Am J Transl Res 2022;14(8):5941-5951
www.ajtr.org /ISSN:1943-8141/AJTR0145338

Original Article

Epigenetic dynamics of H4K20me3 modification during oocyte maturation and early reprogramming of somatic cell nuclear transfer goat embryos

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Received July 14, 2022; Accepted August 22, 2022; Epub August 25, 2022; Published August 30, 2022

Abstract: Objective: We examined the epigenetic dynamics of histone H4K20 trimethylation (H4K20me3), a repressive signature in heterochromatin, during goat oocyte meiosis and the reprogramming of somatic cell nuclear transfer (NT) embryos through the first three cell divisions. Methods: Following NT, oocytes were treated with parthenogenetic activation (PA), by 5 μM calcium ionophore A23187 for 5 min followed by incubation in 2.0 mM 6-dimethylaminopurine with 5 μg/mL cycloheximide for 4 h. NT embryos up to 8-celled stage were incubated with H4K20me3 antibody. Results: Immunofluorescence microscopy revealed the existence of a persistent H4K20me3 signature during oocyte maturation from germinal vesicle phase to metaphase I, anaphase I, telophase I, and metaphase II, with a gradual reduction in staining intensity. NT embryos at the 2-, 4- and 8-celled stage showed lower H4K20me3 intensity than PA and IVF embryos ($P < 0.05$). Conclusion: These results indicate that NT embryos exhibit insufficient H4K20me3 modification compared with IVF and PA embryos during early reprogramming, suggesting the existence of a resistant memory of differentiated cell nuclear architecture. These findings help unravel the epigenetic mechanism of histone H4K20me3 in goat nuclear transfer reprogramming.

Keywords: Goat, nuclear transfer, epigenetic modification, H4K20me3, meiosis

Introduction

Xenopus was the first animal cloned by somatic cell nuclear transfer (NT), which is the transfer of a somatic cell genome into an enucleated oocyte, demonstrating that every cell in the body contains the same genes [1]. Since then, NT has successfully generated live clones in many mammalian species, including sheep [2], mice [3], cattle [4, 5], and goats [6], but its efficiency is as low as 1-3% due to incomplete reprogramming [7, 8]. Thus far, major reasons for failure are the aberrant epigenetic regulation of gene expression at reprogramming resistant regions of the genome due to barriers such as H3K9me3 [9], H3K27me3 [10, 11], and H3K4me3 [12] epigenetic modifications, DNA methylation and aberrant *Xist* gene activation [11, 13] in cloned embryos. Histone trimethyl Lys 20 (H4K20me3) is an epigenetic histone H4 modification which is generated by a histone methyltransferase (HMT) variegation 4-20 (Suv4-20h2) adding a methyl group onto H4K20me2 [14]. H4K20me3, which is dynamically regulated by both Suv4-20h2 and histone demethylase hHR23 proteins, is a heterochromatin appearing late in development that is associated with the transcription of repetitive elements [15, 16]. However, it is unknown whether H4K20me3 is involved in the process of oocyte meiosis from the germinal vesicle (GV) phase to metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII).

During NT reprogramming, a highly differentiated somatic nucleus is transferred into a recipient oocyte at MII for nuclear remodeling, including nuclear envelope breakdown and sub-

sequent premature chromosome condensation (PCC), which may be induced by maturation-promoting factor in oocyte cytoplasm [17, 18]. However, the epigenetic modification pattern of H4K20me3 during NT reprogramming is unknown, especially in large domestic goats. After fertilization, transcription of the zygote genome in goat embryos, referred to as zygotic genome activation (ZGA), occurs during the second and third cell cycle of embryo cleavage, resulting in 4- to 8-celled embryos, similar to cattle [19], rabbits [20], and humans [21]. Recently, aberrant DNA and histone methylation (i.e., H3K4me2/3 and H3K9me3) were observed in cloned goat embryos during ZGA [22]. Therefore, it is important to understand the presence of H4K20me3 in goat NT embryos during ZGA transition and explore whether H4K20me3 modification is an epigenetic barrier to reprogramming.

In the present study, we investigated the dynamic pattern of H4K20me3 modification at different meiotic phases during goat oocyte IVM as well as NT embryos during early cleavage (i.e., up to the 4- to 8-celled stage) and ZGA.

Material and methods

Chemicals and reagents

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Oocyte collection and IVM

Yangtze River white goats, a native Chinese goat breed, were used for this study. During the goat breeding season (October to December), ovaries were collected from a local slaughterhouse in the Jiangsu province of China. The procedures were followed by the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). All animal use procedures were approved by the Animal Care and Use Committee of Nanjing Normal University (IACUC-20201209). Ovaries were maintained in saline solution at 30°C during their transport to the university laboratory. Ovaries were rinsed with pre-warmed (30°C) saline solution, and large follicles were aspirated by an 18-gauge needle to collect cumulus-oocyte complexes (COCs). The remaining ovary tissue containing small-sized follicles was sliced with surgical blades in D-PBS-based oocyte aspirate plus (Renova Life Inc., College Park, MD) supplemented with 3 IU/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Renova Life Inc.). IVM was performed according to our published procedures [23]. Briefly, COCs with two or more layers of cumulus cells were collected and washed in 10% (v/v) fetal bovine serum (FBS; SH0070.03, Hyclone, Logan, UT) M199 (Gibco, Grand Island, NY) containing Earle's salts, L-glutamine, 2.2 g/l sodium bicarbonate, and 25 mM HEPES. The maturation medium was 10% FBS M199 containing 0.5 μg/ml ovine FSH (NIDDK), 5.0 μg/ml ovine LH (NIDDK), 1.0 μg/ml estradiol (E-8875), and antibiotics. To promote goat oocyte maturation, maturation medium was supplemented with 100 μM cysteamine, 10 μM small molecule Y27632 (Renova Life Inc.), and 10 IU/ml leukemia inhibitory factor (LIF). Twenty to 25 COCs were transferred into droplets of maturation medium (75 μl) and incubated at 38.5°C in 5% CO_2 in a humidified atmosphere for 24 h. COCs post-IVM were denuded by 3 min incubation in 5 mg/ml hyaluronidase D-PBS and stripping with a fine pore pipette (with a diameter close to that of an oocyte). Denuded oocytes were characterized as MI, AI, TI, or MII (i.e., oocytes with a first apparent polar body (PB) seen under a stereomicroscope) and further used for H4K20me3 immunostaining.

In vitro fertilization (IVF)

IVF was routinely performed according to the protocol by An et al. [23]. Briefly, spermatozoa were washed in 8 ml TALP sperm washing medium with 3 mg/ml bovine serum albumin (BSA) by centrifugation at 300 × g for 10 min. The washed sperm pellet was resuspended for a second centrifugation. The supernatant was removed carefully, and the sperm pellet was resuspended in 0.5 ml washing solution for the subsequent IVF procedure. Matured COCs of 25 COCs were allocated per 50-μl droplet of pre-equilibrated fertilization medium. An approximate 50 μl of sperm suspension, giving a final sperm concentration of 10×10^6/ml, was added to each oocyte-containing fertilization droplet. Oocytes and washed sperm were incubated for 18 h at 38.5°C in 5% CO_2 in a humidified atmosphere. Presumptive zygotes we-

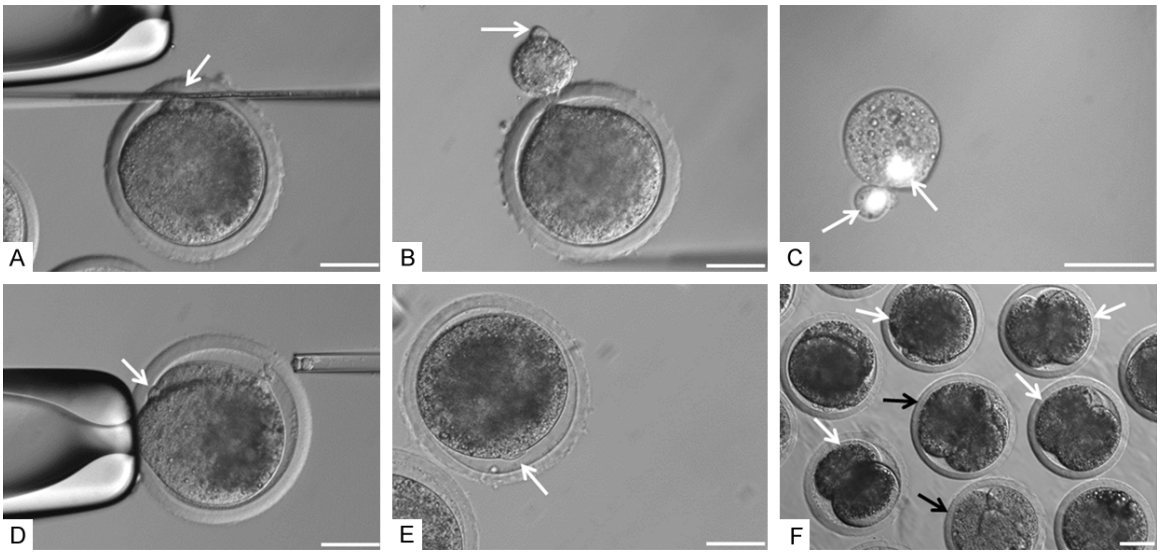


Figure 1. Somatic cell NT with IVM goat oocytes. A. Enucleation of a mature MII oocyte. The first PB (arrow) along with a portion of cytoplasm was pressed out through an incision made in the zona pellucida using a microneedle. B, C. The PB and a small part of the cytoplast containing the MII chromosome set was confirmed by fluorescence microscopy. D. An intact donor cell was transferred into the perivitelline space of an enucleated oocyte (arrow) prior to membrane fusion. Close contact between the donor cell and recipient oocyte was assured by micromanipulation to achieve a higher rate of cell fusion. E. One hour after an electrical stimulus, the donor cell membrane fused with the oocyte cytoplasm (arrow). F. Goat NT embryos cleaved to 2-celled (white arrows) and 4-celled (black arrows) stages after IVC for 48 h. Scale bar = 70 μm.

re denuded and transferred into 50 μl drops of SAR-IVC medium (Renova Life Inc. College Park, MD) supplemented with 6 mg/ml BSA, covered with mineral oil, and incubated at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 48 hours. Two celled embryos were collected after 24 hours of IVC and 4- to 8-celled embryos at 48 h after IVC were selected for H4K20me3 immunostaining.

Donor cell preparation, NT, activation, and embryo IVC

Freshly collected goat cumulus cells served as somatic cell nuclear donors for NT as described by Sung et al. [24]. Briefly, cumulus cells were washed once in Ca^{2+}- and Mg^{2+}-free D-PBS (14190-144, Gibco) supplemented with 10% polyvinylpyrrolidone 40 and centrifuged at 1000 × g for 10 min. Cumulus cell pellets were then treated with trypsin-EDTA for 5 min at 37°C. Trypsinized cumulus cells were suspended into individual cells in 10% FBS-containing DMEM and maintained at 37°C for at least 30 min prior to NT.

NT micromanipulations were performed using our standard procedure [24-26]. Briefly, during enucleation, a small cut on the zona pellucida was made near the PB by a glass needle under a microscope (**Figure 1A**), and approximately one-eighth of the surrounding oocyte cytoplasm together with the PB was squeezed by pressing with the needle (**Figure 1B**). The extruded cytoplasm and PB were individually separated from the oocyte, transferred into separate micro-droplets containing 10 μg/ml Hoechst 33342 for 10 min, and then examined for MII chromosomes under a fluorescent microscope (Olympus IX71, Japan) (**Figure 1C**). Only oocytes with extruded cytoplasts that contained MII plates were used for NT. A donor cell with a diameter of 15-18 μm was aspirated into a micro-pipette with an outer diameter of 20 μm and transferred into the perivitelline space of an enucleated goat oocyte. Close contact between the donor cell and enucleated oocyte was obtained to optimize fusion (**Figure 1D**). Donor cell-cytoplasm pairs were preincubated in 0.3 M mannitol supplemented with 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ for 1.5 min. Pairs were then transferred into a 1-mm-wide electrical chamber containing the same fusion medium. Cell fusion was induced by applying three direct current pulses of 3.2 kV/cm for a duration

of 20 μsec each using a BTX 2001 Electro Cell Manipulator (Biotechnologies & Experimental Research Inc., San Diego, CA). After electric fusion, NT oocytes were incubated for 2 h at 38.5°C to induce cell membrane fusion (**Figure 1E**) and further nuclear remodeling. PA was performed with 5 μM of the calcium ionophore A23187 followed by incubation for 4 h in 2.0 mM 6-dimethylaminopurine (D-2629), 5 μg/ml cycloheximide (C-6255), and 10% FBS M199. Fused NT oocytes were transferred into 50-μl drops of 6 mg/ml BSA synthetic oviductal fluid medium, covered with mineral oil, and incubated at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 48 h to examine the first three cell cycle cleavages (**Figure 1F**). The rate of 2- to 8-celled stage was evaluated. Only 4- to 8-celled NT embryos underwent H4K20me3 immunostaining, whereas 1- or 2-celled embryos were discarded due to retardation or delayed cleavage.

Immunofluorescence microscopy

Oocytes at different meiosis phases (GV, AI, TI, MI, or MII) and NT embryos were fixed with fresh 4% paraformaldehyde in DPBS for 10 min and stored in D-PBS at 4°C until immunostaining processing. After washing in D-PBS for 10 min, permeabilization was achieved by treatment of 0.5% Triton-X 100 for 15 min and washing in 0.25% DPBS/Tween 20 (PBST) for 30 min at room temperature. Incubation in D-PBS supplemented with 2% BSA for 1 h at room temperature was used to block nonspecific binding sites. Immunostaining was performed by incubation with primary monoclonal antibody against H4K20me3 (Cat. 2372, ABclonal, Wuhan, China, dilution 1:100) followed by washing in PBST three times for 15 min at room temperature. Incubation with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Cat. AS053, ABclonal, Wuhan, China, dilution 1:300) was performed for 1 h at 37°C. Oocytes and NT embryos were washed in PBST twice for 30 min at room temperature. Finally, embryos were stained with 4,6-diamidino-2-phenylindole (DAPI; 100 ng/ml; D9564) for 10 min and mounted on slides with 50% glycerol in D-PBS. Image processing and quantification of H4K20me3 staining was performed using fluorescence microscopy (Olympus BX53, Japan) with an exposure time of 500 ms. ImageJ (v1.8.0; National Institutes of Health) was used to perform intensity analysis of the images. Images were first converted to 16-bit greyscale, and the background value was eliminated by the background subtract function. Nuclear intensities of integrated fluorescent images were measured by manually outlining all nuclei of NT embryos at 1-, 2-, 4-, and 8-celled stages. IVF and PA embryos at different stages until the third cell cycle served as controls for NT embryos.

Experimental design

Effect of calcium ionophore (A23871) stimulation on activation of NT oocytes: We tested the effect of A23871 stimulation on the efficiency of activation and further cleavage after NT. Oocytes were treated with 5 μM A23187 for 5 min in the dark and transferred into activation medium with 2.0 mM 6-dimethylaminopurine (D-2629), 5 μg/ml cycloheximide (C-6255), and 10% FBS M199. After 1 h of incubation in activation medium, oocytes were treated a second time with 5 μM A23187 for 5 min and continued to be incubated in activation medium for another 3 h before selection for IVC. Oocyte lysis and cleavage were assessed after activation and after 48 h IVC, respectively.

Statistical analysis

Data on oocyte lysis, germinal vesicle breakdown (n = 22), MI-to-TI (n = 19,20,20), and MII (n = 24) during IVM; cleavage rate of embryos at 48 h post IVC; and H4K20me3 fluorescence intensity of NT (the number of 1-, 2-, 4-, 8-celled was 22, 21, 19, 16), IVF (the number of 1-, 2-, 4-, 8-celled was 23, 23, 21, 19) and PA (the number of 1-, 2-, 4-, 8-celled was 22, 21, 20, 18) embryos were analyzed using SPSS software (SPSS 20.0, Chicago, IL). Percentage data for each replicate were arcsine-transformed and subjected to one-way ANOVA, and means were compared using Fisher's least significant difference tests.

Results

Effect of A23871 stimulation on activation of NT goat oocytes

We first examined the effect of repeated stimulation with A23187, a calcium ionophore, on NT oocyte lysis and cleavage. After one A23187 stimulus, lysis rate was similar between PA

Table 1. Effect of calcium ionophore A23187 stimulation on the cleavage of PA and NT goat embryos in vitro

Treatment	Type of oocyte	No. stimulus	No. oocytes	No. replicates	Lysed % (Mean ± SEM)	Cleavage % (Mean ± SEM)	
						Total	4- to 8-celled
A23187	PA	0	54	4	0.0[a]	24.7 ± 7.3[a,b]	8.7 ± 3.2[a,b]
		1	102	4	10.6 ± 3.1[a]	60.0 ± 6.8[c]	38.9 ± 3.7[c]
		2	88	4	36.5 ± 5.0[b]	41.2 ± 0.9[b,c]	24.0 ± 1.0[d]
	NT	0	74	4	11.8 ± 8.0[a]	10.4 ± 5.1[a]	2.8 ± 2.8[b]
		1	103	4	12.6 ± 4.2[a]	40.8 ± 4.6[b,c]	16.2 ± 1.1[a,d]
		2	109	4	51.0 ± 9.0[b]	24.1 ± 2.5[a,b]	8.6 ± 0.4[a,b]

a, b, c, d Values with different superscript letters within columns are significantly different ($P < 0.05$). Two hours after electrical fusion, NT oocytes were stimulated once or twice with A23187 with an interval of 1 h. NT oocytes were then transferred into activation medium for 4 h and cultured in BSA synthetic oviductal fluid medium at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 for 48 h. Cleaved NT embryos were examined by immunofluorescence microscopy after incubation with anti-H4K20me3 antibody. MII oocytes were treated with the same activation protocol (PA) and served as NT controls. NT, nuclear transfer; PA, parthenogenetic activation.

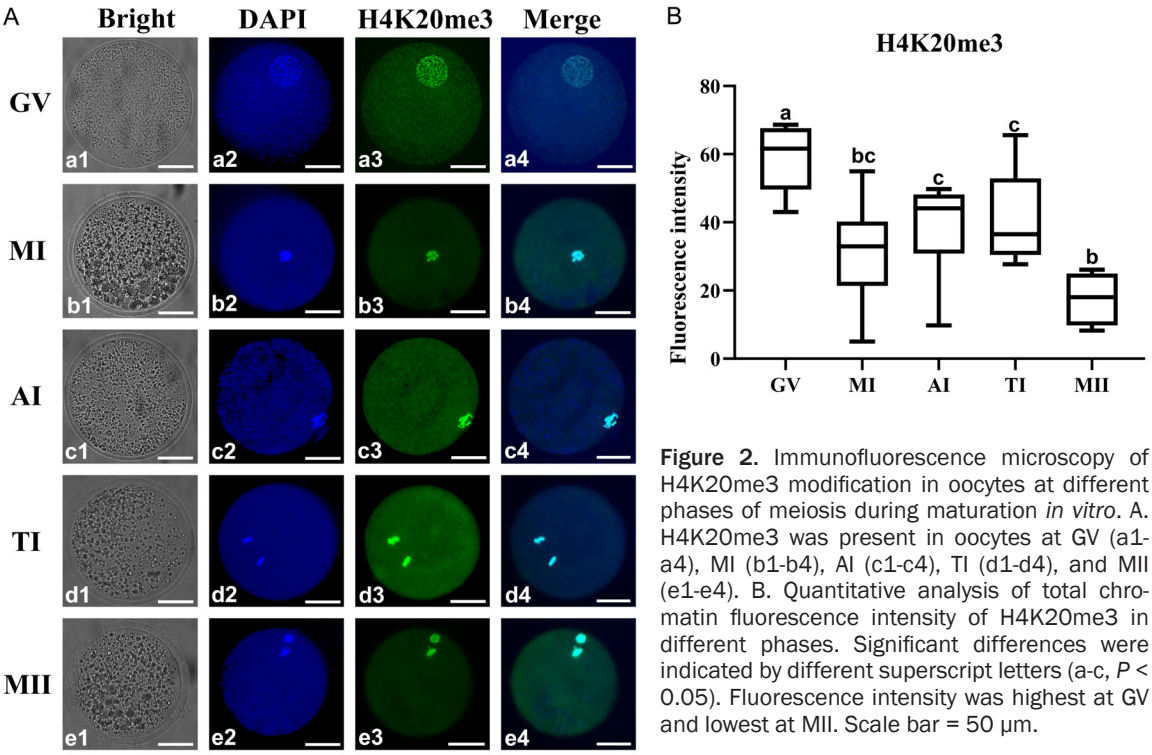


Figure 2. Immunofluorescence microscopy of H4K20me3 modification in oocytes at different phases of meiosis during maturation *in vitro*. A. H4K20me3 was present in oocytes at GV (a1-a4), MI (b1-b4), AI (c1-c4), TI (d1-d4), and MII (e1-e4). B. Quantitative analysis of total chromatin fluorescence intensity of H4K20me3 in different phases. Significant differences were indicated by different superscript letters (a-c, $P < 0.05$). Fluorescence intensity was highest at GV and lowest at MII. Scale bar = 50 μm.

(10.6%) and NT (12.6%) oocytes ($P > 0.05$; **Table 1**), and a second A23187 stimulus increased lysis rate in both PA (36.5%) and NT (51.0%) oocytes ($P < 0.05$). By comparing within each PA and NT group, one A23187 stimulation increased the total cleavage rate (2- to 8-celled stages) (PA 60.0%, NT 40.8%) and the rate of 4- to 8-celled PA (38.9%) and NT embryos (16.2%) ($P < 0.05$). Furthermore, by comparing between NT and PA group, both one and two A23187 stimuli resulted in a lower 4- to 8-celled cleavage rate for NT embryos than for PA embryos (one A23187: NT 16.2% vs. PA 38.9%; twice A23187: NT 8.6 vs. PA 24.0%) ($P < 0.05$) (**Table 1**).

H4K20me3 dynamics in meiosis during goat oocyte maturation

Immunofluorescence microscopy showed that H4K20me3 was present in oocytes at GV phase prior to IVM (**Figure 2A**, a1-a4). At MI, the

chromosome set was condensed (**Figure 2A**, b1-b4), and the metaphase plate began to separate toward AI (**Figure 2A**, c1-c4). At TI, the metaphase chromosome set was completely apart (**Figure 2A**, d1-d4) and finally matured to extrude a PB and MII metaphase plate (**Figure 2A**, e1-e4). H4K20me3 intensity was highest at GV phase and lowest at MII (**Figure 2B**; $P < 0.05$), with a similar moderate and reduced intensity across MI, AI, and TI ($P > 0.05$).

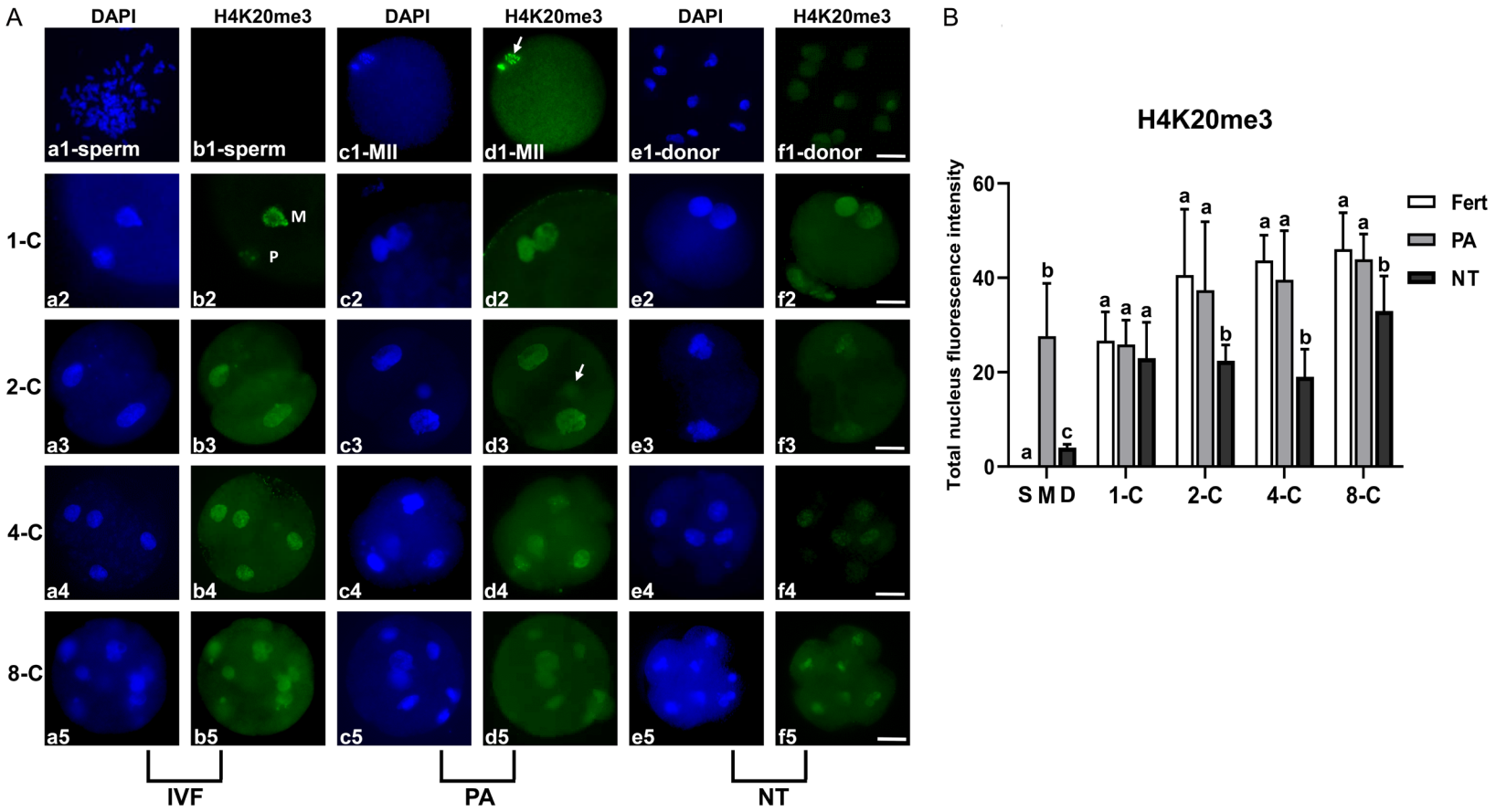
Dynamic pattern of H4K20me3 modification in NT goat embryos

To examine the pattern of H4K20me3 modification during NT embryo development, NT oocytes were remodeled in M199 medium for 2 h and subjected to activation for 4 h, and fused oocytes underwent IVC for 48 h, after which oocytes and cleaved embryos were collected for immunostaining (**Figure 3A**). After remodeling and activation, NT oocytes formed two pseudo-pronuclei (PPN) that carried H4K20me3 staining, and maintained its intensity at 2-celled stage. After IVC for 48 h, NT embryos that cleaved to the 4- to 8-celled stage showed weaker H4K20me3 staining. As activation controls, MII oocytes were subjected to the same activation process as NT oocytes; these oocytes formed two PPN 4 h after activation and developed to the 4- to 8-celled stage 48 h after IVC. H4K20me3 staining was observed in the PPN of these PA oocytes and through the 4- to 8-celled stage (**Figure 3A**). Donor nuclei showed low baseline H4K20me3 staining intensity, which began to increase and reached a peak after activation. MII oocytes showed higher H4K20me3 staining intensity than donor nuclei and NT embryos after activation (NT 2 h). Sperm showed no H4K20me3 staining while sperm derived paternal PN carried weaker signature (**Figure 3B**). After IVC 48 h, fertilized goat embryos at 4-, 8-celled stages maintained constant H4K20me3 staining (**Figure 3B**). However, intensity analysis revealed that cleaved NT embryos (2-, 4-, 8-celled) showed a significantly decreased H4K20me3 intensity compared to that IVF and PA embryos ($P < 0.05$) (**Figure 3B**). Furthermore, IVF and PA embryos showed similarly intensity after IVC for 48 h (**Figure 3B**).

Discussion

Our study reveals the dynamic profile of H4K20me3 modification across oocyte meiosis and during reprogramming in NT. The persistent presence of H4K20me3 modification in oocytes at different meiotic phases (GV to MII) suggests that it plays an important role in remodeling oocyte nuclear chromatin structure and regulating gene expression during meiosis. H4K20 and H3K9 trimethylation are considered to be important components of repressive gene expression at constitutive heterochromatin [14, 27] and regulators of the transcription of repetitive DNA elements [15]. H4K20me3 dynamics are controlled by functional interplay between histone Suv4-20 methyltransferase [16] and H4K20 demethylases [15]. During meiosis, when bivalent homologous chromosomes with synapsis in prophase (i.e., GV phase) enter MI, the chromosome set is remodeled into a higher-order structure in the compacting process. We found the highest H4K20me3 intensity at GV phase and lowest intensity at MII, reflecting a reduced H4K20me3 signature after the completion of meiosis. This H4K20me3 signature co-existed with highly DAPI-stained regions, where DNA is highly folded. A previous study reported that H4K20me3 existed in the female pronucleus and polar bodies but not in the male pronucleus of mouse zygotes, providing evidence of the existence of a maternal pattern of H4K20me3 modification during meiosis [16]. The dynamics of H4K20me3, a constitutive heterochromatin hallmark, reflects the conformation of its inherent chromatin structure [12, 14, 16]. Repressive chromatin is interspersed with active chromatin in the mammalian genome [28, 29]. The pattern of H4K20me3 is maintained in an evolutionary conserved manner and serves to regulate position-effect variegation [12, 30].

Successful nuclear reprogramming by somatic cell NT has been demonstrated by the generation of more than a dozen species of cloned animals, although the reasons for its low efficiency and related inherent reprogramming mechanisms remain to be completely revealed. Toward this end, incomplete global epigenetic modifications were recently found to be barriers to reprogramming and cloning success, including H3K9me3 [9], H3K27me3 [11, 31], H3K4me3 [12], imprinting genes [32], X-chromosome inactivation [13], and DNA methylation [11, 22, 33]. At least some of these barriers can be overcome, for example, H3K9me3 removal by its demethylase overexpression and Suv39h1/2 knockdown improve NT efficiency



Figue 3. Dynamic pattern of H4K20me3 in goat NT embryos. A. Negative immunostaining of H4K20me3 in sperm (a1-b1), positive in MII oocyte (c1-d1) and cumulus donor cells (e1-f1). After IVF, one-celled fertilized goat embryo showed strong intensity in maternal PN and weak staining in paternal PN (a2-b2). The constant staining was shown at 2-celled (a3-b3), 4-celled (a4-b4), and 8-celled (a5-b5), respectively. After activation, PA oocytes showed staining at PPN (c2-d2), 2-celled (c3-d3), 4-celled (c4-d4) and 8-celled (c5-d5), respectively. The donor nucleus was present as 2 PPN in the oocyte 4 h after activation (Act 4 h; e2-f2). A cloned embryo cleaved into 2-celled (e3-f3), 4-celled (e4-f4) and 8-celled (e5-f5) stage with lower H4K20me3 intensity. B. Quantitative analysis of total nuclear fluorescence intensity of H4K20me3 in IVF, PA, and cloned embryos after NT, activation, and IVC. Different a, b letters indicated significant differences ($P < 0.05$). H4K20me3 intensity in donor cells was lower than that in MII oocytes. The intensity in NT embryos was similar to IVF and PA embryos at one celled stage, but it was significantly lower than both IVF and PA embryos from 2-celled, 4-celled and 8-celled stage, respectively ($P < 0.05$). 1-C, one celled; 2-C, 2-celled; 4-C, 4-celled; 8-C, 8-celled; M, MII oocytes; D, donor celled; Fert, fertilized. Scale bar = 50 μm.

in both mice (KDM4D) [9] and humans (KDM4A) [34]. The combined use of demethylases KDM4B and KDM5B greatly improves mouse cloned blastocyst rate and birth rate [12]. Furthermore, inactivation of DNA methyltransferases rescues remethylation defects in mouse NT embryos [33].

The role that H4K20me3 plays in the reprogramming of an oocyte to an introduced somatic nucleus has not been well understood. The first three cell cycles are critical for successful reprograming, as ZGA occurs at the 4- to 8-celled stage in humans [35], rabbits [20], and goats [21]. We chose to investigate H4K20me3 profile in PA goat oocytes because NT embryos are activated in the same manner when an oocyte receives a differentiated somatic nucleus. We found that inactivated oocytes and PA embryos maintained a constant intensity of H4K20me3 from the MII to an 8-celled embryo stage. This pattern is in contrast to that observed by Wongtawan et al. [16], who showed that the H4K20me3 signal was undetectable from zygotes to blastocysts. When we performed immunostaining with the same H4K20me3 antibody in mouse oocytes, zygotes, 2-celled embryos, 4-celled embryos, 8-celled embryos, morula, and blastocysts, we detected the presence of H4K20me3 in other stages except for the male pronucleus in zygotes containing a PB and 2-, 4-celled (Du, unpublished data). We have validated the H4K20me3 antibody, therefore, this discrepancy might be due to differential sensitivity of the antibody used for experiments. Theoretically, H4K20me3, a conserved constitutive heterochromatin mark and repressive regulator of gene expression, is maintained in the repressive structure region of heterochromatin, which is a feature of centromeres and telomeres [28]. We found that the donor nucleus carried a weak H4K20me3 signal compared with that in the chromosomes of MII oocytes. After its transfer into oocyte cytoplasm, the nucleus underwent a series of remodeling changes, including nuclear swelling and PCC, reflecting a dramatic exchange or replacement of oocyte protein/RNA components oocyte with donor nuclear chromatins. However, H4K20me3 intensity in NT embryos was lower than that in PA embryos, indicating insufficient nuclear H4K20me3 modification in goat NT embryos. This implies that the donor cumulus nucleus maintained a resistant memory of its H4K20me3 pattern even though it was remodeled or reprogramed by oocyte mechanisms. Indeed, epigenetic memory is often present in NT embryos. For example, *Xenopus* cloned blastula embryos derived from muscle donor nuclei express memory of the muscle gene marker MyoD in the neuroectoderm (i.e., nerve/skin cell lineage) and endoderm (i.e., intestine linage) to an excessive extent in about half of all embryos [36]. Therefore, it is necessary to first erase this H4K20me3 memory in donor nuclei, such as via histone demethylase, and then reestablish the oocyte/embryonic pattern.

It is interesting to compare fertilized embryos with NT embryos, as they represent a natural reprogramming and development event, an oocyte is activated during fertilization by the penetration of sperm, which cannot be achieved by NT. We found that intensity of H4K20me3 from 2-, 4- to 8-celled embryos was similar between IVF and PA groups, but both groups carried much higher intensity than that of NT group. In addition, the total cleavage rate of cloned embryos (40.8%) and rate of on-schedule embryos (4- or 8-celled after 48 h IVC, 16.2%) was inferior to those of PA embryos (60.0% and 38.9%, respectively). This developmental inferiority may be due to different reprogramming barriers, such as aberrant H3K9me3, H3K27me3, or H3K4me3 modification or DNA hypermethlyation. Overexpression of ten-eleven translation 3 (*Tet3*) in donor cells can correct abnormal DNA hypermethylation in cloned embryos [37]. Also, whereas lncRNA can represses KDM5B expression and impede the H3K4me3 demethylation process, both overexpression of KDM5B and knock down of lncRNA can overcome the H3K4me3 barrier in goat NT embryos [38].

It is important to explore whether H4K20me3 regulates the reprogramming of cloned embryos. In particular, dynamic H4K20me3 events occurring in cloned embryos during the first three cell cycles are critical for elucidating and understanding the cellular and molecular mechanisms of remodeling and reprogramming. Recently, two histone H4K20 demethylases were found to demethylate the H4K20me3 signature [15]. This provides a useful tool for erasing H4K20me3 in donor nuclei established

with a particular differentiation memory in specifically differentiated cumulus donor cells and allowing oocytes to reestablish a modification pattern necessary for reprogramming and further competent development, not only for cleavage but also for preimplantation and term development. Thus, future studies are needed to comprehensively compare reprogramming events between goat NT embryos and PA or fertilized embryos during preimplantation development (i.e., morula and blastocysts) as well as their competent development potential.

Different activation protocols have been applied for PA in goat NT studies [22, 39, 40], all of which used ionomycin combined with other reagents. In the present study, we examined the effect of the calcium ionophore A23187 (5 μM for 5 min) on activation of both NT embryos and MII oocytes as controls. A23187 releases the intracellular calcium pool in oocytes to mimic their activation by the penetration of sperm during fertilization [41]. One stimulation with A23187 was sufficient to activate oocytes. However, two stimulations with A23187 had a detrimental effect on oocyte survival, indicating that multiple stimulations to release calcium ions via artificial activation with A23187 may be fatal to oocytes.

In summary, our study reveals the dynamic pattern of H4K20me3 epigenetic modification, a repressive histone trimethylation signature in heterochromatins, during oocyte maturation, PA, IVF and NT reprogramming during the first three cell cycles. We found that H4K20me3 remains a constant signature during oocyte meiosis (GV to MII), PA and IVF. However, H4K20me3 modification was insufficient in NT embryos compared with PA and IVF embryos, indicating the existence of a resistant memory of differentiated cell nuclear architecture. These findings provide insight into the mechanism of histone H4K20 trimethylation in reprogramming, regulation, and function during somatic cell NT in goats.

Acknowledgements

This study was supported in part by grants from the Natural Science Foundation of China (Grant No. 31872353, 32072732, 31340041, and 31471388) and Priority Academic Program Development of Jiangsu Higher Education Institutions to FD and a grant from the Natural Science Foundation of China (Grant No. 3170-1285) to LA.

Disclosure of conflict of interest

None.

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