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Loss of methylation imprint of *Snrpn* in postovulatory aging mouse oocyte

Xing-Wei Liang [a,b], Jia-Qiao Zhu [b], Yi-Liang Miao [b], Jing-He Liu [b], Liang Wei [b], Sheng-Sheng Lu [a], Yi Hou [b], Heide Schatten [c], Ke-Huan Lu [a], Qing-Yuan Sun [b,*]

[a] *Animal Reproduction Institute, Guangxi Key Laboratory of Subtropical Bioresource Conservation and Utilization, Guangxi University, Nanning 530004, China*
[b] *State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*
[c] *Department of Veterinary Pathobiology, University of Missouri-Columbia, MO 65211, USA*

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ABSTRACT

Prolonged residence of postovulatory oocyte in the oviduct or prolonged culture *in vitro* can lead to oocyte aging, which significantly affects pre- and post-implantation embryo development. In this study, we employed bisulfite sequencing and COBRA methods to investigate the DNA methylation status of differentially methylated regions (DMRs) of *Snrpn* and *Peg1/Mest*, two maternally imprinted genes, in postovulatory oocytes aged *in vivo* and *in vitro*. The results showed that *Snrpn* DMR was clearly demethylated in oocytes aged *in vivo* at 29 h post-hCG and in denuded oocytes aged *in vitro* for the same time period. However, *Peg1/Mest* did not show any demethylation in all aged groups at 29 h post-hCG. These data indicate that oocytes undergo time-dependent demethylation of *Snrpn* DMR during the process of postovulatory aging.

The fertilizable life span of mammalian oocytes ranges from 12 to 24 h, and even shorter in certain species [1]. Mouse oocytes are ovulated at the MII stage at around 12 h after the LH-surge and are expected to be fertilized within 6 h after ovulation [2]; these oocytes can be fertilized *in vivo* for about 15 h postovulation [3]. However, prolonged time of postovulatory oocytes residing in the oviduct or prolonged culture *in vitro* will lead to oocyte aging and may subsequently result in abnormal development of embryos/fetuses [4]. In clinical applications, insemination of aged oocytes by intracytoplasmic sperm injection (ICSI) that is oftentimes used when IVF procedures fail, may affect developmental potential [5,6].

As reviewed by Tarin et al. [7], aging of postovulatory oocytes prior to fertilization may not only affect the development of pre- and post-implantation embryos/fetuses but also the later lives of the offspring. Aging of postovulatory oocytes can decrease the oocytes' fertilization potential [8] and increase the loss of early pregnancy [4]. In addition, offspring derived from aged oocytes suffer from retarded sensorimotor integration during pre-weaning development, increased spontaneous motor activity, higher emotional distress [9], decreased reproductive fitness and have decreased longevity [10]. However, the mechanisms underlying these outcomes are largely still unknown. Thus, studies of the molecular mechanisms that occur during aging in postovulatory oocytes are needed and may have important clinical significance.

It is well known that genomic imprinting plays important roles in the regulation of fetal growth, development, placental function, and postnatal behavior [11–13]. The establishment of parent-specific methylation imprints during oogenesis has been well documented [14–16]. DNA methyltransferase Dnmt3a and Dnmt3b are responsible for the establishment of methylation imprints, and Dnmt1 can methylate the newly synthesized DNA molecules [17,18]. Once the methylation imprints are established, they will be maintained, even during the epigenetic reprogramming after fertilization, while genomic DNA undergoes global demethylation [19–21]. Hence, parent-specific methylation imprints may play an important role during genomic reprogramming, which is an essential factor that determines developmental potential [22].

However, the dynamics of DNA methylation imprints during aging of postovulatory oocytes is poorly understood although recently, Imamura et al. [23] reported the dynamic changes of DNA methylation of *Peg1/Mest* during postovulatory oocyte aging *in vitro*. Tarin et al. [7] proposed that aging of postovulatory oocytes *in vivo* and *in vitro* may share common properties, and particular traits emerge depending on the environmental conditions during the process of oocyte aging. It has been reported that the process of oocyte aging is retarded during *in vitro* culture compared with that of oocytes residing in the oviduct for the same period of time [24]. In addition, cumulus cells can accelerate the aging of postovulatory oocytes during *in vitro* culture [25]. However, the dynamics of methylation imprints in aged cumulus-enclosed oocytes remain unclear. To obtain further insights into the dynamics of methylation imprints during the aging process of postovulatory oocytes, we determined the methylation status of the DMRs of

* Corresponding author. Fax: +86 10 64807050.
E-mail address: sunqy@ioz.ac.cn (Q.-Y. Sun).

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Snrpn and *Peg1/Mes*t employing the methods of bisulfite DNA sequencing (BSP) and COBRA in oocytes aged *in vivo* and cumulus-enclosed oocytes (CEOs) and denuded oocytes (DOs) aged *in vitro*.

Materials and methods

Animals. All procedures described were reviewed and approved by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences. Six- to eight-week-old Kunming strain mice were used for all experiments.

Preparation of aged oocytes. MII oocytes were obtained from the oviducts of females superovulated by intraperitoneal injection (I.P.) of 10 IU of PMSG (Tianjin Animal Hormone Factory, Tianjin, China) 48 h later with 10 IU of hCG (Tianjin Animal Hormone Factory, Tianjin, China). Freshly ovulated and *in vivo* postovulatory aged oocytes were collected into M2 medium (Sigma) at 13, 21, and 29 h after hCG injection.

For *in vitro* aged oocytes, samples were collected into M2 medium 13 h after the hCG injection. CEOs were incubated directly in M16 medium (Sigma) for 8 or 16 h (corresponding to 21 and 29 h post-hCG, respectively) at 37 °C under 5% CO_2/air. DOs were obtained by removing cumulus cells in M2 medium containing 0.3% hyaluronidase (Sigma) and then cultured under the same incubation conditions.

DNA isolation and bisulfite modification. Before DNA isolation, the cumulus cells were removed. We selected non-fragmented and 'healthy'-looking oocytes for methylation analysis [15]. The selected oocytes were briefly exposed to acid M2 medium [26] to remove the zona pellucida together with the remaining attached cumulus cells and then washed extensively with M2 medium to prevent contamination with somatic cells.

The DNA from two independent batches of 500–700 oocytes for each treatment was isolated using TIANamp Micro DNA Kit (Tiangen, China). DNA was treated using the Methylamp DNA Modification Kit (Epigentek, USA) according to the manufacturer's instructions. Two separate bisulfite modification treatments were performed for each DNA sample.

PCR amplification, cloning and sequencing. The following steps were conducted according to our previous report with minor modifications [27]. To obtain PCR products, two individual nested PCRs were carried out using 2 μl bisulfite-treated DNA in the first round PCR of 25 μl reaction system and 6 μl of the first round PCR products as templates in the second round PCR of 50 μl reaction system. All reactions contained 0.4 mM primers, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, and 1.25 U of rTaq Hotstart polymerase (TaKaRa, Japan). The PCRs were performed with a Peltier Thermal Cycler-100 (MJ Research) using the following programs. The program for the first round was 1 cycle at 94 °C for 6 min; 35 cycles of 94 °C for 1 min, 45 (*Snrpn*) or 50 °C (*Peg1/Mest*) for 2 min, 72 °C for 3 min; and 1 cycle of 72 °C for 10 min. For the second round PCR, the program was 1 cycle at 94 °C for 4 min; 30 cycles at 94 °C for 1 min, 47 (*Snrpn*) or 50 °C (*Peg1/Mest*) for 1 min, and 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. The primers were synthesized as previously reported [14]. Products of the second round PCR were then recovered and gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Purified fragments were subcloned into T-vectors (TaKaRa, Japan). The clones confirmed by PCR were selected for DNA sequencing using an automatic sequencer (ABI Prism-77). Four independent amplification experiments were carried out for each treatment. We sequenced 4–5 clones from the each independent set of amplification and cloning.

Analysis by COBRA. Half of all purified PCR products used for cloning and sequencing from the four repeats in each treatment were pooled together and some of them were digested with restriction enzyme *BstU I* and *BstB I* for *Snrpn* and *Peg1/Mest*, respectively. The digested fragments were electrophoresed on 2.5% agarose gels.

Results

Methylation analysis by BSP

We used the BSP method to examine the methylation patterns of *Snrpn* and *Peg1/Mes*t DMRs during postovulatory aging of mouse oocytes. In *in vivo*, freshly (13 h after hCG) (Fig. 1A) ovulated oocytes and in those collected 21 h after hCG (Fig. 1B), DMR of *Snrpn* was fully methylated. However, oocytes collected 29 h after hCG showed evident demethylation (Fig. 1C). Next, we examined the DNA methylation patterns of *Snrpn* in postovulatory DOs and CEOs aged *in vitro*. Sequencing results demonstrated that *Snrpn* was fully methylated in CEOs at 21 h (Fig. 1F) and 29 h (Fig. 1G) post-hCG and in DOs at 21 h post-hCG (Fig. 1D). However, demethylation appeared in DOs at 29 h post-hCG (Fig. 1E). In contrast to *Snrpn,* sequencing results of *Peg1/Mes*t showed that DMR of the gene not only in postovulatory oocytes *in vivo* at 13, 21, and 29 h post-hCG (Fig. 2A, B, and C), but also in DOs (Fig. 2D and E) and CEOs (Fig. 2F and G) cultured *in vitro* at 21 and 29 h post-hCG were fully methylated.

Methylation analysis by COBRA

To ensure that the sequencing results from a limited number of templates accurately reflect the overall methylation pattern for these DMRs in the collected oocytes populations and to confirm further that the cloning was not biased toward either treated or untreated templates, we carried out COBRA [28] on oocyte DNA, cutting the DNA with enzymes that can only cleave the methylated templates: this was performed by carrying out restriction digests on the same bisulfite-treated PCR amplification products that were used for the cloning and sequencing shown in Figs. 1 and 2. We digested the pooled amplified PCR products (described in Materials and methods) of *Snrpn* and *Peg1/Mes*t with *BstU I* and *BstB I*, respectively. Digestion of fragments with enzyme *BstU I* resulted in the appearance of cleaved and uncleaved products in *in vivo* aged oocytes collected at 29 h post-hCG and in *in vitro* aged DOs at the same period, but all fragments of the other groups were completely cleaved (Fig. 3A). This indicates that the digestion results are consistent with the sequencing results (Fig. 1). We also digested amplified products of *Peg1/Mes*t with *BstB I* and found that all the products were completely cleaved (Fig. 3B), indicating that DMR of *Peg1/Mes*t was fully methylated during aging of postovulatory oocytes.

Discussion

In the present study, bisulfite sequencing results show that a subset of postovulatory *in vivo* aged oocytes collected at 29 h post-hCG and *in vitro* culture of DOs at 29 h post-hCG exhibit demethylation of *Snrpn*. In contrast to *Snrpn*, *Peg1/Mes*t maintains hypermethylation of the DMR during postovulatory oocyte aging *in vivo* and *in vitro* up to 29 h post-hCG. Sequencing results are confirmed further by COBRA analysis.

A previous investigation has shown that the methylation imprint at the *SNRPN* loci is not established in the freshly ovulated human oocytes but during or after fertilization [29]. However, Geuns et al. [30] found that the *SNRPN* has gained the full methylation imprint at the GV stage and maintains the pattern in the late oocyte stage. Similarly, Lucifero [14] found that full acquisition of the methylation imprint of *Snrpn* was completed at 20 h post-hCG which corresponds to 6–7 h after ovulation. Our sequencing results are consistent with the findings of the latter two reports. Imamura et al. [23] reported that *Peg1/Mes*t DMR has undergone demethylation at 42 h post-hCG under *in vitro* conditions, however, the *in vivo* situation is unclear. We showed that freshly ovulated oocytes, *in vivo* oocytes collected at 21 h post-hCG and *in vitro* cultured oocytes (both DOs and CEOs) at the same time periods exhibited full methylation of *Snrpn*. Prolonging the *in vivo* culture time from 21 h to 29 h post-hCG and *in vitro* culture of DOs for the same period caused *Snrpn* demethylation in a subset of oocytes. Interestingly, we found that *Snrpn* DMR stayed fully methylated in CEOs at 29 h post-hCG, although *in vivo* oocytes and *in vitro* DOs exhibited demethylation at the same time period.

Lucifero et al. [15] have demonstrated that DMR of *Peg1/Mes*t was hypermethylated in the fully grown GV oocytes isolated from 25 days postpartum (dpp) follicles, and the whole *de novo* methylation process was completed rapidly from 15 dpp to 25 dpp oocytes. In addition, their previous investigations showed the DMR of *Peg1/Mes*t was fully methylated in the postovulatory mouse MII oocytes obtained from oviducts 20 h post-hCG [14]. However,

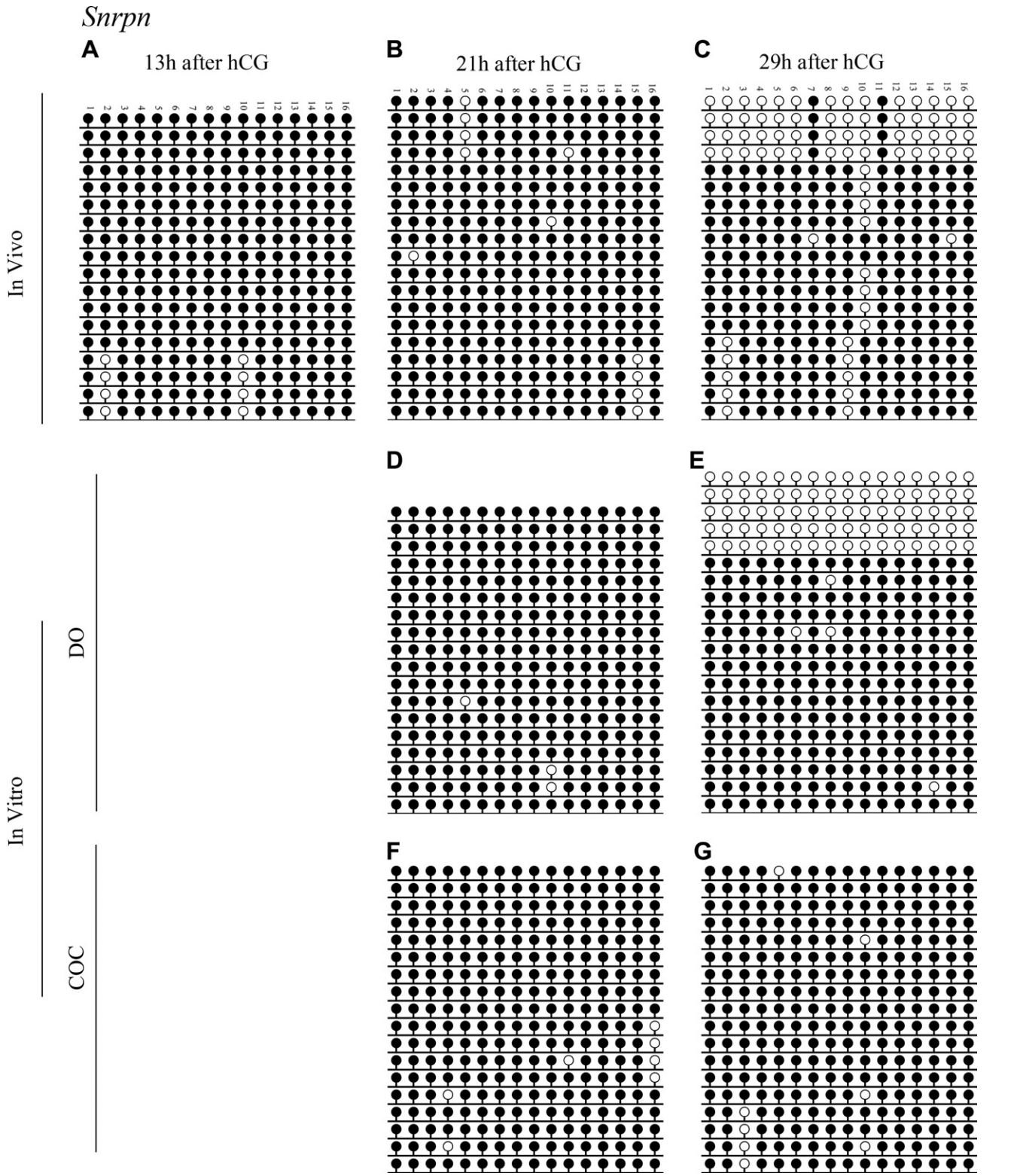

Fig. 1. Cytosine methylation profiles of *Snrpn* DMR during postovulatory aging of mouse oocytes *in vivo* and *in vitro*. (A, B, and C) *In vivo* oocytes collected 13, 21, and 29 h after hCG, respectively. (D,E) and (F,G) DOs and CEOs cultured *in vitro* for 8 h and 16 h, respectively (equals to 21 and 29 h after hCG). Each line presents an individual clone allele, with open circle for non-methylated CpG site and filled circles for methylation cytosines. The CpG sites are numbered from 1 to 16.

Imamura et al. [23] have found that the CpG methylation pattern was heterogeneous in the fully grown GV and freshly ovulated oocytes 14 h post-hCG. These freshly ovulated oocytes may acquire the hypermethylation pattern after *in vitro* culture for 8 h, which corresponds to 22 h post-hCG. Our results showed that CpG loci of the postovulatory oocytes at 21 h post-hCG or culture *in vitro* for the same time period were fully methylated. Aging the oocytes *in vivo* until 29 h post-hCG and prolonging the *in vitro* culture time of DOs and CEOs for 16 h (corresponding to 29 h post-hCG) did not change the hypermethylation pattern of *Peg1/Mest*.

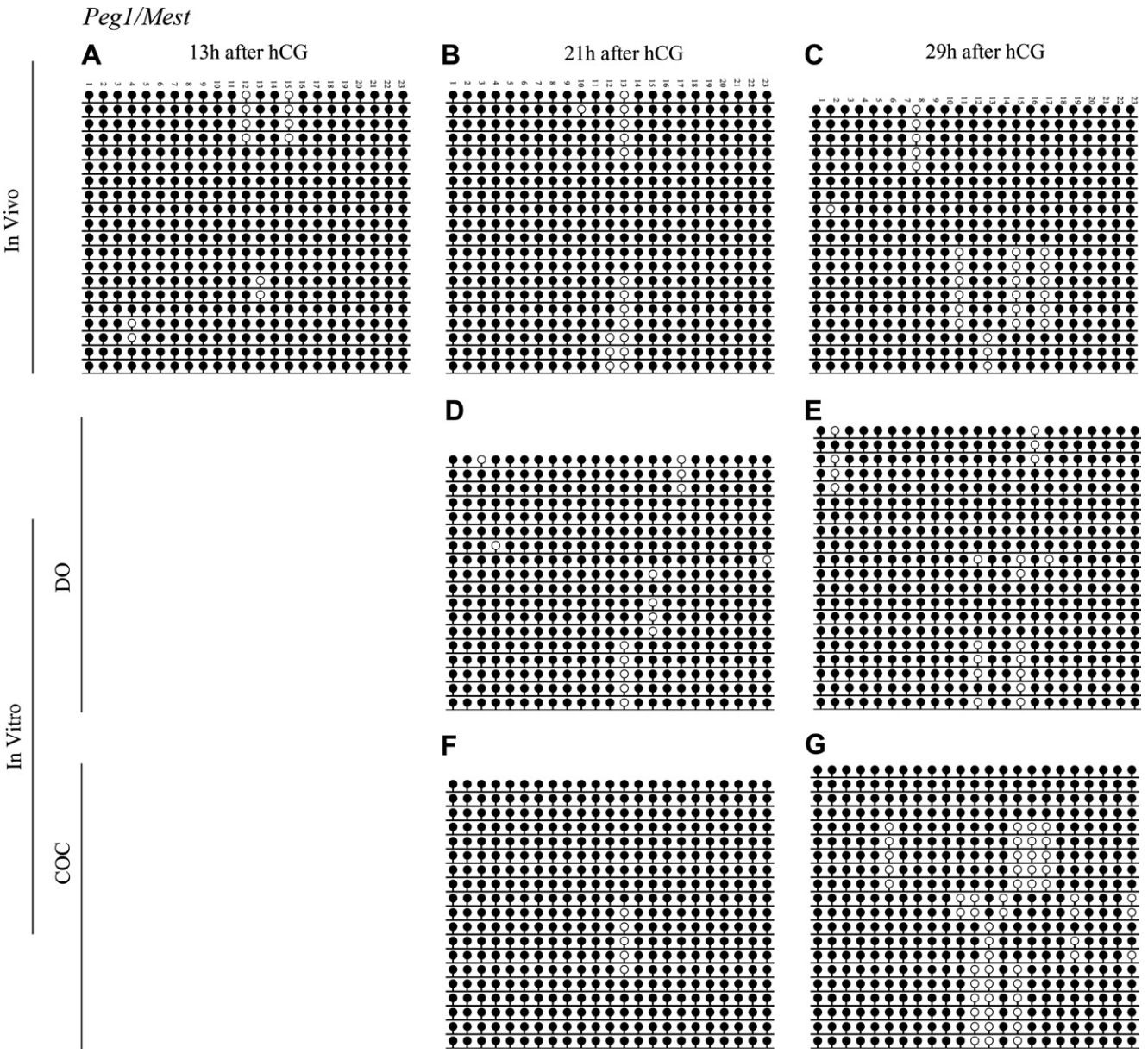

Fig. 2. Cytosine methylation profiles of *Peg1/Mest* DMR during postovulatory aging of mouse oocytes *in vivo* and *in vitro*. (A, B, and C) Postovulatory *in vivo* oocytes collected at 13, 21, and 29 h post-hCG. (D,E) and (F,G) DOs and CEOs cultured *in vitro* for 8 h and 16 h, respectively (equals to 21 and 29 h after hCG). Each line presents an individual clone allele, with open circle for non-methylated CpG site and filled circles for methylation cytosines. The CpG sites are numbered from 1 to 23.

Although previous results [23], together with ours, suggest that demethylation of imprinted genes occurs during the aging process of postovulatory oocytes, the direct molecular biological evidence contributing to the phenomenon is still unclear. Hamatani et al. [31] have found that gene expression patterns are altered in oocytes obtained from old mice. These altered genes including the transcript levels of methyltransferase Dnmtl0, Dnmtls, and Dnmt3L were downregulated. So, we proposed that it may be that the normal transcript levels of methyltransferase were disturbed during the aging process of postovulatory oocytes. The decreased activities of methyltransferase subsequently led to demethylation in those aged oocytes. However, the activities of methyltransferase in the postovulatory aged oocytes still need to be determined.

Despite the fact that aging of postovulatory oocytes has deleterious effects on offspring derived from aged oocytes [4,9,32], hypotheses to explain the mechanism(s) by which aged oocytes may affect offspring are scarce. Tarin [33] proposed a mechanism based on the 'the oxygen radical mitochondrial injury hypothesis of aging [34] to explain the effects of oocyte aging on subsequent viability and potential for embryo and fetal development. This mechanism places emphasis on a key role for senescence-related oxygen radical damage to mitochondrial DNA, proteins and lipids. The results of our present experiments and previous observations by Imamura et al. [23] that imprinted genes exhibit demethylation during the aging process of postovulatory oocytes, together with oxygen radical damage to mitochondrial DNA in aged oocytes may further explain the serious consequences on offspring derived from aged oocytes. This conclusion is supported by the fact that imprinted genes account for the requirement of both maternal and paternal genomes in normal development and play significant roles in regulating embryo growth [35,36]. Aberrant expression of imprinted genes has been linked to abnormal development and human diseases [37].

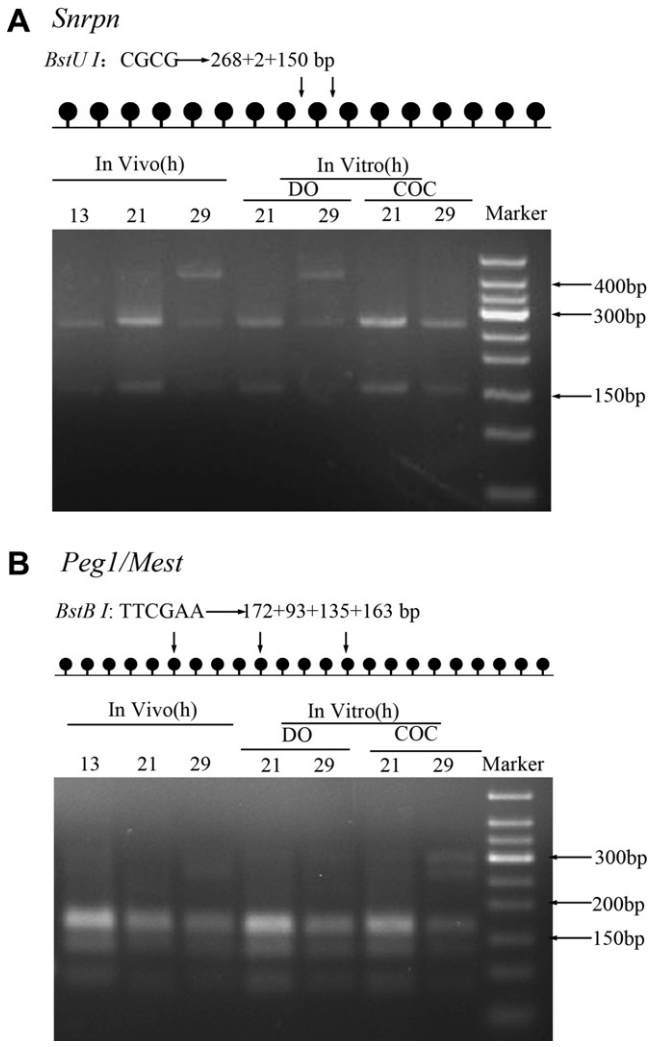

Fig. 3. Overall methylation profiles of the DMRs in postovulatory aging oocytes analyzed by COBRA. The same bisulfite-treated DNA amplified products used for Figs. 1 and 2 were cleaved by restriction enzymes *BstU I* and *BstB I* for *Snrpn* (A) and *Peg1/Mest* (B), respectively. Cleavage CpG sites and the sizes of digested products are indicated on the top. The enzymes only cut the fragments when the specific CpG site was methylated. Sizes of the marker are indicated on the right.

It should be noted that, although we did not find loss of DNA methylation of imprinted genes during the aging process of CEOs culture *in vitro*. It does not indicate that it is safe to extend the *in vitro* culture time of CEOs before fertilization. The roles of surrounding cumulus cells in maturation, ovulation, and fertilization of oocytes have been extensively studied [38,39]. Cumulus cells have also been found to accelerate postovulatory oocyte aging *in vitro* [25]. We should take into account the molecular, biochemical, cellular, and functional changes during the aging process of postovulatory oocytes. Our results suggest that individual imprinted genes undergo time-independent demethylation during the postovulatory oocyte aging process *in vivo* and *in vitro*. These results could have important implications for efficacy and safety for postovulatory aged oocytes in clinical applications.

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