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EMBRYO BIOLOGY

Loss of methylation of *H19*-imprinted gene derived from assisted reproductive technologies can be mitigated by cleavage-stage embryo transfer in mice

Shuqiang Chen[1] · Meizi Zhang[2] · Li Li[1] · Ming Wang[1] · Yongqian Shi[1] · Hengde Zhang[1] · Bin Kang[1] · Na Tang[3] · Bo Li[1]

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Abstract

Purpose Studies on rodents have shown that assisted reproductive technologies (ARTs) are associated with perturbation of genomic imprinting in blastocyst-stage embryos. However, the vulnerable developmental window for ART influence on the genomic imprinting of embryos is still undetermined. The purpose of this study was to establish the specific embryonic development stage at which the loss of methylation of *H19* imprinting control regions (ICRs) was caused by ART occurrence. Additionally, we explored protocols to safeguard against possible negative impacts of ART on embryo *H19* imprinting.

Methods Mouse embryos were generated under four different experimental conditions, divided into four groups: control, in vitro culture (IVC), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). The methylation levels of *H19* ICR of the grouped or individual embryos were analyzed by bisulfite-sequencing PCR.

Results Our data showed that the loss of methylation of *H19* ICR in mouse blastocysts was inflicted to a similar extent by IVC, IVF, and ICSI. Specifically, we observed a significant loss of methylation of *H19* ICR between the mouse 8-cell and morula stages. In addition, we revealed that the transfer of mouse embryos generated by ARTs in the uterus at the 8-cell stage induced the occurrence of methylation patterns in the blastocysts closer to the in vivo ones.

Conclusions Our findings indicate that the loss of methylation of *H19* ICR caused by ARTs occurs between the 8-cell and the morula stages, and the transfer of cleavage embryos to the uterus mitigates the loss methylation of *H19* derived by mice ARTs.

Keywords Assisted reproductive technologies (ARTs) · Genomic imprinting · Imprinting control regions (ICRs) · Loss of methylation

Introduction

Genomic imprinting is a unique epigenetic phenomenon in which genes are expressed in a parent-of-origin-specific manner that only occurs in mammals [1, 2]. Imprinted genes are frequently found in clusters containing differentially methylated regions (DMRs) [1, 3]. For germline DMRs, DNA methylation is established during oogenesis or spermatogenesis and maintained upon the fertilization and throughout the development [1, 3]. Germline DMRs usually act as imprinting control regions (ICRs) that can function over a long-range distance to control the parental origin-specific gene expression/repression of imprinted genes in the cluster [1, 3]. Disruption of the ICR causes a loss of imprinting that can lead to changes in the expression of the imprinted genes, resulting in severe consequences for the development of the mammalian fetus and

Shuqiang Chen and Meizi Zhang contributed equally to this work.

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✉ Na Tang
tnlb2000@126.com

✉ Bo Li
lbtn2000@126.com

[1] Department of Obstetrics and Gynecology, Tangdu Hospital, the Fourth Military Medical University, Xi'an 710038, China

[2] Reproductive Medicine Center, Tianjin Frist Central Hospital, Tianjin 300192, China

[3] Shaanxi Institute for Food and Drug Control, Xi'an 710038, People's Republic of China

placenta [3, 4]. In humans, imprinting defects could cause the Prader–Willi syndrome (PWS), Beckwith–Wiedemann syndrome (BWS), Silver–Russell syndrome (SRS), and Angelman syndromes [3]. For example, normally, paternal ICR1 of the *H19/IGF2* cluster is hypermethylated, whereas maternal ICR1 is hypomethylated [3, 5]. Hypomethylation of *the H19/IGF2* ICR1 cluster occurs in SRS, whereas while hypermethylation of ICR1 is found in approximately 10% of the Beckwith–Wiedemann syndrome (BWS) patients [3, 5].

Assisted reproductive technologies (ARTs) include all fertility treatment and procedures in which both eggs and embryos are handled, and over six million children are born this way worldwide [6]. In general, ART procedures involve follicular stimulation, in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo in vitro culture (IVC), and vitrification coincide with the timing of DNA methylation reprogramming [7]. In humans, ART pregnancies are associated with a number of maternal and fetal health risks, including stillbirth, preterm birth, low birth weight, abnormal placentation, and other pregnancy complications [8, 9]. Studies of ART-borne children suggested that ART was associated with an increased incidence of epigenetics abnormalities [10, 11]. ARTs also have been linked to imprinting perturbations that lead to an increased risk of PWS, BWS, and Angelman syndromes [3]. Previous studies have demonstrated that imprinting is sensitive to environmental influences and can be disrupted during the preimplantation development by ARTs [12, 13]. Research in mouse models established that superovulation perturbed the genomic imprinting of both maternally and paternally expressed genes in blastocyst-stage embryos [14, 15]. In vitro culture of mouse preimplantation embryos resulted in a loss of imprinting of *H19*, small nuclear ribonucleoprotein N (*Snrpn*), and paternally expressed 3 (*Peg3*) at the blastocyst stage [16, 17]. IVF was found to change the DNA methylation of the growth factor receptor-bound protein 10 (*Grb10*) in mouse blastocysts [18]. Additionally, ICSI can cause a reduction in the number of trophoblastic and inner cell mass cells at the blastocyst stage and induce a loss of imprinting in multiple somatic tissues in adult mice [19, 20]. Together, these earlier human and mouse observations indicate that the embryo genomic imprinting is sensitive to ARTs and may have long-lasting harmful consequences. As the number of children born by ART continues to increase, it is critical to understand at which specific stage of the embryonic development the imprinting disorders caused by ART occur. Such findings would facilitate the development of new protocols that safeguard against possible negative impacts on embryo genomic imprinting.

In this study, using mouse as animal model and *H19* as methylation marker, we initially compared the effects of different ART procedures that included IVC, IVF, and ICSI on the *H19* and *Snrpn* imprinting at the blastocyst stage. Then, we explored at which specific stage of the embryonic development the loss of methylation of *H19* caused by ART occurred. We also analyzed whether loss of methylation of *H19* is a common phenomenon in blastocysts derived from ART and explored new protocols to safeguard against possible negative impacts in embryo *H19* imprinting. Our results showed that the loss of methylation of *H19* ICR in mouse blastocysts is inflicted to a similar extent by IVC, IVF, and ICSI. Specifically, we observed a significant loss methylation of *H19* at the morula stage. In addition, we established that the transfer of embryos generated by ART to the uterus at the 8-cell stage resulted in the occurrence of *H19* methylation patterns closer to those in in vivo blastocysts. These findings indicate that loss of methylation of *H19* caused by ARTs occurred between the 8-cell and the morula stages, and the transfer of cleavage embryos into the uterus may partly prevent aberrant *H19* imprinting derived from ARTs in mice.

Materials and methods

Animals and ethics

The SPF grade CD-1 (ICR) outbred mice were used for obtaining the embryos. All animals were provided with nesting material and housed in cages that were maintained under a constant 12-h light/dark cycle at 21 °C–23 °C with free access to food and water. The study was reviewed and approved by the Ethics Committee of Animal and Medicine of the Tangdu Hospital of The Fourth Military Medical University, Xi'an, China (Approval identification number: TDLL-2018-03-47) and was conducted in accordance with the guidelines of the Committee on the Use of Live Animals in Teaching and Research of the Tangdu Hospital of The Fourth Military Medical University.

Experimental design

Mouse embryos were generated under four different experimental conditions: (1) in vivo group (control group): eggs were fertilized and developed in vivo and then recovered by flushing the oviducts or uterine horns with modified human tubal fluid medium; (2) IVC group (in vitro culture group): eggs were fertilized in vivo, collected at the one-cell stage, and cultured in vitro in KSOM + AA (potassium simplex optimization medium with amino acids, Millipore); (3) IVF group (in vitro fertilization group): eggs were fertilized in vitro and then in vitro cultured in KSOM + AA; (4) ICSI group (intracytoplasmic sperm injection group): eggs were fertilized by ICSI and then in vitro cultured in KSOM + AA.

Embryos of the control group

CD-1 females were mated with CD-1 males, and mating was determined by the presence of a vaginal plug the following

morning (day 0.5). The 2-cell-, 8-cell-, and morula-stage embryos were flushed from the oviduct of the females at 48, 72, and 84 h post-hCG, respectively. The blastocyst-stage embryos were recovered by flushing from the uterus of the females at 96 h post-hCG.

Superovulation

For IVC-, IVF-, and ICSI-derived embryos, oocytes were obtained by superovulation. Females were superovulated by intraperitoneal injection of 5.0 IU pregnant Mare Serum Gonadotropin (PMSG, ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel). After 48 h, the mice were injected with 5.0 IU human chorionic gonadotropin (hCG, Millipore, Billerica, MA, USA).

In vitro fertilization

Conventional IVF was performed using human tubal fluid (HTF, Millipore, Billerica, MA, USA) medium as described previously [21]. Briefly, sperm from young adult male CD-1 males was squeezed out of freshly isolated mouse cauda epididymidis in HTF using a sterile forceps and allowed to "swim out" for at least 30 s in an incubator at 37 °C under 5.0% CO_2 and 95% humidified air for 1–2 h for capacitation in HTF. The preincubated and capacitated sperm suspension was added to the freshly ovulated cumulus–oocyte complexes to obtain a concentration of 1–2 × 10^6/mL of motile sperm, as determined using a hemocytometer. Then, the sperm and oocytes were co-cultured for 8 h in HTF at 37 °C in 5% CO_2 and 95% humidified air.

Intracytoplasmic sperm injection

ICSI was performed as described previously [22]. In brief, mature oocytes were collected from gonadotropin-stimulated CD1 females, and spermatozoa were recovered from the cauda epididymis of CD1 males and suspended in HEPES-CZB medium for 20 min. A drop of sperm suspension was then mixed with 12% (w/v) polyvinylpyrrolidone (PVP) in HEPES-CZB, and the head of a single sperm was detached from the tail using a Piezo-driven pipette (PrimTech, Ibaraki, Japan) and then injected into each oocyte. The microinjected oocytes were allowed to rest in the manipulation drop of M2 medium for at least 10–15 min. The embryos with transparent cytoplasm were judged to be survival and were then cultured in KSOM + AA.

Embryo culture

Further, 24 h post-hCG, in vivo fertilized eggs, in vitro fertilized eggs, or ICSI eggs, as determined by the presence of two pronuclei, were cultured under optimized culture conditions (KSOM + AA; Millipore) and their developmental efficiency assessed. The embryo was cultured at different embryonic stages in mineral oil at 37 °C with 20% O_2 and 5% CO_2.

Embryo transfer recipients

Six-week-old Kunming females were mated with vasectomized Kunming males 2 days before the embryo transfer. Mating was determined by the presence of a vaginal plug, and embryonic day zero (E0) was assumed to be midnight. In the morning after mating, females were checked for the presence of a vaginal plug, and its identification led to the prediction of pseudopregnancy of day 0.5. Then, the 8-cell embryos were transferred to the uterine horns of the pseudopregnant females on day 1.5, according to standard procedures.

DNA extraction and modification with sodium bisulfate

Embryos derived from the four groups were collected at different stages of embryonic development. Total DNA was extracted from 150 pooled 2-cell embryos, 80 pooled 8-cell embryos, 50 pooled morula embryos, or 30 pooled blastocyst embryos from the individual of the of IVC, IVF, ICSI, and control groups, respectively. The experiment was repeated three times. DNA extraction was performed for each individual sample using the QIAamp DNA Investigator Kit (Qiagen GmbH, Hilden, Germany). The extracted DNA was then treated and modified with sodium bisulfite procedure using the EpiTect Bisulfite kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Bisulfite converts unmethylated cytosines to uracil, whereas 5-methylcytosines (5-MeCs) remain unaltered. EpiTect Control DNA (human), unmethylated (Qiagen GmbH, Hilden, Germany), was used for standardized and reliable control reactions for each bisulfite conversion reaction. Only sequences with 95% of non-CpG cytosines converted, without unconverted cytosines adjacent to CpGs, were validated.

DNA isolation and bisulfite mutagenesis for individual embryos

Bisulfite mutagenesis with agarose embedding was conducted on single embryos as described previously [16]. Individual embryos were lysed with SDS lysis buffer. Next, the lysed embryos were embedded in 2% low-melting point agarose (Sigma) under mineral oil at 95 °C. DNA/agarose beads were allowed to solidify for 10 min on ice. Oil was removed, and denaturation of DNA was performed in 0.1 M NaOH (Sigma) at 37 °C for 15 min with shaking. Then, agarose beads were placed in 2.5 M bisulfite solution (0.125 M hydroquinone (Sigma), 3.8 g sodium hydrogen sulfite (Sigma), 5.5 mL of water, 1 mL 3 M NaOH) at 50 °C for 3.5 h to allow the occurrence of bisulfite mutagenesis. After the incubation, the agarose beads were washed once in TE pH 7.5 and

desulfonated with 0.3 M NaOH at 37 °C for 15 min with shaking. The agarose beads were washed three times with TE pH 7.5 and three times with sterile water. Further, the beads were incubated in oil at 65 °C, followed by the addition of approximately 60 μL of prewarmed double distilled water. The agarose beads were then mixed by pipetting, and 20 μL of diluted agarose was added to one Ready-To-Go PCR bead (GE) containing gene-specific primers and 1 μL of 240 ng/mL of tRNA as a carrier. PCRs were divided into three parts allowing three independent PCR reactions to be completed for each gene analyzed.

Methylation analysis

Bisulfite-treated DNA was subsequently used as a template for polymerase chain reaction (PCR) amplification. The nested primer sequences and the associated information for each gene are presented in Supplemental Table 1. The PCR program consisted of a denaturing step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 45 s at 55 °C, and 60 s at 72 °C, with a final extension of 5 min at 72 °C. The amplification products were purified using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Next, purified PCR products were cloned with the pBackZero-T Vector (TaKaRa Bio, Shiga, Japan), following the manufacturer's instructions. More than 50 clones were sequenced for each sample and gene analyzed to obtain a representative number of DNA strands. The methylation statuses of all CpGs present in the sequences were analyzed manually using BiQ Analyzer software (Max Planck Institute, Munich, Germany). The methylation levels across the region of analysis were determined by calculating the number of methylated CpG/total number of CpG for each individual CpG site as a percentage. Total DNA methylation for each gene was calculated as a percentage of the total number of methylated CpG/the total number of CpG dinucleotides.

Statistical analysis

For each group, the mean of the percentages obtained from the different stage embryos (the patterns calculated for each type of DNA methylation and then averaged) and the standard deviation (SD) were analyzed using Microsoft H Excel H analysis. Statistical analyses were performed using raw data (number of clones) obtained from different stage embryos of different groups using one-way ANOVA. The least significant difference (LSD) post-hoc test was employed to examine the significant differences between the groups. Results were considered to be statistically significant at $P < 0.05$.

Results

Loss of methylation of H19 ICR in blastocysts is inflicted to a similar extent by different ART procedures

We initially measured the methylation levels of 16 CpG units in *H19* ICR blastocysts derived from the control, IVC, IVF, and ICSI groups, respectively. We found that the mean methylation levels of the *H19* ICR were significantly decreased in the blastocysts from the IVC, IVF, and ICSI groups as compared with the control group (0.5053 ± 0.0422 vs. 0.2529 ± 0.0312, 0.2585 ± 0.0268, and 0.2272 ± 0.0162, respectively; $P < 0.05$; Fig. 1a, b). However, no significant difference was observed among the IVC, IVF, and ICSI groups ($P > 0.05$; Fig. 1a, b).

With regard to the 16 CpGs of the *Snrpn* ICR, the average methylation levels of the IVC, IVF, and ICSI groups were significantly decreased compared with the control group (0.4896 ± 0.006 vs. 0.43965 ± 0.0012, 0.42416 ± 0.01476, and 0.34375 ± 0.01068, correspondingly; $P < 0.05$; Fig. 2a, b), and the methylation level was significantly lower in the ICSI group than in the IVC and IVF groups ($P < 0.05$; Fig. 2a, b).

Loss of methylation of H19 ICR is a common phenomenon occurring in each blastocyst generated by ART

Next, we analyzed whether loss of methylation is a common phenomenon in blastocysts derived from ART. We assessed the methylation levels of the *H19* ICR in single blastocysts derived from the IVC by bisulfite mutagenesis with agarose embedding. We found that the mean methylation levels of CpG sites of the *H19* ICR in each IVC blastocysts were significantly lower than those of the control group (0.5053 ± 0.0422 vs. 0.2451 ± 0.0182, 0.2495 ± 0.0219, 0.2818 ± 0.0096, 0.2494 ± 0.0129, 0.2588 ± 0.0125, 0.2578 ± 0.0125, 0.2572 ± 0.0079, 0.2635 ± 0.0244, 0.3026 ± 0.0068, and 0.2919 ± 0.0147, respectively; $P < 0.05$; Fig. 3a–k). However, no significant difference was observed among the IVC blastocysts ($P > 0.05$; Fig. 3a–k). The results suggested that loss of methylation is a common phenomenon occurring in each blastocyst generated by IVC.

Significant loss of methylation of the H19 ICR derived from ART emerged at the morula stage

So far, however, the key developmental stage of ART-induced embryonic loss of methylation of imprinted genes was not clear. We initially measured the methylation levels of *H19* ICR in 2-cell embryos derived from the control, IVC, IVF,

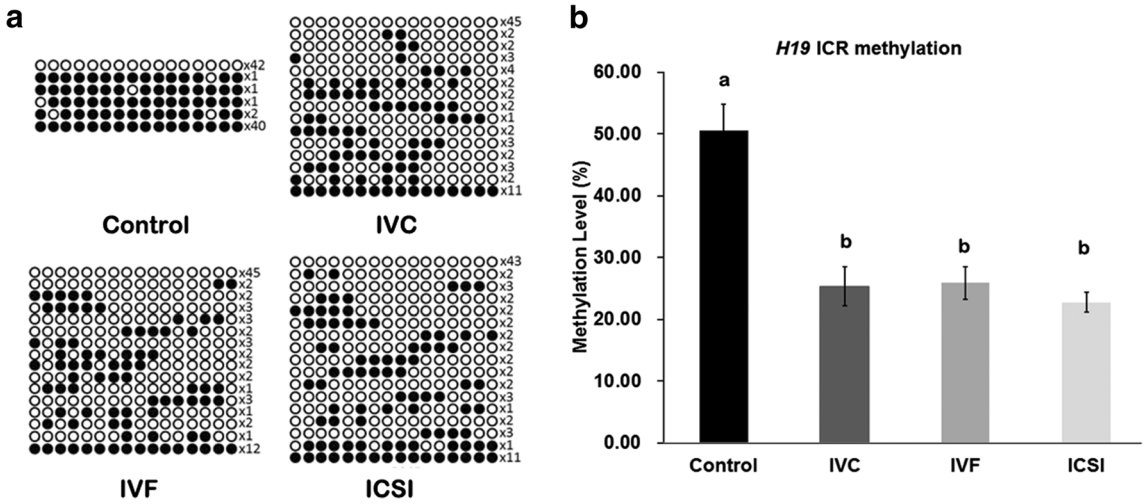

Fig. 1 Methylation of the *H19* ICR in mouse blastocysts. **a** The methylation status of *H19* in mouse blastocysts derived from the control, IVC, IVF, and ICSI groups. Filled circles represent methylated CpG dinucleotides, whereas the unfilled circles represent unmethylated CpGs. Each line denotes an individual strand of DNA. Clones with identical methylation patterns and non-CpG conversion rates representing the same DNA strand were included once. **b** The methylation levels of *H19* in mouse blastocysts derived from the control, IVC, IVF, and ICSI groups. The percentage of methylation is indicated above each set of DNA strands and was calculated as the number of methylated CpGs/total number of CpG dinucleotides. Bars marked with the same letter indicate no significant difference among the groups, whereas the bars marked without a same letter indicate that there is a significant difference ($P < 0.05$) between the groups

and ICSI groups. However, no significant difference among the four groups was established, as in the 2-cell embryos (0.5064 ± 0.0123 vs. 0.4966 ± 0.0113, 0.5038 ± 0.0079, 0.499 ± 0.01; $P > 0.05$; Fig. 4a, b). We compared the methylation levels of *H19* ICR in 8-cell embryos derived from the control, IVC, IVF, and ICSI groups. However, there was no significant difference among the four groups (0.4981 ± 0.0127 vs. 0.4912 ± 0.0115, 0.493 ± 0.0155, 0.4875 ± 0.0126; $P > 0.05$; Fig. 4a, c). As in the morula embryos, the mean methylation levels of the *H19* ICR in the control, IVC, IVF, and ICSI groups were 0.4978 ± 0.0157 vs. 0.378 ± 0.018, 0.3848 ± 0.0125, and 0.3701 ± 0.0103, correspondingly, and compared with the control group, the methylation levels of *H19* ICR in the IVC, IVF, and ICSI groups were significantly lower ($P < 0.05$; Fig. 4a, d). Nevertheless, there were no differences among the IVC, IVF, and ICSI groups. We observed similar DNA methylation patterns in the IVC, IVF, and ICSI groups, that is, the DNA methylation loss occurred in the morula embryo stage, with statistical significance ($P < 0.05$; Fig. 4a, d).

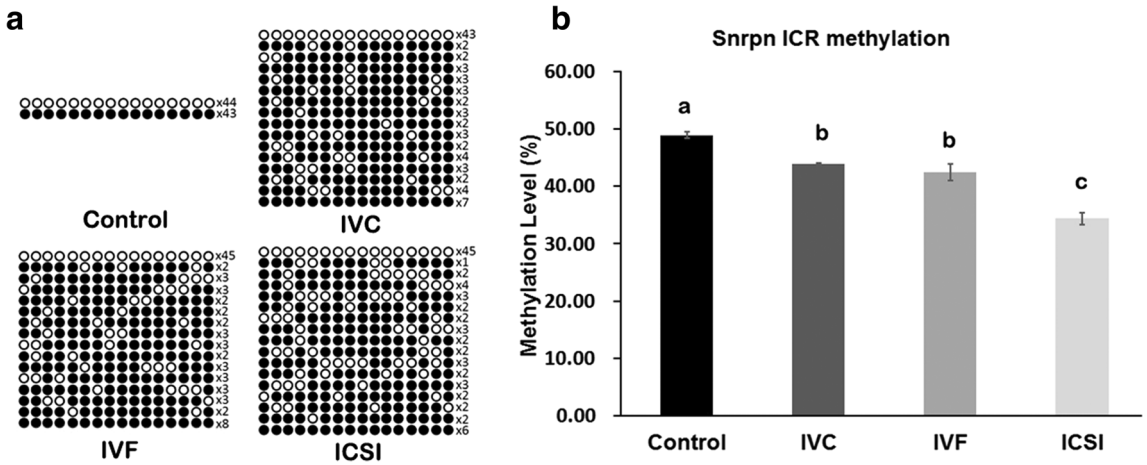

Fig. 2 Methylation of the *Snrpn* ICR in mouse blastocysts. **a** The methylation status of *Snrpn* in mouse blastocysts derived from the control, IVC, IVF, and ICSI groups. Unmethylated CpGs are represented as empty circles, whereas methylated CpGs are depicted as filled circles. Each line denotes an individual strand of DNA. Clones with identical methylation patterns and non-CpG conversion rates representing the same DNA strand were included once. **b** The methylation levels of *Snrpn* in mouse blastocysts derived from the control, IVC, IVF, and ICSI groups. The percentage of methylation is indicated above each set of DNA strands and was calculated as the number of methylated CpGs/total number of CpG dinucleotides. Bars marked with the same letter indicate the absence of significant difference among the groups, whereas the bars marked without the same letter indicate is the presence of a significant difference ($P < 0.05$) among the groups

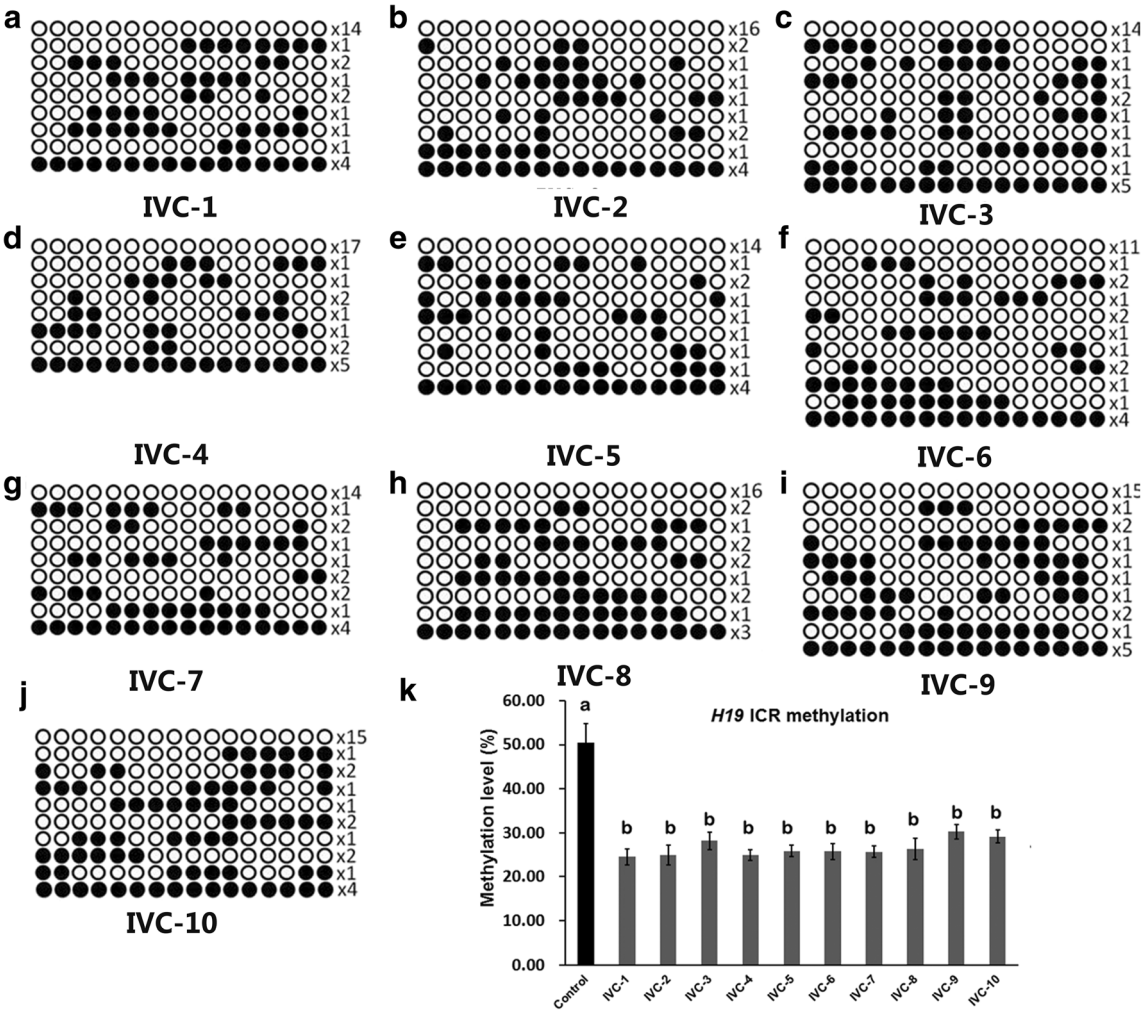

Fig. 3 Methylation of the *H19* ICR in each mouse blastocyst derived from IVC. **a**–**j** The methylation status of *H19* in each mouse blastocysts derived from IVC. Methylation status of individual DNA strands in the *H19* ICR in each blastocyst derived from IVC. Unmethylated CpGs are represented as empty circles, whereas methylated CpGs are depicted as filled circles. Each line denotes an individual strand of DNA. Clones with identical methylation patterns and non-CpG conversion rates representing the same DNA strand were included once. **k** The methylation levels of *H19* in each mouse blastocysts derived from IVC. The percentage of methylation is indicated above each set of DNA strands and was calculated as the number of methylated CpGs/total number of CpG dinucleotides. Bars marked with the same letter indicate the lack of significant difference among the groups, whereas the bars marked without the same letter denote the presence of significant differences ($P < 0.05$) among the groups

Transfer 8-cell embryos generated by ART to the uterus results in the methylation patterns of the H19 ICR closer to in vivo blastocysts

In addition, we transferred embryos generated by IVC to the uterus at the 8-cell stage (8-cell ET) to determine whether the loss of methylation in blastocyst caused by ART could be reduced by cleavage-embryo transfer (Fig. 5a). The methylation levels of *H19* ICR from two individual blastocysts produced by IVC or 8-cell ET were compared with their in vivo counterparts. We found the methylation level of *H19* ICR in 8-cell-ET group was significantly increased than the IVC group (0.4073 ± 0.022 vs. 0.2529 ± 0.0312, $P < 0.01$; Fig. 5b–d). Although the methylation level of *H19* ICR in 8-cell-ET group was still lower than that of the in vivo blastocysts (0.4073 ± 0.022 vs. 0.5053 ± 0.0422; $P < 0.05$; Fig. 5d), it was significantly higher than that of the blastocysts in the IVC group.

Discussion

ART has been correlated with aberrant methylation at the blastocyst stage [14, 15, 23, 24]. Using a mouse model, we demonstrated that the loss of methylation of *H19* in blastocysts is induced to a similar extent by IVC, IVF, and ICSI. Specifically, we observed a significant loss of methylation of *H19* between the 8-cell and the morula stages in ART embryos. In addition, we demonstrated that blastocysts derived from the transfer of the 8-cell-stage embryos generated by

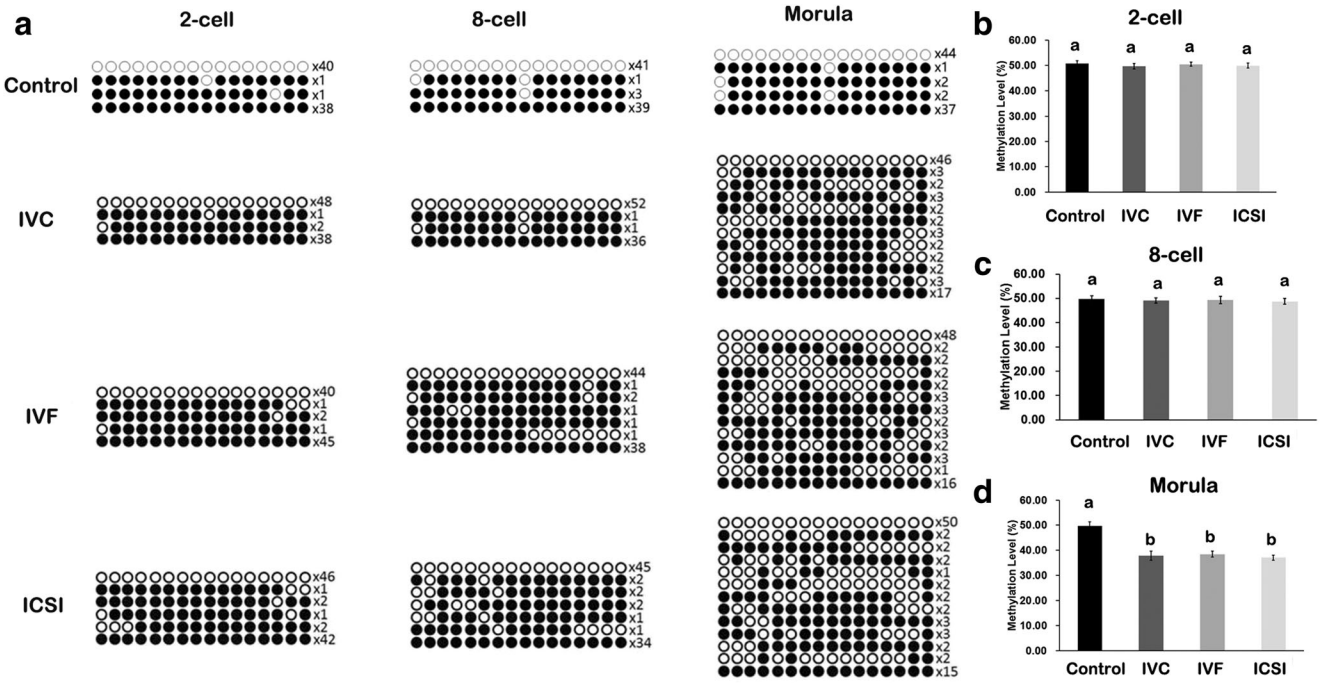

Fig. 4 Methylation of *H19* ICR in 2-cell, 8-cell, and morula-stage embryos derived from the control, IVC, IVF, and ICSI groups. **a** The methylation status of *H19* ICR in 2-cell, 8-cell, and morula-stage embryos derived from the control, IVC, IVF, and ICSI groups. Unmethylated CpGs are represented as empty circles, whereas methylated CpGs are depicted as filled circles. Each line denotes an individual strand of DNA. Clones with identical methylation patterns and non-CpG conversion rates representing the same DNA strand were included once. **b**–**d** The methylation levels of *H19* ICR in 2-cell, 8-cell, and morula-stage embryos were derived from the control, IVC, IVF, and ICSI groups. The percentage of methylation is indicated above each set of DNA strands and was calculated as the number of methylated CpGs/total number of CpG dinucleotides. Bars marked with the same letter indicate no significant difference among the groups while bars marked without a same letter indicate that there is a significant difference ($P < 0.05$) among the groups

ART to the uterus resulted in a higher methylation level of *H19* compared to IVC in the methylation. However, the methylation was still lower than that of the in vivo developed blastocyst. These results suggested that loss of *H19* methylation derived from assisted reproductive technologies could be mitigated by cleavage-stage embryo transfer in mice.

In addition, in vitro embryo culture has been demonstrated to induce perturbation of imprinted methylation in preimplantation embryos [14, 16]. Consistent with previous studies, we report loss of methylation at the *H19* and *Snrpn* ICR following in vitro culture to the blastocyst stage. Moreover, we found the mean methylation levels of the *H19* and *Snrpn* ICR were significantly decreased in blastocysts from the IVF and ICSI groups compared to the control group. We observed similar levels of *H19* ICR methylation loss following IVC, IVF, as well as ICSI. This suggests that the loss of *H19* ICR methylation may account for the disruption of imprinted methylation maintenance in the embryo culture rather than the process of fertilization. The methylation loss in *Snrpn* ICR was more pronounced in the ICSI group than in the IVC and IVF groups. These results indicate that the ICSI manipulation significantly reduced the methylation of the *Snrpn*, especially specifically as compared to that of IVC and IVF.

We found that the loss of methylation in *H19* was a common phenomenon that occurred for each blastocyst generated using IVC in mice. There is a common concern that DNA methylation errors could persists in individuals conceived using ART and eventually lead to long-term health risks. Studies have demonstrated that the DNA methylation errors persisted in the extraembryonic tissues (the placenta and yolk sac), but not in the fetal tissues at the midgestation stage of mice [17, 23, 25]. A study in bovine conceptuses also demonstrated that the majority of the methylation differences induced by ART occurred in the trophectoderm, with the embryonic disc showing almost no alterations [26]. A study to determine whether loss of methylation imprints occurs selectively in the trophectoderm (TE) demonstrated that the inner cell mass (ICM) had a similar loss in the methylation to DNA from intact blastocysts in mice [23]. Our study suggests that the loss of methylation may occur randomly in blastocysts, regardless of ICM or TE. A possible explanation for the loss of methylation that selectively occurs in the placenta and yolk sac rather than the fetus that was subjected to ART manipulations may be due to different DNA methylation dynamics between the embryonic and extraembryonic tissues [27, 28]. After implantation, de novo methylation was observed in the embryonic but not in the extraembryonic tissues; hence, the

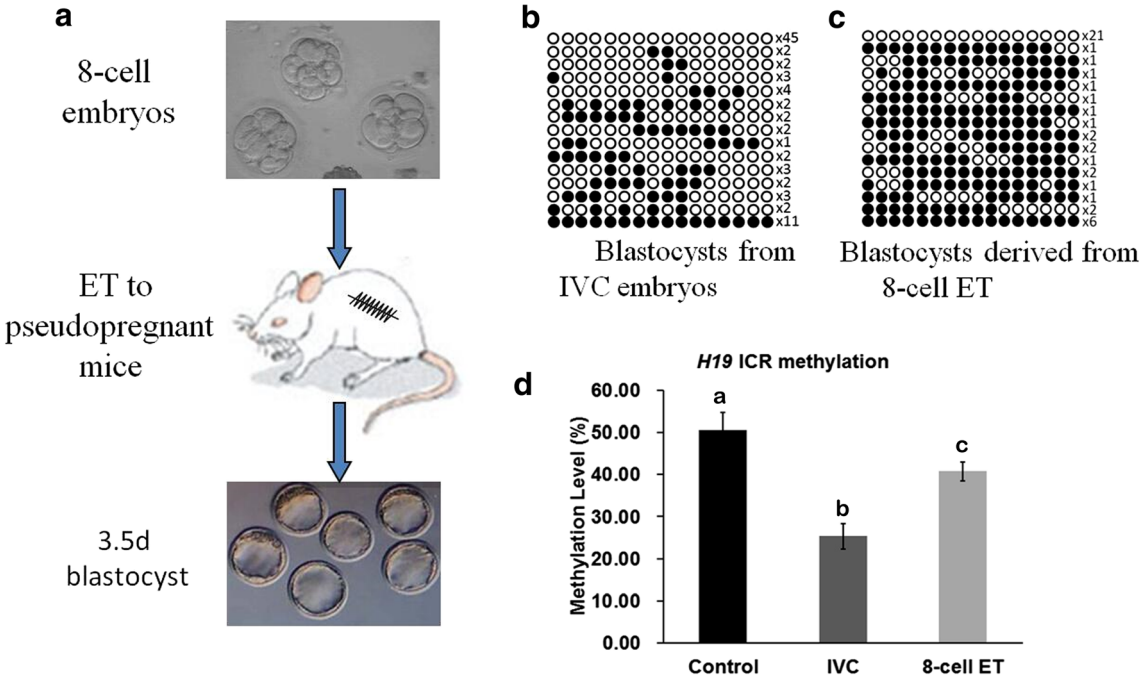

Fig. 5 Methylation of *H19* ICR in blastocysts derived from IVC or 8-cell ET. **a** Transfer 8-cell embryos generated by IVC to the uterus results in ET blastocysts. **b**, **c** The methylation status of *H19* ICR in mouse blastocysts derived from the IVC and 8-cell ET groups. Unmethylated CpGs are represented as empty circles, whereas methylated CpGs are depicted as filled circles. Each line denotes an individual strand of DNA. Clones with identical methylation patterns and non-CpG conversion rates representing the same DNA strand were included once. **d** The methylation levels of *H19* ICR in mouse blastocysts derived from the control, IVC, and 8-cell ET groups. Percent methylation is indicated above each set of DNA strands and was calculated as the number of methylated CpGs/total number of CpG dinucleotides. Bars marked with the same letter indicate no significant difference among the groups, whereas the bars marked without the same letter indicate that there is a significant difference ($P < 0.05$) among the groups

placenta and the yolk sac remain relatively hypomethylated as compared to the embryonic cells [27, 28].

The loss of methylation of imprinted genes is a common phenomenon observed in blastocysts generated using ART [14, 16]. However, the vulnerable developmental window in which ART can influence the methylation patterns of embryos is still unknown. Previous studies using limiting dilution bisulfite (pyro) sequencing have established no dramatic differences in the methylation of *H19*, *Snrpn*, and *Igf2r* between 2-cell mouse embryos derived from ART and non-ART [29]. Studies on the effects of superovulation on imprinted methylation revealed that imprinted methylation in oocytes was not perturbed. This suggests that superovulation did not induce imprinted methylation loss in mouse oocytes [15, 30]. However, studies have demonstrated that superovulation results in loss of imprinted methylation at the blastocyst stage in mice [14, 15]. This suggests that superovulation may affect imprinted methylation maintenance mechanism in preimplantation embryos which may result in loss of imprinted methylation of blastocysts in mice. We found that the imprinted methylation levels at the *H19* ICR were similar in 2-cell and 8-cell embryos in the control, IVC, IVF, and ICSI groups, in which the oocytes were obtained by spontaneous ovulation. However, compared to the control group, the methylation levels of *H19* ICR in the IVC, IVF, and ICSI groups were significantly lower at the morula stage. These results indicate that the decreased in the methylation levels of *H19* in the morula were caused by embryo manipulation that disrupted the imprinted methylation maintenance between the 8-cell and morula stages. However, there were no differences in *H19* methylation levels in the morula between IVC, IVF, and ICSI groups. This suggested that the different fertilization methods did not change the methylation levels of *H19*, suggesting that the vulnerable time window for ART to influence methylation of *H19* maybe between the 8-cell and morula stage. Whether loss of methylation of other imprinted genes induced by ART occurring at the same stage needs to be determined.

In the present study, the embryo culture was performed routinely at atmospheric oxygen concentration (20% O_2). However, the concentration of oxygen in the female reproductive tract was more likely from 2 to 8% [31]. In all species tested, including humans and mice, a lower oxygen concentration has been beneficial for the embryo culture, particularly when the embryos were cultured to the blastocyst stage [32, 33]. The use of oxygen as an electron acceptor results in production of more reactive oxygen species (ROS) and leads to oxidative stress [34]. Studies have shown that the ROS levels had a stepwise increase along with the elevation in the oxygen concentration in the embryo culture [35, 36]. Evidence exists

that the damage induced by supraphysiological ROS levels affects the DNA structure, which suggests that ROS could affect the epigenetic marks [34, 37]. Moreover, differences were found in the gene expression and DNA methylation in embryos cultured in 5% and 20% oxygen [38, 39]. Waal et al. found that placenta from embryos cultured at 20% O_2, but not at 5% O_2, had significantly different methylation patterns at the *Peg3* ICR from those of in vivo–conceived placenta [17]. However, concerning the *H19/Igf2* ICR, the embryos from both the 5% and 20% O_2 culture groups differed from the controls, with no significant differences between the embryos cultured at 5% and those at 20% oxygen concentration [17]. In addition, de Waal et al. found that culturing embryos at 20% O_2 did not induce a statistically significant more epigenetic abnormalities in the embryonic or placental tissues than those cultured at 5% O_2 [17]. Studies conducted by different groups at various oxygen concentrations established that culturing embryos at oxygen concentrations of 5% and 20% caused the loss of embryonic imprinting and led to similar abnormal phenotypes in the fetus and the placenta [14, 16, 21, 40]. These results suggested that the maintenance methylation at ICRs was not adversely impaired by a high oxygen tension during the embryo culture.

From the 8-cell to the morula stage, the rate of the cell division was found to increase concurrently with developmental events, such as compaction, cell polarity, and first lineage segregation [41]. In parallel, there was a significant elevation in the glucose consumption via aerobic glycolysis [34]. After compaction, the embryo underwent a transition from pyruvate-based metabolism, characterized by low oxidative levels, to a more active utilization of glucose, combined with an increase in oxygen consumption in response to the changes in its developmental needs [34]. Glucose, pyruvate, and oxygen consumption rose significantly with the morula compaction and blastocyst formation [42]. A growing body of evidence indicates that the culture environment influences the embryonic metabolism in significant subtle ways [34, 43]. Compared to in vivo embryos, in vitro embryos produce more lactate and have higher oxidative rates, which suggested the presence of higher metabolic rates of in vitro embryos [42, 44]. These studies indicated that embryo culture media might be stressful or might modify embryo metabolism to produce an abnormal profile [44, 45]. Emerging studies have shown that key metabolites, including those produced intracellularly or the ones imported from the culture media, can substantially influence histone and/or DNA epigenetic marks [43, 46]. Metabolic co-factors modulate the activity of epigenetic modifiers and have the capacity to establish long-lasting alterations to the epigenetic landscape [43, 46]. The loss of methylation of *H19* between the 8-cell and the morula stage may be partly due to changes in the embryo metabolism caused by the in vitro culture conditions.

As imprinting disorders have been linked to ART, it is essential to improve ART procedures to prevent epigenetic disorders. Animal studies have shown that fertilization and growing embryos in media supplemented with fluids from the female reproductive tract results in epigenetic patterns of embryos being closer to naturally conceived embryos [47]. This result indicates that secretions from the female reproductive tract minimize the imprinting disorders induced by ARTs. Our study revealed that although the methylation levels of *H19* ICR in the blastocysts of the 8-cell-ET group were lower than those in the in vivo–derived blastocysts, they were significantly higher than the IVC. These results indicate that transferring mouse embryos generated using ARTs to the uterus at the 8-cell stage could ameliorate the loss of *H19* methylation but cannot prevent the loss of methylation in blastocysts. The maintenance of methylation of imprinted genes at the 8-cell to morula stage may require fluids from the reproductive tract. Although the use of mouse models is valuable in the analysis of the effects of ART on genomic imprinting in oocytes and embryos, it is essential to assess their effects in humans. This is especially important as the epigenetic regulation mechanisms in gametes and early embryos are distinct between humans and mice [28]. Previous studies using human embryos have shown a high frequency of imprinted methylation errors in *SNRPN*, *KCNQ1OT1*, and *H19* in day-3 embryos and blastocysts. Both loss and gain of imprinted methylation were observed previously [48–50], which is different from the multi-locus loss of imprinting observed in mouse embryos. However, there were several limitations of the studies performed using ART human embryos. First, these investigations lacked naturally conceived controls, which was unavoidable. In addition, due to the limited availability of donor embryos, these examinations were conducted on a low number of embryos or on poor quality human embryos. The embryos analyzed were usually donated by infertile patients. Hence, it was difficult to distinguish between abnormal imprinted methylations caused by ART from the inherited. Embryos were usually transferred into the uterus at the cleavage or blastocyst stage in IVF clinics [51]. The live birth and clinical pregnancy rates were higher after blastocyst transfer than after cleavage embryo transfer [51]. Performing further analysis and comparison of imprinting errors between human cleavage and blastocyst embryos is needed to determine safer ART for the prevention of epigenetic disorders.

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Compliance with ethical standards

Ethical approval The present study was reviewed and approved by the Ethics Committee of Animal and Medicine of the Tangdu Hospital of The Fourth Military Medical University (Approval identification: TDLL-2018-03-47) and was conducted in accordance with the guidelines from the Committee on the Use of Live Animals in Teaching and Research of the Tangdu Hospital of The Fourth Military Medical University.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Peters J. The role of genomic imprinting in biology and disease: an expanding view. Nat Rev Genet. 2014;15(8):517–30.
2. Ferguson-Smith AC. Genomic imprinting: the emergence of an epigenetic paradigm. Nat Rev Genet. 2011;12(8):565–75.
3. Elhamamsy AR. Role of DNA methylation in imprinting disorders: an updated review. J Assist Reprod Genet. 2017;34(5):549–62.
4. Hamada H, Okae H, Toh H, Chiba H, Hiura H, Shirane K, et al. Allele-specific methylome and transcriptome analysis reveals widespread imprinting in the human placenta. Am J Hum Genet. 2016;99(5):1045–58.
5. Jurkiewicz D, Kugaudo M, Skorka A, Smigiel R, Smyk M, Ciara E, et al. A novel IGF2/H19 domain triplication in the 11p15.5 imprinting region causing either Beckwith-Wiedemann or Silver-Russell syndrome in a single family. Am J Med Genet A. 2017;173(1):72–8.
6. Hunter P. The long-term health risks of ART: epidemiological data and research on animals indicate that in vitro fertilization might create health problems later in life. EMBO Rep. 2017;18(7):1061–4.
7. van Montfoort AP, Hanssen LL, de Sutter P, Viville S, Geraedts JP, de Boer P. Assisted reproduction treatment and epigenetic inheritance. Hum Reprod Update. 2012;18(2):171–97.
8. Palomba S, Homburg R, Santagni S, La Sala GB, Orvieto R. Risk of adverse pregnancy and perinatal outcomes after high technology infertility treatment: a comprehensive systematic review. Reprod Biol Endocrinol. 2016;14(1):76.
9. Luke B. Pregnancy and birth outcomes in couples with infertility with and without assisted reproductive technology: with an emphasis on US population-based studies. Am J Obstet Gynecol. 2017;217(3):270–81.
10. El Hajj N, Haertle L, Dittrich M, Denk S, Lehnen H, Hahn T, et al. DNA methylation signatures in cord blood of ICSI children. Hum Reprod. 2017;32(8):1761–9.
11. Litzky JF, Deyssenroth MA, Everson TM, Armstrong DA, Lambertini L, Chen J, et al. Placental imprinting variation associated with assisted reproductive technologies and subfertility. Epigenetics. 2017;12(8):653–61.
12. Drobna Z, Henriksen AD, Wolstenholme JT, Montiel C, Lambeth PS, Shang S, et al. Transgenerational effects of bisphenol A on gene expression and DNA methylation of imprinted genes in brain. Endocrinology. 2018;159(1):132–44.
13. Menezo Y, Dale B, Elder K. The negative impact of the environment on methylation/epigenetic marking in gametes and embryos: a plea for action to protect the fertility of future generations. Mol Reprod Dev. 2019. https://doi.org/10.1002/mrd.23116.
14. Market-Velker BA, Fernandes AD, Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. Biol Reprod. 2010;83(6):938–50.
15. Velker BAM, Denomme MM, Krafty RT, Mann MRW. Maintenance of Mest imprinted methylation in blastocyst-stage mouse embryos is less stable than other imprinted loci following superovulation or embryo culture. Environ Epigenet. 2017;3(3):dvx015.
16. Market Velker BA, Denomme MM, Mann MR. Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. Biol Reprod. 2012;86(5):143 1-16.
17. de Waal E, Mak W, Calhoun S, Stein P, Ord T, Krapp C, et al. In vitro culture increases the frequency of stochastic epigenetic errors at imprinted genes in placental tissues from mouse concepti produced through assisted reproductive technologies. Biol Reprod. 2014;90(2):22.
18. Chen X, Huang Y, Huang H, Guan Y, Li M, Jiang X, et al. Effects of superovulation, in vitro fertilization, and oocyte in vitro maturation on imprinted gene Grb10 in mouse blastocysts. Arch Gynecol Obstet. 2018;298(6):1219–27.
19. de Waal E, Yamazaki Y, Ingale P, Bartolomei M, Yanagimachi R, McCarrey JR. Primary epimutations introduced during intracytoplasmic sperm injection (ICSI) are corrected by germline-specific epigenetic reprogramming. Proc Natl Acad Sci U S A. 2012;109(11):4163–8.
20. Giritharan G, Li MW, Di Sebastiano F, Esteban FJ, Horcajadas JA, Lloyd KC, et al. Effect of ICSI on gene expression and development of mouse preimplantation embryos. Hum Reprod. 2010;25(12):3012–24.
21. Chen S, Sun FZ, Huang X, Wang X, Tang N, Zhu B, et al. Assisted reproduction causes placental maldevelopment and dysfunction linked to reduced fetal weight in mice. Sci Rep. 2015;5:10596.
22. Yoshida N, Perry AC. Piezo-actuated mouse intracytoplasmic sperm injection (ICSI). Nat Protoc. 2007;2(2):296–304.
23. Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, et al. Selective loss of imprinting in the placenta following preimplantation development in culture. Development. 2004;131(15):3727–35.
24. Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D, et al. Assisted reproductive technology affects developmental kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos. BMC Dev Biol. 2007;7:116.
25. Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. Hum Mol Genet. 2008;17(1):1–14.
26. O'Doherty AM, McGettigan P, Irwin RE, Magee DA, Gagne D, Fournier E, et al. Intragenic sequences in the trophectoderm harbour the greatest proportion of methylation errors in day 17 bovine conceptuses generated using assisted reproductive technologies. BMC Genomics. 2018;19(1):438.
27. Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol. 2002;241(1):172–82.
28. Hanna CW, Demond H, Kelsey G. Epigenetic regulation in development: is the mouse a good model for the human? Hum Reprod Update. 2018;24(5):556–76.
29. El Hajj N, Trapphoff T, Linke M, May A, Hansmann T, Kuhtz J, et al. Limiting dilution bisulfite (pyro)sequencing reveals parent-specific methylation patterns in single early mouse embryos and bovine oocytes. Epigenetics. 2011;6(10):1176–88.
30. Denomme MM, Zhang L, Mann MR. Embryonic imprinting perturbations do not originate from superovulation-induced defects in DNA methylation acquisition. Fertil Steril. 2011;96(3):734–8 e2.
31. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil. 1993;99(2):673–9.

32. Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF. Reprod BioMed Online. 2016;32(2):137–41.
33. Kelley RL, Gardner DK. Individual culture and atmospheric oxygen during culture affect mouse preimplantation embryo metabolism and post-implantation development. Reprod BioMed Online. 2019;39(1):3–18.
34. Chason RJ, Csokmay J, Segars JH, DeCherney AH, Armant DR. Environmental and epigenetic effects upon preimplantation embryo metabolism and development. Trends Endocrinol Metab. 2011;22(10):412–20.
35. Belli M, Zhang L, Liu X, Donjacour A, Ruggeri E, Palmerini MG, et al. Oxygen concentration alters mitochondrial structure and function in in vitro fertilized preimplantation mouse embryos. Hum Reprod. 2019;34(4):601–11.
36. Goto Y, Noda Y, Mori T, Nakano M. Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radic Biol Med. 1993;15(1):69–75.
37. Bomfim MM, Andrade GM, Del Collado M, Sangalli JR, Fontes PK, Nogueira MFG, et al. Antioxidant responses and deregulation of epigenetic writers and erasers link oxidative stress and DNA methylation in bovine blastocysts. Mol Reprod Dev. 2017;84(12):1296–305.
38. Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertil Steril. 2006;86(4 Suppl):1252–65 65 e1–36.
39. Li W, Goossens K, Van Poucke M, Forier K, Braeckmans K, Van Soom A, et al. High oxygen tension increases global methylation in bovine 4-cell embryos and blastocysts but does not affect general retrotransposon expression. Reprod Fertil Dev. 2016;28(7):948–59.
40. de Waal E, Vrooman LA, Fischer E, Ord T, Mainigi MA, Coutifaris C, et al. The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model. Hum Mol Genet. 2015;24(24):6975–85.
41. Coticchio G, Lagalla C, Sturmey R, Pennetta F, Borini A. The enigmatic morula: mechanisms of development, cell fate determination, self-correction and implications for ART. Hum Reprod Update. 2019;25:422–38. https://doi.org/10.1093/humupd/dmz008.
42. Leese HJ. Metabolism of the preimplantation embryo: 40 years on. Reproduction. 2012;143(4):417–27.
43. Harvey AJ. Mitochondria in early development: linking the microenvironment, metabolism and the epigenome. Reproduction. 2019;157(5):R159–R79.
44. Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. BioEssays. 2002;24(9):845–9.
45. Scott R 3rd, Zhang M, Seli E. Metabolism of the oocyte and the preimplantation embryo: implications for assisted reproduction. Curr Opin Obstet Gynecol. 2018;30(3):163–70.
46. Zhang J, Zhao J, Dahan P, Lu V, Zhang C, Li H, et al. Metabolism in pluripotent stem cells and early mammalian development. Cell Metab. 2018;27(2):332–8.
47. Canovas S, Ivanova E, Romar R, Garcia-Martinez S, Soriano-Ubeda C, Garcia-Vazquez FA, et al. DNA methylation and gene expression changes derived from assisted reproductive technologies can be decreased by reproductive fluids. eLife. 2017;6. https://doi.org/10.7554/eLife.23670.
48. White CR, Denomme MM, Tekpetey FR, Feyles V, Power SG, Mann MR. High frequency of imprinted methylation errors in human preimplantation embryos. Sci Rep. 2015;5:17311.
49. Khoueiry R, Ibala-Romdhane S, Al-Khtib M, Blachere T, Lornage J, Guerin JF, et al. Abnormal methylation of KCNQ1OT1 and differential methylation of H19 imprinting control regions in human ICSI embryos. Zygote. 2013;21(2):129–38.
50. Ibala-Romdhane S, Al-Khtib M, Khoueiry R, Blachere T, Guerin JF, Lefevre A. Analysis of H19 methylation in control and abnormal human embryos, sperm and oocytes. Eur J Hum Genet. 2011;19(11):1138–43.
51. Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. Cochrane Database Syst Rev. 2016;6:CD002118.