Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Normal epigenetic inheritance in mice conceived by in vitro fertilization and embryo transfer*

Lei LI[1,2], Fang LE[1], Li-ya WANG[1], Xiang-rong XU[1], Hang-ying LOU[1], Ying-ming ZHENG[1], Jiang-zhong SHENG[3], He-feng HUANG[1], Fan JIN[†‡1]

([1]*Centre of Reproductive Medicine, Women's Hospital, School of Medicine, Zhejiang University, Hangzhou 310006, China*)
([2]*Department of Gynecologic Oncology, Henan Cancer Hospital, Zhengzhou 450003, China*)
([3]*Department of Cell Biology, School of Medicine, Zhejiang University, Hangzhou 310006, China*)
[†]E-mail: jinfan@zju.edu.cn

Received Nov. 28, 2010; Revision accepted July 27, 2011; Crosschecked Sept. 7, 2011

Abstract: An association between assisted reproductive technology (ART) and neurobehavioral imprinting disorders has been reported in many studies, and it seems that ART may interfere with imprint reprogramming. However, it has never been explored whether epigenetic errors or imprinting disease susceptibility induced by ART can be inherited transgenerationally. Hence, the aim of this study was to determine the effect of in vitro fertilization and embryo transfer (IVF-ET) on transgenerational inheritance in an inbred mouse model. Mice derived from IVF-ET were outcrossed to wild-type C57BL/6J to obtain their female and male line F2 and F3 generations. Their behavior, morphology, histology, and DNA methylation status at several important differentially methylated regions (DMRs) were analyzed by Morris water maze, hematoxylin and eosin (H&E) staining, and bisulfite genomic sequencing. No significant differences in spatial learning or phenotypic abnormality were found in adults derived from IVF (F1) and female and male line F2 and F3 generations. A borderline trend of hypomethylation was found in *H19* DMR CpG island 3 in the female line-derived F3 generation (0.40±0.118, P=0.086). Methylation status in *H19/Igf2* DMR island 1, *Igf2* DMR, KvDMR, and *Snrpn* DMR displayed normal patterns. Methylation percentage did not differ significantly from that of adults conceived naturally, and the expression of the genes they regulated was not disturbed. Transgenerational integrity, such as behavior, morphology, histology, and DNA methylation status, was maintained in these generations, which indicates that exposure of female germ cells to hormonal stimulation and gamete manipulation might not affect the individuals and their descendents.

Key words: Differentially methylated regions (DMRs), In vitro fertilization and embryo transfer (IVF-ET), Central nervous system (CNS), Neurobehavioral imprinting disorders, Transgenerational epigenetic inheritance
doi:10.1631/jzus.B1000411 **Document code:** A **CLC number:** R394

1 Introduction

A growing number of reports suggest that the use of assisted reproductive technology (ART), such as in vitro fertilization (IVF), could cause aberrant DNA methylation and result in de novo DNA methylation and histone methylation (Li *et al*., 2005). In particular, several reports have suggested that ART might lead to neurobehavioral imprinting disorders, including Angelman syndrome (Ørstavik *et al*., 2003), Beckwith-Weidemann syndrome (Cox *et al*., 2002), and Silver-Russell syndrome (Bliek *et al*., 2006). Some studies also demonstrated that exposure to endocrine disruptors such as vinclozolin, at generation age or during gonadal sex determination, could promote the development of a variety of different

‡ Corresponding author
* Project supported by the National Basic Research Program (973) of China (No. 2007CB948104), the National Natural Science Foundation of China (No. 81070532), and the Zhejiang Provincial Natural Science Foundation of China (No. Z207021)
© Zhejiang University and Springer-Verlag Berlin Heidelberg 2011

adult-onset diseases, including breast tumors, prostate diseases, kidney diseases, and immune abnormalities (Gluckman and Hanson, 2004; Anway *et al*., 2005; Schneider *et al*., 2008). These disease states can develop in a transgenerational manner (Painter *et al*., 2008).

It was stated that exposure to methoxychlor in the uterus resulted in decreased male fecundity in rats, as exemplified by a dearth of spermatogenic cells in the treated animal (Anway *et al*., 2006). The phenotype can persist into the next two generations. These animals have increased levels of cholesterol, enhanced tumor development, and abnormalities in several tissues. Notably, these effects correlate with altered DNA methylation in the male germ line, occurring in both non-coding and coding DNA sequences. These studies suggest that the epigenome, once affected by a detrimental environment, might escape the normal reprogramming that occurs early in development. These epigenetic changes can be transmitted to subsequent generations (Nilsson *et al*., 2008).

Concern should be raised over whether the situations mentioned above could happen to the human ART-born population. As the ART manipulation time coincides with how long gametic methylation imprints must be maintained, potential problems could arise as a result of hormonal stimulation, embryo manipulation, cryopreservation, or culture. Can imprinting disease or aberrant DNA methylation status caused by gamete manipulation be inherited by the next generation, even transgenerationally? To date, it seems that we know little about the molecular nature of transgenerational inheritance of the epigenetic state as related to ART (Chan *et al*., 2006; Hitchins *et al*., 2007). This is an under-investigated area of reproductive biology, which calls for more intensive research into the possible effects and for the recognition of this issue as an emergent problem.

In order to examine the possible transgenerational effects induced by IVF and to investigate whether controlled ovarian hyperstimulation (COH), early perturbations to embryo culture, and embryo transfer have unknown long-term consequences on neuro-behavioral, morphological, and DNA methylation inheritance, we established an IVF-born C57BL/6J mouse model. Behavior, morphology, and histology were examined in three consecutive generations. We also focused on the methylation statuses of several important differentially methylated regions (DMRs), aiming to verify the validity of the hypothesis mentioned above.

2 Materials and methods

2.1 Production of ART-born mice and their descendants

All treatment protocols were approved by the University Animal Care Committee according to institutional guidelines for animal experimentation. Six- to seven-week-old females C57BL/6J with a body weight of 20–25 g were superovulated by intraperitoneal injection of 10 U of pregnant mare serum gonadotropin (PMSG; GEN's, Hangzhou, China) followed by 10 U human chorionic gonadotrophin (HCG) 50 h later.

The epididymides were surgically isolated from eight- to ten-week-old males and placed in warmed modified human tubal fluid (MHTF) medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% synthetic serum supplement. After transfer to a new tube containing 1 ml human tubal fluid (HTF) (Irvine Scientific), the epididymal tissue was pressured to let the sperm swim out, and placed for 1.5 h in an incubator to allow capacitation. Next, 13–14 h after HCG injection, oocytes were dissected out of the ampulla region of the oviduct and washed with MHTF solution. Oocytes were then fertilized for 4–6 h (final sperm concentration: 1×10^6–2.5×10^6 ml^{-1}) and were moved to a fresh drop of media. Then 8–12 embryos at the morula stage were transferred into the uterine horns of a pseudopregnant foster mother at the third day of pseudopregnancy (3pdc), which was then mated to vasectomized males according to standard procedures (Chin and Wang, 2001).

To study possible transgenerational effects, F1 generation males or females were crossed with wild-type females or males in order to obtain F2 male and female line descendants. Outcrosses of the F2 generation to wild-type were used at the age of two months to obtain F3 generation. Naturally conceived mice were made by crossing one male and one female. All animals were housed, fed, and maintained under the same conditions with regulated light-dark cycles (14-h light/10-h dark, starting from 6:00 a.m. each day).

2.2 Spatial learning and behavioral studies

Spatial learning and behavioral studies were initiated at 6–7 weeks of age for each group (n=12) and were assayed by using the hidden platform version of the Morris water maze test (Zhenghua Biologic Apparatus Facilities Co., Ltd., Huaibei, China). A pool (150 cm in diameter, 50 cm in depth) was filled with 22–24 °C water to a depth of 30 cm. Water was rendered opaque with non-toxic white putty. The invisible white escape platform (16 cm in diameter, submerged 1 cm under water) was positioned in the center of the imaginary southwest quadrant, 20 cm from the wall, and remained in a fixed position. Subjects' movements were recorded by a computer connected to the video camera placed above the pool.

During each trial, one mouse was released into the water facing the wall of the pool from pseudo-randomly chosen cardinal compass points (North, East, South, and West). Randomization ensured that all positions were sampled before a given position was repeated. In all trials, mice were allowed to swim until 70 s (maximum latency) had elapsed. Starting the following day, trials were performed each day for five consecutive days (Morris, 1984; Luuk *et al*., 2009).

2.3 Morphological and histological analyses

At 40–42 d of age, eight mice from each generation, including four males and four females, were killed, and other mice were subsequently crossed to obtain the next generation. Some viscera, including the brain, heart, liver, lung, kidney, spleen, testis, and ovary, were excised and weighed. Samples were fixed in 10% buffered formalin and embedded in paraffin wax. Then, 4–6 mm thick sections were cut and stained with hematoxylin and eosin (H&E) and then observed under a light microscope.

2.4 Methylation analyses of *H19/Igf2* DMR, KvDMR, and *Snrpn* DMR

DNA was isolated from the cerebrum by proteinase K digestion followed by phenol chloroform extraction and ethanol precipitation (Qiagen, Valencia, CA, USA). MethPrimer software was used for the identification of CpG islands and designs of primers for *H19/Igf2* DMR island 1, *H19/Igf2* DMR island 3, *Igf2* DMR, *Snrpn* DMR, and KvDMR (Li and Dahiya, 2002). Then 1 μg DNA was processed for bisulfite sequencing analysis using the EpiTect Bisulfite kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. DNA amplification was performed in one or two rounds of polymerase chain reaction (PCR). PCR was performed in 25 μl reaction systems consisting of 80–100 ng of DNA, 2 mmol/L $MgCl_2$, PCR buffer, 0.2 mmol/L each of oligonucleotide primer (Sangon, Shanghai, China), and 1 U Hotsart DNA polymerase (TaKaRa, Dalian, Liaoning, China). Thermocycling conditions consisted of an initial 5-min denaturation at 94 °C, followed by 35 cycles of 94 °C for 45 s, 56 to 62 °C for 45 s, and 72 °C for 45 s, and finally a 10-min extension at 72 °C. PCR products were gel-purified and ligated into the pMD19-T simple vector (TaKaRa) at 16 °C for 2 h in accordance with the manufacturer's instructions. Plasmids were transformed into competent *Escherichia coli* DH-5α. Transformed bacteria were spread onto lysogeny broth (LB) agar plates containing 100 μg/ml ampicillin and 50 μl of 10 mg/ml X-gal, and incubated overnight at 37 °C. A single strain was inoculated into 2 ml LB liquid medium containing 100 μg/ml ampicillin and grown overnight. In this study, 10–20 samples were sequenced from each mouse that showed the expected band size. Primer sequences for the DMRs are shown in Table 1.

2.5 Quantitative real-time reverse transcription (RT)-PCR

Allelic expressions of seven imprinted genes regulated by the sequenced DMRs were further determined (n=12 each group). Three of these genes, namely *H19*, *Cdkn1c*, and *Ube3a*, are expressed from the maternal alleles, whereas *Igf2*, *Snrpn*, *Kcnq1ot1*, and *Peg3* are expressed from the paternal chromosomes. Total RNA was prepared from cerebrum using the TaKaRa RNeasy® Mini kit according to the provided protocol. cDNA was synthesized by reverse transcription of 500 ng of total RNA using an oligodeoxythymidine primer and the TaKaRa RT kit. Real-time PCR was carried out in a 10-μl reaction system containing 5 μl SYBR Premix Ex Taq™, 0.2 μl PCR forward primer (10 μmol/L), 0.2 μl PCR reverse primer (10 μmol/L), 0.2 μl ROX reference dye, and 1 μl of the cDNA sample. Amplification was carried out on an ABI7900 instrument. After an initial denaturing step at 95 °C for 10 min, PCR was

performed for 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Expressions were calculated by using *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) as a reference gene. The fold-change was calculated using the comparative cycle threshold (C_T) method. The data were expressed as mean±standard deviation (SD). Primer sequences for these imprinting genes are shown in Table 2.

2.6 Statistical analysis

All computations were analyzed using SPSS software (Version 17.0 for Windows; SPSS, Chicago, IL). Fisher's exact test and chi-square test were used to compare the proportion of outliers, and median methylation values were compared with one-way analysis of variance (ANOVA) (Dunnett or SNK tests). Box plots were generated using the default parameters of SPSS. A *P*-value of 0.05 was considered to be statistically significant.

3 Results

3.1 Spatial learning, memory test, and morphological and histological investigations

With shortened latencies in the Morris water maze, all groups of mice were able to swim normally and located on the hidden platform during each trial. No differences in spatial learning memory ability as assayed by the Morris water maze test were found in adults derived from IVF (F1 generation) and male or female line-derived F2 and F3 generations as compared with adults conceived naturally (P>0.05) (Fig. 1). Morphologic and routine histological studies were performed on F1–F3 generations, and results were compared with those of naturally conceived mice. No obvious phenotypic abnormality or defect, such as small eye, brachyury, short ear, or extra toes, was observed from Weeks 1 to 7. No statistically significant reduction or increase in the weight of

Table 1 Differentially methylated regions (DMRs) and primer sequences used for bisulfite sequencing

Gene	Primer sequence	PCR product size (bp)	CpG number
H19/Igf2 DMR island 1	F: 5′-TTTTTGGGTAGTTTTTTTAGTTTTG-3′	153	7
	R: 5′-ACACAAATACCTAATCCCTTTATTAAAC-3′		
H19/Igf2 DMR island 3	F: 5′-AAGGAGATTATGTTTTATTTTTGGA-3′	211	12
	R: 5′-CCCACAACATTACCATTTATAAATTC-3′		
Igf2 DMR	F: 5′-TGTTTTGTGGAATTTTTAGGTAGGT-3′	238	13
	R: 5′-CCCCAAATCAAAAAATAAATAATCTC-3′		
KvDMR	OF: 5′-GTGTGATTCTACTTGGAGAG-3′	481	30
	OR: 5′-GTGGCCAGCACCAAGGTAGTAGTGAGTGG-3′		
	IF: 5′-GGTTAGAAGTAGAGGTGATT-3′		
	IR: 5′-ATAGAAGTAGGGGTGGTTTTG-3′		
Snrpn DMR	OF: 5′-TATGTAATATGATATAGTTTAGAAATTAG-3′	419	16
	OR: 5′-AATAAACCCAAATCTAAAATATTTTAATC-3′		
	IF: 5′-AATTTGTGTGATGTTTGTAATTATTTGG-3′		
	IR: 5′-ATAAAATACACTTTCACTACTAAAATCC-3′		

F: forward; R: reverse; OF: outside forward; OR: outside reverse; IF: inside forward; IR: inside reverse

Table 2 Investigated imprinting genes that DMRs controlled and their real-time RT-PCR primers

Gene	Accession number	Primer sequence	Product size (bp)
H19	NR_001592.1	F: 5′-GCACTAAGTCGATTGCACTGG-3′	193
		R: 5′-GCCTCAAGCACGGCCACA-3′	
Igf2	NM_010514.2	F: 5′-AGCCCATTTGGCTGGACA-3′	135
		R: 5′-AGGTGCCTGCATCAAGGTGAC-3′	
Kcnq1ot1	NM_008434.2	F: 5′-GGGTAGAGCCTGACTCCTTCATTC-3′	115
		R: 5′-TAGGGTGGACAGTGGACAATCC-3′	
Peg3	NM_008817.2	F: 5′-AAGCCCTTGGGTGTGAGCA-3′	131
		R: 5′-CCACTTCGGCTCATGTCGTC-3′	
Ube3a	NM_011668.2	F: 5′-ACCGAATGGCCACAGCTTGTA-3′	147
		R: 5′-TGCAGGCCTCATTTCCACAG-3′	
Snrpn	NM_013670.3	F: 5′-GGATTAGCAGGCCCTGTCGA-3′	131
		R: 5′-TGCCTACAGGTGGAGGTGGA-3′	
Cdkn1c	NM_009876.4	F: 5′-AGAGAACTGCGCAGGAGGAC-3′	127
		R: 5′-TCTGGCCGTTAGCCTCTAAA-3′	
GAPDH	NM_008084	F: 5′-TGACGTGCCGCCTGGAGAAA-3′	98
		R: 5′-AGTGTAGCCCAAGATGCCCTTCAG-3′	

total body compared with wild-type littermates was observed at six weeks of age. Organs including the brain, heart, liver, lung, kidney, spleen, testis, and ovary were grossly normal on histological analysis and microscopic studies using formalin-fixed paraffin sections (data not shown).

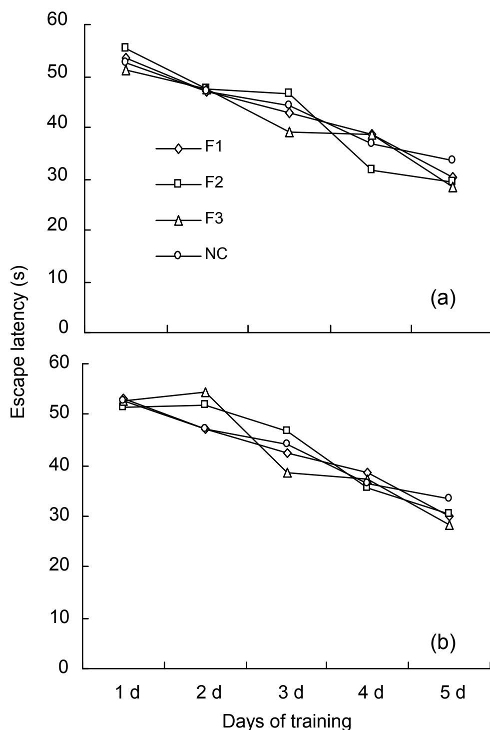

Fig. 1 Spatial learning and memory test
Mice derived from IVF and their female (a) and male (b) line-derived F2 and F3 generations exhibit normal spatial learning, as determined by using the hidden platform version of the Morris water maze (n=12). NC: naturally conceived mice

3.2 Bisulfite genomic sequencing

Bisulfite treatment and PCR amplification were followed directly by sequencing in the F1 generation, male and female line-derived F2 and F3 generations, and the control group. We analyzed paternally methylated *H19/Igf2* DMR island 1, *H19/Igf2* DMR island 3, and maternally methylated *Igf2* DMR, *Snrpn* DMR, and KvDMR. *H19* DMR is unmethylated on the maternal allele, which allows binding by the insulator protein CCCTC-binding factor (CTCF). CTCF blocks enhancer access to the *Igf2* promoter, resulting in the silencing of *Igf2* on the maternal allele. There are three CpG islands at *H19* DMR, among which island 3 serves as the binding site. For each DMR, a number of CpG loci varying in length between 7 and 30 sequences were analyzed: 7 CpG loci located in *H19* DMR CpG island 1, 12 CpG loci located in *H19* DMR CpG island 3, 13 CpG loci located in *Igf2* DMR, 30 CpG loci located in KvDMR, and 16 CpG loci located in the *Snrpn* DMR. The amount of methylated CpGs in the F1–F3 generations and the control was close to 50% of the total methylated+unmethylated CpGs in all DMRs tested, which was in agreement with the theoretical value expected for DMRs, as DNA methylation only presents in one of the two parental alleles.

The methylation statuses and the numbers of methylated CpGs in F1 and female line-derived F2–F3 generations at these DMRs were comparable to the levels observed in controls, except for *H19* DMR CpG island 3 and *Igf2* DMR, which revealed a tendency towards hypomethylation in the F3 and F1 generations (0.40±0.118 and 0.42±0.065, respectively), but statistical analysis could not reveal significant differences in the methylation percentage (P=0.244 and P=0.086, respectively; Fig. 2). This hypomethylation tendency disappeared from the F2 and F3 generations in *Igf2* DMR.

To further investigate the methylation patterns of imprinted genes in the male germ-line, the outcross of IVF-born F1 generation males with a wild-type female was performed. The methylation patterns of the five target regions mentioned above were analyzed. The amount of methylated CpGs was close to the theoretical value of 50% of the total CpGs in all the control regions. Methylation index did not differ from that of the control offspring, revealing little variability in methylation pattern at these DMRs. The median methylation percentages of DMRs are shown in Table 3.

3.3 Real-time RT-PCR for DMR-regulated gene expression

The expressions of genes regulated by these DMRs (*H19*, *Igf2*, *Kcnq1ot1*, *Cdkn1c*, *Ube3a*, *Snrpn*, and *Peg3*) were analyzed by real-time RT-PCR. For example, on the maternal allele where KvDMR is normally methylated, the *Kcnq1ot1* gene is silenced, thereby allowing the expressions of the flanking genes. It is noteworthy that a good correlation was found between the expression level of the imprinted genes analyzed and the expression level of the control

gene *GAPDH*. We failed to identify evidence of perturbation in the expressions of other imprinted genes analyzed in brain tissues from the F1 generation or female and male line-derived F3 generation, as our results indicated that expression did not vary (P>0.05; Fig. 3). The expression patterns correlated with the methylation status in these generations, which further validated the sequenced DMR data. This result indicates that effects of the IVF procedure on expression may be trifling and that the tiny differences in DNA methylation do not affect the expression they control and regulate among IVF-conceived offsprings, which likely reflects the general health of the descendants. The data are expressed as means of fold change±SD from three independent RNA preparations.

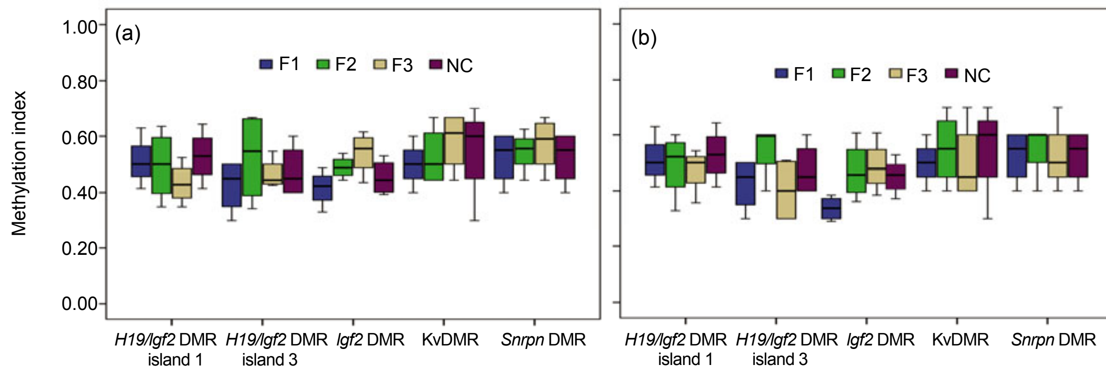

Fig. 2 Bisulfite genomic sequencing of DMRs

The box-plot representations of the methylation patterns of paternally methylated *H19/Igf2* DMR island 1 and island 3 and maternally methylated *Igf2* DMR, *Snrpn* DMR, KvDMR in F1, female (a) and male (b) line-derived F2 and F3 generations, and naturally conceived (NC) mice. The median is represented by horizontal lines. The bottom of the box indicates the 25th percentile and the top the 75th percentile. No significant differences were observed in methylation degree (P>0.05)

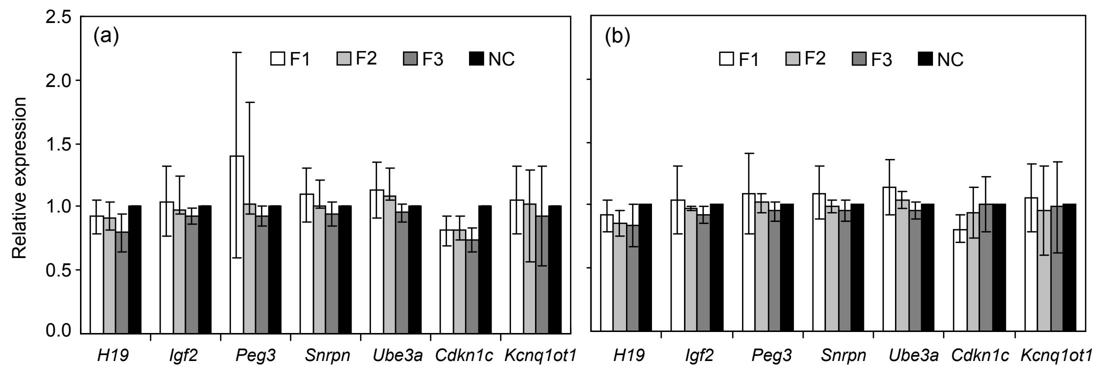

Fig. 3 DMR-regulated imprinted gene expression

Factorial gene expressions of imprinted genes *H19*, *Igf2*, *Kcnq1ot1*, *Cdkn1c*, *Ube3a*, *Snrpn*, and *Peg3* in brain tissues from F1 generation, female (a) and male (b) line-derived F2 and F3 generations, and naturally conceived (NC) mice. Data are expressed as mean±SD (n=12)

Table 3 Methylation percentages of DMRs determined by bisulfite sequencing in F1 generation, male and female line-derived F2 and F3 generations, and naturally conceived (NC) mice

Mice	Methylation percentage (%)				
	H19/Igf2 DMR CpG island 1	*H19/Igf2* DMR CpG island 3	*Igf2* DMR	KvDMR	*Snrpn* DMR
F1	0.51±0.089	0.43±0.096	0.42±0.065	0.50±0.082	0.53±0.096
F2[a]	0.49±0.117	0.55±0.099	0.47±0.104	0.55±0.129	0.55±0.100
F3[a]	0.48±0.081	0.40±0.118	0.49±0.092	0.50±0.141	0.53±0.126
F2[b]	0.50±0.125	0.53±0.162	0.49±0.039	0.53±0.106	0.55±0.075
F3[b]	0.43±0.073	0.47±0.055	0.54±0.077	0.58±0.106	0.57±0.097
NC	0.53±0.094	0.48±0.096	0.45±0.063	0.55±0.173	0.53±0.096

[a] Female-line derived F2 and F3 generations; [b] Male-line derived F2 and F3 generations. Data are expressed as mean±SD

4 Discussion

Transgenerational epigenetic inheritance is known to occur in plants and invertebrates, but evidence in vertebrates, especially in humans, is still scarce. Transgenerational epigenetic inheritance in vertebrates can be divided in two types: heritable epigenetic modifications that influence morphological appearance and modifications that impact on disease susceptibility, both of which can be altered by environmental factors (Richards, 2006). There are several specific examples, both in mice and humans, where non-mutagenic, environmental insults to either parent, such as those caused by the consumption of endocrine-disrupting chemicals, can cause unexpected transgenerational phenotypic changes in offspring (Jirtle and Skinner, 2007; Xing *et al*., 2007; Nadeau, 2009). Thus, epigenetics provides a means of understanding how environmental factors might alter heritable changes without changing the DNA sequence and understanding origins of some diseases that cannot be explained by conventional genetic mechanisms. If the environment can impact on parts of the epigenome and these epigenetic changes can be inherited across generations, the way we think about the inheritance in ART-related subpopulations would be significantly altered. Recent reports demonstrated that among infertile men enrolled for IVF or intracytoplasmic sperm injection (ICSI), the rates of chromosomal aberrations are markedly increased above the population baseline, and epigenetic alterations could be a significant cause for infertility (Marques *et al*., 2004; Kobayashi *et al*., 2007; Filipponi and Feil, 2009). In particular, a growing number of reports suggest that ART itself might lead to epigenetic defects and increase the risk of epigenetic diseases such as Angelman syndrome and Beckwith-Weidemann syndrome. These factors together should raise our speculation as to whether these imprinting diseases or susceptible statuses related to ART descendants can be inherited transgenerationally (Whitelaw and Whitelaw, 2008; Chao *et al*., 2009), but this analysis was necessarily conducted in an animal model system because no similar analysis has been feasible in humans to date.

In the present study, we first investigated IVF-born mice and their descendants in terms of behavior, morphology, and histology because an increased risk of cerebral palsy and birth defects in IVF singletons were observed (Lidegaard *et al*., 2005; Olson *et al*., 2005). DNA methylation was also found to be dynamic in regulating the adult nervous system. This cellular mechanism is a crucial step in memory formation (Miller and Sweatt, 2007). For these neurobehavioral analyses, we used the hidden platform test. This assay allows for an easy identification of memory and behavioral malfunction in mice. Accordingly, it is possible to repetitively and noninvasively monitor the development of individual IVF mice related to naturally conceived mice over consecutive time points. As compared with adults conceived naturally, no specific neurobehavioral alterations were found among adults derived from ART and their F2 and F3 generations, which suggests that IVF may not alter their neurological function. A series of studies also suggested that IVF/ICSI per se does not increase the risk of severe cognitive impairment (i.e., mental retardation) or neuromotor handicaps in humans (Middelburg *et al*., 2008; 2009). We also did not find a significantly increased risk for major birth defects in adult mice conceived by IVF or the next two generations when compared with natural conception, but there are still concerns related to risks of specific types of defects after ART, and more well-conducted observational studies are needed.

DNA methylation occurs throughout the genome, but is functionally most relevant when present in sequences rich in CpG dinucleotides, called CpG islands. Several human syndromes have been associated with aberrant methylation at DMRs. Beckwith-Weidemann syndrome, for example, showed loss of methylation at KvDMR. DNA methylation is also associated with transcriptional silencing because it can directly inhibit the binding of transcription factors or regulators or indirectly recruit methyl-CpG binding proteins (MBPs), such as CTCF in *H19* DMR island 3. Therefore, we focused on DNA methylation patterns of several important DMRs and CpG islands. Contrary to some previous reports that superovulation can affect several paternal and maternal DMRs and that the related changes were still present in *H19* and *Snrpn* in the second generation (Stouder *et al*., 2009), we did not observe obvious changes in the methylation at either of the DMRs examined, which suggests that IVF may not disturb transgenerational epigenetic reprogramming or DNA methylation integrity in the

central nervous system. The expressions of these DMR-regulated genes were further investigated by real-time RT-PCR. For example, maternally expressed *H19* and paternally expressed *Igf2* are regulated by *H19/Igf2* DMR. Several maternally expressed genes (*Cdkn1c*, *Kcnq1*, *Ascl2*, *Slc22a18*, and *Phlda2*) and one paternally expressed gene (*Kcnq1ot1*) are regulated by KvDMR. No obvious differences were found in their mRNA expressions. Our results suggest that the expressions of imprinted genes were not disturbed, which correlated with their methylation status.

Even though our data show that ART-born mice and their descendants did not show transgenerational phenotypic changes, and that DNA methylation integrity was maintained, it is obvious that the present study was preliminary. We only examined the possible transgenerational influence in somatic tissue (the brain). As we only focused on the methylation states of specific genomic sites, we must also consider the possibility of other effects, and genome-wide scans of DNA methylation with bisulfite sequencing can be used for future study. It is possible that methylation in a different region of the DMR is of equal importance for the maintenance of imprinting, and therefore, we cannot conclude that methylation is not affected. We do not know whether the mouse is a suitable model system for ART in humans (Skinner, 2008). Other clinical procedures used in ART, such as ICSI and in vitro maturation (IVM), should be investigated. Further analysis is necessary before we can provide a definitive answer.

Acknowledgements

The authors would like to thank the staff at the Women's Reproductive Health Laboratory of Zhejiang Province for their assistance with the experiment.

References

Anway, M.D., Cupp, A.S., Uzumcu, M., Skinner, M.K., 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, **308**(5727):1466-1469. [doi:10.1126/science.1108190]

Anway, M.D., Leathers, C., Skinner, M.K., 2006. Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. *Endocrinology*, **147**(12):5515-5523. [doi:10.1210/en.2006-0640]

Bliek, J., Terhal, P., van den Bogaard, M.J., Maas, S., Hamel, B., Salieb-Beugelaar, G., Simon, M., Letteboer, T., van der Smagt, J., Kroes, H., *et al*., 2006. Hypomethylation of the *H19* gene causes not only Silver-Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype. *Am. J. Hum. Genet*., **78**(4):604-614. [doi:10.1086/502981]

Chan, T.L., Yuen, S.T., Kong, C.K., Chan, Y.W., Chan, A.S., Ng, W.F., Tsui, W.Y., Lo, M.W., Tam, W.Y., Li, V.S., *et al*., 2006. Heritable germline epimutation of *MSH2* in a family with hereditary nonpolyposis colorectal cancer. *Nat. Genet*., **38**(10):1178-1183. [doi:10.1038/ng1866]

Chao, M.J., Ramagopalan, S.V., Herrera, B.M., Lincoln, M.R., Dyment, D.A., Sadovnick, A.D., Ebers, G.C., 2009. Epigenetics in multiple sclerosis susceptibility: difference in transgenerational risk localizes to the major histocompatibility complex. *Hum. Mol. Genet*., **18**(2):261-266. [doi:10.1093/hmg/ddn353]

Chin, H.J., Wang, C.K., 2001. Utero-tubal transfer of mouse embryos. *Genesis*, **30**(2):77-81. [doi:10.1002/gene.1036]

Cox, G.F., Bürger, J., Lip, V., 2002. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am. J. Hum. Genet.*, **71**(1):162-164. [doi:10.1086/341096]

Filipponi, D., Feil, R., 2009. Perturbation of genomic imprinting in oligozoospermia. *Epigenetics*, **4**(1):27-30. [doi:10.4161/epi.4.1.7311]

Gluckman, P.D., Hanson, M.A., 2004. Living with the past: evolution, development and patterns of disease. *Science*, **305**(5691):1733-1736. [doi:10.1126/science.1095292]

Hitchins, M.P., Wong, J.J., Suthers, G., Suter, C.M., Martin, D.I., Hawkins, N.J., Ward, R.L., 2007. Inheritance of a cancer-associated *MLH1* germ-line epimutation. *N. Engl. J. Med*., **356**(7):697-705. [doi:10.1056/NEJMoa064522]

Jirtle, R.L., Skinner, M.K., 2007. Environmental epigenomics and disease susceptibility. *Nat. Rev. Genet*., **8**(4):253-262. [doi:10.1038/nrg2045]

Kobayashi, H., Sato, A., Otsu, E., Hiura, H., Tomatsu, C., Utsunomiya, T., Sasaki, H., Yaegashi, N., Arima, T., 2007. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Hum. Mol. Genet*., **16**(21):2542-2551. [doi:10.1093/hmg/ddm187]

Li, L.C., Dahiya, R., 2002. MethPrimer: designing primers for methylation PCRs. *Bioinformatics*, **18**(11):1427-1431. [doi:10.1093/bioinformatics/18.11.1427]

Li, T., Vu, T.H., Ulaner, G.A., Littman, E., Ling, J.Q., Chen, H.L., Hu, J.F., Behr, B., Giudice, L., Hoffman, A.R., 2005. IVF results in de novo DNA methylation and histone methylation at an *Igf2-H19* imprinting epigenetic switch. *Mol. Hum. Reprod*., **11**(9):631-640. [doi:10.1093/molehr/gah230]

Lidegaard, O., Pinborg, A., Andersen, A.N., 2005. Imprinting diseases and IVF: Danish national IVF cohort study. *Hum. Reprod*., **20**(4):950-954. [doi:10.1093/humrep/deh714]

Luuk, H., Plaas, M., Raud, S., Innos, J., Sütt, S., Lasner, H., Abramov, U., Kurrikoff, K., Kõks, S., Vasar, E., 2009. Wfs1-deficient mice display impaired behavioural adaptation in stressful environment. *Behav. Brain Res.*, **198**(2):334-345. [doi:10.1016/j.bbr.2008.11.007]

Marques, C.J., Carvalho, F., Sousa, M., Barros, A., 2004. Genomic imprinting in disruptive spermatogenesis. *Lancet*, **363**(9422):1700-1702. [doi:10.1016/S0140-6736(04)16256-9]

Middelburg, K.J., Heineman, M.J., Bos, A.F., Hadders-Algra, M., 2008. Neuromotor, cognitive, language and behavioural outcome in children born following IVF or ICSI—a systematic review. *Hum. Reprod. Update*, **14**(3):219-231. [doi:10.1093/humupd/dmn005]

Middelburg, K.J., Heineman, M.J., Bos, A.F., Pereboom, M., Fidler, V., Hadders-Algra, M., 2009. The Groningen ART cohort study: ovarian hyperstimulation and the in vitro procedure do not affect neurological outcome in infancy. *Hum. Reprod.*, **24**(12):3119-3126. [doi:10.1093/humrep/dep310]

Miller, C.A., Sweatt, J.D., 2007. Covalent modification of DNA regulates memory formation. *Neuron*, **53**(6):857-869. [doi:10.1016/j.neuron.2007.02.022]

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods*, **11**(1):47-60. [doi:10.1016/0165-0270(84)90007-4]

Nadeau, J.H., 2009. Transgenerational genetic effects on phenotypic variation and disease risk. *Hum. Mol. Genet.*, **18**(R2):R202-R210. [doi:10.1093/hmg/ddp366]

Nilsson, E.E., Anway, M.D., Stanfield, J., Skinner, M.K., 2008. Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. *Reproduction*, **135**(5):713-721. [doi:10.1530/REP-07-0542]

Olson, C.K., Keppler-Noreuil, K.M., Romitti, P.A., Budelier, W.T., Ryan, G., Sparks, A.E., van Voorhis, B.J., 2005. In vitro fertilization is associated with an increase in major birth defects. *Fertil. Steril.*, **84**(5):1308-1315. [doi:10.1016/j.fertnstert.2005.03.086]

Ørstavik, K.H., Eiklid, K., van der Hagen, C.B., Spetalen, S., Kierulf, K., Skjeldal, O., Buiting, K., 2003. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *Am. J. Hum. Genet.*, **72**(1):218-219. [doi:10.1086/346030]

Painter, R.C., Osmond, C., Gluckman, P., Hanson, M., Phillips, D.I., Roseboom, T.J., 2008. Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. *BJOG*, 1**15**(10):1243-1249. [doi:10.1111/j.1471-0528.2008.01822.x]

Richards, E.J., 2006. Inherited epigenetic variation—revisiting soft inheritance. *Nat. Rev. Genet.*, **7**:395-401. [doi:10.1038/nrg1834]

Schneider, S., Kaufmann, W., Buesen, R., van Ravenzwaay, B., 2008. Vinclozolin—the lack of a transgenerational effect after oral maternal exposure during organogenesis. *Reprod. Toxicol.*, **25**(3):352-360. [doi:10.1016/j.reprotox.2008.04.001]

Skinner, M.K., 2008. What is an epigenetic transgenerational phenotype? F3 or F2. *Reprod. Toxicol.*, **25**(1):2-6. [doi:10.1016/j.reprotox.2007.09.001]

Stouder, C., Deutsch, S., Paoloni-Giacobino, A., 2009. Superovulation in mice alters the methylation pattern of imprinted genes in the sperm of the offspring. *Reprod. Toxicol.*, **28**(4):536-541. [doi:10.1016/j.reprotox.2009.06.009]

Whitelaw, N.C., Whitelaw, E., 2008. Transgenerational epigenetic inheritance in health and disease. *Curr. Opin. Genet. Dev.*, **18**(3):273-279. [doi:10.1016/j.gde.2008.07.001]

Xing, Y., Shi, S., Le, L., Lee, C.A., Silver-Morse, L., Li, W.X., 2007. Evidence for transgenerational transmission of epigenetic tumor susceptibility in *Drosophila*. *PLoS Genet.*, **3**(9):1598-1606. [doi:10.1371/journal.pgen.0030151]