman correlation coefficient) of the fertilized, XIST-KO and wild-type embryos.

(C) GO analysis of genes inactivated in wild-type embryos while activated in XIST-KO embryos.

(D) Hierarchical cluster analysis of IVF, XIST-deficient and wild-type cell-derived embryos. Yellow and purple represent higher and lower gene expression levels, respectively.

(E) Relative expression levels of H3K9me3 demethylases (Kdm4A/B) and methyltransferases (SUV39h1 and EDTB1) in XIST-KO, wild-type and IVF embryos by qRT-PCR (n = 3 biological replicates, Error bars indicate SEM, *p < 0.05, **p = 0.01, two-tailed unpaired Student’s t test).

(F) Immunofluorescent staining of global H3K9me3 in fertilized embryos, parthenogenetic embryos, wild-type embryos, and XIST-null embryos. Three independent experiments. Scale bars, 100 µm.
Figure 4. The Influence of Addition of Kdm4A on the Quality of NT Embryos

(A) Kdm4A, Kdm4B, Suv39h1, and SETDB1 expression pattern in the early stage of in vivo fertilized embryos.

(B) Kdm4A injection greatly increased blastocyst rate and helped in vitro culture embryos develop to the hatched stage. Scale bar, 500 μm.

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Kdm4A mRNA was injected into 428 embryos at 5 hr after activation, and the remaining 374 embryos served as non-injected controls (Table S4). We followed the early developmental process of these SCNT embryos, and found that Kdm4A addition significantly increased the blastocyst rate (+Kdm4A 129/368, 35% versus control 92/367, 25%, n = 6) (p < 0.01) (Table S4), although it did not affect the development of the two- and four-cell embryo formation (Figure S4C). Addition of Kdm4A also increased in the number of hatched embryos (Figures 4B and 4D) and the cell number per blastocyst after in vitro culture with serum on day 6 (+Kdm4A 110 versus control 53, p < 0.001) in the Kdm4A-injection group (Figures 4C and 4E).

We transferred 654 Kdm4A-injected embryos and 485 control NT embryos into the same 8 surrogates, 4 of them were pregnant, and 3 piglets coming Kdm4A-injected embryos were delivered. The in vivo developmental capacity of the NT embryos after injection of H3K9me3 demethylase Kdm4A was not enhanced (3/654, 0.46%) (Table S4).

We further examined if addition of Kdm4A could influence Xist expression. The fertilized, XIST-KO and wild-type cell-derived embryos were used as control. First, immunostaining revealed the presence of global H3K9me3 in the one-cell-stage embryos, but not in the two-cell-stage and blastocyst-stage embryos in Kdm4A-injected embryos (Figure 4D). Moreover, Kdm4A addition greatly increased Kdm4A while it reduced SUV39h1 expression level of day 6 in vitro blastocysts (Figure 4E). By contrast, Kdm4A, but not Kdm4B or Kdm4D addition, significantly elevated Xist expression level (Figure 4F) in wild-type cell-derived embryos, but not affect that in the embryos derived from the XIST-null donor cells (Figure 4G). Chromatin immunoprecipitation (ChIP)-qPCR further revealed that in the donor fibroblasts, XIST promoter was enriched with H3K9me3 (Figure 4H). Bisulfite sequence analysis showed that the DNA methylation level substantially decreased by Kdm4A addition on the XIST loci (+Kdm4A 10% versus control 34.5%) (Figure 4I), which would lead to Xist upregulation.

We next compared the embryo transcriptome of all groups in genome-wide scale by Spearman correlation analysis and PCA analysis. The results revealed that transcription pattern of Kdm4A injection embryos were similar to XIST-null embryos rather than to IVF embryos and wild-type cell-derived embryos (Figures S4F and S4G). Linear correlation analysis revealed that the correlation factor between Kdm4A addition embryos and IVF embryos was 0.929 (R), very close to that of XIST-KO embryos (R = 0.92) (Figure S4H). Genes up- and downregulated in Kdm4A addition embryos also distributed across all the chromosomes (Figures S4I and S4J). Compared with XIST-KO embryos, the number of specially upregulated genes were decreased in Kdm4A addition embryos (XIST-KO 608 versus Kdm4A 375), while the number of specially downregulated genes in Kdm4A addition embryos was increased, even though it was fewer than in the control embryos (Kdm4A 628 versus XIST-KO 154 versus control 1,091) (Figure S4K). The up- and downregulated genes in Kdm4A addition embryos were also distributed across every chromosome (Figure S4L).

DISCUSSION

In the present study, we systematically analyzed the gene expression profile of porcine pre- and post-implantation embryos and found that fertilized, SCNT embryos featured different global transcriptome patterns. The level of change in the normal embryos was smaller than that in the abnormal ones. There were more CUGs in the normal fetuses than those in abnormal fetuses (475 versus 267 in post-implantation fetuses). GO analysis revealed that the genes that failed to be activated in the abnormal fetuses were enriched in embryo development pathways. By contrast, the CUGs in the abnormal fetuses seemed involved in different functions, such as metabolism. The difference in the total number of CDGs between the abnormal and normal fetuses (805 versus 120 in post-implantation fetuses) appeared much more striking than the number of upregulated genes. These CDGs existed not only in the X chromosome, but also in autosomes.
particular, Xist, a long non-coding RNA gene, showed highly ectopic expression (among the top ten) in abnormal fetuses. Consistently, the PRC2 gene expression profile of retarded embryonic day 35 (E35) and E30 fetuses was similar to each other rather than to normal cloned fetuses and fertilized fetuses on E30 and E35.

Previous studies reported that the ectopic Xist expression specially caused chromosome-wide gene downregulation on the X chromosome in mouse cloned embryos (Matoba et al., 2011). However, in pigs, the overall decrease in the expression of the genes in cloned embryos not only happened on the X chromosome but also on the other autosomes. In the mouse, the ectopic Xist expression in cloned embryos was corrected autonomously after implantation in both embryonic and extraembryonic regions. However, in pig, highly anomalous Xist expression persisted to post-implantation stage in abnormal fetuses. This may be due to a species difference of Xist imprinting pattern. In the mouse, Xist is imprinted at the pre-implantation stages of development, and later is randomly mono-allelically expressed, while, in rabbits and humans, imprinting of Xist was not observed, even at pre-implantation stages (Okamoto et al., 2011). For pigs, a Xist imprinting pattern has as yet not been established but is probably similar to that of rabbits and humans, since an overall decrease in the expression of the genes was found on both the X chromosome and autosomes.

Compared with the IVF-derived embryos, as in the mouse, SCNT embryos exhibited significantly higher XIST transcription levels at the morula stage. The XIST gene is not expressed in the Xa of somatic cells because the XIST gene promoter is enriched with H3K9me3. When the somatic donor cells are injected into oocytes, H3K9me3 loss for chromatin decondensation is a necessary condition for reprogramming differentiated cells into pluripotent ones, inevitably causing derepression of XIST. Therefore, reduction of XIST expression will help the development of cloned embryos. In mouse, both the deletion of XIST in the donor cells (Inoue et al., 2010) (Marahrens et al., 1997) and the anti-XIST siRNA injection at the one-cell-stage embryos (Matoba et al., 2011) could effectively inhibit abnormal XIST expression at the morula stage and markedly increase the mouse cloning efficiency. In the pig SCNT embryos, however, a previous report showed that siRNA-mediated XIST repression only slightly improved the survival rate of cloned pig embryos (Zeng et al., 2016). The silencing effect of anti-XIST siRNA injected at the one-cell stage needed to be maintained for at least 3 days in mouse and 5 days in pig. These durations correspond to the time required for developing from the one-cell stage to the morula stage for the two species. Injected siRNA may degrade before morula formation in the pig (Amarzguioui et al., 2003; Holen et al., 2002; Tuschl, 2002). In the same study, the authors employed several measures to promote the silencing effect of injected anti-XIST siRNA. These measures included increasing the concentration of injected anti-XIST siRNA by 10-fold; using chemically modified anti-XIST siRNA and short hairpin RNA, which were expected to provide a more persistent and stable gene silencing effect than siRNA (Gu et al., 2011); and postponing the injection of anti-XIST agents from the one-cell to the two- or four-cell stage. However, all these methods could still not effectively suppress XIST expression at the morula stage in the cloned pig embryos (Zeng et al., 2016). In addition, Oikawa et al. (2013) found that the RNAi-mediated knockdown of XIST in mouse SCNT embryos does not rescue the impaired development of female cloned mouse embryos (Oikawa et al., 2013). Notably, our qRT-PCR results showed that the abnormal upregulation of XIST in the pig existed, not only in the early stage of the pre-implantation embryos but also in the post-implantation stage. This finding is consistent with previous results on XIST expression in abnormal fetuses from post-implantation and post-natal-stage embryos (liang et al., 2008; Yuan et al., 2014). Thus, contrary to the effect in mouse, the RNAi treatment of oocytes is not applicable for improving SCNT embryo development in pigs.

In the present study, we choose to disrupt the XIST gene function of porcine donor cells by inserting an EGFP CDS and a puromycin-resistance cassette downstream of the XIST promoter. Consequently, the XIST deficiency of donor cells could enhance blastocyst rate and significantly increase average cell number per blastocyst. We also confirmed that the reconstructed embryos from the XIST-nullified cells exhibited a higher in vivo long-term developmental competency than the embryos derived from the wild-type cells. This conclusion is convincing because we attained four piglets plus seven normal fetuses out of 530 XIST-deficient embryos (2.07%) when an equal number of embryos, derived from male XIST-deficient cells and wild-type cells, were transferred into the same surrogates. By contrast, we only achieved two piglets plus one normal fetus out of as many as 953 wild-type cell-derived embryos (0.3%). The cloning efficiency under the use of XIST-deficient cells as donor nuclei was 6.9 times higher than that under the use of wild-type cells. This result is consistent with the finding of similar works on mice (Inoue et al., 2010; Matoba et al., 2011). The XIST-null and normal wild-type fetuses shared highly similar transcriptomes, including the total numbers of up- and downregulated genes and the expression levels of both paternally expressed (DLK1 and IGF2) and maternally expressed (H19) genes. Hence, the normal gene expression of cloned embryos normalized by XIST nullification in the donor cells could persist to the post-implantation stage and result in enhanced cloning efficiency.
The mechanism of the beneficial influence of XIST deficiency on the development of early-stage cloned embryos is poorly understood. Our results showed that the nullification of the XIST gene of donor cells for SCNT could normalize the aberrant expression of many genes in the early stage of cloned embryos. The expression levels of pluripotency-regulating genes, such as GATA4 and ZFP42, were elevated, whereas those of pluripotency repressor genes were downregulated in the XIST-deficient embryos. DNA demethylation levels of some transcription factors and imprinted genes, including POU5F1, XIST, and IGF2, which can reflect reprogramming completion degree, were decreased in the embryos derived from XIST-deficient cells and became more identical to those in IVF embryos. The expression levels of genes related to histone modification in cloned embryos, which are considered to play important roles in embryo development (Diao et al., 2014; Gao et al., 2010; Park et al., 2011; Zhou et al., 2014) and in reprogramming differentiated cells into pluripotent stem cells (Chen et al., 2013; Rais et al., 2013), became similar to those of the fertilized embryos. The H3K9me3 level, which was considered as a main epigenetic barrier of reprogramming (Chen et al., 2013), became lower and more identical to the level of the IVF embryos in the XIST-null embryos than in the control embryos. Among all the H3K9me3 regulators, H3K9me3 demethylase Kdm4A showed most prominently high-expression levels in the XIST-null embryos. More notably, H3K9me3 methyltransferase Suv39h1, which was expressed in somatic cells and wild-type cell-derived cloned embryos, but not in IVF embryos, became undetectable in XIST-null embryos. All the above molecular changes are favorable for the development of cloned embryos.

A previous study on mice demonstrated that the inactivation of histone demethylases accounts for the arrest of cloned embryo development, and the addition of histone demethylases Kdm4b and Kdm5b in one-cell-stage embryos to reduce H3K9me3 and H3K4me3 levels, respectively, could improve both pre- and post-implantation developmental capacity of cloned embryos (Liu et al., 2016). For pigs, we tentatively injected Kdm4A, Kdm4B, and Kdm4D into one-cell-stage cloned embryos and found that Kdm4A was able to improve development of pre-implantation stage embryos. However, the in vivo long-term developmental capacity of the NT embryos after injection of H3K9me3 demethylase Kdm4A was not enhanced. The addition of Kdm4A could result in some positive changes prone to cloned embryo development at the molecular level, such as no H3K9me3 methyltransferase SUV39h1 expression or reduction of global H3K9me3 levels.

Figure 5. A Model Demonstrating the Influence of XIST on Pig SCNT Embryo Development

Many SCNT embryos undergo growth retardation in the post-implantation stage because of incomplete reprogramming. H3K9me3 and DNA methylation are the barriers impeding the reprogramming process. In the early pre-implantation stage of the NT embryo development, H3K9me3 loss for chromatin decondensation seems inevitable. Global H3K9me3 level decreases (as Kdm4A injection) and DNA demethylation in XIST promoter during reprogramming, which causes XIST elevation and thus hinders embryo developmental capacity of the NT embryos. Through XIST gene nullification, the global H3K9me3 level and the DNA methylation level in specific locus are substantially reduced and result in enhanced embryo competency to develop full term.
However, addition of Kdm4A significantly elevated XIST expression, thus hindering the developmental capacity of pig NT embryos.

In summary, our results demonstrated that nullification of the XIST gene in donor cells can normalize aberrant gene expression in cloned embryos and enhance long-term development capacity of embryos, which were mediated by downregulation of H3K9me3 levels (Figure 5). Injection of Kdm4A into NT embryos, on the one hand, can improve oocyte reprogramming with increased blastocyst rate and total cell number per blastocyst. However, addition of Kdm4A significantly elevated XIST expression, which would hinder the developmental capacity of pig NT embryos. Therefore, injecting Kdm4A mRNA into reconstructed SCNT embryos, such as that in mouse, may not applicable for generating genome-modified pig models.

EXPERIMENTAL PROCEDURES

All experimental protocols involving the use of pigs were approved by the Institutional Animal Care and Use Committee at Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences (Animal Welfare Assurance no. A5748-01).

RNA-Seq Data Process

Illumina bcl2fastq (v.1.8.4) was used for base calling. Reads were trimmed for adaptor sequence, and masked for low-complexity or low-quality. Then, the clean reads were mapped to pig genome by RSEM (rsem-1.2.4) (Li and Dewey, 2011), genome files used SGSC Sscrofa10.2/susScr3, gene referenced file came from NCBI. Transcripts per million normalized counts were used for the downstream analysis. PCA, hierarchical cluster, etc., were performed by local Python scripts based on scikit-learn, Matplotlib, Seaborn, etc.

Chromatin Immunoprecipitation-qPCR

The procedure of ChIP was carried out as described previously (Chen et al., 2013). Basically, pig fetal fibroblasts suspended in fibroblast culture medium were crosslinked with 1% formaldehyde for 10 min at room temperature. Then, using 125 mM glycine to quench formaldehyde; subsequently, cells were washed twice with cold PBS. Cells were then lysed in ChIP buffer A for 10 min at 4°C. Samples were centrifuged at 1,400 × g for 5 min at 4°C. Pellets were resuspended in ChIP buffer B for 10 min at 4°C and were sheared by sonication. Sheared chromatin was centrifuged to discard the pellets, and the supernatant was diluted with ChIP IP buffer. Chromatin was incubated with protein A or G beads conjugated to anti-H3K9me3 antibodies (Abcam, ab8898) or rabbit IgG (Abcam, ab37415) overnight at 4°C in ChIP buffer. After immunoprecipitation, beads were washed with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris-EDTA buffer. DNA was extracted and used for analysis. The sequences of all primers used in this study are given in the Supplemental Information (Table S2).

Statistical Analyses

Values are reported as the means ±SEM. The p values were calculated by Student’s t test, p < 0.05 was considered statistically significant. All graphs were plotted with GraphPad Prism software.

ACCESSION NUMBERS

Data from this study are available in GSE107302.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.12.015.

AUTHOR CONTRIBUTIONS

D.R., K.W., X.L., and L.L. designed the study. D.R., J.P., Y.Y., J.Z., F.C., W.G., H.W., Z.Z., Q.J., H.S., J.X., H.S., X.Y.Y., and X.Y. performed the experiments. D.R. and X.W. analyzed the data. Z.O., B.Z., Q.Z., C.L., N.F., and N.L. performed SCNT. Z.L. and Y.Z. performed the embryos transfer experiments. D.R., K.W., and L.L. prepared the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res. 31, 589–595.
Bao, S., Miyoshi, N., Okamoto, I., Jenuwein, T., Heard, E., and Azim Surani, M. (2005). Initiation of epigenetic reprogramming of the X chromosome in somatic nuclei transplanted to a mouse oocyte. EMBO Rep. 6, 748–754.
Brockdorff, N. (2017). Polycomb complexes in X chromosome inactivation. Philos. Trans. R. Soc. Lond. B Biol. Sci. 372.
Cao, S., Han, J., Wu, J., Li, Q., Liu, S., Zhang, W., Pei, Y., Ruan, X., Liu, Z., Wang, X., et al. (2014). Specific gene-regulation networks
during the pre-implantation development of the pig embryo as revealed by deep sequencing. BMC Genomics 15, 4.

Cerase, A., Pintacuda, G., Tattermusch, A., and Avner, P. (2015). Xist localization and function: new insights from multiple levels. Genome Biol. 16, 166.

Chen, J., Liu, H., Liu, J., Qi, J., Wei, B., Yang, J., Liang, H., Chen, Y., Chen, J., Wu, Y., et al. (2013). H3K9 methylation is a barrier during somatic cell reprogramming into iPSCs. Nat. Genet. 45, 34–42.

Chung, Y.G., Matoba, S., Liu, Y., Eum, J.H., Lu, F., Jiang, W., Lee, J.E., Sepiliani, V., Cha, K.Y., Lee, D.R., et al. (2015). Histone demethylation expression enhances human somatic cell nuclear transfer efficiency and promotes derivation of pluripotent stem cells. Cell Stem Cell 17, 758–766.

da Rocha, S.T., and Heard, E. (2017). Novel players in X inactivation: insights into Xist-mediated gene silencing and chromosome conformation. Nat. Struct. Mol. Biol. 24, 197–204.

Diao, Y.F., Oqani, R.K., Li, X.X., Lin, T., Kang, J.W., and Jin, D.I. (2014). Changes in histone H3 lysine 36 methylation in porcine oocytes and preimplantation embryos. PLoS One 9, e100205.

Gao, Y., Hyttel, P., and Hall, V.J. (2010). Regulation of H3K27me3 and H3K4me3 during early porcine embryonic development. Mol. Reprod. Dev. 77, 540–549.

Gu, S., Jin, L., Zhang, F., Huang, Y., Grimm, D., Rossi, J.J., and Kay, M.A. (2011). Thermodynamic stability of small hairpin RNAs highly influences the loading process of different mammalian Argonautes. Nat. Struct. Mol. Biol. 18, 9209–9213.

Holen, T., Amarigouliou, M., Wiiger, M.T., Babaie, E., and Prydz, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. 30, 1757–1766.

Huan, Y., Zhu, J., Huang, B., Mu, Y., Kong, Q., and Liu, Z. (2015). Trichostatin A rescues the disrupted imprinting induced by somatic cell nuclear transfer in pigs. PLoS One 10, e0126607.

Huang, Y., Ouyang, H., Yu, H., Lai, L., Pang, D., and Li, Z. (2013). Efficiency of porcine somatic cell nuclear transfer – a retrospective study of factors related to embryo recipient and embryos transferred. Biol. Open 2, 1223–1228.

Inoue, K., Kohda, T., Sugimoto, M., Sado, T., Ogonuki, N., Matoba, S., Shiura, H., Ikeda, R., Mochida, K., Fuji, T., et al. (2010). Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. Science 330, 496–499.

Jiang, L., Lai, L., Samuel, M., Prather, R.S., Yang, X., and Tian, X.C. (2008). Expression of X-linked genes in deceased neonates and surviving cloned female piglets. Mol. Reprod. Dev. 75, 265–273.

Lai, L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., Samuel, M., Bonk, A., Rieke, A., Day, B.N., et al. (2002). Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295, 1089–1092.

Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. BMC Bioinformatics 12, 323.

Liu, W., Liu, X., Wang, C., Gao, Y., Gao, R., Kou, X., Zhao, Y., Li, J., Wu, Y., Xiu, W., et al. (2016). Identification of key factors conquering developmental arrest of somatic cell cloned embryos by combining embryo biopsy and single-cell sequencing. Cell Discov. 2, 16010.

Mao, J., Tessanne, K., Whitworth, K.M., Spate, L.D., Walters, E.M., Samuel, M.S., Murphy, C.N., Tracy, L., Zhao, J., and Prather, R.S. (2012). Effects of combined treatment of MG132 and scriptaid on early and term development of porcine somatic cell nuclear transfer embryos. Cell Reprogram. 14, 385–389.

Mao, J., Zhao, M.T., Whitworth, K.M., Spate, L.D., Walters, E.M., O’Gorman, C., Lee, K., Samuel, M.S., Murphy, C.N., Wells, K., et al. (2015). Oxamflatin treatment enhances cloned porcine embryonic development and nuclear reprogramming. Cell Reprogram. 17, 28–40.

Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997). Xist-deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev. 11, 156–166.

Matoba, S., Inoue, K., Kohda, T., Sugimoto, M., Mizutani, E., Ogou-nuki, N., Nakamura, T., Abe, K., Nakano, T., Ishino, E., et al. (2011). RNAi-mediated knockdown of Xist can rescue the impaired post-implantation development of cloned mouse embryos. Proc. Natl. Acad. Sci. USA 108, 20621–20626.

Matoba, S., Liu, Y., Lu, F., Iwabuchi, K.A., Shen, L., Inoue, A., and Zhang, Y. (2014). Embryonic development following somatic cell nuclear transfer impeded by persisting histone methylation. Cell 159, 884–895.

Meissner, A., and Jaenisch, R. (2006). Mammalian nuclear transfer. Dev. Dyn. 235, 2460–2469.

Ogura, A., Inoue, K., and Wakayama, T. (2013). Recent advances in cloning by somatic cell nuclear transfer. Philos. Trans. R. Soc. Lond. B Biol. Sci. 368, 20110329.

Oikawa, M., Matoba, S., Inoue, K., Kamimura, S., Hirose, M., Ogou-nuki, N., Shiura, H., Sugimoto, M., Abe, K., Ishino, E., et al. (2013). RNAi-mediated knockdown of Xist does not rescue the impaired development of female cloned mouse embryos. J. Reprod. Dev. 59, 231–237.

Okamoto, I., Patrat, C., Thepot, D., Peynot, N., Fauque, P., Daniel, N., Diabangouaya, P., Wolf, J.P., Renard, J.P., Duranthon, V., et al. (2011). Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development. Nature 472, 370–374.

Park, K.E., Johnson, C.M., Wang, X., and Cabot, R.A. (2011). Differential developmental requirements for individual histone H3K9 methyltransferases in cleavage-stage porcine embryos. Reprod. Fertil. Dev. 23, 551–560.

Park, K.W., Cheong, H.T., Lai, L., Im, G.S., Kuhholzer, B., Bonk, A., Samuel, M., Rieke, A., Day, B.N., Murphy, C.N., et al. (2001). Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim. Biotechnol. 12, 173–181.

Rais, Y., Zviran, A., Geula, S., Gafni, O., Chomsky, E., Viiukov, S., Mansour, A.A., Caspi, I., Kupalnik, V., Zerbib, M., et al. (2013). Deterministic direct reprogramming of somatic cells to pluripotency. Nature 502, 65–70.

Rodriguez-Osorio, N., Urrego, R., Cibelli, J.B., Ellertsen, K., and Memili, E. (2012). Reprogramming mammalian somatic cells. Theriogenology 78, 1869–1886.
Schultz, R.M. (2002). The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum. Reprod. Update 8, 323–331.

Tuschl, T. (2002). Expanding small RNA interference. Nat. Biotechnol. 20, 446–448.

Yuan, L., Wang, A., Yao, C., Huang, Y., Duan, F., Lv, Q., Wang, D., Ouyang, H., Li, Z., and Lai, L. (2014). Aberrant expression of Xist in aborted porcine fetuses derived from somatic cell nuclear transfer embryos. Int. J. Mol. Sci. 15, 21631–21643.

Zeng, F., Huang, Z., Yuan, Y., Shi, J., Cai, G., Liu, D., Wu, Z., and Li, Z. (2016). Effects of RNAi-mediated knockdown of Xist on the developmental efficiency of cloned male porcine embryos. J. Reprod. Dev. 62, 591–597.

Zhao, M.T., Rivera, R.M., and Prather, R.S. (2013). Locus-specific DNA methylation reprogramming during early porcine embryogenesis. Biol. Reprod. 88, 48.

Zhou, N., Cao, Z., Wu, R., Liu, X., Tao, J., Chen, Z., Song, D., Han, F., Li, Y., Fang, F., et al. (2014). Dynamic changes of histone H3 lysine 27 acetylation in pre-implantational pig embryos derived from somatic cell nuclear transfer. Anim. Reprod. Sci. 148, 153–163.