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The Histone Demethylase JMJD2C Is Stage-Specifically Expressed in Preimplantation Mouse Embryos and Is Required for Embryonic Development[1]

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ABSTRACT

Epigenetic modifications play a pivotal role in embryonic development by dynamically regulating DNA methylation and chromatin modifications. Although recent studies have shown that core histone methylation is reversible, very few studies have investigated the functions of the newly discovered histone demethylases during embryonic development. In the present study, we investigated the expression characteristics and function of JMJD2C, a histone demethylase that belongs to the JmjC-domain-containing histone demethylases, during preimplantation embryonic development of the mouse. We found that JMJD2C is stage-specifically expressed during preimplantation development, with the highest activity being observed from the two-cell to the eight-cell stage. Depletion of JMJD2C in metaphase II oocytes followed by parthenogenetic activation causes a developmental arrest before the blastocyst stage. Moreover, consistent with a previous finding in embryonic stem (ES) cells, depletion of JMJD2C causes a significant down-regulation of the pluripotency gene *Nanog* in embryos. However, contrary to a previous report in ES cells, we observed that other pluripotency genes, *Pou5f1* and *Sox2*, are also significantly down-regulated in JMJD2C-depleted embryos. Furthermore, the depletion of JMJD2C in early embryos also caused significant down-regulation of the *Myc* and *Klf4* genes, which are associated with cell proliferation. Our data suggest that the deregulation of these critical genes synergistically causes the developmental defects observed in JMJD2C-depleted embryos.

developmental biology, early development, embryo, embryonic development, histone demethylase, JMJD2C, RNAi

INTRODUCTION

Epigenetic modifications, including DNA methylation, histone modifications, and X chromosome inactivation, have generally been accepted as important for early embryonic development. Within a short period of time following fertilization, the paternal genome has been found to be actively demethylated, and this phenomenon has been proposed to be important for the epigenetic reprogramming of condensed sperm chromosomes [1–4].

Histone modifications are catalyzed by different types of enzymes. Histone deacetylase 1 (HDAC1) has been found to play an important role during mouse embryo development [5]. Moreover, preimplantation development was greatly reduced when HDAC1 expression was down-regulated by RNAi [6]. Histone methylation is considered very stable and plays important roles in embryo development. Methylation of the core histone H3K9 is an important epigenetic modification that is involved in heterochromatin formation and repression of gene expression [7, 8]. Many histone methyltransferases responsible for mediating histone H3K9 methylation, including EHMT2 (G9a) and SUV39H, have been shown to be essential for embryo survival and the regulation of pluripotency genes.

Accumulating evidence has clearly shown that histone methylation is reversible, and many histone demethylases have been characterized by different laboratories [9–19]. However, very few studies have investigated the functions of the different histone demethylases during embryo development.

Demethylation of methylated histone H3K9 could be carried out by a group of histone demethylases, and these demethylases might play different roles in a variety of organisms. KDM3A (JHDM2A), which is one of the demethylases that could catalyze demethylation of dimethylated histone H3K9 [20], has recently been proven to be essential for maintaining male fertility and for inhibiting the development of obesity [21, 22].

The JMJD2 family of histone demethylases, including JMJD2A, JMJD2B, JMJD2C, and JMJD2D, demethylates trimethylated H3K9 and H3K36 [16, 17]. JMJD2C was first identified as an oncogene that is amplified in esophageal cancer cell lines [23] and is preferentially expressed in undifferentiated embryonic stem (ES) cells [24]. Overexpression of JMJD2C can delocalize HP1 and reduce heterochromatin in vivo, whereas inhibition of JMJD2C expression decreases cell proliferation [13].

Recently, evidence has shown that JMJD2C is a target gene of POU5F1 and that it regulates NANOG expression in ES cells to contribute to the maintenance of pluripotency [25]. Many important transcription factors are expressed in both early embryos and ES cells. Because the ES cell lines are derived from early embryos, i.e., the blastocyst stage, they might share similar pluripotency regulatory mechanisms. Nevertheless, early embryo development is a process that transmits totipotency to pluripotency, and intrinsic regulation plays a dominant role, whereas both intrinsic and external signals are important for the maintenance of pluripotency of ES

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cells [26], which may lead to subtle differences in gene regulation between early embryos and ES cells.

In the present study, we aimed to investigate the expression patterns of the JMJD2 family of histone demethylase, JMJD2Cc, and its function in early embryo development. The depletion of JMJD2C in preimplantation embryos, which is stage-specifically expressed, leads to an arrest in embryo development before the blastocyst stage. Moreover, we observed down-regulation of some pluripotency-related genes, including *Nanog* (which is similar to the result in ES cells), *Pou5f1* (which is different from the result in ES cells), *Sox2*, *Klf4*, and *Myc*.

MATERIALS AND METHODS

Animals and Chemicals

The specific pathogen-free grade hybrid mice, B6D2F1 (C57BL/6×DBA/2; Charles River), were housed in the animal facility of the National Institute of Biological Sciences in Beijing, China. All studies adhered to procedures that are consistent with the National Institute of Biological Sciences Guide for the care and use of laboratory animals. All inorganic and organic compounds were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

Oocyte Collection, Parthenogenetic Activation, Intracytoplasmic Sperm Injection, and Embryo Culture

Female B6D2F1 mice (8–10 wk old) were superovulated by sequentially injecting 7 IU each of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). Metaphase II (MII) oocytes collected 14 h after hCG injection were used for double-strand RNA microinjection. Parthenogenetic activation of the RNAi-injected MII oocytes was performed by culturing the oocytes in calcium-free Chatot-Ziomek-Bavister medium supplemented with 10 mM strontium and 5 μg/ml of cytochalasin B for 6 h. To investigate the expression pattern of histone demethylases in the oocytes and early embryos, the germinal vesicle stage oocytes were collected from the ovaries of female mice 44 h after eCG injection. To perform intracytoplasmic sperm injection (ICSI), a single sperm head was microinjected into the MII oocyte assisted with a Piezo-drill micromanipulator. To collect the zygotes, the successfully mated female mice were sacrificed 20 h after hCG injection, and zygotes were collected from the oviducts. The embryos, produced by either fertilization or parthenogenetic activation, were cultured in KSOM medium (MR-121-D; Chemicon).

Localization of JMJD2C in Preimplantation Mouse Embryos

To examine the localization of JMJD2C in mouse embryos, we first constructed an in vitro transcription vector for *Jmjd2c* with an EGFP tag driven by the T3 promoter. We used R1 ES cell cDNA as a template to amplify the *Jmjd2c* full-length coding sequence linked with a restriction enzyme site using the primer set 5′-CCGGCTCGAGATGGAGGTGGTGGAGGTGGA-3′ and 5′-CCGGGAGCTCCTGTCTCTTCTGACACTTCT-3′, and then ligated it into the pBT3 expression vector that contains a T3 promoter at the 5′ end and an EGFP tag at the 3′ end. The mMESSAGE mMACHINE Kit (Ambion) was used to carry out the in vitro transcription reaction. Following the Ambion kit protocol, 6 μl of the PCR template was used per 20-μl reaction. After *Jmjd2c_EGFP* was in vitro transcribed, 1 μl of TURBO RNase-free DNase was added to each 20-μl reaction, and the *Jmjd2c_EGFP* mRNA was microinjected into the cytoplasm of MII oocytes using a Piezo-drill micromanipulator. We examined the GFP signal using a live-cell imaging system (Olympus).

Double-Stranded RNA Preparation and Microinjection

The DNA template for *Jmjd2c* double-stranded RNA (dsRNA) was amplified from R1 ES cell cDNA with the primers 5′-AATTAACCCTCAC TAAAGGGAGACATGGAAGTAACCTTGAGCC-3′ and 5′-AAT TAACCCTCACTAAAGGGAGAACTTCATGAGAAGGGACACC-3′. The GFP dsRNA was amplified from the pEGFP-N1 vector by primers 5′-AATTAACCCTCACTAAAGGGAGAATGGTGAGCAAGGGCGAGGA-3′ and 5′-AATTAACCCTCACTAAAGGGAGATTACTTGTACAGCTCGTC CA-3′. Purification of the DNA template of dsRNA was performed using the PCR DNA and gel band purification kit (GE Health). The mMESSAGE mMACHINE Kit was used for the in vitro transcription reaction. Following the Ambion kit protocol, 6 μl of PCR template was used per 20-μl reaction. After the in vitro transcription reaction, 1 μl of TURBO RNase-free DNase was added to each 20-μl reaction. The dsRNA was aliquoted and stored at −80°C. Double-strand RNA was microinjected into the cytoplasm of MII oocytes using a Piezo-drill micromanipulator. Oocytes injected with GFP dsRNA and water were used as control.

RNA Extraction, PCR, and Real-Time RT-PCR

Oocytes and embryos (50–100) of different stages were collected for examination of the JMJD2C expression profile. About 150 embryos from both the dsRNA-mediated knockdown group and the control group were separately collected 48 h after injection. Total RNAs were extracted from the samples using the PicoPure RNA isolation kit (ARCTURUS). All the extracted RNAs were reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (18080–051; Invitrogen). To analyze the JMJD2C expression profile by PCR during mouse oocyte maturation and embryo development, the reverse-transcribed RNAs were used as templates in a PCR reaction containing TaKaRa Ex Hot Start Taq (TaKaRa DRR006A). To quantify the JMJD2C expression profile and the gene expression differences between the knockdown group and the control group, real-time RT-PCRs were performed on an ABI Prism 7500 using an SYBR Green Pre-mix (ABI). H2AFZ was used as control for the quality and amount of reverse-transcribed RNA. All the primers used in this study are listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org).

Immunofluorescent Staining

Oocytes and embryos were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature and then permeabilized with 1% Triton X-100 in PBS for 20 min. Then, all the samples were incubated in a blocking solution (3% bovine serum albumin in PBS) for at least 1 h. Immunocytochemistry staining was performed by incubating the fixed samples with primary antibodies for 60 min, followed by incubation with secondary antibodies conjugated to FITC for 60 min. Finally, the DNA was stained with 4′,6-diamidino-2-phenylindole (1 μg/ml), and all the samples were mounted on slides in anti-fade solution. At least 10 oocytes were processed for each separate sample, and the experiments were replicated at least three times. The slide-mounted, stained oocytes were observed on an LSM 510 META confocal microscope (Zeiss) using the excitation wavelengths of 488 nm and 405 nm. The channel signals were collected sequentially. For each experiment, the same detector gain, amplifier offset, and pinhole parameters were used. All collected images were assembled using Adobe Photoshop software (Adobe Systems) without adjusting the contrast and brightness of any of the images.

Western Blotting

To investigate the protein expression differences between the knockdown group and the control group at 82 h after hGC injection, total protein was collected from 250 embryos into SDS sample buffer and boiled at 100°C for 5 min. After being cooled on ice and centrifuged at 12 000 × g for 5 min, the samples were kept at −80°C until use. Total proteins were separated by SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane at 4°C. The transferred PVDF membrane was then blocked in Tris-buffered saline Tween-20 (TBST) buffer containing 5% nonfat milk overnight at 4°C. The blocked PVDF membranes were separately incubated with a 1:500 dilution of a mouse monoclonal anti-Oct3/4 antibody (SC-5279; Santa Cruz), a 1:2000 dilution of a rabbit polyclonal anti-trimethyl-histone H3 (Lys9; 07442; Upstate), and a 1:2000 dilution of a mouse monoclonal anti-α-tubulin (T6199; Sigma) overnight at 4°C. After three washes with TBST, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (NA931; GE) and an HRP-conjugated goat anti-rabbit secondary antibody (NA934; GE) for 2 h at room temperature. The membranes were extensively washed with TBST three times and then processed with the ECL detection system (Amersham).

Statistical Analysis

The Student t-test was used to evaluate the difference between groups, and differences of $P < 0.05$ were considered significant.

RESULTS

JMJD2C Is Stage-Specifically Expressed in Preimplantation Mouse Embryos

To determine the function of the JMJD2 family of histone demethylases in early embryonic development, the expression

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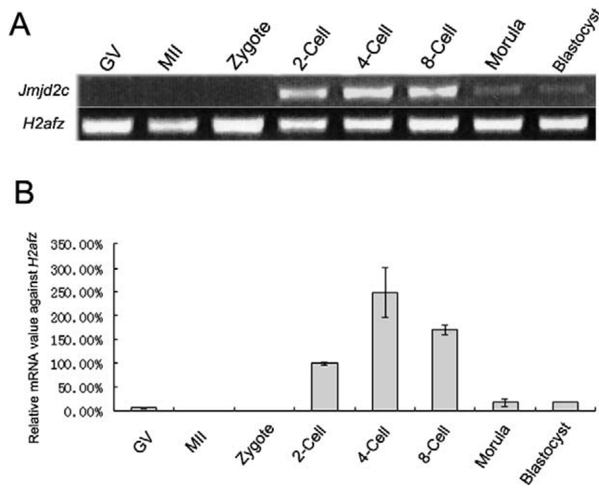

FIG. 1. Expression characteristics of *Jmjd2c* in early mouse embryonic development. **A**) PCR result of the *Jmjd2c* expression pattern during early mouse embryonic development. *H2afz* was used as a control gene to quantify the cDNA amount and quality. **B**) Real-time PCR was further applied to detect the *Jmjd2c* expression pattern during early mouse embryonic development. The levels of the transcripts were normalized against *H2afz*. Data are presented as the mean ± SEM.

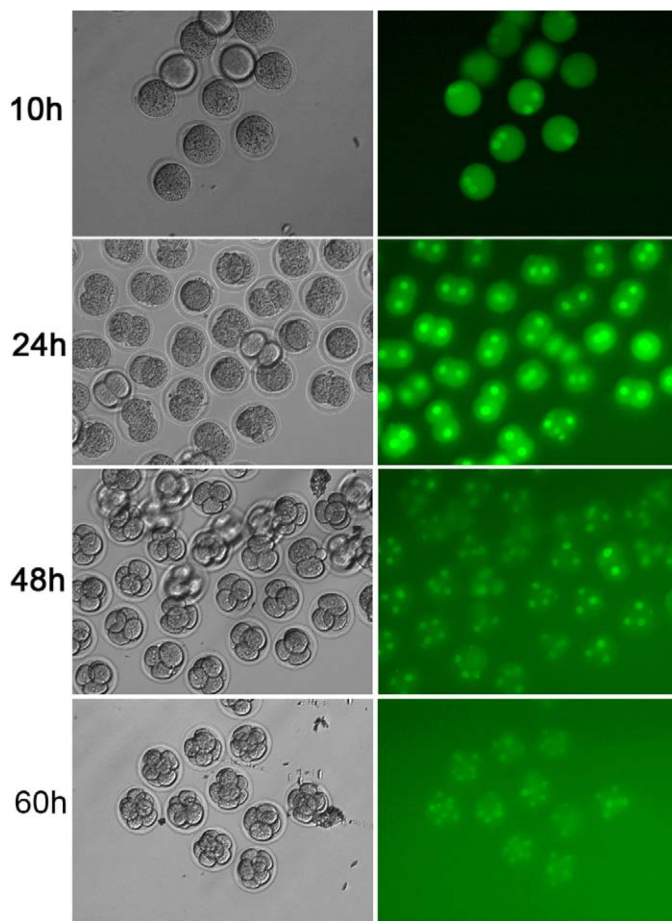

FIG. 2. Localization of JMJD2C in preimplantation mouse embryos. MII oocytes were injected with *Jmjd2c_EGFP* mRNA, parthenogenetically activated, then cultured for 10, 24, 48, or 60 h, and the GFP signal was visualized using a live cell-imaging system. JMJD2C could be observed localizing in the nuclei of early embryos. Original magnification ×200.

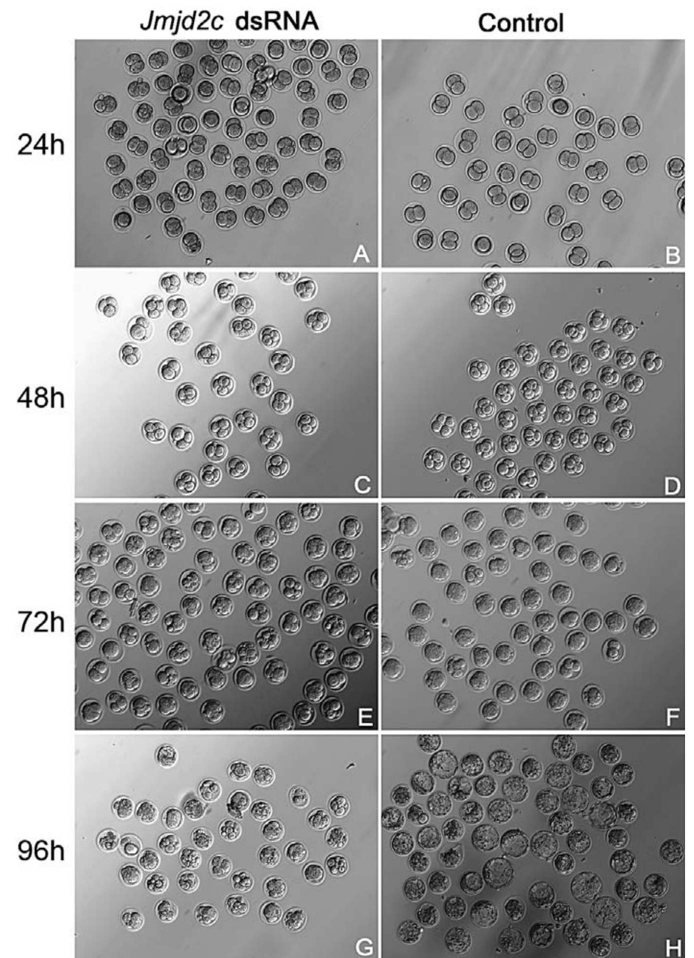

FIG. 3. Effect of RNAi-mediated depletion of *Jmjd2c* on early embryonic development. MII oocytes were injected with either water (control) or *Jmjd2c* dsRNA, parthenogenetically activated, then cultured for 24, 48, 72, or 96 h after injection and scored for development. **A**–**D**) No developmental difference could be observed between the dsRNA and the control groups until the four-cell stage. **E**–**H**) The dsRNA-knockdown embryos exhibited a serious developmental arrest from the eight-cell stage to the morula stage, and very few embryos in the knockdown group developed to the blastocyst stage. Original magnification ×100.

characteristics of these demethylases in preimplantation embryos were first investigated. As shown in Figure 1A, the RT-PCR results indicate that the expression of JMJD2C could be observed only from the two-cell stage. Furthermore, quantitative PCR (qPCR) data show that the *Jmjd2c* mRNA level increases at the two-cell stage and reaches a peak at the four-cell stage. Subsequently, the expression level of *Jmjd2c* decreases from the eight-cell stage and becomes expressed at a low level at the morula and blastocyst stages (Fig. 1B). Since no commercial antibodies are available for JMJD2C, we constructed a vector for in vitro transcription of a GFP-tagged *Jmjd2c* mRNA, which was then injected into the MII oocytes followed by pathenogenetic activation to observe the localization of JMJD2C in early embryos. As shown in Figure 2, localization of the JMJD2C protein in the pronuclei could be observed 10 h after activation. Subsequently, JMJD2C, which could be visualized by the GFP signal tag, was localized within the nuclei of the two- to eight-cell-stage embryos, though the fluorescence intensity became weaker along with cell division. Therefore, we concluded that, based on the live-cell imaging results, JMJD2C localizes to the nuclei during early embryonic

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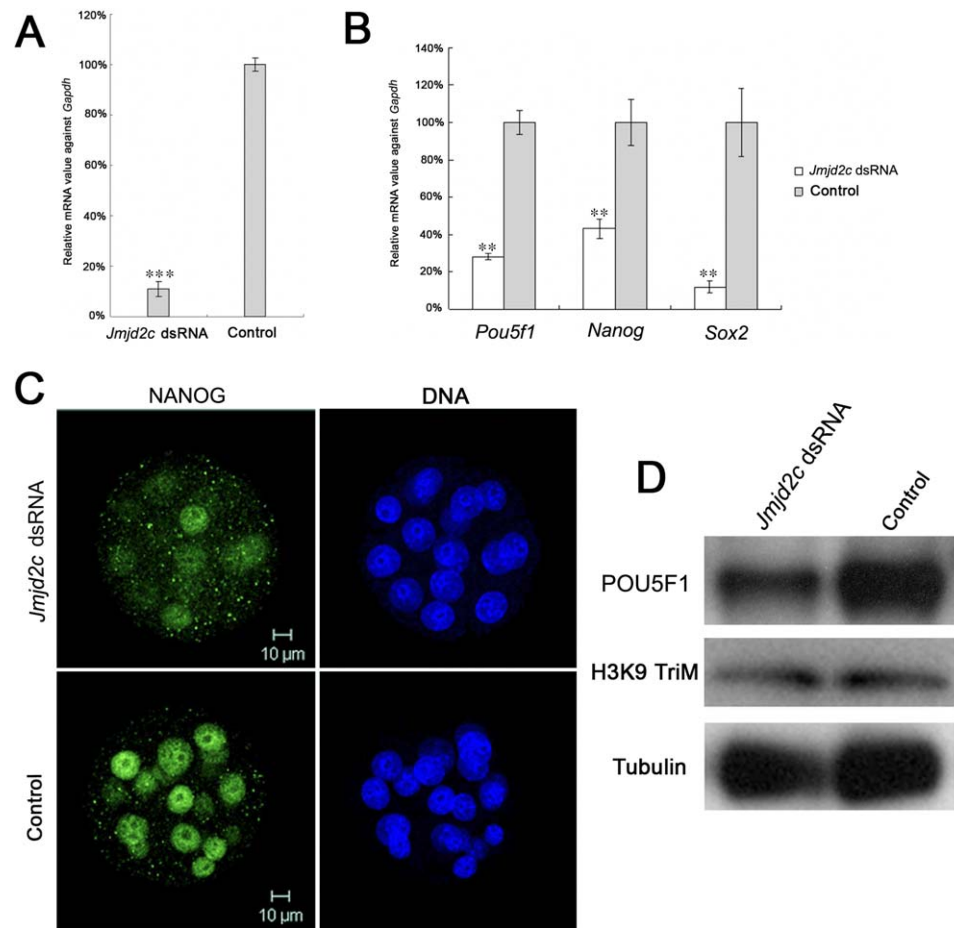

FIG. 4. Knockdown of *Jmjd2c* down-regulates the expression of the pluripotency genes *Pou5f1* and *Nanog* in early embryos. **A**) Quantitative real-time PCR analysis of *Jmjd2c* expression after knockdown using dsRNA targeting a *Jmjd2c*-specific region of the transcript. Forty-eight hours after the injection, the embryos were collected at the four-cell stage. The levels of the transcripts were normalized against *Gapdh*. Data are presented as the mean ± SEM. **B**) Real-time PCR analysis of pluripotency gene expression. The levels of the transcripts were normalized against *Gapdh*. Data are presented as the mean ± SEM. **C**) Immunostaining of the *Jmjd2c* dsRNA-knockdown and control embryos at the morula stage using an anti-NANOG antibody. **D**) Western blot analyses of the *Jmjd2c*-knockdown and control embryos using anti-POU5F1 and anti-H3K9Me3 antibodies. An anti-tubulin antibody was used for the loading controls. ***$P < 0.001$; **$P < 0.01$.

development. Based on the observed expression characteristics, we speculated that JMJD2C may play a role in early embryo development.

JMJD2C Is Required for Early Embryo Development

Next, we sought to investigate the function of JMJD2C in early embryo development. Gene silencing mediated by dsRNA was carried out in the present study using an established protocol [27]. The dsRNA, which was designed to the *Jmjd2c* coding sequence, was microinjected into the MII oocytes. Then, the surviving oocytes were parthenogenetically activated to initiate embryo development. As shown in Figure 3, A–D, no obvious difference was observed early on between the dsRNA-injected embryos and the control embryos, which were injected with either GFP dsRNA or distilled water. Most of the embryos continued to develop up to the four-cell stage with a normal morphology. However, the dsRNA-injected embryos exhibited a developmental arrest after the four-cell stage, and most of the embryos did not develop past the morula stage, whereas the control parthenotes developed to the blastocyst stage with high efficiency (Fig. 3, E–H). In fact, the mRNA expression of Jmjd2c was greatly reduced in the dsRNA-injected embryos (Fig. 4A). The results of co-injection of *Jmjd2c* mRNA and dsRNA also indicated that down-regulation of JMJD2C protein expression could be observed (Supplemental Fig. S1). As shown in Table 1, over 82% of the control embryos developed to the blastocyst stage, whereas only 13% of the dsRNA-injected embryos reached the blastocyst stage. The results indicate that JMJD2C plays an important role in early embryo development and that depletion of JMHD2C expression in early embryos impairs preimplantation embryo development. Similarly, we also investigated the embryo development of the *Jmjd2c* dsRNA-knockdown MII oocytes followed by ICSI. Our results clearly indicate that knockdown of *Jmjd2c* in fertilized embryos also impaired the early embryo development (Table 2).

JMJD2C Regulates NANOG Expression in Early Embryos

Based on a previous finding that JMJD2C could directly regulate NANOG expression in ES cells to maintain ES cell self-renewal, we next sought to determine whether JMJD2C also regulates NANOG expression in early embryos. The qPCR analysis shows that expression of *Jmjd2c* is greatly increased from the two-cell stage, and our previous data also indicates that expression of *Nanog* mRNA is initiated at the four-cell stage (data not shown). The expression data indicate that the expression of *Jmjd2c* and *Nanog* are temporally connected. Next, late four-cell-stage embryos produced from both *Jmjd2c* dsRNA-injected oocytes and control oocytes were collected to investigate the mRNA expression of *Nanog* by qPCR analysis. As shown in Figure 4A, more than 80% of the endogenous *Jmjd2c* mRNA was eliminated by the dsRNA, and, coincidently, *Nanog* expression was markedly decreased, which is consistent with the results observed in ES cells (Fig. 4B). As expected, the down-regulation of *Nanog* expression was also observed in the *Jmjd2c* dsRNA-injected eight-cell- and morula-stage embryos. Since the NANOG protein is detectable only from the eight-cell stage, we then performed immunofluorescent staining to detect whether the down-regulation of NANOG protein expression could be observed

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TABLE 1. Phenotype obtained following injection of *Jmjd2c* dsRNA into MII oocytes followed by parthenogenetic activation.

Group	No. of embryos	No. of blastocysts developed (%)*
Jmjd2c dsRNA	519	70 (13.5%)[a]
Water	417	344 (82.5%)[b]
GFP dsRNA	170	129 (75.9%)[b]

*The *P* value between a and b <0.001; the *P* value between water group and *GFP* dsRNA group >0.1

in the *Jmjd2c* dsRNA-injected morulae. As shown in Figure 4C, the NANOG staining fluorescence intensity was greatly reduced in the dsRNA embryos compared to the control embryos. All these data suggest that *Nanog* expression is regulated by the histone demethylase *Jmjd2c* in early embryo development, which is consistent with the previous findings in ES cells.

Knockdown of Jmjd2c *Down-Regulates* Pou5f1 *and* Sox2 *Expression*

It has previously been shown that *Jmjd2c* is a downstream target of the master regulator, *Pou5f1,* in ES cells, which in turn regulates NANOG activity [25]. Therefore, we sought to address whether the expression of *Pou5f1* and another pluripotency gene, *Sox2*, is affected in the *Jmjd2c* dsRNA knockdown embryos. By performing qPCR analyses, we found that the *Pou5f1* mRNA expression level decreased by more than 80% in the knockdown group than in the control embryos at the four-cell stage (Fig. 4B). To determine whether the POU5F1 protein expression was also decreased in the *Jmjd2c* dsRNA-knockdown embryos, we performed a Western blot analysis for POU5F1 protein expression in embryos. The result clearly showed that the POU5F1 protein level decreased dramatically in the dsRNA-knockdown embryos 82 h after dsRNA injection (Fig. 4D). We then investigated the expression of the other pluripotency gene, *Sox2*, in *Jmjd2c* dsRNA-knockdown embryos. Similar to the results obtained for *Pou5f1* expression, the *Sox2* mRNA expression was also seen to dramatically decrease in the embryos subjected to *Jmjd2c* dsRNA injection (Fig. 4B). Interestingly, similar to the results that have previously been published [13], the global H3K9me3 was not significantly affected by JMJD2C depletion (Fig. 4D). Similarly, global H3K36me3 showed no changes in the JMJD2C depletion embryos (Supplemental Fig. S2). Therefore, dsRNA injection-mediated JMJD2C depletion in early embryos results in a down-regulation of the expression of several pluripotency genes, including *Pou5f1*, *Nanog,* and *Sox2*.

Knockdown of Jmjd2c *Down-Regulates the Expression of Some Oncogenes*

Since *Jmjd2c*, which functions as a transcription regulator, was first identified as an oncogene that is frequently amplified

TABLE 2. Phenotype obtained following injection of *Jmjd2c* dsRNA into MII oocytes followed by ICSI.

Group	No. of embryos	No. of blastocysts developed (%)*
Jmjd2c dsRNA	67	14 (20.9%)
Water	66	34 (51.5%)

*The *P* value between the two groups <0.05.

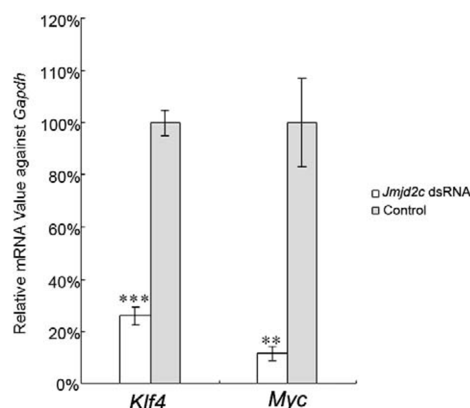

FIG. 5. Knockdown of *Jmjd2c* down-regulates the expression of the oncogene, *Myc*, and the transcription factor, *Klf4*, in four-cell-stage embryos. Quantitative real-time PCR was used to analyze the expression level of *Myc* and *Klf4* after knockdown by dsRNA targeting a *Jmjd2c*-specific region of the transcript. Forty-eight hours after injection, the embryos were collected at the four-cell stage. The levels of the transcripts were normalized against the control embryos injected with water. Data are presented as the mean ± SEM. ***$P < 0.001$; **$P < 0.01$.

in esophageal cancer cell line [23], we then sought to investigate whether the depletion of *Jmjd2c* in early mouse embryos could interfere with the expression of other oncogenes. We chose to investigate only the oncogenes that are highly expressed in early mouse embryos to more readily detect the effect of dsRNA depletion of *Jmjd2c* on the expression of these genes. By performing a qPCR analysis, we observed that *Klf4* expression is decreased by 75% in *Jmjd2c* dsRNA-knockdown embryos compared to the control embryos (Fig. 5). Moreover, expression of *Myc* is decreased by more than 90% in the *Jmjd2c*-knockdown embryos compared to the control embryos (Fig. 5). Down-regulation of these genes might, in part, contribute to the cell cytokinesis disruption that occurs in *Jmjd2c* dsRNA knockdown embryos.

DISCUSSION

In the present study, we investigated the expression profile and function of the histone demethylase JMJD2C in preimplantation mouse embryos. We showed that JMJD2C is stage-specifically expressed in preimplantation mouse embryos from the two- to eight-cell stage. Moreover, the depletion of *Jmjd2c* in early embryos by microinjection of dsRNAs leads to an embryo developmental arrest before the blastocyst stage. Consistent with a previous finding in ES cells, our data indicate that depletion of *Jmjd2c* markedly down-regulates *Nanog* expression in early embryos. Furthermore, two other pluripotency genes, *Pou5f1* and *Sox2*, and two cellular proliferation-related genes, *Klf4* and *Myc*, are also dramatically down-regulated in *Jmjd2c*-knockdown embryos. Similar developmental defects have been observed in the ICSI-produced embryos when Jmjd2c is depleted. Our study provides the first evidence to suggest that the histone demethylase JMJD2C plays a critical role in the preimplantation development of mouse embryos.

Depletion of gene-specific expression by dsRNA injection has frequently been applied to investigate the function of a gene in early embryo development [27]. For example, knocking down the expression of *Mos* in oocytes by dsRNA injection results in a premature extrusion of the second polar body, which is similar to the phenotype observed in null mutant mice. In the present study, our data clearly indicated

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that the expression of *Jmjd2c* could be efficiently knocked down using dsRNA-mediated gene silencing in early embryos.

Accumulating evidence indicates that epigenetic modifications play critical roles in mammalian embryo development. Core histone H3K9 methylation is an important epigenetic modification that is involved in heterochromatin formation and gene silencing. The histone methyltransferases, including EHMT2 and SUV39H, are responsible for catalyzing this modification. Knockout of *Ehmt2* leads to loss of H3K9 methylation in the euchromatin and a developmental and growth arrest at Embryonic Day 8.5 (E8.5) [28]. Double knockout of *Suv39h1* and *Suv39h2* results in a loss of H3K9 methylation in the heterochromatin, polyploidy in MEF cells, chromosome pairing defects during spermatogenesis, male sterility, and death of some of the double-mutant embryos at E14.5 [29]. More recently, many histone demethylases have been characterized and shown to be capable of removing H3K9 methylation. JHDM2A, an H3K9me2/1-specific demethylase, has recently been shown to play an important role in spermatogenesis. *Jhdm2a*-deficient mice exhibit postmeiotic chromatin condensation defects, and it has been found that JHDM2A directly binds to and controls the expression of the transition nuclear protein 1 (*Tnp1*) and protamine 1 (*Prm1*) genes, the products of which are required for the packaging and condensation of sperm chromatin [21]. Additionally, the loss of *Jhdm2a* function results in obesity and hyperlipidemia in mice [22].

The JMJD2 family of histone demethylases, including JMJD2A, JMJD2B, JMJD2C, and JMJD2D, belong to the JmjC-domain containing histone demethylases. This group of histone demethylases possesses the capacity to revert trimethylated H3K9 and H3K36 in mammalian cells. In the present study, we provide evidence that indicates that all the demethylases exhibit stage-specific expression characteristics during early embryonic development (Supplemental Fig. S3). However, only JMJD2C appears to play an important role in preimplantation embryonic development. The depletion of *Jmjd2c* by dsRNA in early embryos deteriorates embryonic development prior to the blastocyst stage. Consistent with a previous study in which *Jmjd2c* knockdown in ES cells resulted in decreased expression of *Nanog* and impaired self-renewal of the ES cells [25], depletion of *Jmjd2c* in early embryos markedly decreases the expression of *Nanog*. However, in contrast to a previous report in which *Pou5f1* was shown to directly regulate *Jmjd2c* in ES cells, down-regulation of two other important pluripotency genes, *Pou5f1* and *Sox2*, was also observed in *Jmjd2c*-depleted embryos. POU5F1, NANOG, and SOX2 form the pluripotency regulatory circuitry in ES cells, and we believe that a negative regulatory feedback must exist in early embryos. The decreased expression of *Nanog* might induce down-regulation of *Pou5f1* and *Sox2* synergistically. However, the mechanism by which this may occur is currently unknown and deserves further investigation in the future.

Apart from the pluripotency genes, the expression status of some of the critical oncogenes was also investigated in *Jmjd2c* depletion embryos, since *Jmjd2c* was originally discovered as an oncogene. Interestingly, we found that the expression level of *Myc* and *Klf4* is also significantly decreased after the depletion of *Jmjd2c* in early embryos. *Myc* was one of the first proto-oncogenes discovered in human cancers [30]. *Myc* functions are necessary and sufficient for the entry of most cells into the DNA synthetic phase of the cell cycle [31–33], and MYC family members are transcription factors that activate cell cycle progression, block cellular differentiation, and are implicated in many human malignancies [34]. Results from previous studies have shown that *Myc*–/– fetuses die by E10.5, with defects in growth and in cardiac and neural development [35]. However, studies employing in vitro culture of embryos have shown that inhibition of *Myc* expression from the two-cell stage causes embryonic developmental arrest at the eight-cell/morulae stage [36], which is similar to the phenotype that was observed in the present study when *Jmjd2c* was depleted in early embryos. KLF4 belongs to the Krüppel-like factors (KLFs), zinc-finger proteins that contain amino acid sequences that resemble those of the Drosophila embryonic pattern regulator, Krüppel [37]. KLF4 can function both as a tumor suppressor and an oncogene [38]. The expression level of *Klf4* is very high in early embryos, and the down-regulation of *Klf4* following depletion of *Jmjd2c* might be detrimental to cell cycle progression in early mouse embryos.

Taken together, our study provides the first evidence that indicates that the histone demethylase JMJD2C is stage-specifically expressed during preimplantation embryo development and that depletion of *Jmjd2c* in early embryos causes a developmental arrest before the blastocyst stage, though this effect might be indirect given that the global H3K9 methylation was not altered in the *Jmjd2c* knockdown embryos. The down-regulation of pluripotency genes, including *Nanog*, *Pou5f1*, and *Sox2*, and of cellular proliferation-related genes is proposed to, in part, contribute to the defects observed in the *Jmjd2c* knockdown embryos.

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