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ORIGINAL RESEARCH

The effects of postovulatory aging of mouse oocytes on methylation and expression of imprinted genes at mid-term gestation

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ABSTRACT: Previous studies by others and ourselves have suggested that the methylation pattern of imprinted genes in oocytes is altered during postovulatory aging. The purpose of the current study was to evaluate the effects of postovulatory aging of mouse oocytes on methylation and expression of imprinted genes at the mid-gestation development stages. Proestrous females were artificially inseminated at 13 h (fresh-oocyte group) or 22 h (aged-oocyte group) post-hCG. Estrous females were mated with males as a control group. On dpc (day post coitus) 10.5 of development, embryos and placentas were collected and DNA and RNA were extracted, respectively. Methylation and total expression of *Igf2r* and *H19* was investigated by quantitative analysis of methylation by PCR and quantitative real-time RT–PCR, respectively. Our results showed no significant changes of methylation in the differentially methylated region (DMR) and total expression of *Igf2r* in embryos and placentas, and no significant changes in methylation of *H19* in embryos at dpc 10.5 of development compared with the control group regardless of artificial insemination of fresh or aged oocytes. In contrast, placentas of the aged-oocyte group exhibited significantly lower methylation levels in the *H19* DMR. Furthermore, we observed that the increased expression of *H19* in placentas of the aged-oocyte group was associated with significant hypomethylation of *H19* DMR. These results suggest that postovulatory aging of mouse oocytes has adversed effects on methylation and expression of *H19* in placentas at the mid-gestation development stage.

Key words: embryo / imprinting / mouse oocyte / placenta / postovulatory aging

Introduction

It has been well established that several morphological and cellular changes occur during the process of oocyte aging, which results in oocyte quality decline. As a consequence, problems associated with fertilization of aged oocytes may occur not only during pre- and post-implantation embryonic development but also during the lifetimes of offspring (Tarin *et al.*, 2000; Fissore *et al.*, 2002; Miao *et al.*, 2009). These long-term consequences are observed in F0 females that have lower pregnancy rates, shortened gestation length, decreased litter size, as well as higher perinatal death of pups compared with normal fertilization of fresh oocytes (Tarin *et al.*, 1999). As a consequence, the resultant F1 offspring are also affected by postovulatory aging of oocytes as demonstrated by increased number of growth-retarded pups, delayed development of the righting reflex, higher spontaneous motor activity and higher emotional distress (Tarin *et al.*, 1999). Furthermore, the resultant F2 pups suffer from teratogenic defects, higher pre-weaning mortality and decreased body weight at weaning (Tarin *et al.*, 2002). In humans, a previous study revealed that reinsemination of failed-fertilized IVF oocytes is more efficient when it is performed soon after the initial conventional insemination (Nagy *et al.*, 2006). In contrast, postovulatory aging of ooctyes prior to fertilization results in early pregnancy failure in humans (Wilcox *et al.*, 1998).

Genomic imprinting involves the formation of an epigenetic 'mark' at specific loci in a parent-of-origin-specific manner such that genes are expressed monoallelically (Reik *et al.*, 2001). A small group of genes that undergo such a process are termed imprinted genes. Genomic imprinting is a multi-step process that is erased in the primordial germ cells of the embryo, and sex-specific methylation patterns are established during gametogenesis (Reik *et al.*, 2001). Such sex-specific methylation established in the matured female and male

gametes must be maintained throughout the life of an organism following fertilization. The majority of imprinted genes have a differentially methylated region (DMR), with sex-specific methylation patterns inherited from the gametes (Lucifero *et al.*, 2002; Li *et al.*, 2004). Through modulating the DNA methylation of DMR, imprinted genes achieve allelic expression. Correct expression of imprinted genes is essential for proper regulation of embryo growth, placental functions and neurobehavioral processes. The opposite also takes place; for example, epigenetic change in *Igf2r* is associated with fetal overgrowth after sheep embryo culture (Young *et al.*, 2001).

Previous studies have shown that methylation alterations in imprinted genes are associated with the oocyte aging. For example, Imamura *et al.* (2005) found that *Peg1* showed demethylation in mouse oocytes at 42 h post-hCG. Another study from our laboratory also demonstrated that postovulatory aging of mouse oocytes resulted in methylation imprinting loss of *Snrpn* at 29 h post-hCG (Liang *et al.*, 2008). All these results suggest that methylation of imprinted genes in oocytes is sensitive to postovulatory oocyte aging.

As discussed already, not only is it important to establish sex-specific DNA methylation during gametogenesis, these patterns must be properly maintained during subsequent development, which is essential for successful pregnancy outcome. Despite results from previous studies suggesting that postovulatory aging of oocytes prior to fertilization results in altered methylation of imprinted genes in oocytes (Imamura *et al.*, 2005; Liang *et al.*, 2008), the effects of postovulatory oocyte aging on genomic imprinting during pre- and post-implantation development are still unknown. In the present study, we designed experiments to investigate how postovulatory aging of mouse oocytes affects genomic imprinting at mid-gestation development. We evaluated the methylation status of DMR and the expression of *Igf2r* and *H19* in dpc (day post coitus) 10.5 embryos and placentas. Proestrous females were artificially inseminated (AI) at 13 h (1 h of ovulation, termed fresh-oocyte group) or 22 h (10 h of ovulation, termed aged-oocyte group) post-hCG. Estrous females were mated with males as a control group.

Materials and Methods

Animals and embryos collection

Male institute for cancer research (ICR) mice (3–4 months old) and females (8–12 weeks old) were used in all experiments. Mice were provided by the Beijing Vital River Experimental Animals Centre and maintained at a 14L:10D photoperiod in a temperature-controlled room. All procedures described were reviewed and approved by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences.

Estrous females were mated to males to produce *in vivo* dpc 10.5 concepti. A detailed procedure for producing the dpc 10.5 concepti derived from fresh- and aged-oocytes by AI was based on previous publications with minor modifications (Leckie *et al.*, 1973; Tarin *et al.*, 1999). Briefly, proestrous females were AI using a mixture of spermatozoa from 2 to 4 randomly selected males at 13 or 22 h post-hCG (5 IU) injection. Modifications of the AI procedures included mainly (i) the phase of the estrous cycle was examined by vaginal cytology; and (ii) the females after AI were mated to vasectomized males instead of mechanical stimulation of the vagina. Concepti were collected at dpc 10.5. To avoid contamination of trophoblastic tissue with the fetuses, the membrane outside the concepti was completely split with a pair of fine forceps. The embryos and placentas were bisected and snap-frozen in liquid nitrogen.

Methylation level analysis by quantitative analysis of methylation by PCR

DNA was extracted from one half of bisected embryos or placentas using DNA Tissue Kit (Tiangen, China) according to the manufacturer's instructions. The methylation levels were determined using quantitative analysis of methylation (qAMP) as described previously with modifications (Oakes *et al.*, 2006; Lopes *et al.*, 2009). Briefly, fetal and placental DNA (200 ng/enzyme) in 20 μl reaction volume was digested with methylation-sensitive (*Hha*I) and methylation-dependent (*McrBC*) restriction enzyme, respectively. Then incubation of the DNA at 37°C for 3 h, and 65°C for 20 min was performed to inactivate the restriction enzyme. The resultant-digested DNA fragments were diluted by adding 30 μl nuclear-free H_2O and then used as templates for real-time RT–PCR using the SYBR kit (Kangwei, China). The samples were analyzed in triplicate by qAMP, employing a Rotor-Gene 6000 Real-Time PCR instrument (Qiagen). The percent of methylation for CpG sites was calculated as previously described for *Hha*I, % methylation = 100 $[e^{-0.7(\Delta C_t)}]$; for *McrBC*, % methylation = 100 $[1-e^{-0.7(\Delta C_t)}]$. ΔC_t was based on the cycle threshold (C_t) values between digested and undigested sham aliquots. Primers used for qAMP were synthesized according to Lopes *et al.* (2009) and were designed to span ~150 bps, covering part of the DMR.

Total expression analysis by quantitative real-time RT–PCR

RNA was extracted from the other half of bisected embryos or placentas using RNA Tissue Kit (Tiangen, China) according to the manufacturer's instructions. cDNA was prepared using High-Capacity cDNA Reverse Transcription Kits (ABI, USA), random primers and 1 μg of total RNA.

Quantitative Real-time RT–PCR was carried out to assay the total amount of mRNA in embryos from different groups. The primers for total expression analysis of *Igf2r* and *H19* were synthesized according to the method reported by Varrault *et al.* (2006) and Wang *et al.* (2010), respectively. The samples were analyzed in triplicate, and the threshold cycle was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Wang *et al.*, 2010). Real-time RT–PCR was performed using an ABI 7500 fast Real-Time PCR systems (ABI). The expression level were calculated using the $2^{-}\Delta\Delta C_t Z$ method as previously described (Livak and Schmittgen, 2001; Wang *et al.*, 2010).

Statistical analysis

Data were analyzed using the SPSS 13.0 software package (Cary, NC, USA). Methylation level of DMR from different groups was determined as normally distributed, with homogeneity of variance; they were compared by one-way analysis of variance. Expression levels were determined as normally distributed and without homogeneity of variance; they were compared with Dunnett's T3 for multiple comparisons. A probability level of $P < 0.05$ was considered significant.

Results

Methylation status of *Igf2r* and *H19* DMR in embryos

Methylation was determined by using DNA sample digestion with appropriate methylation-sensitive (*Hha*I) and methylation-dependent (*McrBC*) restriction enzymes followed by quantitative real-time PCR.

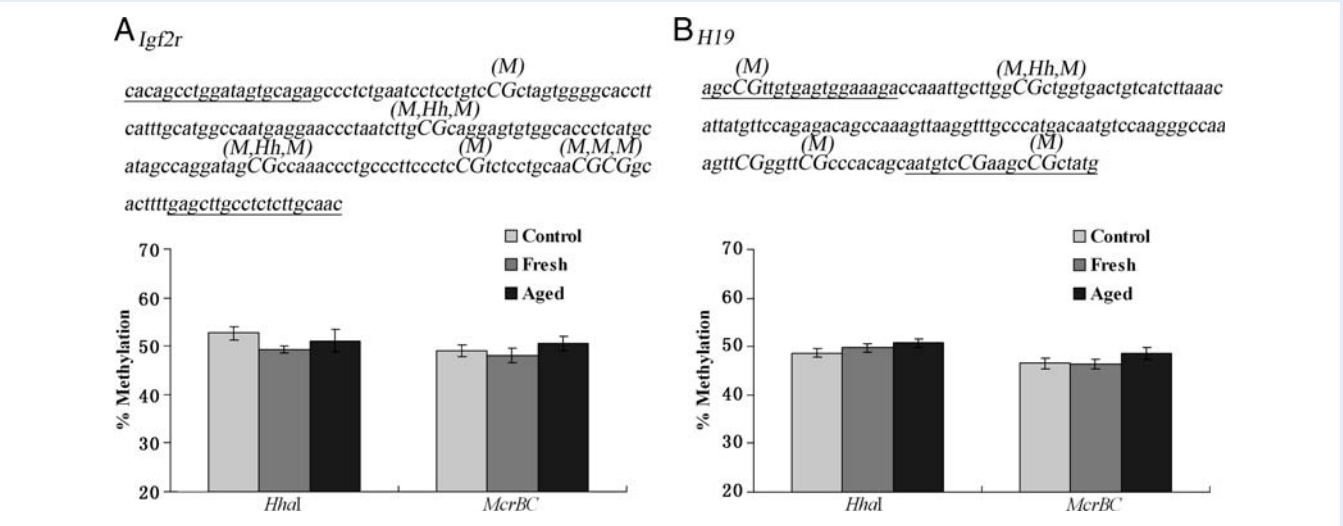

Figure 1 Quantitative DNA methylation analysis in DMRs of imprinted genes *Igf2r* (**A**) and *H19* (**B**) in embryos on 10.5 dpc of gestation using qAMP. Dpc 10.5 embryos were collected ($n = 17$ from four females, $n = 17$ from four females and $n = 18$ from five females for control, fresh- and aged-oocyte group, respectively). DNA was isolated and digested with *Hha*I (Hh) or *McrBC* (M) and amplified using quantitative real-time PCR. Primer-binding sites are underlined and the CpGs loci analyzed by *Hha*I and *McrBC* restriction enzyme are displayed for both DMRs. *In vivo* control; fresh, AI at 13 h post-hCG injection; aged, AI at 22 h post-hCG injection. The data are presented as mean ± SE for each enzyme applied.

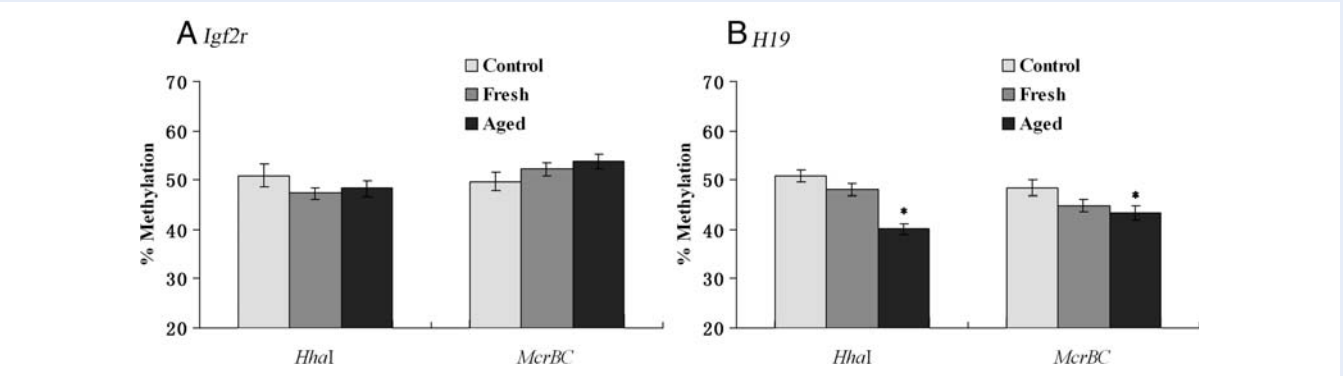

Figure 2 Quantitative DNA methylation analysis in DMRs of imprinted genes *Igf2r* (**A**) and *H19* (**B**) in placentas on 10.5 dpc of gestation using qAMP. Dpc 10.5 placentas ($n = 17$ from four females, $n = 17$ from four females and $n = 18$ from five females for control, fresh- and aged-oocyte group, respectively). DNA was isolated and digested with *Hha*I or *McrBC* and amplified using quantitative real-time PCR. Primer-binding sites and CpGs loci analyzed by *Hha*I and *McrBC* restriction enzyme are displayed in Fig. 1. *In vivo* control; fresh, AI at 13 h post-hCG injection; aged, AI at 22 h post-hCG injection. The data are presented as mean ± SE for each enzyme applied. *$P < 0.05$, statistically significant compared with the control.

The results showed that *Igf2r* was close to the expected 50% methylation in all enzyme sites assayed (Fig. 1A). There was no significant difference among the groups. Similar to *Igf2r*, methylation of *H19* in all groups was also close to the expected 50% (Fig. 1B). These results suggest that methylation of *Igf2r* and *H19* partial DMR is properly acquired/maintained in the embryos derived from AI of fresh or aged oocytes at the mid-gestation development.

Methylation status of *Igf2r* and *H19* DMR in placentas

Our results demonstrated that methylation of *Igf2r* DMR in placentas was close to the expected 50% in all groups (Fig. 2A). For *H19*, the average methylation levels were 50.49 ± 1.24, 48.13 ± 1.26 and 40.16 ± 1.02% in *Hha*I sites and 48.46 ± 1.46, 44.87 ± 1.13 and 43.73 ± 1.4% in *McrBC* sites in groups of control, fresh- and aged-oocyte, respectively (Fig. 2B). In the control group, methylation of *H19* in *Hha*I and *McrBC* sites was close to the expected 50%. Although methylation in these sites was slightly lower than 50% in the fresh-oocyte group, they showed no significant difference compared with the control group. However, compared with the control, methylation of *H19* in the evaluated sites in placentas was significantly decreased in the aged-oocyte group but not in the fresh-oocyte group. The results suggest that methylation of *Igf2r* is properly acquired/maintained in the placentas derived from aged oocytes at mid-gestation development. In contrast, although the methylation pattern

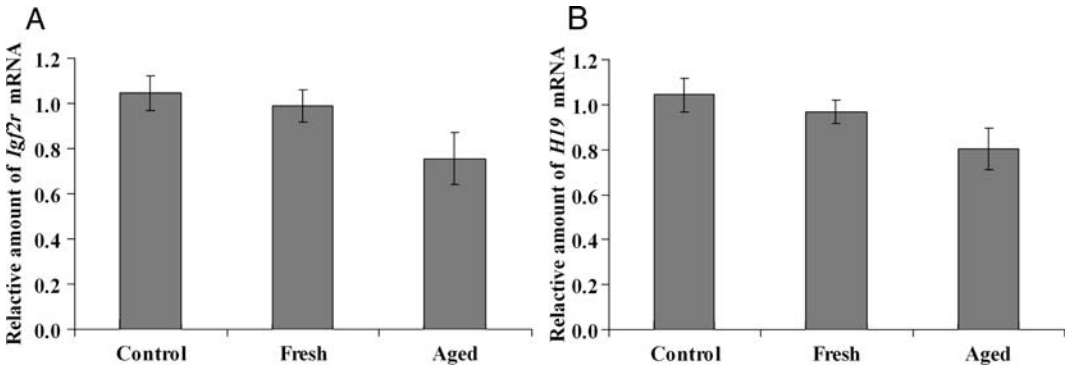

Figure 3 Analysis of *Igf2r* (**A**) and *H19* (**B**) expression in embryos on Day 10.5 of gestation ($n = 17$ from four females, $n = 17$ from four females and $n = 18$ from five females for control, fresh- and aged-oocyte group, respectively). Quantitative real-time RT–PCR was applied to assay the total amount of *Igf2r* and *H19* mRNA relative to the housekeeping gene *Gapdh*. The fold-change was calculated using the comparative C_T method. *In vivo* control; fresh, AI at 13 h post-hCG injection; aged, AI at 22 h post-hCG injection. The data are presented as mean ± SE.

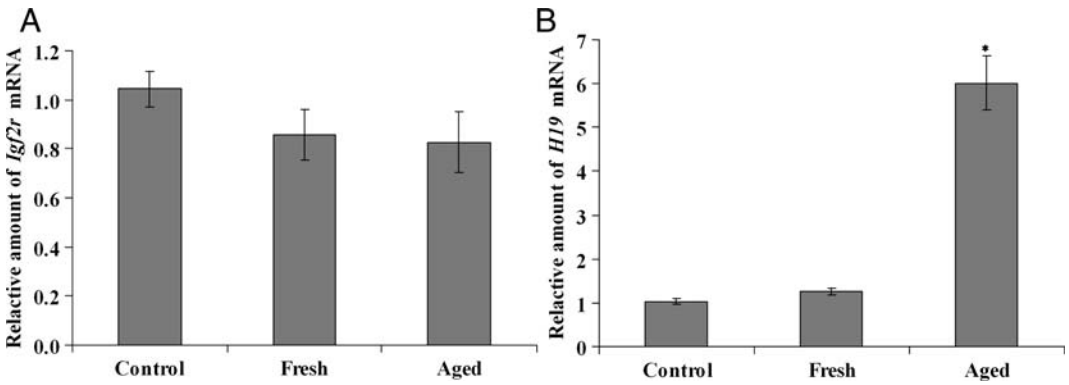

Figure 4 Analysis of *Igf2r* (**A**) and *H19* (**B**) expression in placentas on Day 10.5 of gestation ($n = 17$ from four females, $n = 17$ from four females and $n = 18$ from five females for control, fresh-oocyte and aged-oocyte group, respectively). Quantitative real-time RT–PCR was applied to assay the total amount of *Igf2r* and *H19* mRNA relative to the housekeeping gene *Gapdh*. The fold-change was calculated using the comparative C_T method. *In vivo* control; fresh, AI at 13 h post-hCG injection; aged, AI at 22 h post-hCG injection. The data are presented as mean ± SE. *$P < 0.05$, statistically significant compared with the control.

of *H19* is established in sperm, it becomes disturbed by fertilization of postovulatory aged oocytes by mid-term gestation.

Expression levels of the imprinted genes *Igf2r* and *H19* in embryos and placentas

To examine the effects of postovulatory oocyte aging on the expression of imprinted genes in the resultant concepti at 10.5 days of gestation, the total expression levels were determined by quantitative real-time RT–PCR. For the embryos, the results showed that the total expression levels of *Igf2r* (Fig. 3A) and *H19* (Fig. 3B) had no significant differences among all groups. For the placentas, while we did not observe any differences in the total expression levels of *Igf2r* (Fig. 4A) in all groups, the expression of *H19* (Fig. 4B) (1.03 ± 0.06; 1.26 ± 0.07; 5.92 ± 0.59 in groups of control, fresh- and aged-oocyte, respectively) in the aged-oocyte group was significantly increased when compared with the control group. The results suggest that the expression of *Igf2r* in both embryos and placentas, as well as that of *H19* in embryos is not affected by postovulatory aging of oocytes. However, the expression of *H19* in placentas is sensitive to postovulatory aging of oocytes at mid-gestation development.

Discussion

Despite the strong association of imprinted genes with embryonic growth and development as well as placental functions, little is known about the possible epigenetic effects of postovulatory aging of oocytes. In the present study, we investigated the effects of postovulatory aging of oocyte on methylation and expression of imprinted genes in both the embryo and the placenta at the mid-gestation development. We did not observe significant effects of postovulatory aging of oocytes on methylation and expression of *Igf2r* in either embryos or placentas, nor that of *H19* in embryos on dpc 10.5 of gestation. In

contrast, methylation and expression of *H19* in the placentas of the aged-oocyte group was adversely affected.

Previous studies have shown that the derivatives of inner cell mass (ICM; embryo) and trophectoderm (TE; placenta) responded differently to the environmental changes or manipulation during post-implantation development. For example, placentas are more susceptible to disruption of imprinted genes than embryos resulting from *in vitro* embryo culture (Mann *et al.*, 2004; Rivera *et al.*, 2008), embryo manipulation (Rivera *et al.*, 2008) as well as hormonal stimulation (Fortier *et al.*, 2008). In our current study, we also found that methylation of *H19* in the placenta are more sensitive to disruption by post-ovulatory aging of oocytes than in the embryo. We speculate that this could be explained in part by difference of position—outer versus inner—since placenta is the derivative of outer TE, while embryo is the derivative of ICM (Mann *et al.*, 2004; Rivera *et al.*, 2008).

Igf2r is maternally imprinted and paternally expressed (Stoger *et al.*, 1993), its methylation pattern is established in metaphase II oocytes and must be maintained at subsequent development after fertilization (Wutz *et al.*, 1997). Results analyzed by applying qAMP showed that there are no obvious differences in the methylation levels of *Igf2r* in the embryo or placenta in all groups. Our results are similar to those reported by Lopes *et al.* (2009), who found that the *Igf2r* methylation level in embryos is close to 50%, using the same analysis method. The current results suggest that imprinting of *Igf2r* is not affected during the process of postovulatory aging. Furthermore, it is properly maintained in concepti during mid-gestation development. However, we cannot rule out the possibility that proper establishment and maintenance of imprinting in other maternally methylated genes is affected by postovulatory aging of oocytes since there are several other maternally imprinted genes including *Peg1* (Imamura *et al.*, 2005) and *Snrpn* (Liang *et al.*, 2008), that have lost methylation imprinting in aged oocytes. In addition, only partial DMR was assayed in the present study.

Imprinting pattern of *H19* is established in the male germline and has to be maintained during early embryonic development (Tremblay *et al.*, 1997). We observed that *H19* showed normal methylation levels in embryos in all groups. However, methylation levels of *H19* DMR were significantly lower in the placentas of the aged-oocyte group compared with the control counterparts. It appears that *H19* imprinting in the placenta is highly sensitive to perturbation by postovulatory aging of oocytes. Manipulations of sperm during AI procedures may affect the imprinting of *H19*. However, this potential factor is ruled out since the methylation levels in the placentas of the fresh-oocyte group display no significant differences compared with the *in vivo* counterparts. Alternatively, we speculate that the declined oocyte quality taking place in the process of postovulatory aging may compromise the ability of oocytes to correctly maintain the methylation imprinting in the placenta during the mid-gestation development.

Furthermore, we assayed the total mRNA expression of *Igf2r* and *H19*. Interestingly, our results demonstrated that total mRNA expression levels were consistent with the methylation status. Our results showed that methylation and expression of *Igf2r* in the embryos and placentas, as well as expression of *H19* in the embryos, displayed no significant differences among all groups. In addition, elevated total expression of *H19* in the placentas of the aged-oocyte group was compatible with hypomethylation of DMR. The elevated total expression of *H19* could be explained in part by hypomethylation in DMR, as observed in the current study.

In summary, although we cannot analyze the methylation status and expression of each parental allele (without using a hybrid strain of mouse), the results of our current study indicate that postovulatory aging of mouse oocytes adversely affects the methylation and expression of *H19* in placentas during mid-gestation development. Our findings combined with earlier findings reporting deleterious effects on progeny outcome associated with postovulatory aging of oocytes call for further studies to verify the safety issues concerning fertilization and development of oocytes after postovulatory aging.

Authors' roles

X.W.L. designed and performed the experiments, analyzed the data and wrote the paper; Z.J.G., L.W. and L.G. performed the experiments and analyzed the data; H.S. revised the paper; Z.M.H. and Q.Y.S. designed the experiments, analyzed the data and revised the paper.

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References

Fissore RA, Kurokawa M, Knott J, Zhang M, Smyth J. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction* 2002;**124**:745–754.

Fortier AL, Lopes FL, Darricarrere N, Martel J, Trasler JM. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Hum Mol Genet* 2008;**17**:1653–1665.

Imamura T, Kerjean A, Heams T, Kupiec JJ, Thenevin C, Paldi A. Dynamic CpG and non-CpG methylation of the Peg1/Mest gene in the mouse oocyte and preimplantation embryo. *J Biol Chem* 2005; **280**:20171–20175.

Leckie PA, Watson JG, Chaykin S. An improved method for the artificial insemination of the mouse (*Mus musculus*). *Biol Reprod* 1973;**9**:420–425.

Li JY, Lees-Murdock DJ, Xu GL, Walsh CP. Timing of establishment of paternal methylation imprints in the mouse. *Genomics* 2004; **84**:952–960.

Liang XW, Zhu JQ, Miao YL, Liu JH, Wei L, Lu SS, Hou Y, Schatten H, Lu KH, Sun QY. Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. *Biochem Biophys Res Commun* 2008;**371**:16–21.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–408.

Lopes FL, Fortier AL, Darricarrere N, Chan D, Arnold DR, Trasler JM. Reproductive and epigenetic outcomes associated with aging mouse oocytes. *Hum Mol Genet* 2009;**18**:2032–2044.

Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM. Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* 2002; **79**:530–538.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, Bartolomei MS. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004; **131**:3727–3735.

Miao YL, Kikuchi K, Sun QY, Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum Reprod Update* 2009;**15**:573–585.

Nagy ZP, Rienzi LF, Ubaldi FM, Greco E, Massey JB, Kort HI. Effect of reduced oocyte aging on the outcome of rescue intracytoplasmic sperm injection. *Fertil Steril* 2006;**85**:901–906.

Oakes CC, La Salle S, Robaire B, Trasler JM. Evaluation of a quantitative DNA methylation analysis technique using methylation-sensitive/dependent restriction enzymes and real-time PCR. *Epigenetics* 2006; **1**:146–152.

Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;**293**:1089–1093.

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**:1–14.

Stoger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H, Barlow DP. Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. *Cell* 1993;**73**:61–71.

Tarin JJ, Perez-Albala S, Aguilar A, Minarro J, Hermenegildo C, Cano A. Long-term effects of postovulatory aging of mouse oocytes on offspring: a two-generational study. *Biol Reprod* 1999;**61**:1347–1355.

Tarin JJ, Perez-Albala S, Cano A. Consequences on offspring of abnormal function in ageing gametes. *Hum Reprod Update* 2000;**6**:532–549.

Tarin JJ, Perez-Albala S, Perez-Hoyos S, Cano A. Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. *Biol Reprod* 2002;**66**:495–499.

Tremblay KD, Duran KL, Bartolomei MS. A 5′ 2-kilobase-pair region of the imprinted mouse H19 gene exhibits exclusive paternal methylation throughout development. *Mol Cell Biol* 1997;**17**:4322–4329.

Varrault A, Gueydan C, Delalbre A, Bellmann A, Houssami S, Aknin C, Severac D, Chotard L, Kahli M, Le Digarcher A *et al*. Zac1 regulates an imprinted gene network critically involved in the control of embryonic growth. *Dev Cell* 2006;**11**:711–722.

Wang Z, Xu L, He F. Embryo vitrification affects the methylation of the H19/Igf2 differentially methylated domain and the expression of H19 and Igf2. *Fertil Steril* 2010;**93**:2729–2733.

Wilcox AJ, Weinberg CR, Baird DD. Post-ovulatory ageing of the human oocyte and embryo failure. *Hum Reprod* 1998;**13**:394–397.

Wutz A, Smrzka OW, Schweifer N, Schellander K, Wagner EF, Barlow DP. Imprinted expression of the Igf2r gene depends on an intronic CpG island. *Nature* 1997;**389**:745–749.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genet* 2001;**27**:153–154.