CARM1 is heterogeneous in mouse four-cell embryo and important to blastocyst development

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Abstract

Coactivator-associated arginine methyltransferase 1 (CARM1) is a type I arginine methyltransferase that methylates the arginine residues of histone and nonhistone. *Carm1* regulates various cellular processes, including transcriptional regulation, mRNA processing, cellular proliferation, and differentiation. Blastomeres with high *Carm1* expression levels show cleavage tendency to inner cell mass (ICM) in mouse embryos. However, details about the factors for CARM1 distribution in mouse early embryos and the role of *Carm1* in blastocyst development remain unclear. Here, the endonuclear distribution of CARM1 protein was heterogeneous between blastomeres from the late four-cell stage to the blastocyst stage. The heterogeneity of CARM1 distribution in blastomeres at the late four-cell stage was randomly obtained from two-cell stage embryos. From the four-cell stage to morula, CARM1 in individual blastomere remained heterogeneous. In the blastocyst stage, CARM1 protein level in ICM was much higher than that in trophoblast. We found that microRNA (miRNA) miR-181a is an important regulator for *Carm1* distribution at the late four-cell stage. The ratio of heterogeneous embryos was reduced in all the embryos when miR-181a was inhibited. CARM1 inhibition reduced the level of symmetrical histone H3 arginine-26 dimethylation and impaired blastocyst development. Silencing *Carm1* reduced cell number and increased cell apoptosis at the blastocyst stage. These results show a CARM1 heterogeneous distribution from the four-cell embryos to the blastocysts. miR-181a regulates the control of CARM1 heterogeneous distribution in the four-cell-stage embryos, and CARM1 is an important protein in regulating blastocyst development.

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Introduction

Coactivator-associated arginine methyltransferase 1 (CARM1, also known as PRMT4) belongs to the type I PRMT family that catalyzes the transfer of methyl groups from S-adenosyl-L-methionine to the N of arginine residues on R17 and R26 sites in histone H3 and nonhistone proteins, including SRC-3, CBP/p300, RNA pol II, and Sox2 (Schurter *et al.* 2001, Xu *et al.* 2001, Yadav *et al.* 2003, Sims *et al.* 2011, Zhao *et al.* 2011). *Carm1* regulates various cellular functions, including mRNA processing and stability, transcription, and translation (Ma *et al.* 2001, Bedford & Richard 2005, Cook *et al.* 2006). *Carm1* overexpression is associated with various cancers, such as breast tumors, prostate adenocarcinomas, and liver cancers, due to its methylation on histone- and cancer-associated transcription factors, including NF- κ B, p53, and E2F1 (Hong *et al.* 2004, Bedford & Clarke 2009, Cheng *et al.* 2013, Osada *et al.* 2013). Therefore, CARM1 can be a potential target for cancer therapy. CARM1 is closely related to the pluripotency in embryonic stem (ES)

cells and transactivates stem-associated transcription factors, including *Oct4* and *Sox2* (Wu *et al.* 2009, Xu *et al.* 2013). When *Carm1* is overexpressed, ES cells can increase its resistance to differentiation; otherwise, ES cells will differentiate into specific lineages when *Carm1* is depleted (Wu *et al.* 2009). Morula transition to blastocyst is associated with cell differentiation and blastomere fate decision. However, the mechanism of the effect of *Carm1* on this process is poorly understood.

Blastomeres undergo major changes in developmental potency during mouse preimplantation embryonic development. The heterogeneity of embryonic blastomeres arises as early as the four-cell stage, thereby exhibiting differences in developmental potency (Fujimori *et al.* 2003, Piotrowska-Nitsche *et al.* 2005, Torres-Padilla *et al.* 2007). Meanwhile, the spatial arrangement of an individual blastomere at the four-cell stage affects epigenetic modifications, transcriptional activity, and embryonic–abembryonic axis in blastocysts (Piotrowska-Nitsche & Zernicka-Goetz 2005, Torres-Padilla *et al.* 2007). Through epigenetic marker analysis,

Torres-Padilla *et al.* (2007) revealed that H3R26me2, which is correlated with cell fate and potency, is heterogeneous at the four-cell mouse embryo stage. The blastomere with overexpressed *Carm1* contributes to the high H3R26me2 level and directs its potency to the inner cell mass (ICM). Conversely, the blastomere that is potentially part of the differentiation phenotype has low H3R26me2 and CARM1 levels. CARM1 regulates lineage specification by suppressing cell polarity and reducing the expression of *Cdx2*, which is a trophoderm-specific gene (Parfitt & Zernicka-Goetz 2010). *Carm1*-knockout embryos are reduced in size (Yadav *et al.* 2003). In addition, mice with *Carm1* depletion die perinatally and show defective T cell and lung development and adipogenesis (Yadav *et al.* 2003, 2008, Kim *et al.* 2004, 2010). Therefore, *Carm1* is important in modulating the developmental potency of blastomeres and predisposing the differentiation of fetal tissues. miRNAs are 21–23 nucleotide noncoding RNAs that are generally base-paired with the 3'-UTRs of their target mRNAs to induce degradation and inhibit their translation. miRNAs are involved in a broad range of biological processes, such as embryonic development, cell proliferation, apoptosis, and stem cell renewal and pluripotency (Sinkkonen *et al.* 2008, Goossens *et al.* 2013, Chen *et al.* 2017, Dernowsek *et al.* 2017). Tang *et al.* (2007) reported that maternally inherited miRNAs are essential to mouse embryonic development at the earliest stages following Dicer maternal knockdown (KD). The effect of individual miRNA on embryonic development has been widely discussed (Renthal *et al.* 2010, Liu *et al.* 2012, Zhang *et al.* 2015, Tan *et al.* 2016). miR-181a, which is closely related to cell proliferation, development, and cancer, is a member of the miR-181 family (Zhang *et al.* 2013, 2018, Xu *et al.* 2014). Xu *et al.* (2013) reported that miR-181c targets *Carm1* 3'-UTR to promote human embryonic stem cell differentiation. However, the mechanism of the effect of miRNAs on the epigenome of an early embryo remains unclear. Whether miRNAs participate in the regulation of mouse embryonic heterogeneity also needs to be elucidated.

In this report, *Carm1* heterogeneity is shown at the mouse four-cell-stage embryos and is not predetermined in two-cell embryos. miR-181a is a key factor for regulating the heterogeneous distribution of *Carm1* at the four-cell stage. As an arginine methylase, *Carm1* is responsible for H3R26me2s construction. As a transcription factor, it is essential for normal blastocyst formation and hatching. These findings indicate the clear localization and function of *Carm1* in mouse preimplantation embryos.

Materials and methods

Embryo work

Kunming strain mice used in this study were purchased from the Experimental Animal Center of Xi'an Jiaotong University

(Xi'an, China). The mice were maintained on a 12/12 h light/dark cycle and 50–70% humidity with free access to food and water at the Laboratory Animal Facility of the College of Veterinary Medicine, Northwest A&F University. All animal works and experiments were approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University.

Female mice aged 6–8 weeks were superovulated by an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin, followed by an intraperitoneal injection of 5 IU human chorionic gonadotropin (hCG) after 48 h. Female mice were individually mated with Kunming male mice after being injected with hCG. Zygotes enclosed in cumulus masses were released from the ampullae, and cumulus cells were removed by digestion with M2 media containing 1 mg/mL hyaluronidase (Sigma, H3506) for 2–3 min at 37°C. Embryos were collected and cultured in fresh KSOM medium (Caisson, IVL04) at 37°C under 5% CO₂. Lastly, two-cell, four-cell, eight-cell, morula, and blastocyst-stage embryos were collected after 22–26, 48–50, 60–65, 70–75, and 96–100 h of culture, respectively. The zona pellucida was removed using Tyrode's solution (Sigma). Embryos were treated at two-cell or morula stage with a chemical inhibitor against CARM1 (Millipore) to inhibit CARM1 activity, and the concentration of CARM1 inhibitor was selected according to the published article (Goolam *et al.* 2016).

RNA isolation and quantitative real-time PCR

Pooled embryos ($n = 100$ embryos per sample) were lysed with Cells-to-Signal™ Kit (Ambion Co., Austin, TX, USA) according to the manufacturer's instructions, and first-strand cDNA was directly synthesized with SuperScript® III CellsDirect cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. After synthesis, we used the ABI StepOnePlus PCR system (Applied Biosystems) to perform the qRT-PCR experiment with the first-strand cDNAs. The results were normalized to *PPIA* mRNA level. Data were expressed as fold change = $2^{-\Delta\Delta Ct}$. Each reaction was run in triplicate with at least three independent replicates.

Mature miRNA samples ($n = 100$ embryos per sample) were reverse-transcribed with miScript II RT (Qiagen). To quantify the miRNA, we also examined the mature miRNA expression through qRT-PCR by using miScript SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. The expression level of each miRNA species was normalized to that of U6. The primer sequences are listed in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

Cell culture

Mouse NIH/3T3 and F9 cells were maintained and grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum. All cell media were supplemented with 50 U/mL penicillin (Invitrogen) and 50 µg/mL streptomycin (Invitrogen). The cells were cultured at 37°C in a humidified 5% CO₂/95% air incubator.

Western blot analysis

Total cell lysates were prepared in a 1× sodium dodecyl sulfate buffer (Sun *et al.* 2018). Different cells were lysed with radioimmunoprecipitation assay buffer (Beyotime, P0013B). Proteins were electrophoresed on 12% acrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk/Tris-buffered saline Tween-20 for 4 h at room temperature (RT) and then incubated with the primary antibody overnight at 4°C. Primary antibodies are shown in Supplementary Table 2. All secondary antibodies were purchased from Beyotime Company (Beyotime, China).

Immunofluorescence staining

Cells and pooled embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, washed, and permeabilized in PBS containing 0.2% Triton X-100 for 15 min at RT. After treatment with 5% bovine serum albumin (BSA) in PBS for 2 h at RT, the samples were incubated with the primary antibody diluted in PBS containing 1% BSA overnight at 4°C, washed twice, and incubated with the corresponding secondary antibodies for 2 h at RT. Subsequently, nuclei were stained with DAPI (Beyotime, C1005) for 5 min. Primary antibodies used here are shown in Supplementary Table 2. All secondary antibodies were purchased from Beyotime Company (Beyotime). The samples were observed under a Nikon eclipse Ti-S (Nikon) or a Zeiss Axio Observer D1 microscope (Carl Zeiss, Inc.). Multiple images were exposed to IMAGEJ for processing, intensity measurements, and cell counting. Fluorescence intensity was quantified by normalizing to DAPI by using the built-in IMAGEJ function. Data were normalized with respect to background levels.

siRNA, miRNA, and mRNA microinjection

The miRNA mimic, miRNA inhibitor, and mouse *Carm1*-specific siRNA were diluted in nuclease-free water to final concentrations of 800 nM, 25 µM, and 40 µM, respectively. The aliquots of 5–10 µl of the siRNA/miRNA were microinjected into the cytoplasm of mouse zygotes in M2 medium (Sigma-Aldrich, M7167) using an Eppendorf micromanipulator under a Leica inverted microscope and then cultured in fresh KSOM medium. Controls were injected with the negative control siRNA. Zygotes cultured for 24 h (two-cell stage) were collected to detect knockdown efficiency by qRT-PCR. siRNA sequence used to silence *Carm1* is as follows: 5'-CCUGGUGAAGAGUAAACAAUdTdT-3'.

Enhanced fluorescent green protein (EGFP) cDNA was cloned into T7-driven vectors and synthesized using the mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies) according to the manufacturer's instruction. The storage concentration of each mRNA was optimized to 800 ng/µL. For two-cell embryo injection, 5–10 µl of EGFP mRNA was randomly injected into one two-cell embryo blastomere. Then, two-cell embryos were cultured in fresh KSOM medium.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

We carried out apoptosis assays to detect apoptotic cells in 4-day blastocysts using the DeadEnd Fluorometric TUNEL System (Promega) in accordance with the methods of a previous study (Su *et al.* 2012).

Blastocyst outgrowth culture

Here, 0.1% gelatin was pipetted into wells of a 48-well plate, which was then placed in an incubator for 10 min. Subsequently, the gelatin was discarded and left at RT for 10 min. Then, the stem cell culture medium containing LIF was added to the wells of the 48-well plate and incubated to equilibrate the medium. The zona pellucida (ZPs) of the 4-day blastocysts were removed by incubating them for 10–30 s in 100 µL of prewarmed Tyrode's solution until the ZPs were dissolved, as observed under a microscope. The blastocysts were immediately transferred to a wash dish containing MEM-HEPES. Then, one blastocyst was transferred to a well of the prepared blastocyst outgrowth culture plate and then cultured at 37°C/5% CO₂ for 72 h. The blastocysts were examined under a microscope after 72 h culture.

Statistical analysis

Each experiment was repeated at least thrice. In embryonic development experiments, the number of examined embryos was indicated (*n*). The data were presented as means ± s.d. Student's *t*-test was used to compare the two groups. We used SPSS, version 19.0 (IBM Corporation) for analyses. Significance was accepted at *P* < 0.05.

Results

Expression pattern of *Carm1* mRNA in early embryos

We examined the mRNA expression levels of *Carm1* in mouse preimplantation embryos by qRT-PCR analysis to reveal the expression profile of *Carm1*. During the preimplantation embryonic stage, *Carm1* was predominantly expressed at two-cell stage, and the level decreased from the four-cell stage (Fig. 1A). Meanwhile, the *Carm1* expression level in the germinal vesicle and meiosis II stage was significantly lower than that in the one-cell stage. *Carm1* was activated at the zygote stage. We then detected the *Carm1* expression level between the early one-cell-stage embryos and late one-cell-stage embryos (Fig. 1B). *Carm1* expression increased at the late one-cell stage possibly coinciding with the minor wave of zygotic genome activation (Hamatani *et al.* 2004).

CARM1 protein distribution was heterogeneous at late four-cell stage

CARM1 distribution at each stage of mouse embryo preimplantation has not been elucidated well to date.

Figure 1 Expression pattern of *Carm1* in mouse preimplantation embryos. (A) qRT-PCR analysis of *Carm1* was performed in GV, MII oocytes, zygotes, two-cell embryos, four-cell embryos, eight-cell embryos, morula, and blastocyst. (B) mRNA level of *Carm1* in early one-cell stage and late one-cell stage. Bar indicates the s.d. ** $P < 0.01$.

We analyzed the CARM1 protein levels from the zygote stage to the blastocyst stage via immunostaining. As shown in Fig. 2A, CARM1 showed mainly nuclear distribution, except at the meiosis II stage. From zygote to early four-cell stage, the CARM1 protein was evenly distributed in the nucleus at each stage. From the beginning of the late four-cell stage, the CARM1 protein in the nucleus was heterogeneously distributed. Meanwhile, the CARM1 protein in the cytoplasm of each stage was almost equally distributed. Thus, the CARM1 ratio in the nucleus and cytoplasm (Nucl./Cyto.) was adopted for the analysis of asymmetry. The Nucl./Cyto. ratio in preimplantation is shown in Fig. 2B. In the blastocyst stage, the CARM1 protein level in the ICM was higher than that in the trophoblast (Supplementary Fig. 1A). The CARM1 heterogeneity at the early and late four-cell stages was analyzed and is shown in Fig. 2C. At the early four-cell stage, a small part of embryos (23.1%, $n=26$) displayed heterogeneity in CARM1 distribution. After the development for 10–12 h, 72.7% ($n=44$) of embryos revealed CARM1 distribution heterogeneity in the nucleus. According to the data of single-cell sequencing (Goolam *et al.* 2016), we analyzed the mRNA levels of the four blastomeres at the four-cell stage. The expression pattern of *Carm1* mRNA was consistently highly variable, and the expression level between the highest and lowest expressing blastomeres across all four-cell embryos greatly varied (Fig. 2D; $n=40$ cells, 10 embryos). In most cases, at least one blastomere in each embryo expressed an extremely low or no *Carm1* mRNA level (Fig. 2E; Goolam *et al.* 2016). Then, we examined the protein level in each blastomere of a four-cell embryo (Fig. 2F). The cellular heterogeneity in CARM1 protein level was consistent with its mRNA expression level (Fig. 2G; $n=10$). The CARM1 protein expression was also variable at the eight-cell stage, and the statistical results are shown in Supplementary Fig. 1B.

Heterogeneous CARM1 distribution was not predetermined in two-cell embryos

We performed microinjection experiments to trace the dynamic CARM1 distribution from two-cell stage embryos to provide additional insights into CARM1 heterogeneity in four-cell stage blastomeres. *EGFP* mRNA was randomly injected into a one blastomere of the two-cell stage embryos (Fig. 3A). The CARM1 protein distribution in four-cell-stage blastomeres with or without *EGFP* was analyzed by IF. Embryos with two blastomeres with high CARM1 expression are shown in Fig. 3B (rows two and three; 30.6%, $n=85$). Row 4 represents the situations that two blastomeres with high CARM1 expression came from different blastomeres of two-cell-stage embryos (45.9%, $n=85$). The results demonstrated the random *Carm1* source from two-cell embryos. Torres-Padilla *et al.* (2007) reported that *Carm1* overexpression in a two-cell blastomere can result in the contribution of that cell predominantly to the ICM of the blastocyst stage. Then, two-cell-stage embryos with one blastomere injected with *EGFP* mRNA were developed into the blastocyst stage. As shown in Fig. 3C, the *EGFP* distribution in the blastocyst can be divided into the following four patterns: evenly distributed, half of the blastocyst, mainly in ICM, and mainly in TE. The proportion of each pattern is shown in Fig. 3C. The *Carm1* expression level in the ICM of the blastocyst was considerably higher than that in TE (Supplementary Fig. 1A). Thus, the *EGFP* distribution injected into one of the two-cell blastomeres is inconsistent with the CARM1 distribution. Similar result was obtained when embryos with one two-cell stage blastomere was injected with *EGFP* developed into the morula stage (Fig. 3D). The blastomere injected with *EGFP* did not contribute to the inner or outer cells only.

miRNA-181a regulated *Carm1* expression and heterogeneous distribution

Considering the dynamic changes in the CARM1 protein level during the four-cell embryonic development, we then quantified the mRNA levels of *Carm1* between the early and late four-cell embryos. As shown in Fig. 4A, the *Carm1* mRNA level in early four-cell embryos was higher than that in the late four-cell embryos. Xu *et al.* (2013) reported that miR-181c targets *Carm1* 3'-UTR to promote human ES cell differentiation. The 3'-UTR sequences were also mostly conserved in different species (Fig. 4B). This result prompted us to consider whether miR-181 regulates *Carm1* expression in mouse embryos. The mature transcripts of the four members of the miR-181 family were quantified from one- to eight-cell embryos (Fig. 4C). Only the miR-181a level significantly increased from one- to eight-cell embryos. This outcome was consistent with the decrease in *Carm1* mRNA level from one- to eight-cell embryos. The miR-181a level was higher at the late stage than

Figure 2 CARM1 heterogeneity analysis at four-cell stage. (A) Freshly collected oocytes and various early embryos were processed for immunostaining with *Carm1*. Scale bar=20 μ m. (B) Nuclear/cytoplasm (Nucl./Cyto.) ratio of *Carm1* fluorescence intensity at each embryonic stage. $**P < 0.01$, $*P > 0.05$. Error bars represent s.d. (C) Proportion of heterogeneous distribution of *Carm1* in early ($n=26$) and late ($n=44$) four-cell stage embryos. (D) Average relative *Carm1* mRNA levels in four-cell embryos. (E) Relative *Carm1* mRNA expression in all individual four-cell embryos. (F) *Carm1* immunofluorescence in late four-cell embryos. Scale bar=20 μ m. (G) Fluorescence analysis of *Carm1* in Nucl./Cyto. ratio per individual four-cell embryo blastomere ($n=10$). Error bars represent s.d. $**P < 0.01$.

Figure 3 Developmental potential of one blastomere in two-cell embryo. (A) Diagram showing the experiment design. A randomly selected single two-cell blastomere was microinjected with *EGFP* mRNA. (B) Embryos injected with *EGFP* developed at the four-cell stage. Analysis of *Carm1* heterogeneity from two-cell stage embryos. The first row represents *Carm1* that was evenly distributed in the four-cell stage embryos (23.5%, $n=85$). The second and third rows represent the two blastomeres with low (12.9%, $n=85$) or high (17.6%, $n=85$) *Carm1* expression coming from the same blastomere of the two-cell stage embryos. The remaining four-cell embryos represent the two blastomeres with high or low *Carm1* expression coming from two different blastomeres of two-cell-stage embryo (line four; 45.9%, $n=85$). Arrowheads indicate the highest blastomere expression. Asterisks indicate the lowest blastomere expression. Scale bar = 20 μ m. (C) Representative blastocysts from the GFP-injected embryos. The ICM position is indicated with a dashed line. Scale bar = 20 μ m. (D) Representative morulas from the GFP-injected embryos. Scale bar = 100 μ m.

that at the early stage of four-cell embryos (Fig. 4D). Subsequently, we constructed the luciferase reporter vectors that have either wild-type (wt) or a mutant (mut) 3'-UTR of *Carm1*, with three-point mutations in the seed sequences of the predicted miR-181a-binding sites as the negative control (Fig. 4E). The two resulting constructs were cotransfected with a miR-181a mimic into the mouse cell line NIH/3T3. The luciferase activities significantly decreased after the miR-181a

coexpression with the *Carm1* wt 3'-UTR compared with the negative control (Fig. 4F). The endogenous *Carm1* mRNA level was also suppressed in response to miR-181a mimic and enhanced in response to miR-181a inhibitor in mouse embryos (Fig. 4G). As demonstrated earlier, miR-181a directly targeted *Carm1* 3'-UTR sequences and promoted *Carm1* mRNA degradation to some extent. We performed immunostaining analysis to determine whether miR-181a affects CARM1 protein

Figure 4 miR-181a directly regulated *Carm1* expression. (A) *Carm1* expression levels at the early and late four-cell stages. Error bars represent s.d. * $P < 0.05$. (B) 3'-UTR sequence of *Carm1* targeted by miR-181a was conserved in different species. (C) Expression levels of miR-181 family from zygote to eight-cell stage. Error bars represent s.d. (D) Expression levels of miR-181a in the early and late four-cell stages. Error bars represent s.d. * $P < 0.05$. (E) Consequential pairing of miRNA-181a and its target binding sites in wt or mut *Carm1* 3'-UTR are shown. (F) Relative luciferase activity in NIH/3T3 cells cotransfected with miR-181a and reporter constructs containing wt or mut *Carm1* 3'-UTR. * $P < 0.05$, * $P > 0.05$. (G) Relative *Carm1* mRNA levels in embryos were determined by qRT-PCR after injection with miR-181a mimic. Bars indicate the s.d. ** $P < 0.01$. (H) *Carm1* protein levels in four-cell stage embryos were detected by immunostaining after the injection with miR-181a mimic or inhibitor. Scale bar = 20 μ m. (I) *Carm1* immunofluorescence in late four-cell embryos injected with miR-181a ($n = 10$) or control inhibitor ($n = 8$). Fluorescence was quantified and normalized to the nucleus with the strongest staining per individual four-cell embryo. Scale bar = 20 μ m. Bars indicate the s.d. (J) Analysis of the *Carm1* heterogeneity between embryos injected with miR-181a inhibitor ($n = 74$) and the control group ($n = 67$). Error bars represent s.d. ** $P < 0.01$.

level or its heterogeneity in the late four-cell stage embryos. The result revealed that the CARM1 protein levels of the four-cell embryos decreased in response to

miR-181a mimic injection and increased in response to miR-181a inhibitor injection (Fig. 4H). Meanwhile, the rate of CARM1 heterogeneity significantly decreased in

the miR-181a inhibitor-injected four-cell embryos (Fig. 4I and J; $71.0 \pm 4.0\%$, the control group; $38.0 \pm 3.8\%$, the miR-181a inhibitor-injected group). These results suggested that miR-181a was a key regulator for *Carm1* expression and heterogeneous distribution in four-cell stage mouse embryos.

Symmetric H3R26 dimethylation was catalyzed by CARM1

CARM1 is a type I PRMT that catalyzes asymmetrically dimethylated protein substrates on arginine residues (Chen *et al.* 2000, Ma *et al.* 2001, Di Lorenzo & Bedford 2011). CARM1 catalyzed the asymmetrical dimethylation R26 site in histone H3 (H3R26me2a) (Schurter *et al.* 2001, Zhang *et al.* 2017). However, the enzyme responsible for the construction of symmetrical dimethylation R26 site in histone H3 (H3R26me2s) remains unclear. The difference between H3R26me2a and H3R26me2s is shown in Supplementary Fig. 2A. Here, we further investigated whether H3R26me2s is catalyzed by CARM1. First, we performed IF to reveal H3R26me2s distribution in early mouse embryos (Fig. 5A). The heterogeneous distribution of H3R26me2s was detected at the four-cell stage embryos, not in two-cell stage embryos (Supplementary Fig. 2B and C). Considering the similar distribution between *Carm1* and H3R26me2s in four-cell stage embryos, we next performed Western blot and IF using two cell lines to confirm whether H3R26me2s was catalyzed by CARM1. CARM1-specific inhibitor 1-benzyl-3,5-bis-(3-bromo-4-hydroxybenzylidene) piperidin-4-one (called 'inhibitor') was used to inhibit the enzymatic activity of CARM1 (Supplementary Fig. 2D) (Goolam *et al.* 2016). At the cellular level, CARM1 inhibitor potently inhibited H3R26me2s (Fig. 5B and C). Through *Carm1* overexpression and silencing experiments, we also found that CARM1 is responsible for H3R26me2 construction (Fig. 5D). Then, we treated the two-cell embryos with CARM1 inhibitor. All embryos treated with 4.5 or 9.0 μ M inhibitor were arrested at the two-cell stage (Fig. 5E). Subsequently, we treated the morula embryos with 9 μ M inhibitor for 24 h. Approximately half of the embryos were arrested at the morula stage, and the remaining embryos developed into the early blastocyst stage (Fig. 5E). Meanwhile, the embryos treated with dimethyl sulfoxide (DMSO) can develop into the late blastocyst stage. We also treated early two-cell stage embryos with an inhibitor for 16 h to collect the embryos. Then, the late two-cell embryos treated with an inhibitor or DMSO was collected for the analysis of H3R26me2s level. Meanwhile, early blastocysts treated with an inhibitor or DMSO underwent the same analysis. As shown in Fig. 5F, the H3R26me2s level decreased in inhibitor-treated embryos, especially in the two-cell embryos. (O'Brien *et al.* 2010) reported that embryos with *Carm1* loss can survive until birth and respond to stimulus. Conversely,

our results showed that embryos treated with CARM1-specific inhibitor almost arrested at the two-cell or morula stage. The difference between our results and the findings of O'Brien's study was significant. We predicted that CARM1 inhibitor can affect cell survival in addition to inhibiting H3R26me2s. At the cellular level, some NIH/3T3 cells exhibited morphotype change when treated with CARM1 inhibitor (Supplementary Fig. 2E). CARM1 inhibitor treatment resulted in the change in nuclear organization in cells (Supplementary Fig. 2F). Similar results that are consistent with AMI-1 treatment, a specific inhibitor of arginine methylation (Supplementary Fig. 2G), were observed in mouse embryos (Torres-Padilla *et al.* 2007). Abnormal cell division was also detected in cell lines and embryos, and large and small nuclei were contained in one cell (Supplementary Fig. 2H and I). The abnormal cell division cannot be detected in cells or embryos treated with DMSO. The results above suggested that CARM1 inhibition can cause abnormal cell division and nuclear organization, which may be the reasons for embryonic developmental retardation.

Silencing *Carm1* reduced developmental potential of mouse blastocyst

The findings mentioned above revealed that H3R26me2s was catalyzed by CARM1. The H3R26me2s localization was similar with CARM1 expression at the blastocyst stage (Fig. 6A). The CARM1 and H3R26me2s signal levels in the ICM were higher than those in TE. The H3R26me2s signal dramatically weakened, in which the chromatin was condensed (Fig. 6B). The high H3R26me2s signal in the ICM indicated that it may play a specific role in blastocyst development. Considering the CARM1 heterogeneity in early embryos, we explored the role of *Carm1* in mouse embryonic development. We injected *Carm1* siRNA to zygotes to downregulate *Carm1* and then cultured in KSOM medium. We first confirmed *Carm1* knockdown (KD) efficiency using qRT-PCR (Supplementary Fig. 3A). As shown in Fig. 6C, the difference in the blastocyst ratio between the si*Carm1*-injected and control groups was statistically insignificant ($80.7 \pm 2.6\%$, the control group; $73.7 \pm 4.8\%$, the si*Carm1*-injected group). However, blastocyst size in these two groups was significantly variable. Thus, we performed experiments to explore the developmental potential of blastocyst injected with si*Carm1*. Then, the 4-day blastocysts were collected for TUNEL assay (Fig. 6D) and anti-CDX2 immunostaining (Fig. 6E). The results showed an apparent increase in apoptotic rates in the si*Carm1*-injected embryos. The total cell number in si*Carm1*-injected blastocysts was apparently reduced. The ICM:TE ratio was also reduced in the si*Carm1*-injected blastocysts. We then analyzed the expression level of *Nanog* and *Sox17*, which are the markers of epiblast and primitive endoderm, respectively. As

Figure 5 CARM1 was responsible for H3R26me2s construction. (A) Freshly collected early embryos from the four-cell to blastocyst stage were processed for immunostaining with H3R26me2s. Scale bar=20 μm. (B and C) H3R26me2s protein levels were determined by immunostaining (B) and immunoblotting (C) in NIH/3T3 or F9 cells, which were individually treated with DMSO or CARM1 inhibitor. Scale bar=10 μm. (D) H3R26me2s detection in the *Carm1* overexpression group and the *Carm1* knockdown group. (E) Developmental arrest was shown when two-cell embryos or morula were cultured in the medium containing CARM1 inhibitor. Scale bar=100 μm. (F) H3R26me2s protein levels were determined by immunostaining in embryos treated with DMSO or CARM1 inhibitor. Scale bar=20 μm.

Figure 6 CARM1 was essential for normal blastocyst development. (A) Distribution patterns of CARM1 and H3R26me2s were comparatively determined by immunostaining in blastocyst. Scale bar=20 μ m. (B) Distribution pattern of H3R26me2s in the nucleus at the blastocyst stage. Scale bar=2 μ m. (C) Comparison of blastocyst ratio among siCarm1- ($n=82$) and siControl-injected embryos ($n=73$). Scale bar=100 μ m. Error bars indicate s.d. $^{\#}P>0.05$. (D) Representative image of TUNEL-positive cells in mouse blastocysts after injection with siCarm1 ($n=10$) and siControl ($n=9$). Bars indicate the s.d. $^*P<0.05$. (E) CDX2 and DAPI immunostaining in blastocysts developed from siCarm1- ($n=10$) and siControl-injected groups ($n=10$). Box plots show the total cell number and ICM:TE ratio of each blastocyst in each group. Scale bar=20 μ m. Bars indicate the s.d. $^*P<0.05$, $^{**}P<0.01$. (F) Representative images of Nanog and Sox17 in mouse blastocysts after injection with siCarm1 ($n=10$) and siControl ($n=8$). Scale bar=20 μ m. (G) Fluorescence intensities of Nanog and Sox17 were analyzed and shown as box plots. Bars indicate the s.d. $^*P<0.05$, $^{**}P<0.01$. (H) Representative image of outgrowth blastocysts treated with DMSO ($n=8$) or CARM1 inhibitor ($n=8$). Relative outgrowth area between these two groups is shown as box plot (right panel). Scale bar=20 μ m. Bars indicate the s.d. $^*P<0.05$.

shown in Fig. 6F and G, *Nanog* expression level was significantly reduced in si*Carm1*-injected embryos, and the *Sox17* expression level was slightly increased. Then, we collected E4.0 embryos to perform an outgrowth experiment in analyzing the outgrowth potential of embryos, in which *Carm1* was silenced. We collected D4.0 embryos injected with siControl or si*Carm1* individually and cultured the two groups in a stem cell culture medium for 3 days. The capacity for embryos injected with si*Carm1* to initiate spreading over the culture surface was lower than that of the control group (Fig. 6H). Considering the relationship between the H3R26me2s and nuclear organization in early embryos (Supplementary Fig. 2), we explored whether *Carm1* affects the H3K4me3 and H3K9me3 levels, which are closely related to chromatin structure and transcription regulation. As shown in Supplementary Fig. 3B, H3K4me3 level but not H3K9me3 was reduced when CARM1 was inhibited by a CARM1-specific inhibitor, which may be one reason for the change in nuclear organization and transcription imbalance in blastocyst when CARM1 was inhibited.

Discussion

In this study, the heterogeneous CARM1 distribution at the four-cell stage was not predetermined, and the role of *Carm1* for the further development of blastocyst is important. The following are the basis for this conclusion. (1) CARM1 was heterogeneously distributed at the late four-cell stage, and heterogeneity was not predetermined at the two-cell stage; (2) at the four-cell stage, miR-181a participated in the translation regulation of *Carm1* mRNA and may be one of the regulators for heterogeneous CARM1 protein distribution; (3) CARM1 was responsible for H3R26me2s modification; and (4) CARM1 was important for further blastocyst development (Fig. 7).

The first cell-fate determination in mammalian embryos has gained considerable research interest. In addition, the time the blastomere heterogeneity first arises and how this condition may occur remained debatable to date. The blastomeres of the early mammalian embryo seem to be nearly equivalent in their developmental potency until the generation of the inside and outside cells (Yamanaka *et al.* 2006,

Rossant & Tam 2009, Wennekamp *et al.* 2013). However, molecular heterogeneity is already exhibited in the four-cell stage embryos, thereby showing the differences in developmental potential toward either ICM or TE of the four blastomeres (Piotrowska-Nitsche *et al.* 2005, Wu *et al.* 2009, Goolam *et al.* 2016, White *et al.* 2016). Meanwhile, two emerging studies have provided new evidence that heterogeneity is identified at the two- to four-cell stage (Hupalowska *et al.* 2018, Wang *et al.* 2018). The heterogeneity of CARM1 protein has two seemingly contradictory views. Torres-Padilla *et al.* (2007) reported that the CARM1 heterogeneity in individual blastomeres first appeared at the four-cell stage, but the most recently published article showed that CARM1 protein showed asymmetric expression at the two-cell stage (Hupalowska *et al.* 2018). These findings are slightly contradictory. In our experiments, CARM1 was found in the whole blastomere of each embryo stage and showed uniform distribution within the nucleus or cytoplasm. The CARM1 protein distribution in the nucleus was higher than that in the cytoplasm. Thus, the ratio of CARM1 staining in the nucleus and cytoplasm was adopted to analyze the CARM1 protein heterogeneity. Consistent with previous reports by Torres-Padilla *et al.* (2007), CARM1 protein showed asymmetrical distribution in four-cell stage embryos in our study. However, we found that most embryos which showed heterogeneity in CARM1 distribution were at the late four-cell stage.

In our experiments, the two blastomeres at the four-cell stage with high or low CARM1 protein levels did not show indications that they came from the same two-cell stage embryo blastomere. The two blastomeres with the highest and lowest CARM1 staining can come from one of the blastomeres of the two-cell embryos. In addition, the cell fate decision of the two-cell blastomeres has been controversial (Piotrowska *et al.* 2001, Chrościcka *et al.* 2004, Motosugi *et al.* 2005). According to the results shown in Fig. 3, our findings supported Chrościcka A's research. Both blastomeres of the mouse two-cell embryo have the potential to develop into ICM or TE of the blastocyst (Chrościcka *et al.* 2004). Considering the above points, CARM1 is heterogeneous at the two-cell stage and can promote ICM formation (Torres-Padilla *et al.* 2007,

Figure 7 Schematic representation of the proposed model illustrating the heterogeneous distribution of CARM1 in preimplantation embryos. In four-cell-stage embryos, miR-181a participates in the expression and distribution of CARM1. In blastocyst stage, CARM1 is highly distributed in ICM and regulates gene expression important for normal blastocyst development. The green color represents the expression level of CARM1.

Hupalowska *et al.* 2018). Each two-cell embryo blastomere should have a distinguishable fate in the blastocyst (Piotrowska *et al.* 2001). This phenomenon seems to be relatively contradictory to our results. We speculated that the research difference has the following possibilities: (1) differences in research methods and analytical methods we mentioned above, (2) differences in mouse strains, and (3) possible varying *Carm1* expression level in one blastomere, accompanied with the change in spatial and polarity through embryonic development. Meanwhile, the mRNA level in each blastomere of the four-cell stage also showed heterogeneity. Xu *et al.* (2013) reported that miR-181c is a *Carm1* regulator. MiR-181c had low expression levels in early embryos (Fig. 4C). Here, our research showed that miR-181a was one of the regulators for *Carm1* expression and heterogeneity in the four-cell-stage embryos. When miR-181a was inhibited, the proportion of heterogeneous embryos was dramatically lower than that of the control group (Fig. 4J). To the best of our knowledge, this work is the first time to reveal the regulatory relationship between miRNA and embryo heterogeneity. However, approximately 38% of embryos were still heterogeneously distributed at the late four-cell stage when miR-181a was inhibited. Thus, other factors may participate in the regulation of CARM1 heterogeneous distribution in the late four-cell stage embryos. Wang *et al.* (2018) reported LincGET functions as a scaffold to recruit diffusible *Carm1* in the nucleus. Hupalowska *et al.* (2018) reported that the association of the CARM1 with paraspeckles can affect proper lineage allocation in mouse preimplantation embryos. *Carm1* expression and distribution in a four-cell stage blastomere may be regulated by the inherent molecular or CARM1's partner, such as miRNA, lncRNA, and other proteins. However, the exact mechanism and the original effector for the heterogeneity need further investigation.

Histone arginine methylation is a key regulator for cell fate decision in the early mouse embryo, and CARM1 is an asymmetrically arginine methylase. Here, our research showed that CARM1 can also catalyze sDMA formation on H3R26. H3R26me2a, which is a symmetrical dimethylation on H3R26, was also catalyzed by CARM1. The distribution model of H3R26me2a in mouse early embryos was unclear, and the functional difference between these two modifications also needs further studies.

Next, we focused on the effects of *Carm1* on blastocyst developmental quality and potential. Although the blastocyst rate was unaffected, the blastocyst quality in the *Carm1* KD group was worse than that of the control group. *Carm1* KD embryos were delayed in their developmental size and formed blastocysts with reduced numbers of cells, in which the abnormal *Nanog* and *Sox17* expression occurred. The outgrowth area was also smaller in the *Carm1*-silenced

group than that of the control group, thereby indicating that the developmental potential cannot be as good as normal embryos when CARM1 was inhibited. Mice with *Carm1* knockout have given birth although this offspring is smaller than wt littermates and died only after birth (Kim *et al.* 2010). In addition, the evidence we discussed above revealed *Carm1* as an important regulator for cell fate and pluripotency in the early mouse embryo. Thus, *Carm1* may be a full non-essential condition for mouse embryonic development.

Briefly, we elucidated the expression model of *Carm1* in mouse preimplantation embryos and found heterogeneous nuclear distribution of CARM1 at the late four-cell stage. The CARM1 heterogeneity was not predetermined in two-cell embryos. miR-181a was also a key regulator for heterogeneous distribution at the late four-cell stage, and CARM1 was an important protein for mouse preimplantation embryonic development, especially for blastocyst hatching.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0405>.

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

Y Z and H Z S designed and conceived the experiments; H Z S and J M S conducted the oocyte and embryo manipulations; H Z S, X P X, and J M S conducted the molecular experiments; H Z S, F Y W, and J C Z contributed to the bioinformatics analysis; H Z S, J K, and Y Y C conducted the cell manipulations; Y Z and H Z S wrote the manuscript, and all authors reviewed the manuscript.

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