

Active loss of DNA methylation in two-cell stage goat embryos

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ABSTRACT Early mammalian embryos are thought to gain nuclear totipotency through DNA methylation reprogramming (DMR). By this process, DNA methylation patterns acquired during gametogenesis that are unnecessary for zygotic development are erased. The DMR patterns of various mammalian species have been studied; however, they do not seem to have a conserved pattern. We examined early goat embryos to find conforming rules underlying mammalian DMR patterns. Immunocytochemical results showed that the overall level of DNA methylation was not greatly changed during the pronucleus stage. At the two-cell stage, active demethylation occurred and simultaneously affected both parental DNAs, resulting in a global loss of 5-methylcytosine. The level of DNA methylation was lowest in the four-cell stage, with increased *de novo* methylation during the eight-cell stage. Histone H3-lysine 9 was gradually trimethylated in the sperm-derived chromatin, continuing from the pronucleus stage through the two-cell stage. This goat DMR pattern is novel and distinct from the DMRs of other mammalian species. The more mammalian species we included for DMR analysis, the more multifarious patterns we obtained, adding an extra diversity each time to the known mammalian DMR patterns. Nevertheless, the evolutionary significance and developmental consequence of such diverse DMR patterns are currently unknown.

KEY WORDS: DMR, reprogramming, histone methylation, preimplantation development, epigenetics

DNA methylation patterns are relatively stable and heritable, and involved in some specialized biochemical processes, such as allele-specific gene expression (Reik and Walter, 2001), X chromosome inactivation (Heard, 2004), and heritable gene silencing of parasitic sequence elements (Yoder *et al.*, 1997). Notwithstanding the stable and heritable features, DNA methylation patterns go through a reset process at certain thresholds of development, which is necessary to prepare the genome for subsequent development. The conceptual significance of DNA methylation reprogramming (DMR) lies in the removal of the differentiation-associated DNA methylation and re-writing of the epigenetic draft to attain nuclear pluripotency (Kang *et al.*, 2003; Li, 2002; Reik *et al.*, 2001; Rideout *et al.*, 2001). The pre-implantation stage is one stage in which DMR (Park *et al.*, 2007) is necessary.

Pre-implantation stage DMR patterns have been characterized for several mammalian species. The first DMR pattern to be characterized was that of the mouse. In mice, DMR consists of

active DNA demethylation in the male pronucleus (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Santos *et al.*, 2002) and a passive mode of demethylation that mainly affects the female DNA during the cleavage stages (Dean *et al.*, 2001; Mayer *et al.*, 2000; Monk *et al.*, 1987; Oswald *et al.*, 2000; Razin and Shemer, 1995; Rougier *et al.*, 1998; Sanford *et al.*, 1984). When the mouse DMR pattern was first reported, the majority in the field assumed it to be canonical and tacitly took it for granted that other mammalian species might have similar, if not the same, DMR patterns (Kang *et al.*, 2003). Analyses of other mammalian species later, however, have revealed varied DMR patterns. No active demethylation has been detected in sheep (Beaujean *et al.*, 2004a; Beaujean *et al.*, 2004b), rabbits (Beaujean *et al.*, 2004a; Shi *et al.*, 2004), or

Abbreviations used in this paper: 5-azadC, 5-azadeoxycytidine; 5-MeC, 5-methylcytosine; DMR, DNA methylation reprogramming; fPN, female pronucleus; H3K9me3, H3-lysine 9 trimethylation; Pb, polar body.

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Accepted: 13 October 2009. Final author corrected PDF published online: 14 June 2010. Edited by: Makoto Asashima.

pigs (Jeong *et al.*, 2007a; Jeong *et al.*, 2007b). The lack of active demethylation events in these species suggests that the murine DMR pattern should be taken as unique rather than standard. Even more dynamic DMR patterns than that of the mouse has been observed; the bovine DMR pattern includes successive active DNA demethylation and *de novo* DNA methylation during pronucleus development (Park *et al.*, 2007). Taken together, it appears that individual species have their own patterns of DMR. The distinct DMR strategies used by different mammalian species are likely to produce different gene expression profiles, at least temporarily, and may influence the complicated processes of early embryonic development and differentiation.

We examined goat embryos at the pre-implantation stage in an attempt to determine uniform rules underlying mammalian DMR patterns. We found that, at the two-cell stage, an active mode of demethylation affects both parental DNA simultaneously, resulting in a global loss of 5-methylcytosine (5-MeC) in the two-cell nuclei. The DNA methylation level was lowest at the four-cell stage and increased during the eight-cell stage. This DMR pattern

is novel and unique to goat embryos in that the active DNA demethylation occurs in both male and female DNA at a single cleavage stage.

Results

DNA and H3K9 methylation states in goat zygotes derived in vivo

We immunocytochemically examined the global states of DNA methylation and trimethylation at histone H3-lysine 9 (H3K9me3) during early goat development. We found that goat zygotes at different time points of development possessed sufficient 5-methylcytosine (5-MeC) signals in them (Fig. 1A), with the level of the male pronucleus equivalent to, or slightly lower than, that of the female one at later pronucleus stage (Fig. 1Ae and h). That the obvious signals for 5-MeC were present in the male pronucleus implied that DNA demethylation did not occur thoroughly in the zygote stage. When we treated goat zygotes with 5-azadeoxycytidine (5-azadC), we observed no sign of a global loss of DNA methylation in the male goat pronucleus (Fig. 1B); different from the goat zygotes, bovine zygotes as a control revealed evidence of active demethylation in the male pronucleus by the 5-azadC treatment, as shown before (Park *et al.*, 2007). This result indicates the lack of an active DNA demethylation event in the pronucleus-stage goat embryos.

The female pronucleus was shown to harbor intense signals for H3K9me3 (Fig. 1Ac-i). In contrast, the male pronucleus had either no (Fig. 1Ac) or weak H3K9me3 signals (Fig. 1Af-f' and i-i'). These results indicate that the paternal H3K9me3 signals, which are initially absent in

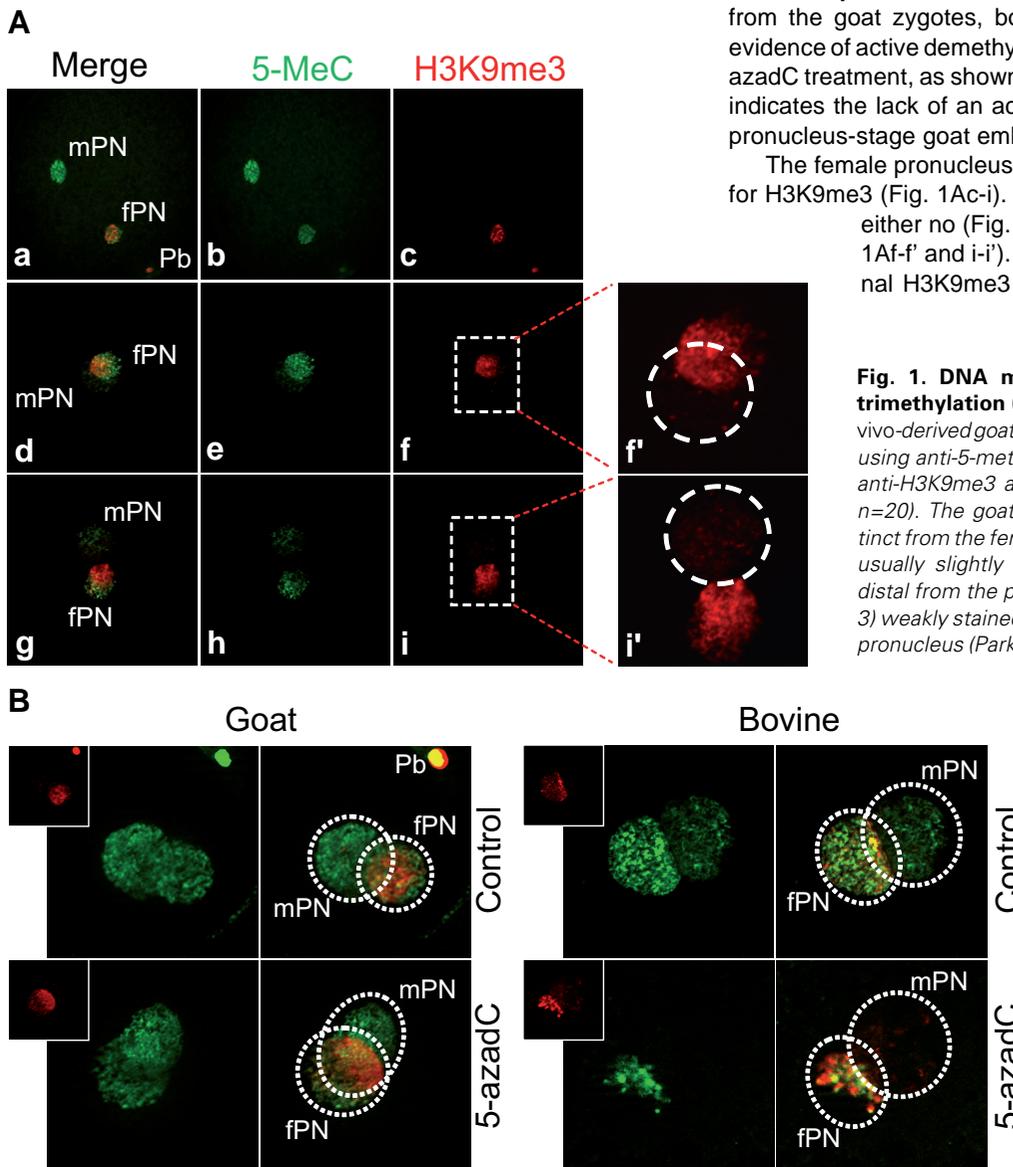


Fig. 1. DNA methylation and histone H3-lysine 9 trimethylation (H3K9me3) in goat zygote pronuclei.

In vivo-derived goat zygotes were fixed before immunostaining using anti-5-methylcytosine (5-MeC) antibody (green) and anti-H3K9me3 antibody (red) (12 hours apart, $n=18$ and $n=20$). The goat male pronucleus is morphologically distinct from the female version and is identified as follows: 1) usually slightly larger than the female counterpart and distal from the polar body; 2) relatively decondensed; and 3) weakly stained for H3K9me3 compared with the female pronucleus (Park *et al.*, 2007). The female pronucleus (fPN)

had stronger H3K9me3 signals than the male pronucleus (mPN). The boxed area in panels (f,i) in (A) are enlarged in panels (f',i'), respectively. (B) 5-azadeoxycytidine (5-azadC) treatment. *In vivo*-derived goat zygotes were cultured in the absence (Control) or presence (5-azadC) of 5 μ M 5-azadC for 12 hours and then fixed for immunostaining. 5-azadC treatment made no difference in the methylation levels of the male goat pronucleus. As a control, bovine zygotes were also included; 5-azadC-treated bovine zygotes contain no 5-MeC signal in the male pronuclei. Insets show H3K9me3 images. Nuclear boundary is indicated as a dashed circle. Pb, polar body.

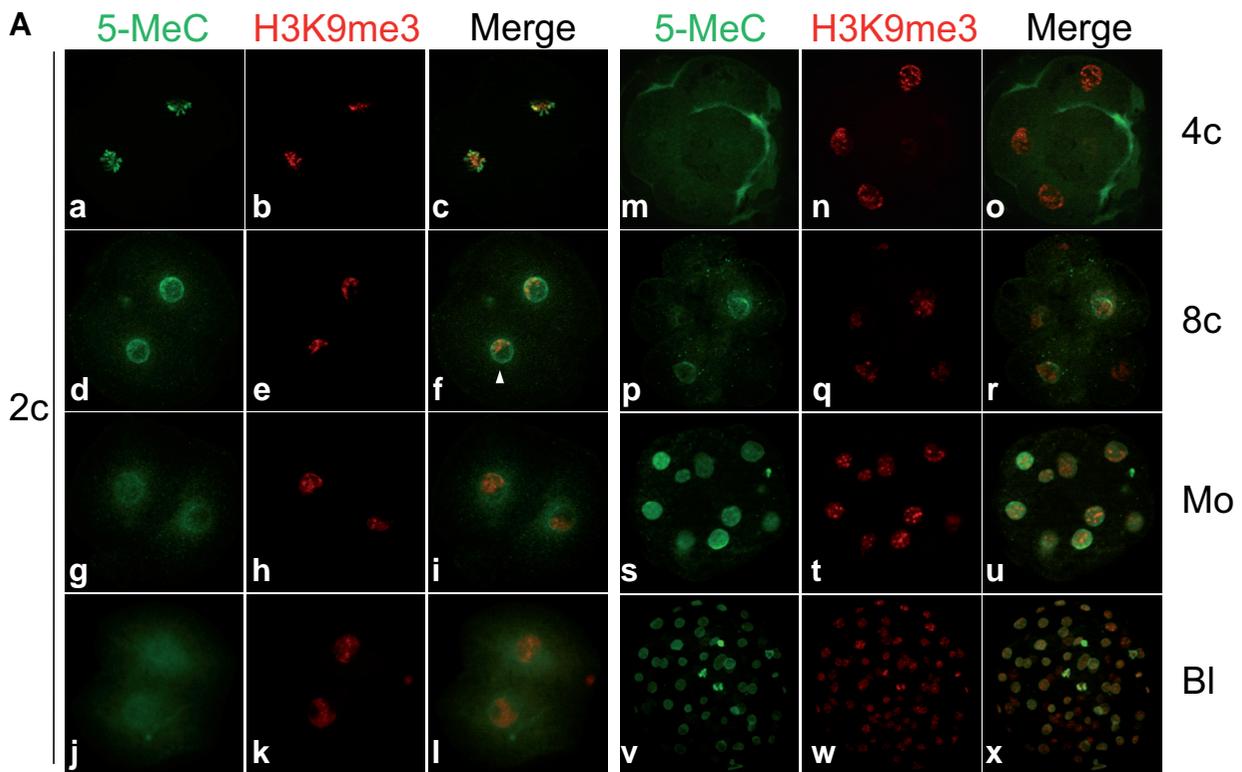


Fig. 2. Features of DNA and H3K9 methylation in cleavage-stage goat embryos. (A) Changes in the DNA methylation and H3K9me3 states. Cleavage-stage goat embryos derived from in vivo fertilization were doubly stained for 5-MeC and H3K9me3. Two-cell stage embryos (2c) were arranged by the extent of H3K9me3 and 5-MeC in the nucleus (b-k). 4c, four-cell

embryo; 8c, eight-cell embryo; Mo, morula; Bl, blastocyst. **(B)** Nuclear compartmentalization of a two-cell nucleus. The two-cell embryonic nucleus shown in panel (f) (arrowhead) was retouched using Photoshop software (version 7) and a plastic wrap filter.

spermic genomic materials, develop gradually after the protamine-histone exchange of chromatin remodeling (McLay and Clarke, 2003). The amount of paternal H3K9me3 was time-dependent, relying on how far the male pronucleus had advanced through the cell cycle. However, the overall levels of paternal H3K9me3 in the goat zygotes were much lower than the maternal level.

Occurrence of active DNA demethylation and H3K9 methylation in two-cell stage goat embryos

The gradually developing H3K9me3 signals in the paternal pronucleus did not reach a level equivalent to the maternal pronucleus. Immunostaining revealed that the nucleus of two-cell stage goat embryos were compartmentalized by H3K9me3 signals, with half the nucleus being methylated and the other half being non-methylated (Fig. 2Ae-h and Fig. 2B). The same nucleus had a relatively even spread of 5-MeC signal to the whole nucleoplasm compared to the H3K9me3 signal. The vacant half of the nucleus, which was assumed to be occupied by the chromatins of male origin, was gradually filled up with H3K9me3 signals as the two-cell embryos advanced (Fig. 2Ak). From the

four-cell stage onwards, the level of H3K9me3 was mostly maintained without significant change (Fig. 2An-w).

As the H3K9me3 signals progressively increased, the 5-MeC levels adjusted in the opposite direction. We noticed that the nuclei of two-cell embryos had a broad spectrum of DNA methylation levels, ranging from distinct (Fig. 2Aa-d) to vague (Fig. 2Ag-j). When two-cell embryos were sorted by the extent of H3K9 trimethylation, which is associated with cell cycle progression, we found that the vivid 5-MeC signals in earlier two cell-stage embryos became fainter at later stages (Fig. 2Aa-j), leaving only trace signals of DNA methylation in the nucleus. Such a change in DNA methylation was ascertained when two-cell embryos were placed side by side with one-cell zygotes; the pronuclei of the one-cell goat zygote had 5-MeC signals, but the two-cell nuclei did not (Fig. 3A).

De novo DNA methylation occurs in eight-cell stage goat embryos

The global loss of 5-MeC during the two-cell stage resulted in the lowest DNA methylation state in the four cell-stage embryos (Fig. 2Am), followed by *de novo* DNA methylation during the eight-

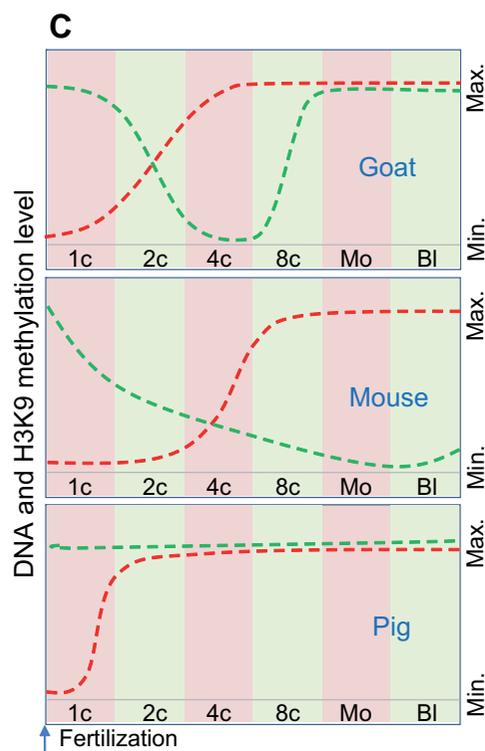
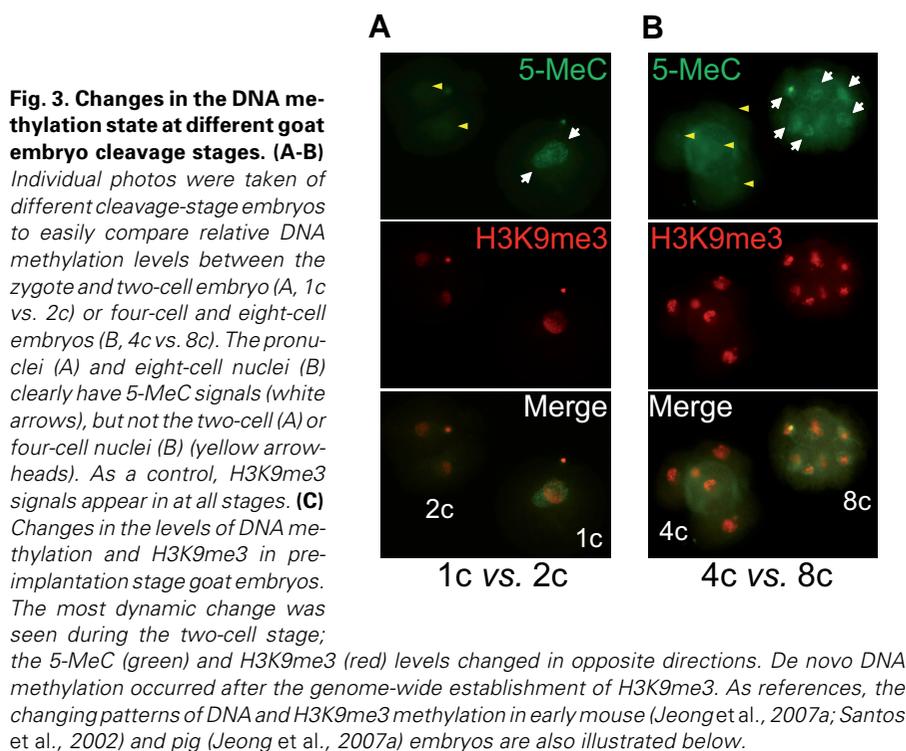
cell stages as 5-MeC signals began to appear in some of the embryonic nuclei (Fig. 2Ap). Figure 3B clearly shows an increase in the 5-MeC level during the transition from the four- to eight-cell stage. DNA methylation signals were eventually present in all embryonic nuclei when the goat embryos reached the morula stage (Fig. 2As). Blastocysts, which consist of two different cell lineages with different developmental potential, such as the inner-cell mass (ICM) and trophectoderm (TE), showed no regional differences in the level of DNA methylation (Fig. 2Av). This pattern of DNA methylation reprogramming differed from those of pig and mouse; the former showed no change in DNA methylation level during the cleavage stages and the latter displayed a progressive demethylation (Jeong *et al.*, 2007a), so none of them resembled the reprogramming pattern of goat embryos. In summary, the global level of 5-MeC was not greatly changed during the pronucleus stage. At the two-cell stage, an active mode of demethylation occurred and both parental DNAs were concurrently affected, resulting in a genome-wide loss of 5-MeC. The level of 5-MeC was lowest at the four-cell stage and increased again during the eight-cell stage via *de novo* DNA methylation. H3K9me3 gradually developed in the sperm-derived chromatin, continuing from the pronucleus stage through the two-cell stage.

Discussion

We recently showed that different mammalian embryos have distinct patterns of DMR at the zygote stage (Park *et al.*, 2007). Type I species, including rodents, undergo active DNA demethylation without global H3K9me3 in the male pronucleus. Type II species maintain the paternal DNA methylation level, and acquired substantial level of H3K9me3, as recently shown in sheep (Beaujean *et al.*, 2004a) and pig zygotes (Jeong *et al.*, 2007a; Jeong *et al.*, 2007b). Bovine zygotes belong to type III species

(Park *et al.*, 2007); the sperm-derived pronucleus looked as if it is partially demethylated. Goat zygotes, looking only at the outside, bear a resemblance to bovine zygotes in that they exhibit fractional demethylation in some of the male pronucleus (Fig. 1e). In spite of the outward similarity of the bovine and goat zygotes, the internal circumstances are quite different; there are completely different DMR processes advancing inward. Bovine zygotes have active demethylation and ensuing re-methylation in the male pronucleus, whereas the goat zygote does not have such a drastic change in the DNA methylation. As shown in Fig. 2A, both active DNA demethylation and re-methylation take place in goat embryos during the cleavage stages. This observation suggests that goat embryos should be re-categorized as a new DMR group of Type-IV.

Zygote-stage mammalian eggs have bundled parental genomes sequestered apart from one another in the pronuclei. The spatial separation between the male and female pronuclei is thought to guarantee global demethylation and intensively affect the male DNA. Thus, the pronucleus stage is assumed to be the most dynamic and relevant period during which the differential methylation and demethylation of the parental DNA take place. In line with this, active DNA demethylation events have been detected in the pronucleus of sperm origin in the mouse, rat, and bovine; therefore, the female genome (and its pan-chromosomal epigenetic states) appears somehow 'protected' as a pronuclear membrane-bound entity from the process of seemingly disastrous global DNA demethylation. However, in this study, we observed full-scale DNA demethylation in the two-cell nuclei (Fig. 2A). Notably, both the female and male genomes were concurrently demethylated within the same nucleus, without the former being spatially isolated from the latter. There are some possible explanations for this observation. Goat embryos may have an escape strategy. Both parental sets of genomes, even if they share the



same nucleoplasmic space, are not necessarily affected by active demethylation processes. The female genome has already been filtered through the pronucleus stage, suggesting that it has sufficient time to secure itself against the upcoming genome-wide demethylation event. On the other hand, the male genome acquires specific indications through the pronucleus stage that are preferentially perceived by the machinery of active DNA demethylation. Alternatively, at a regional level, epigenetically important genomic regions, such as imprinted loci, may possess innate safety devices that ensure the local epigenetic states are protected from destructive modification. Provided that there are such control mechanisms of local DNA methylation states and tactics to evade detrimental epimutations, a global demethylation process occurring simultaneously over the female and male DNA would not necessarily be harmful; instead, simultaneous demethylation would modify both parental DNAs through the same mechanisms using common machinery, so the resulting diploid genome would be regulated in an efficient and coordinate manner.

The timing of H3K9me3 in the spermic chromatin of the goat embryo is also distinct from that of other species. Bovine and porcine sperm-derived chromatins attain sufficient H3K9me3 signals during the pronucleus stage (Jeong *et al.*, 2007b; Park *et al.*, 2007). In mice, the male chromatin does not acquire H3K9me3 signals until the four-cell stage when the embryonic nuclei become enriched with H3K9me3 signals (Liu *et al.*, 2004; Yeo *et al.*, 2005). Regarding the establishment of an H3K9me3 pattern, the goat is not equal to these species. For the sperm-derived chromatin in goat embryos, H3K9me3 began in the zygote stage similar to the bovine and porcine species, but the level of methylation remained low and never increased to the level of the female chromatin (Fig. 2A^f and i'). The methylation state was maintained until the early two-cell stage, and the unbalanced H3K9 trimethylation of the parental genomes produced a compartmentalized nucleus in regards to the H3K9me3 signal (Fig. 2B), as previously seen in two-cell mouse embryos (Yeo *et al.*, 2005). As the embryos advanced to a later two-cell stage, the male H3K9me3 gradually increased to a level comparable to that of female H3K9me3 (Fig. 2Ak). Summarizing the immunostaining results from various mammalian embryos to date, the sperm-derived chromatin might be grossly modified by H3K9me3 during the four-cell stage at the latest. We previously proposed that *de novo* H3K9 methylation directs *de novo* DNA methylation (Park *et al.*, 2007); the results of this study with goat embryos also conform to the proposition that the development of H3K9me3 at the two-cell stage precedes *de novo* DNA methylation at the eight-cell stage (Fig. 3C).

In conclusion, goat embryos exhibit a DMR pattern distinct from the known patterns of other mammalian species; both male and female DNA are concurrently demethylated during the two-cell stage with subsequent *de novo* methylation, mainly during the eight-cell stage. The results agree with previous findings that different mammalian species have their own DMR patterns. The evolutionary significance of the diverse DMR patterns and the developmental consequence of species-specific reprogramming processes are yet to be studied.

Materials and Methods

Antibodies

A monoclonal anti-5-MeC antibody was purchased from Eurogentech (MMS-900S-B). Antibody that specifically recognizes H3K9me3 was pur-

chased from Upstate biotechnologies. Chicken anti-mouse or anti-rabbit IgG secondary antibodies (Alexa-Fluor 488/594-conjugated form, Molecular Probes) were used to detect the individual primary antibodies.

Collection of goat embryos

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. Goat fertilized oocytes were collected from superovulated females 2 to 3-year-old pluriparous Korean native goats (Lee *et al.*, 2000). The estrous cycle of female goats was synchronized by ear implantation of 6 mg norgestomet implants (Synchro-mate-B, Rhone Merieux, Inc., Athens, GA) for 14 days. Superovulation was induced by a combined injection of FSH and hCG. A total of 5.6 mg of FSH (Ovagen, Immuno-Chemical Products, New Zealand) was given to goats over a 4-day period as injection, twice daily, starting 2.5 days before implant removal and continuing to 1 day after removal. A single injection of hCG (100 IU) was given at the time of last FSH injection for the induction of ovulation. Within 24 h following implant removal, the female goats were mated with male goat. Sixty-eight hrs following implant removal, Oviducts of female goats were surgically recovered and flushed in retrograde fashion with sterile PBS as previously reported (Lee *et al.*, 1997).

5-azadeoxycytidine treatment

In vivo-derived goat zygotes were cultured in the presence of 5 μ M 5-azadC (Sigma Aldrich) for 12 hours and then fixed for immunostaining. Procedure for the treatment of bovine zygotes with 5-azadC was described elsewhere (Park *et al.*, 2007). Briefly, the oocytes-spermatozoa complexes were transferred to IVF medium containing 5 μ M 5-azadC eight hours after the commencement of *in vitro* fertilization, and cultured for another 12 hours. The samples were fixed for immunostaining.

Immunostaining early embryos

Immunostaining procedure appeared elsewhere (Jeong *et al.*, 2007b). Briefly, embryos were fixed in 4% formaldehyde in PBS (Gibco) for 30 minutes at 4°C, followed by three times of 20-minute wash in 0.02% Tween 20 (Fisher Scientific) in PBS and permeabilized by 0.25% Triton X-100 (MP Biomedicals) in PBS for one hour at room temperature. The embryos were treated with 4N HCl (Sigma) for 30 minutes at room temperature, neutralized with 0.1 M Tris-HCl (pH 8.0) and blocked for one hour at room temperature in 2% BSA (Sigma), 0.02% Tween 20 in PBS. Primary antibody incubations (diluted by 1:100-300) were carried out in the blocking solution for one hour at 37°C, followed by several washes in 0.02% Tween 20 in PBS. Secondary antibodies (diluted to 1:300-500) were incubated for 30 minutes at room temperature, followed by several washes. Embryos were mounted on Poly-prep slides (Sigma) and, after dry at room temperature, observed with Karl Zeiss Axiovert 200M fluorescence microscope equipped with ApoTome. Most staining experiments were repeated at least three times each with 20 or more embryos. Images were captured digitally using different filter sets and merged using Axiovision (v4.5) or Adobe Photoshop software (v7.0).

Acknowledgement

This work was supported by grants from TDPAF (ARPC) and KRIBB Research Initiative Program (KRIBB).

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