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ORIGINAL RESEARCH

Histone demethylase KDM4A overexpression improved the efficiency of corrected human tripronuclear zygote development

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ABSTRACT: Human zygotes are difficult to obtain for research because of limited resources and ethical debates. Corrected human tripronuclear (ch3PN) zygotes obtained by removal of the extra pronucleus from abnormally fertilized tripronuclear (3PN) zygotes are considered an alternative resource for basic scientific research. In the present study, eight-cell and blastocyst formation efficiency were significantly lower in both 3PN and ch3PN embryos than in normal fertilized (2PN) embryos, while histone H3 lysine 9 trimethylation (H3K9me3) levels were much higher. It was speculated that the aberrant H3K9me3 level detected in ch3PN embryos may be related to low developmental competence. Microinjection of 1000 ng/μl lysine-specific demethylase 4A (KDM4A) mRNA effectively reduced the H3K9me3 level and significantly increased the developmental competence of ch3PN embryos. The quality of ch3PN zygotes improved as the grading criteria, cell number and pluripotent expression significantly increased in response to KDM4A mRNA injection. Developmental genes related to zygotic genome activation (ZGA) were also upregulated. These results indicate that KDM4A activates the transcription of the ZGA program by enhancing the expression of related genes, promoting epigenetic modifications and regulating the developmental potential of ch3PN embryos. The present study will facilitate future studies of ch3PN embryos and could provide additional options for infertile couples.

Key words: KDM4A / corrected human tripronuclear (ch3PN) / H3K9me3 / zygotic genome activation (ZGA) / embryo development

Introduction

Viable human embryos are difficult to obtain for basic scientific research owing to limited sources and ethical constraints (Ehrich *et al.*, 2011). Tripronuclear (3PN) zygotes occur at a rate of 2–5% after conventional IVF (Pieters *et al.*, 1992; Porter *et al.*, 2003; Rosenbusch, 2009). These zygotes commonly form because two sperm enter one oocyte, which could eventually end in abortion, hydatidiform mole and polyploid teratismus; these zygotes are considered unsuitable for clinical transfer and are discarded in clinics (Plachot and Crozet, 1992). Therefore, 3PN zygotes can serve as an alternative source for understanding the mechanisms of human embryo development and analysis of normal euploid human embryonic stem cell lines. Several studies have attempted to restore the diploid status of 3PN zygotes by microsurgical removal of the extra pronucleus (Rawlins *et al.*, 1988; Gordon *et al.*, 1989; Malter and Cohen, 1989; Palermo *et al.*, 1994; Ivakhnenko *et al.*, 2000; Escriba *et al.*, 2006; Gu *et al.*, 2009). The birth of a healthy boy after transferring three embryos that developed from microsurgically corrected human 3PN (ch3PN) zygotes has also been reported (Kattera and Chen, 2003). These results demonstrate that a ch3PN zygote can be reliably corrected by removing the specific extra pronucleus followed by development into a genetically normal embryo. However, ch3PN embryos reaching the blastocyst stage are not

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comparable to normal fertilized embryos; thus, the use of ch3PN zygotes is limited (Chen et al., 2010; Fan et al., 2013; Fan et al., 2014). If more ch3PN embryos can reach the blastocyst stage, the utilization efficiency of ch3PN embryos that had been destined to be discarded would be improved, with favorable ethical implications. However, few efforts have been made to improve the developmental capacity of ch3PN embryos. Only one study has shown that adding the growth factor combination epidermal growth factor (Egf), brain-derived neurotrophic factor (Bdnf) and insulin-like growth factor-1 (Igf-1) to the culture system improved the development efficiency of ch3PN embryos (Fan et al., 2014), but the mechanism(s) underlying the obstacle of ch3PN development remains unknown.

Histone modification is a major factor contributing to the successful development of fertilized embryos, which can affect zygotic genome activation (ZGA) and transcription factor binding ability to ultimately affect gene expression (Swygert and Peterson, 2014; Benveniste et al., 2014). Among these modifications, histone H3 lysine 9 trimethylation (H3K9me3) is associated with heterochromatin and gene transcriptional repression during embryonic fate change (Becker et al., 2016), whereas histone H3 lysine 4 trimethylation (H3K4me3) marks embryonic genome activation and blastocyst cell lineage segregation (Ruthenburg et al., 2007). Lysine-specific demethylase 4A (Kdm4A) demethylates H3K9me3 at promoters marked by H3K4me3 and is crucial for normal preimplantation development and ZGA after fertilization (Sankar et al., 2020). The loss of Kdm4A causes aberrant H3K9me3 that spreads across broad domains of H3K4me3, resulting in insufficient transcriptional activation of genes during ZGA (Sankar et al., 2020). Overexpression of human KDM4A reduces the high levels of H3K9me3 enriched in the reprogramming resistance region (RRR), facilitates ZGA and consequently improves the development of embryos such that they reach the blastocyst stage, as has been shown for human somatic cell nuclear transfer (SCNT) embryos (Chung et al., 2015). Similarly, KDM4D can correct high H3K9me3 levels and improve the developmental capacity of mouse and macaque cloned embryos (Matoba et al., 2014; Liu et al., 2016; Liu et al., 2018). Although KDM4A has been applied to the study of embryo development in different species and has resulted in obvious effects, its function in the embryonic development of ch3PN has not been reported. Therefore, in the present study, we investigated the effects of KDM4A mRNA injection on the developmental competence and embryo quality of ch3PN embryos. Moreover, the associated histone modifications and expression of related genes activated by ZGA were evaluated in these zygotes to explore the potential mechanism of action of KDM4A in injected ch3PN embryos.

Materials and Methods

Ethics statement

The present study was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University and the Peking University Third Hospital (Approval ID: 2019000105). Patients in this study knew and understood that fertilized zygotes would be used and would be voluntarily discarded after signing an informed consent form.

Reagents and chemicals

Unless otherwise noted, all chemicals and reagents used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Collection of 3PN embryos and removal of excess prokaryotes by micromanipulation

Zygotes were from the infertile couples who underwent IVF-embryo transfer (IVF-ET) in our reproductive center between June 2018 and October 2020. The patients were treated with IVF owing to the presence of female oviduct factors. In total, 172 polyspermic zygotes were collected from 38 patients in this study. Fertilization was observed 16–18 h after insemination under a thermostatic inverted microscope. Zygotes with three pronuclei, two polar bodies, regular morphology and a complete zona pellucida were selected. The 3PN zygotes were transferred to a small (30 µl) drop of modified HTF medium (Irvine Scientific, Santa Ana, CA, USA) containing 7.5 µg/ml cytochalasin B (C6762, Sigma). The male pronucleus was excised; the additional male pronucleus was identified according to the previously established standard (Kattera and Chen, 2003). The criteria for identifying male pronuclei included the following: the presence of a prokaryotic sperm tail, a pronucleus size larger than that of the female pronucleus and a greater distance (relative to the female pronuclei) from the second polar body. For the reconstructed diploid fertilized egg, a pronucleus located farther away from the polar body was clearly identified through the eyepiece. With the help of a piezoelectric device (P150, Prime Tech, Japan), a holding pipette and a needle with an internal diameter of 10–12 µm were used to make a hole in the transparent membrane, and the needle was used to remove the pronucleus from the oocyte. ch3PN zygotes were then transferred to G1.5 media (Vitrolife, Gothenburg, Sweden).

mRNA microinjection operation and embryo culture

The transcription of KDM4A was performed according to the instructions of the HiScribe^{TM} T7 Quick High Yield RNA Synthesis Kit (NEB, E2050S). Then, the mRNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. After confirming the integrity of the mRNA by performing electrophoresis, aliquots were stored at −80°C until use. KDM4A mRNA was diluted with RNA-free water to 200 or 1000 ng/µl, and RNA-free water was injected into the embryos of the ch3PN group for control. The mixture was thoroughly mixed and centrifuged at 12 000g for 1 min. Ch3PN fertilized eggs were transferred to microinjection dishes and prepared as droplets for RNA microinjection. RNA was injected into the cytoplasm at ∼1–2 pl per embryo. After injection, the embryos were washed three times with G1.5 embryo culture solution. The first cleavage of the zygote was observed at 27–28 h after fertilization, and the development of embryos was observed at 48 h (D2), 72 h (D3)

and 120 h (D5); if the number of dividing cells was ≥2, then they were defined as early-cleavage embryos. For D3, the embryos were transferred to balanced G2.5 media (Vitrolife) and incubated overnight for further culture. All embryos were maintained in a humidified incubator at 37°C, 5% CO_2 and 5% O_2.

Embryo grading

The ch3PN embryos were graded according to previously established criteria for evaluating eight-cell embryos and blastocysts (Dokras *et al.*, 1993; Xia, 1997). Embryo grading criteria were as follows: for 8G1, there was a uniform blastomere with intact zona pellucida and no fragments; 8G2 had a slightly nonuniform blastomere and <5% fragments; 8G3: blastomere was as for 8G2 with intact zona pellucida and ranged from 5% to 20% fragments; 8G4: blastomere being viable and >50% fragments. The blastocyst grade was as follows: on D5, if the blastocyst cavity was large, the inner cell mass (ICM) cells were closely arranged, and the number of cells was large, then they were BG1; when the volume of the blastocyst cavity expanded to 50–80% and when the ICM had a small amount of cells that loosely accumulated on D5, then they were classified as BG2; and if the blastocyst cavity accounted for <50% of the volume and the ICM had almost no cells on D5, then they were classified as BG3.

Immunofluorescence staining

The embryos were washed three times in PBS-polyvinyl alcohol (1%, wt/vol), fixed with PBS-paraformaldehyde (4%, wt/vol) for 45 min and then permeabilized with PBS/1% Triton X-100 at 37°C for 30 min. The permeabilized embryos were blocked by incubation in PBS/2% bovine serum albumin (wt/vol) for 1 h. Thereafter, the embryos were incubated with a rabbit polyclonal primary antibody against recombinant octamer-binding transcription factor 4 (OCT4) (1:500; sc-5279, Santa Cruz Biotechnology, Inc.), caudal-related homeobox transcription factor 2 (CDX2) (1:200; ab76541, Abcam, Inc.), GATA-binding protein 6 (GATA6) (1:200; #5851, Abcam, Inc.), H3K9me3 (1:200; ab8580, Abcam, Inc.) or H3K4me3 (1; 200; ab8898, Abcam, Inc.) overnight at 4°C. An anti-rabbit IgG fragment (1:500; #8899, #4412, Cell Signaling Technology, Inc.) or anti-mouse IgG fragment (1:500; #8890, #4408, Cell Signaling Technology, Inc.) was then incubated together with the embryos for 1 h at room temperature. After washing three times in PBS, the DNA was counterstained with 10 μg/ml DAPI for 10 min.

The stained embryos were then mounted under a coverslip together with antifading mounting media (P36982, Thermo Fisher, Inc.) to delay photobleaching. The experiment was repeated at least three times, with two or three embryos analyzed per experiment. A 488 nm fluorescein isothiocyanate was used to visualize signals with a fluorescence microscope (Nikon, Tokyo, Japan). Nikon NIS element software (Nikon, Tokyo, Japan) was used to capture and quantify the images.

Quantitative real-time PCR

Total RNA was isolated from ch3PN embryos injected with or without KDM4A (10 embryos per sample) using a Dynabeads mRNA Direct Kit (Life Technologies AS, Oslo, Norway) according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription of mRNA using an oligo (dT) 12–18 primer and a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Real-time PCR amplification was conducted with a QuantiTect SYBR Green PCR kit (Finnzymes, Espoo, Finland) on a RotorGene 2000 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Each real-time PCR mixture consisted of 1 μl of cDNA, 10 μl of SYBR, 0.5 μl of ROX, 7.5 μl of nuclease-free water and 0.5 μl of the appropriate forward and reverse primers (Table I), for a total volume of 20 μl. All tests were conducted in triplicate. Relative gene expression data were analyzed using quantitative real-time PCR and the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Each experiment was repeated more than three times. The data are expressed as the mean ± SEM. Univariate ANOVA was used for data analysis, and SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) statistical software was used for Duncan's multiple range test. The average fluorescence intensity of each nucleus was quantified using Nikon NIS element software. $P < 0.05$ was considered statistically significant.

Results

Removal of one extra pronucleus cannot rescue the developmental competence of 3PN zygotes

ch3PN embryos were derived from 3PN embryos by microsurgical removal of one extra pronucleus (Fig. 1A). The evaluation of developmental competence showed that the cleavage efficiency at the two-cell stage did not differ between two pronuclei (2PN, $n = 24$) IVF human zygotes and the 3PN ($n = 39$) and ch3PN ($n = 44$) embryos (85.37 ± 11.01% vs. 80.06 ± 12.87% and 82.21 ± 9.77%, respectively, $P > 0.05$). However, abnormal cleavage began at the eight-cell stage, which corresponded to the major wave of ZGA in normal embryogenesis in humans (Jukam *et al.*, 2017). Obviously, the 3PN and ch3PN embryos in the eight-cell stage had more fragments, and most of the blastomeres showed significant degeneration before forming blastocysts (Fig. 1B). The eight-cell (44.49 ± 12.79% vs. 41.39 ± 5.67% and 82.48 ± 10.68%, respectively, $P < 0.05$) and blastocyst (58.78 ± 8.93% vs. 6.22 ± 6.02% and 5.58 ± 3.65%, respectively, $P < 0.05$) formation efficiencies were significantly lower in both the 3PN and ch3PN embryos than in the 2PN group (Fig. 1C). This demonstrated that enucleation of one pronucleus from 3PN is essential but not entirely sufficient for overcoming the developmental block of 3PN embryos.

H3K9me3 and H3K4me3 were present at high levels in ch3PN embryos

The global H3K9me3 and H3K4me3 levels were determined in 2PN and ch3PN embryos. At the eight-cell stage, both the H3K9me3 and H3K4me3 levels of the ch3PN embryos were significantly higher

Table I List of primers used for real-time PCR in this study.

Gene name	Primer sequences (5′–3′)	Accession number
Developmental pluripotency associated 2 (*Dppa2*)	F: TGAGGTTCGTGTTTGTGCCTGC R: CCATCAAGTGGTTTTTTCTTTTGC	XM_030245923.1
Developmental pluripotency associated 4 (*Dppa4*)	F: TTTGCTTCAGGGTTTCATCC R: ATCCTCTGCAGCTCCATGTT	XM_011250780.3
Myc	F: AAAGGCTGGAACCCTTGTTT R: GCACCTTGCCTTCAATGAGT	XM_030243266.1
Zinc finger and SCAN domain-containing 4 (*Zscan4*)	F: TCGCTACCGTCGTGACTTCGC R: GCATCCCAGCCTCCGTTATCC	NM_009741.5
Upstream-binding transcription factor like 1 (*Ubtfl1*)	F: CACTCTCAGGAGAGCATTCCA R: CCCAGCATTTCCAGTCTTTG	XM_021185540.2
THO complex 5 (*Thoc5*)	F: CCAGGAGAATGGCAAGAATGAA R: AGGAAGGTAAAGAGCGGGTGAG	XM_021207183.2
Paired pox 5 (*Pax5*)	F: CCAGCCTTTCTACCCAATGT R: ATAGGGCGTCCTTTAGCAGA	XM_029478250.1
Growth factor receptor-bound protein 2 (*Grb2*)	F: AAGCCAAGCTCTGTTCAGCAA R: GAAGGCAATCGTTCTTCTCACT	XM_017312617.2
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F: CAGGTTGTCTCCTGCGACTTCA R: GTGGGTGGTCCAGGGTTTCTTA	NM_001289726.1

F, forward primer; R, reverse primer.

than those in the 2PN embryos (Figs. 2A and B and 3A and B). At the blastocyst stage, there was a significant increase in the fluorescence intensity of H3K9me3 in ch3PN compared to 2PN embryos (Fig. 2C and D), while no statistically significant differences were found in the average fluorescence intensity of H3K4me3 between the 2PN and ch3PN groups (Fig. 3C and D).

Human KDM4A mRNA injection improved the developmental competence of ch3PN embryos

ch3PN human embryos were injected with 200 ($n = 41$) or 1000 ($n = 46$) ng/μl KDM4A (ch3PN-K group) or with RNA-free water (ch3PN group, $n = 44$). There were no significant differences in the cleavage rate between the 200 and 1000 ng/μl ch3PN-K and the ch3PN groups (80.00 ± 16.21% and 81.28 ± 7.58% vs. 82.21 ± 9.77%, respectively, $P > 0.05$). The percentage of embryos developing to the eight-cell stage was higher in the 1000 ng/μl ch3PN-K group than in the 200 ng/μl ch3PN-K or ch3PN group (69.28 ± 5.02% vs. 46.97 ± 6.08% and 41.39 ± 5.67%, respectively, $P < 0.05$). The proportion of embryos that developed to the blastocyst stage was significantly higher in the 1000 ng/μl ch3PN-K group than in the 200 ng/μl ch3PN-K and ch3PN groups (18.16 ± 6.08% vs. 6.29 ± 3.90% and 5.58 ± 3.65%, respectively, $P < 0.05$). However, the development capacity of the ch3PN-K embryos still did not reach the efficiency of the 2PN embryos (Fig. 1C).

KDM4A mRNA injection significantly improved the status of histone H3K9me3 but not H3K4me3 in ch3PN human embryos

To provide a mechanism for the improved developmental capacity of ch3PN human embryos after KDM4A mRNA injection, the levels of H3K9me3 and H3K4me3 were measured. At the eight-cell stage, the fluorescence intensity of H3K9me3 staining was greatly reduced in the embryos injected with KDM4A, which was comparable to that of the 2PN embryos (Fig. 2A and B). At the blastocyst stage, although the fluorescence intensity of H3K9me3 staining in the ch3PN-K group was notably lower than that in the ch3PN group, it was still higher than that in the 2PN blastocytes (Fig. 2C and D). For the other epigenetic marker (H3K4me3), the level of H3K4me3 was statistically lower in the 2PN embryos than in the ch3PN embryos and the level of H3K4me3 was slightly lower in the ch3PN-K embryos than in the ch3PN embryos at the eight-cell stage (Fig. 3A and B), but it did not significantly differ between the 2PN, ch3PN and ch3PN-K groups at the blastocyst stage (Fig. 3C and D).

Overexpression of KDM4A enhanced the quality and developmental pluripotency of human ch3PN embryos

To verify the effects of KDM4A mRNA injection on the quality of ch3PN embryos, the embryos at the eight-cell stage on D3 and at the blastocyst stage on D5 were evaluated using established clinical criteria (Dokras *et al.*, 1993; Xia, 1997). The total cell number of the

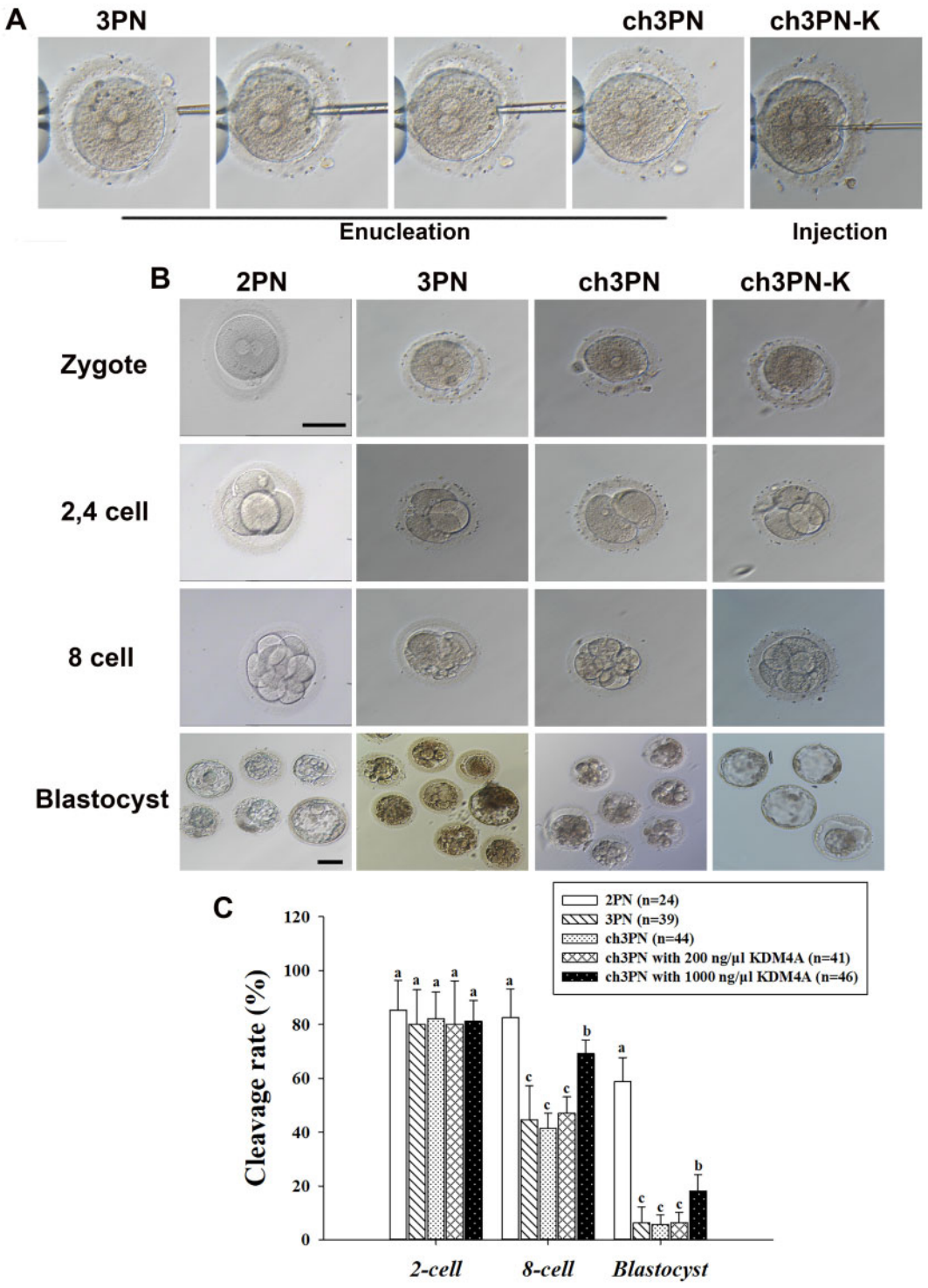

Figure 1. Developmental competence of 2PN, 3PN, ch3PN and ch3PN-K embryos. (**A**) ch3PN derivation from the removal of one pronucleus from a 3PN zygote and KDM4A mRNA injection. 200× magnification. (**B**) Developmental process of 2PN, 3PN, ch3PN and ch3PN-K embryos. Scale bar = 100 μm. (**C**) Developmental efficiency at the eight-cell and blastocyst stages of 2PN, 3PN, ch3PN and ch3PN-K embryos. Each experiment was repeated at least three times with 24–46 oocytes. The data are expressed as the mean ± SEM. Univariate ANOVA was used for data analysis, and SPSS 16.0 statistical software was used for Duncan's multiple range test. The values indicated by different letters are significantly different ($P < 0.05$). 2PN, two pronuclei IVF human zygote; 3PN, tripronuclear zygotes; ch3PN, removal of one pronucleus from a 3PN zygote; ch3PN-K, ch3PN injection with KDM4A; KDM4A, lysine-specific demethylase 4A.

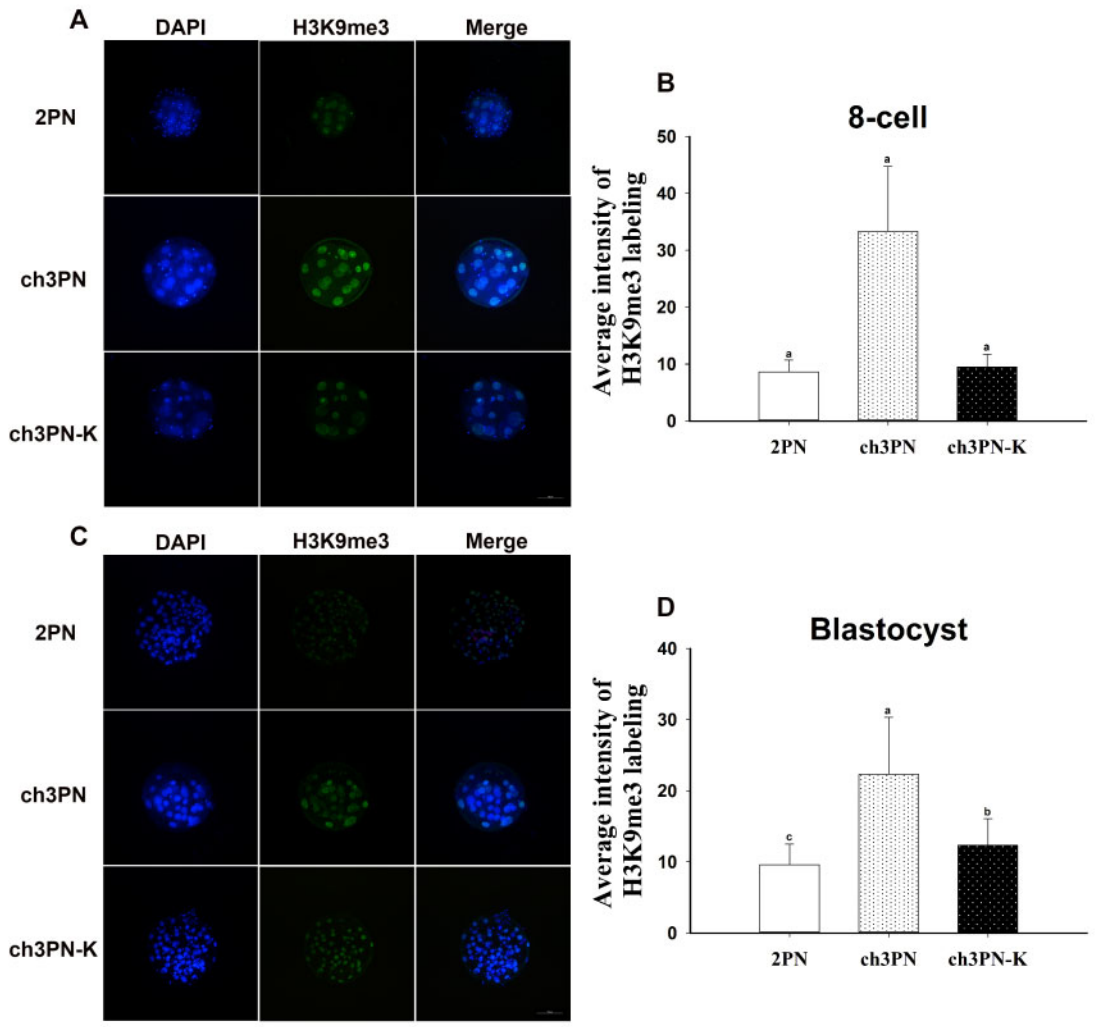

Figure 2. Global H3K9me3 levels in eight-cell and blastocyst embryos. (**A**) Histone H3 lysine 9 trimethylation (H3K9me3) staining (green) in 2PN, ch3PN and ch3PN-K embryos at the eight-cell stage. (**B**) Proportion of fluorescence intensity of H3K9me3 staining in 2PN, ch3PN and ch3PN-K embryos at the eight-cell stages. (**C**) H3K9me3 staining (green) in 2PN, ch3PN and ch3PN-K embryos at blastocyst stage. (**D**) Proportion of fluorescence intensity of H3K9me3 staining in 2PN, ch3PN and ch3PN-K embryos at blastocyst stages. Each sample was counterstained with Hoechst 33342 to visualize the DNA (blue). Scale bar = 50 μm. Each experiment was repeated at least three times with at least 24 oocytes. The data are expressed as the mean ± SEM. Univariate ANOVA was used for data analysis, and SPSS 16.0 statistical software was used for Duncan's multiple range test. The values with different superscripts within groups are significantly different ($P < 0.05$). The experiments were repeated three times, with $n = 2$–3 per group.

blastocysts was also evaluated. For eight-cell stage embryos, the proportion of 8G1 embryos increased in the ch3PN-K group (Fig. 4A) ($P < 0.05$), but the proportion of 8G2 embryos did not increase in the ch3PN-K group compared with the ch3PN group ($P > 0.05$). The blastocyst grades improved following injection of KDM4A, and the number of high-grade blastocysts (BG1 and BG2) increased in the ch3PN-K group ($P < 0.05$, Fig. 4B). The blastocyst cell number was measured in the ch3PN-K and ch3PN groups on D6 after fertilization: the results showed that the total cell number significantly increased in the ch3PN-K group compared with the ch3PN group ($P < 0.05$, Fig. 4E).

To determine the effect of KDM4A overexpression on the developmental pluripotency of ch3PN blastocysts, immunofluorescence staining was performed for the ICM-specific marker molecules *Oct4* and *Gata6* and for the trophectoderm (TE) marker molecule *Cdx2* at the blastocyst stage. The fluorescence intensity of OCT4 and CDX2 staining was significantly higher in ch3PN-K embryos than in ch3PN embryos, indicating that KDM4A mRNA injection enhanced the

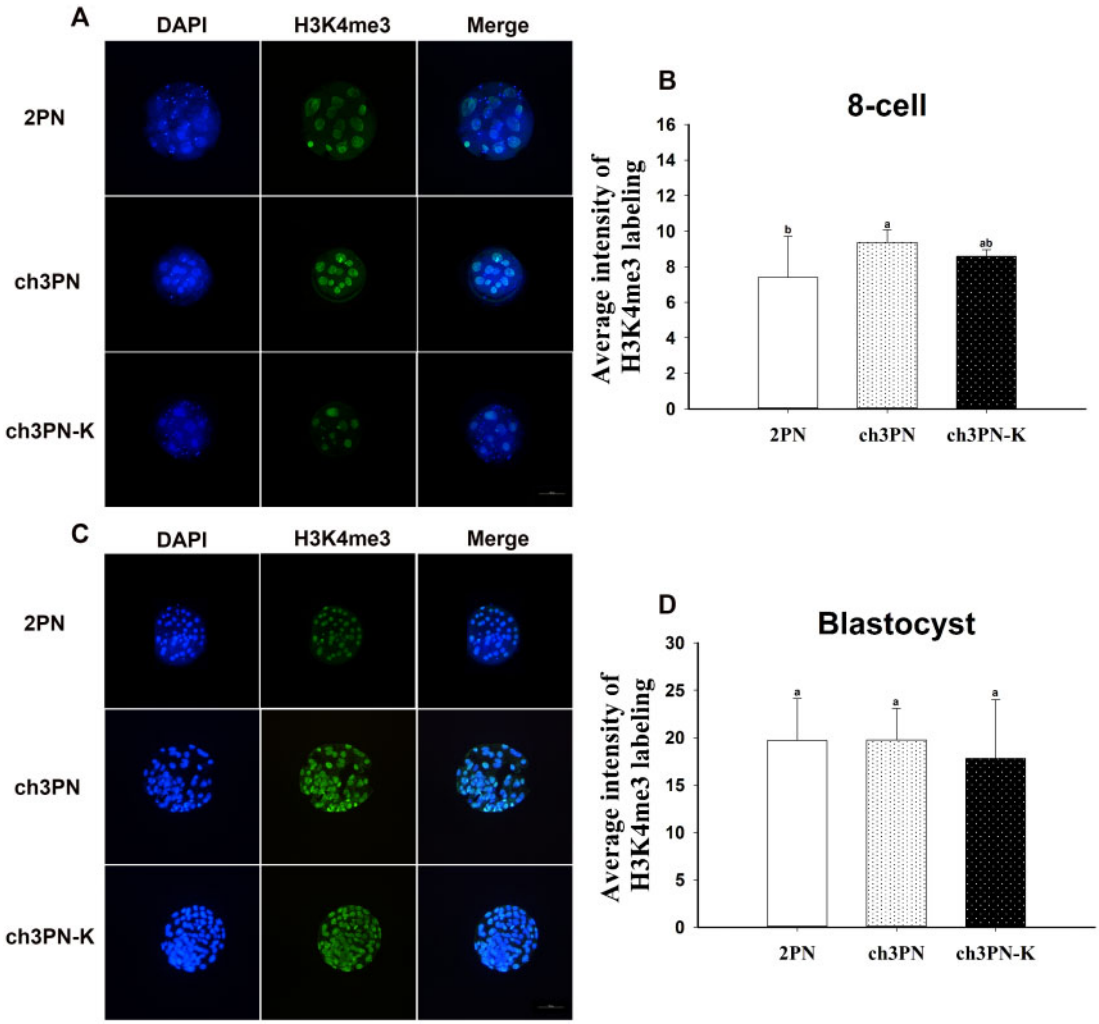

Figure 3. Global H3K4me3 levels in eight-cell and blastocyst embryos. (**A**) H3K4me3 staining (green) in 2PN, ch3PN and ch3PN-K embryos at the eight-cell stage. (**B**) Proportion of fluorescence intensity of H3K4me3 staining in 2PN ($n = 5$), ch3PN ($n = 5$) and ch3PN-K ($n = 5$) embryos at the eight-cell stages. (**C**) H3K4me3 staining (green) in 2PN, ch3PN and ch3PN-K embryos at blastocyst stage. (**D**) Proportion of fluorescence intensity of H3K4me3 staining in 2PN (n=5), ch3PN ($n = 5$) and ch3PN-K ($n = 5$) embryos at blastocyst stages. Each sample was counterstained with Hoechst 33342 to visualize the DNA (blue). Scale bar = 50 μm. Each experiment was repeated at least three times with at least 24 oocytes. The data are expressed as the mean ± SEM. Univariate ANOVA was used for data analysis, and SPSS 16.0 statistical software was used for Duncan's multiple range test. The values with different superscripts within groups are significantly different ($P < 0.05$). The experiments were repeated three times, with $n = 2$–3 per group.

developmental potential of human ch3PN embryos. However, the fluorescence intensity of GATA6 was similar between the ch3PN-K and ch3PN embryos (Fig. 4C and D).

KDM4A mRNA injection increased the expression of ZGA-related genes

To identify candidate genes that were repressed by H3K9me3 and that may be responsible for the poor development of ch3PN embryos, genes previously shown to be significantly upregulated and involved in transcriptional regulation during ZGA were assessed. Based on the quantitative RT-PCR results, the developmental pluripotency-associated genes developmental pluripotency associated 2 (*Dppa2*) and 4 (*Dppa4*) and *Myc* were significantly increased in ch3PN-K embryos compared with ch3PN embryos. Zinc finger and SCAN domain-containing 4 (*Zscan4*), upstream-binding transcription factor like 1 (*Ubtfl1*) and THO complex 5 (*Thoc5*), which are known to be required for normal preimplantation development, were also significantly higher in the ch3PN-K embryos than in ch3PN embryos ($P < 0.05$, Fig. 5). However, the relative expression of Paired Box 5

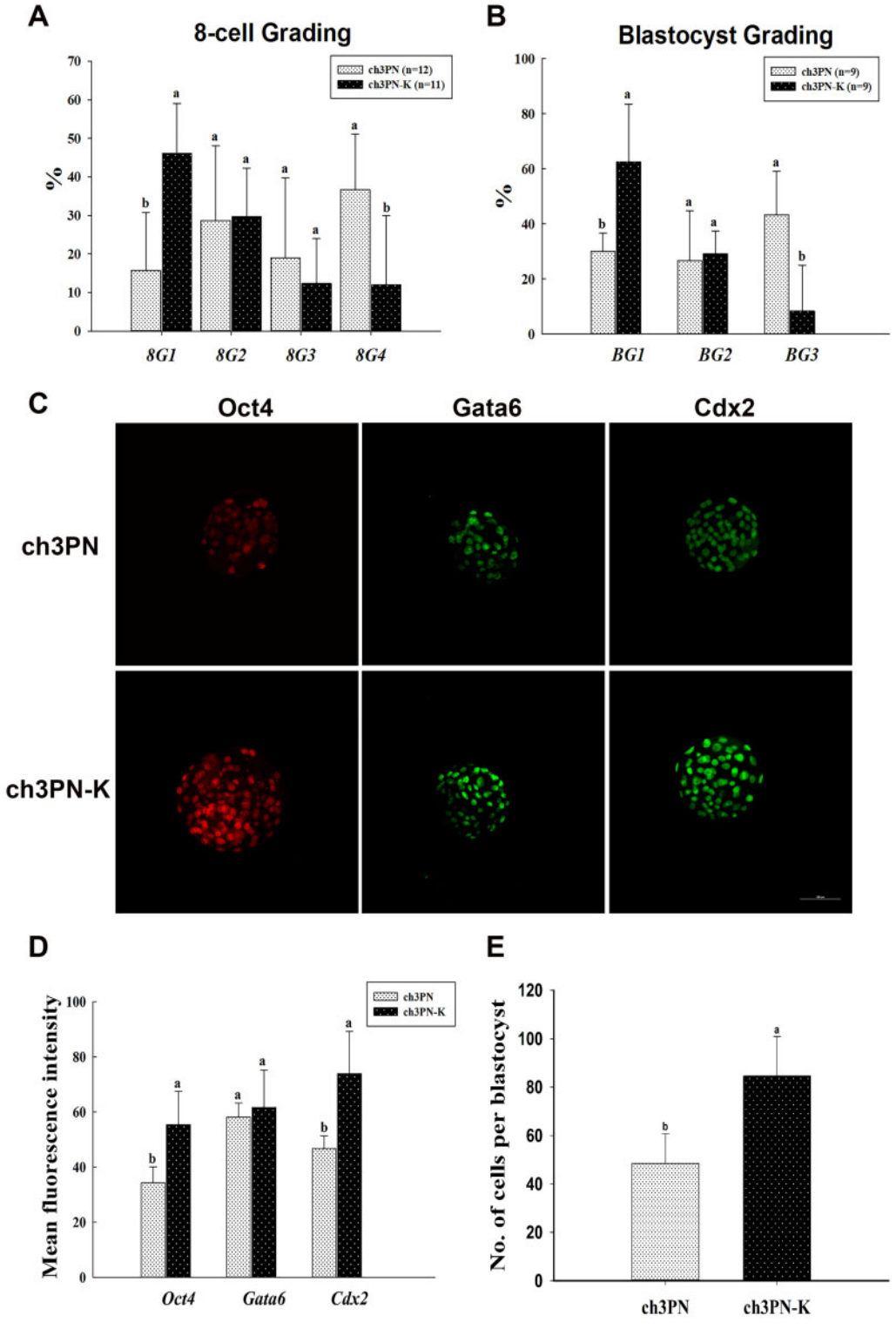

Figure 4. Developmental potential of ch3PN and ch3PN-K embryos. (**A**) Quality grading of eight-cell stage resulting from ch3PN and ch3PN-K zygotes. (**B**) Quality grading of blastocysts resulting from ch3PN and ch3PN-K zygotes. (**C**) Staining of OCT4 (red), GATA6 (green) and CDX2 (green) at the blastocyst stage. (**D**) Proportion of fluorescence intensity of OCT4, GATA6 and CDX2 staining in the ch3PN and ch3PN-K groups at the blastocyst stage. The experiments were repeated three times, with $n = 2$–3 per group. (**E**) Comparison of the total cell numbers in ch3PN blastocysts injected with or without KDM4A. The experiments were repeated three times, with $n = 2$–3 per group. Statistical analyses were performed with Student's *t*-test. The values with different superscripts within groups are significantly different ($P < 0.05$). OCT4; recombinant octamer-binding transcription factor 4; CDX2, caudal-related homeobox transcription factor 2; GATA6, GATA-binding protein 6.

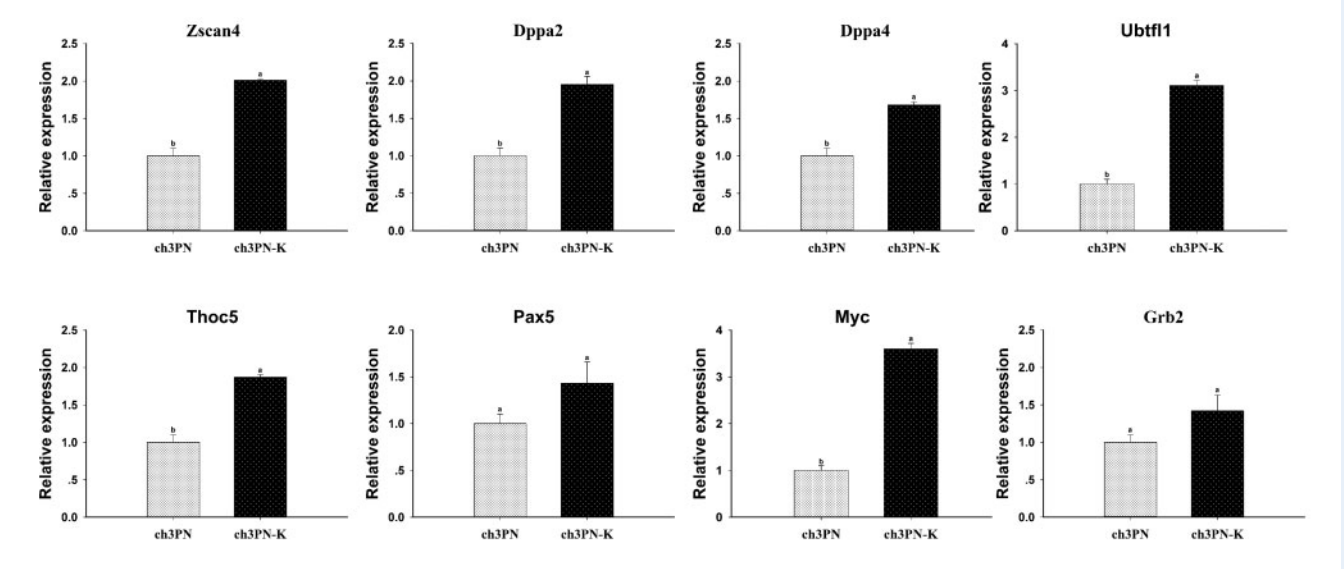

Figure 5. Relative mRNA levels of ZGA-related genes. The relative mRNA expression of the ZGA-related genes developmental pluripotency associated 2 (*Dppa2*) and 4 (*Dppa4*) *Myc*, paired pox 5 (*Pax5*), growth factor receptor-bound protein 2 (*Grb2*), Zinc finger and SCAN domain-containing 4 (*Zscan4*), upstream-binding transcription factor like 1 (*Ubtfl1*) and THO complex 5 (*Thoc5*) was evaluated in ch3PN and ch3PN-K embryos at the eight-cell stage by quantitative RT-PCR. Statistical analyses were performed with Student's *t*-test. [a,b]The values with different superscripts within groups are significantly different ($P<0.05$).

(*Pax5*) and growth factor receptor-bound protein 2 (*Grb2*) was similar in the ch3PN-K and ch3PN embryos. Thus, KDM4A mRNA injection corrected the developmentally important genes of ch3PN during ZGA.

Discussion

Human embryos are difficult to obtain for research because of limited resources and ethical debates (Ehrich *et al.*, 2011). Ch3PN zygotes from abnormally fertilized 3PN zygotes that are destined to be discarded in IVF cycles, are considered an alternative resource for basic scientific research (Rawlins *et al.*, 1988; Gordon *et al.*, 1989; Malter and Cohen, 1989; Palermo *et al.*, 1994; Ivakhnenko *et al.*, 2000; Escriba *et al.*, 2006; Gu *et al.*, 2009; Kattera and Chen, 2003). In our study, the blastocyst development efficiency was not improved by removal of a male pronucleus (Fig. 1C), which was consistent with the results of previous reports (Chen *et al.*, 2010; Fan *et al.*, 2013, 2014). Other studies have shown that the rate of blastocyst formation from corrected zygotes was increased (Jin *et al.*, 2015; Liao *et al.*, 2016). This different developmental competence may be attributed to different micromanipulation and culture systems. However, the results of all these studies were consistent with the developmental competence of ch3PN embryos but this was not comparable to the results of normal fertilized embryos. Previous studies of ch3PN embryos showed that adding the small-molecule compound Egf-Bdnf-Igf-1 to the culture system improved the development efficiency of ch3PN embryos (Fan *et al.*, 2014). This suggested that the developmental limitations of ch3PN can be corrected by different approaches.

Aberrant changes in histone modification are thought to be one of the main epigenetic marks that impede the development of abnormal embryos, particularly during the ZGA process (Zhang *et al.*, 2016; Zheng *et al.*, 2016; Wang *et al.*, 2018). Previous reports have also indicated that removal of a male pronucleus from 3PN human zygotes cannot correct for DNA methylation or H3K9me3 patterns (Chen *et al.*, 2010). In the present study, the H3K9me3 and H3K4me3 levels were much higher in the ch3PN and 3PN embryos than in the IVF embryos at the eight-cell stage, while only H3K9me3, but not H3K4me3, was detected at high levels in the ch3PN embryos at the blastocyst stage (Figs. 2 and 3). We speculated that the aberrant H3K9me3 level detected in the ch3PN embryos may be related to the low developmental competence. This suggests that the normal development of an embryo requires not only a correct diploid genome but also normal histone modification, which regulates the expression of developmental genes during ZGA. The injection of KDM4A mRNA effectively decreased the level of H3K9me3 modification and increased the blastocyst formation rate in ch3PN embryos, similar to that found in other species (Chung *et al.*, 2015; Weng *et al.*, 2020). In mice, injection of KDM4D decreased the H3K9me3 level and eventually increased the developmental competence of cloned embryos (Matoba *et al.*, 2014). In human SCNT embryos, KDM4A mRNA effectively decreased H3K9me3 levels and facilitated ZGA of RRRs (Chung *et al.*, 2015). In macaque monkey studies, injection of KDM4D mRNA and treatment with the histone deacetylase inhibitor, trichostatin A, greatly improved blastocyst development and the pregnancy rate of transplanted SCNT embryos in surrogate monkeys, ultimately yielding two healthy babies (Liu *et al.*, 2018). In fact, we attempted to use KDM4A and KDM4D mRNA to reduce H3K9me3 levels in ch3PN embryos, but H3K9me3 modification was still detected at high levels after

KDM4D overexpression (data not shown), although KDM4D had an apparent effect in mouse and macaque embryos (Matoba et al., 2014; Chung et al., 2015; Liu et al., 2016; Liu et al., 2018). A high level of H3K9me3 is present in the RRRs of nuclear-transfer embryos during ZGA, and removal of H3K9me3 markedly upregulates RRRs and at least partly facilitates ZGA (Chung et al., 2015). For H3K4me3, a slightly decreased level at the eight-cell stage was corrected by KDM4A mRNA injection, although KDM4A is not a demethylase inhibitor that targets H3K4me3 sites (Fig. 3). Previous reports have suggested that Kdm4A-mediated H3K9me3 demethylation and broad domains of H3K4me3 regulate the transcriptional activation of genes, endogenous retroviral elements and chimeric transcripts initiated from long terminal repeats during ZGA (Sankar et al., 2020). This regulation may play a role in H3K9me3 and H3K4me3 mediation in ch3PN embryos injected with KDM4A at the eight-cell stage.

Analysis of single-cell RNA-sequencing datasets of human embryos indicated massive gene expression in eight-cell embryos compared to four-cell embryos (Yan et al., 2013). These genes were enriched for transcriptional regulation, ribosomal biogenesis and RNA processing, suggesting that these genes may play an important role in transcriptional activation during ZGA. According to previous data, all the genes detected in this study, which included *Dppa2*, *Dppa4*, *Myc*, *Zscan4*, *Pax5*, *Grb2*, *Ubtfl1* and *Thoc5*, were expressed at high levels at the eight-cell stage (Yan et al., 2013). Moreover, RRRs with high H3K9me3 levels exist in embryos and repression of H3K9me3 at the ZGA stage regulates gene expression patterns and facilitates embryonic development (Chung et al., 2015). Among genes whose expression was significantly dysregulated by H3K9me3, *Ubtfl1* and *Thoc5* are known to be required for normal preimplantation development (Yamada et al., 2010; Wang et al., 2013). *Ubtfl1* and *Thoc5* were significantly upregulated by KDM4A mRNA injection in eight-cell stage human SCNT embryos, while consistent upregulation was obtained in ch3PN embryos with KDM4A mRNA injection during ZGA (Chung et al., 2015). The developmental pluripotency-associated genes, such as *Dppa2*, *Dppa4* and *Myc*, and the ZGA-related gene *Zscan4* were repressed by H3K9me3 in monkey SCNT embryos (Liu et al., 2018), and Kdm4 injection at least partly corrected the abnormal expression by removal of H3K9me3.

In previous studies, the lower quality of polyspermic zygotes has been shown to result in lower blastocyst formation efficiency and low blastocyst quality (Kawamura et al., 2012). Therefore, improvements in embryo competence can contribute to an increase in embryo quality. Embryo quality was assessed based on embryo grading criteria, cell number and pluripotent expression. The KDM4A mRNA injection significantly improved most grades of eight-cell embryos and blastocysts of the ch3PN embryos (Fig. 4A and B). The other criterion that reflected quality was the cell number of blastocysts. Blastocoel formation is a normal developmental period in which sufficient numbers of cells are generated. In this study, increased blastocyst cell numbers were observed in ch3PN embryos with KDM4A, suggesting that they would probably be successful at forming blastocoels, thereby promoting further embryo development, such as that needed to derive human embryonic stem cells. Successful embryonic development is dependent on the proper expression of specific genes, including *Oct4* and *Cdx2*, which are closely associated with pluripotency and early embryonic development (Yan et al., 2013; Theunissen and Jaenisch, 2017). *Oct4* is necessary for the maintenance of pluripotency of ICM cells and *Cdx2* promotes TE fate, cell-autonomously (Nichols et al., 1998; Ralston and Rossant, 2008). The developmental potential of ch3PN blastocysts overexpressing KDM4A was determined, and the results showed that blastocysts overexpressing KDM4A significantly expressed OCT4 and CDX2 (Fig. 4C and D). These results indicated that embryo KDM4A mRNA injection promoted not only developmental efficiency but also embryo quality in the ch3PN embryos. However, the fluorescence intensity of the primitive endoderm (PE)-specific transcription factor *Gata6* was similar between the ch3PN-K and ch3PN embryos (Fig. 4C and D). ICM cells express either the epiblast (EPI) specific transcription factor *Nanog* or *Gata6*, displaying a mutually exclusive mosaic distribution before the establishment of the PE layer by the late blastocyst stage (Chazaud et al., 2006). Probably because the embryos we tested were in the early blastocyst stage (D5), the change in *Gata6* was not detected.

In summary, H3K9me3 acted as an epigenetic barrier in human ch3PN embryos, and microinjection of KDM4A mRNA effectively reduced the H3K9me3 level and ultimately increased the developmental efficiency of ch3PN embryos. The quality of ch3PN zygotes improved as the grading criteria, cell number and pluripotent expression significantly improved in response to KDM4A mRNA injection. ZGA-related developmental genes were also upregulated. Because various aberrant epigenetic mechanisms may exist during ch3PN embryo development, the use of combinations of small-molecule inhibitors is recommended in future studies.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' Roles

Y.F., Y.Y. and H.-Y.Z. conceived and designed the research. H.-Y.Z., X.-J.K., L.J., P.-Y.Z., H.W. and T.T. conducted the experiments. H.-Y.Z. and X.-J.K. contributed to data interpretation, drafting and critical revision of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors have nothing to disclose.

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