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. Proestrus 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 oocytes 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 H19 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 postimplantation 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; Tarrin 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/μg) in 20 μl reaction volume was digested with methylation-sensitive (HhaI) and methylation-dependent (McBc) 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 H2O 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 HhaI, % methylation = 100 [e^-0.7ΔCt]; for McBc, % methylation = 100 [1 – e^-0.7ΔCt]. ΔCt was based on the cycle threshold (Ct) 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 (Gpdh) (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^-ΔΔCt 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 (HhaI) and methylation-dependent (McBc) restriction enzymes followed by quantitative real-time PCR.
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 HhaI 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 HhaI 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...
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. 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 trophoderm (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 post-ovulatory 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 and performed the experiments, analyzed the data and revised the paper.

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