REPRODUCTION

Methylation dynamics during folliculogenesis and early embryo development in sheep

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Abstract

Genome-wide DNA methylation reprogramming occurs during mammalian gametogenesis and early embryogenesis. Post-fertilization demethylation of paternal and maternal genomes is considered to occur by an active and passive mechanism respectively, in most mammals but sheep; in this species no loss of methylation was observed in either pronucleus. Post-fertilization reprogramming relies on methylating and demethylating enzymes and co-factors that are stored during oocyte growth, concurrently with the re-methylation of the oocyte itself. The crucial remodelling of the oocyte epigenetic baggage often overlaps with potential interfering events such as exposure to assisted reproduction technologies or environmental changes. Here, we report a temporal analysis of methylation dynamics during folliculogenesis and early embryo development in sheep. We characterized global DNA methylation and hydroxymethylation by immunofluorescence and relatively quantified the expression of the enzymes and co-factors mainly responsible for their remodelling (DNA methyltransferases (DNMTs), ten-eleven translocation (TET) proteins and methyl-CpG-binding domain (MBD) proteins). Our results illustrate for the first time the patterns of hydroxymethylation during oocyte growth. We observed different patterns of methylation and hydroxymethylation between the two parental pronuclei, suggesting that male pronucleus undergoes active demethylation also in sheep. Finally, we describe gene-specific accumulation dynamics for methylating and demethylating enzymes during oocyte growth and observe patterns of expression associated with developmental competence in a differential model of oocyte potential. Our work contributes to the understanding of the methylation dynamics during folliculogenesis and early embryo development and improves the overall picture of early rearrangements that will originate the embryo epigenome.
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Introduction

DNA methylation at the 5-position of cytosine (5-mC) in mammals largely takes place in the context of CpGs and plays important roles in diverse processes including transcription regulation, maintenance of genomic integrity, regulation of genomic imprinting and X chromosome inactivation (Jaenisch & Bird 2003). Despite the relative stability of DNA methylation in somatic cells, genome-wide DNA methylation reprogramming occurs twice during the life on an individual: in primordial germ cells and in pre-implantation embryos (Reik 2007). The first extensive demethylation event occurs during gametogenesis, when the pre-existing epigenetic profile of the somatic cells is erased in primordial germ cells, while germ cell- and sex-specific epigenetic patterns are established during the later phases of gametogenesis (Smallwood & Kelsey 2012). The second event of genome-wide epigenetic reprogramming occurs in the embryo soon after fertilization. Such remodelling is required for

starting the new developmental programme of the nascent embryo.

The conventional model for epigenetic reprogramming postulates a parent-specific epigenetic reprogramming of the paternal and maternal genomes during the pre-implantation development of most mammalian species. Up to recent times, the paternal genome was thought to be actively demethylated within a few hours after fertilization in the mouse, rat, pig, bovine and human zygote, whereas the maternal genome would be passively demethylated by a replication-dependent mechanism after the two-cell embryo stage (Mayer *et al.* 2000). Sheep was thought to escape from this model, as the loss of methylation from either pronucleus (PN) was not observed at any point in the first cell cycle in *in vivo*-derived ovine zygotes (Beaujean *et al.* 2004). Recent studies partly defy this theory and suggest that parental pronuclei may share the major mechanisms of active and passive demethylation, which are differently modulated to achieve parent-specific patterns of methylation remodelling (Inoue & Zhang 2011, Shen *et al.* 2014).

Moreover, new dynamics that occur immediately after fertilization were evidenced, including a methylation maintenance during the S phase in the paternal PN of rabbit embryos after an early partial demethylation (Reis Silva *et al*. 2011). The new findings suggest that epigenetic reprogramming may be more complex than previously thought and call for further studies to revisit previous results.

DNA methylation status is largely determined by a balance between the actions of methylating and demethylating enzymes. DNA methylation is established and maintained by DNA methyltransferases (DNMTs) that catalyse the transfer of a methyl group from S-adenosyl-l-methionine to the fifth position of cytosine residues in DNA (Chen & Li 2004). Two distinct mechanisms of DNA methylation activity exist: methylation maintenance and *de novo* methylation (Holliday & Pugh 1975). The first is performed during DNA replication by Dnmt1, which accurately copies the methylation pattern of the existing DNA strand onto the new one (Bestor *et al*. 1988). *De novo* methylation process is mediated by Dnmt3a and Dnmt3b (Okano *et al*. 1998) that are extensively involved in re-methylation during oogenesis (Kaneda *et al*. 2004) and embryo development (Okano *et al*. 1999). Dnmt3L, despite the misnomer, is a catalytically inactive, related protein that modulates Dnm3a and Dnmt3b activity (Bourc'his *et al*. 2001). Conversely, DNA demethylation can take place either passively or actively. Passive demethylation occurs when DNA is replicated without re-methylation of the new DNA filament by DNMT1. The active mechanism, on the other hand, is a highly orchestrated enzymatic process involving ten-eleven translocation (TET) protein-mediated iterative oxidation (Ito *et al*. 2010, He *et al*. 2011) followed by thymine-DNA glycosylase (TDG)-initiated base excision repair (He *et al*. 2011). Although all Tet family members (Tet1–3) possess 5-mC oxidation activity, they show cell- and tissue-specific expression patterns (Ito *et al*. 2010), suggesting different functional roles that are yet to be fully clarified. In accordance with the conventional model for epigenetic reprogramming, several studies have shown that Tet3 is involved in the active demethylation of the paternal genome (Inoue & Zhang 2011, Iqbal *et al*. 2011, Wossidlo *et al*. 2011); however, more recently Tet3 was seen to contribute also to maternal DNA demethylation in zygotes, although to a lesser extent compared to that of paternal DNA (Shen *et al*. 2014).

The activity of methylating and demethylating enzymes is supported by several groups of proteins that act as co-factors and modulate and interpret DNA methylation patterns. These include the family of methyl-CpG-binding domain (MBD) proteins that play an important role in the regulation and interpretation of DNA methylation, chromatin-remodelling processes and gene expression. Currently, the MBD protein family consists of eleven known proteins containing an MBD domain that binds single symmetrically methylated CpG dinucleotides (Ohki *et al*. 2001); they are pivotal in linking methylation with transcriptional activity (Wade 2001) and act mainly as transcriptional repressors, but may also be involved in gene activation or expression modulation. The single members of the MBD family play cell- and developmental-specific roles, and may be involved in stem cell renewal, embryogenesis or differentiation (Hendrich *et al*. 2001). During embryogenesis, MBD proteins have been implicated in both global transcriptional regulation (Klose & Bird 2006) and more specifically in the repression of Oct4, a pluripotency marker essential for early stages of mammalian embryogenesis (Gu *et al*. 2009). MBD1, 3 and 4 were also shown to be expressed in mouse and bovine oocytes (Ruddock-D'Cruz *et al*. 2008).

The molecules that support post-fertilization epigenetic reprogramming, including methylating and demethylating enzymes, are stored in the oocyte during its growth. The growing phase of the oocyte during folliculogenesis is therefore crucial for a proper definition of the epigenome in two ways: on one side, the molecules that will support epigenetic reprogramming during early development are accumulated; on the other side, the re-methylation of the oocyte itself with sex-specific epigenetic patterns occurs during the late stage of the growth. Despite the remodelling that erases most oocyte-specific methylation marks after fertilization, a memory of the epigenetic status of the gamete will be inherited by the nascent embryo, as in the case of imprinted genes (but not exclusively). The epigenetic baggage of the oocyte is therefore essential for its developmental potential; unfortunately, its remodelling often overlaps with potential interfering events such as exposure to assisted reproduction technologies (Young *et al*. 1998, Hansen *et al*. 2005) or environmental changes (i.e. nutrition, pathologies or accidental exposition to various contaminants) (Cortessis *et al*. 2012). Accurate knowledge of the methylation and hydroxymethylation status of the growing oocyte and on the molecules involved in their remodelling is fundamental to gain an overall picture of the early rearrangements that will originate the embryo epigenome.

Here, we analysed DNA methylation and hydroxymethylation during oocyte growth and maturation, in zygotes and early embryos in sheep. We illustrate for the first time hydroxymethylation during oocyte growth and we show different patterns of methylation and hydroxymethylation between the two parental pronuclei, suggesting that male PN undergoes active demethylation also in sheep. In parallel, we relatively quantified the expression of the enzymes mainly responsible for methylation remodelling, and linked the observed patterns with oocyte developmental competence using a differential model of gamete potential.

Materials and methods

All chemicals in this study were purchased from Sigma Chemical unless otherwise stated.

Oocyte recovery

Oocytes were recovered from the ovaries of pre-pubertal (30–40 days of age, body weight 6–10 kg) and adult (4–5 years of age, body weight 35–40 kg) Sarda sheep, collected at a local slaughterhouse and transported to the laboratory within 1 h in Dulbecco's phosphate-buffered saline (PBS) with antibiotics. After washing in fresh medium, ovaries were sliced using a micro-blade and the follicle content was released in TCM-199 medium (with Earle's salts bicarbonate) supplemented with 25 mmol HEPES, 0.1 g/L penicillin, 0.1 g/L streptomycin and 0.1% (w/v) polyvinyl alcohol.

Collection of growing oocytes

Growing oocytes were selected from COCs derived from pre-pubertal donors, showing intact cumulus cell layers and a compact cytoplasm. After removal of cumulus cells by gentle pipetting with a fine bore glass pipette, oocytes were classified on the basis of their major diameter (average of two perpendicular measurements, excluding the zona pellucida) and grouped in three classes: oocytes with 70–90 μm (small; S), 90–110 μm (medium; M) or 110–130 μm (large; L) diameter.

In vitro *maturation*

COCs derived from both pre-pubertal and adult donors, showing several intact cumulus cell layers and a compact cytoplasm, were selected and matured *in vitro* (IVM) in TCM 199 supplemented with 10% heat treated oestrus sheep serum (OSS), 10 μL/mL FSH/LH and 100 μL cysteamine. Then, 30–35 COCs were cultured for 24 h in 5% CO_2 in air at 38.5°C in four-well Petri dishes (Nunclon; Nalge Nunc, Roskilde, Denmark) with 600 μL maturation medium, layered with 300 μL mineral oil. After IVM, oocytes underwent cumulus and corona cells removal by gently pipetting and only oocytes presenting compact cytoplasm and the first polar body were selected.

In vitro *fertilization and embryo development*

Frozen-thawed spermatozoa of one single ram of proven fertility (Sarda breed, 4 years old, body weight 55 kg) were used for all *in vitro* embryo production experiments. Two straws were thawed per IVF and spermatozoa were selected by swim-up technique (1×10^6 spermatozoa*mL). IVM oocytes were incubated in synthetic oviductal fluid (SOF) medium (Tervit *et al.* 1972) with 2% OSS, 1 μg/mL heparin and 1 μg/mL hypotaurine for 22 h at 38.5°C in an atmosphere of 5% CO_2 and 5% O_2 in N2 in four-well Petri dishes (Nunclon, Nalge Nunc). Thereafter, presumptive zygotes were transferred and cultured for 8 days in four-well Petri dishes containing SOF plus essential and non-essential amino acids at oviductal concentrations (Walker *et al.* 1996) +0.4% bovine serum albumin (BSA) under mineral oil, in a maximum humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C (Bogliolo *et al.* 2011). The first cleavage was recorded between 24 and 26 h after fertilization.

As a control for IVF procedure, a pool of embryos in each IVF run was cultured until the blastocyst stage to assess the developmental competence of the fertilized eggs. Only embryos collected from controlled experiments with rates of development to blastocyst stage at day 7 >25% (pre-pubertal) and >40% (adult) were used for the analysis.

Gene expression analysis

Details on gene expression analysis by real-time PCR are described according to the MIQE guidelines (Bustin *et al.* 2009).

Sample collection for gene expression analysis

The RNA samples were isolated from oocytes and embryos obtained during several IVM, IVF and IVC sessions, as given in the following:

- pools of denuded growing oocytes (GO) at different diameter (70/90, 90/110 and 110/130 μm) derived from pre-pubertal (P) animals (5 pools of 30 oocytes per experimental group).
- Pools of denuded germinal vesicles (GV) and IVM metaphase II (MII) oocytes derived from P or adult (A) donors (5 pools of 10 oocytes per experimental group).
- *In vitro* produced (IVP) embryos at two- (2C) and four- (4C)-cell stage derived from P or A donors (5 pools of 10 embryos per experimental group).

Oocytes and embryos were denuded via gentle pipetting in order to completely remove any somatic cell and added to 30 μL RLT buffer (RNeasy Micro Kit, Qiagen), snap frozen in liquid nitrogen and stored at −80°C until RNA isolation.

RNA isolation and reverse transcription

Total RNA was isolated from the groups of oocytes and embryos with the RNeasy Micro Kit (Qiagen) following manufacturer's instructions. Five picogram of luciferase mRNA (Promega) were added to each group prior to RNA extraction to account for RNA loss during the isolation process. During the procedure, RNA was treated with DNase I to exclude any potential genomic DNA contamination. Isolated RNA was eluted in 12 μL RNase-free water and immediately used for reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription was performed in a final volume of 20 μL, consisting of 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 1 mM dNTPs, 2.5 μM random hexamer primers, 0.05 μg oligo (dT), primers, 20 U RNase OUT and 100 U SuperScript III RT (all purchased at Invitrogen). The reaction tubes were incubated at 25°C for 10 min, then at 42°C for 1 h and finally at 70°C for 15 min to inactivate the reaction. One tube without RNA and one with RNA, but without reverse transcriptase, were analysed as negative controls. To quantify the mRNA recovery

Anti-5-methylcytosine mouse mAb (5-mC; EMD Millipore's) and 5-hydroxymethylcytosine Rabbit (5-hmC; Active Motif) antibodies were used. Briefly, the oocytes were fixed in 4% paraformaldehyde for 20 min, treated in 4 M HCl for 1 h and then neutralized in 100 mM Tris–HCl buffer (pH 8.5) for 10 min. Subsequently, oocytes were incubated with propidium iodide (50 µg/mL) in PBS and blocked for non-specific binding in 30% goat serum containing 0.05% Tween 20 in PBS. After extensive washing, the samples were incubated at 4°C overnight with primary antibodies (1:200 dilution) followed with a fluorescein (Alexa Fluor 350 or 488)-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1: 250; Alexa Fluor) at room temperature for 1 h.

Confocal analysis

Immunofluorescence stain was evaluated using a TCS SP5 confocal laser scanning microscope (Leica) and the images were captured with the LAS AF lite application software (Leica). All the pictures were taken approximately in the equatorial z-plane of all developmental stages (GV and MII oocytes, zygotes and 2- and 4-cell stage embryos).

Statistical analysis

Gene expression data were analysed using the MINITAB Release 12.1 software package (Minitab Inc., State College, PA, USA). After testing for normality and equal variance using the Kolmogorov–Smirnov and Levene's tests respectively, transcript data were analysed with general linear model analysis of variance (ANOVA), followed by Tukey's *post hoc* comparisons when P values were significant. Differences were considered significant when $P < 0.05$.

Results

Gene expression during oocyte growth

To investigate DNA methylation dynamics during folliculogenesis, oocyte growth was divided into three main time intervals based on the gamete's largest diameter (70–90, 90–110 and 110–130 µm).

The presence of all analysed transcripts during the investigated time frame of oocyte growth was confirmed. The relative quantification during oocyte growth allowed to discern the stages of oogenesis during which the molecules involved in methylation reprogramming are stored.

To get a better idea on the accumulation of transcripts during this window of oocyte growth, we relatively quantified the expression of four housekeeping genes (*ACTB*, *SDHA*, *YWAHZ* and *RPL1*9) and observed no variations in abundance ($P > 0.1$), suggesting an earlier mRNA storage. Similarly, the transcript levels of *DNMT3A*, the three methyl-CpG-binding proteins (*MBD1*, *MBD3* and *MBD4*) and *TET2* showed no significant variation ($P > 0.05$). Conversely, *DNMT1* and DNMT3B expression increased significantly during the initial growth (*DNMT1*) or during the whole analysed period (*DNMT3B*; Fig. 1) ($P < 0.05$). The accumulation of *TET1* and *TET3* mRNAs is in progress in S GO and is completed by the time oocytes reach the 90–110 µm diameter (M GO).

Gene expression during meiotic progression

Messenger RNAs of evaluated genes were detected in GV and MII oocytes. No transcripts showed significant variation during meiotic progression in oocytes derived from adult (A) donors ($P > 0.05$). Conversely, *TET1*,
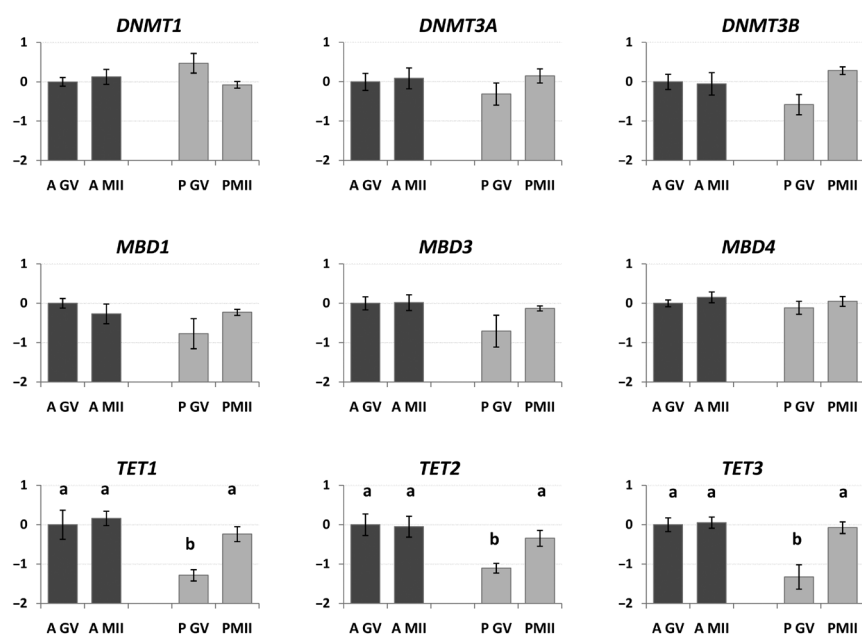
Figure 1 Relative expression of *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1*, *TET2*, *TET3*, *MBD1*, *MBD3* and *MBD4* in ovine growing oocytes (GO) at different diameter derived from pre-pubertal animals: 70–90 (small; S GO), 90–110 (medium; M GO) or 110–130 µM (large; L GO) diameter. The relative quantification of all transcripts was performed after normalization against luciferase mRNA levels and the number of oocytes and embryos (Su *et al*. 2007, Ohsugi *et al*. 2008, Evsikov *et al*. 2009). Relative abundance values are expressed as ΔCq (*Y* axis) and show the mean value ± s.e.m. of five replicates for each stage (each replicate = pool of 30 oocytes). Different letters indicate a significant difference in relative mRNA abundance (ANOVA $P < 0.05$) among the groups.

Figure 2 Relative expression of *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1*, *TET2*, *TET3*, *MBD1*, *MBD3* and *MBD4* in ovine immature GV and IVM MII oocytes derived from adult (A) or pre-pubertal (P) donors. The relative quantification of all transcripts was performed after normalization against Luciferase mRNA levels and the number of oocytes and embryos (Su *et al.* 2007, Ohsugi *et al.* 2008, Evsikov *et al.* 2009). Relative abundance values are expressed as ΔCq (*Y* axis) and show the mean value±s.e.m. of five replicates for each stage (each replicate=pool of 10 oocytes). Different letters indicate a significant difference in relative mRNA abundance (ANOVA *P*<0.05) among the groups.

TET2 and *TET3* expression levels are significantly higher in pre-pubertal derived MII oocytes compared to GVs (Fig. 2) (*P*<0.05). Furthermore, the comparison between pre-pubertal and adult derived gametes shows a significant difference at the GV stage (*P*<0.05), with lower abundance in *P* derived oocytes recovered after oocyte *in vitro* maturation (Fig. 2).

Gene expression in embryos at 2 and 4 cell stage

Transcripts of all genes were detected in both 2C and 4C embryos. No variation in any analysed mRNA level was observed between 2C and 4C adult derived embryos

(*P*>0.05). A significant decrease in *MBD1* and *MBD3* transcript abundance was observed between 2C and 4C embryos derived from pre-pubertal donors (*P*<0.05). *MBD3* expression varies significantly between A and P 4 cell embryos (Fig. 3).

Global DNA methylation and hydroxymethylation analysis

Except for growing oocytes, which were isolated from pre-pubertal ovaries, global DNA methylation and hydroxymethylation analysis were performed on oocytes and embryos derived from both adult and pre-pubertal
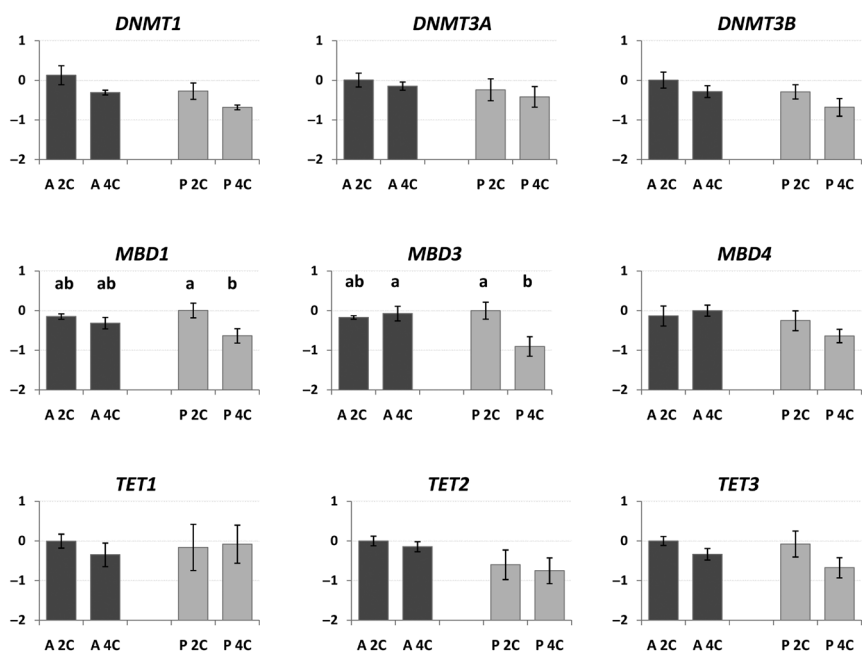
Figure 3 Relative expression of *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1*, *TET2*, *TET3*, *MBD1*, *MBD3* and *MBD4* in ovine *in vitro* produced two- (2C), four-cell (4C), embryos derived from adult (A) or pre-pubertal (P) donors. The relative quantification of all transcripts was performed after normalization against luciferase mRNA levels and the number of oocytes and embryos (Su *et al.* 2007, Ohsugi *et al.* 2008, Evsikov *et al.* 2009). Relative abundance values are expressed as ΔCq (*Y* axis) and show the mean value±s.e.m. of five replicates for each stage of development (each replicate=pool of 10 embryos). Different letters indicate a significant difference in relative mRNA abundance (ANOVA, *P*<0.05) between developmental stages or between donor age.

donors. As no differences were observed between the two sample sets, only images and descriptions of adult derived oocytes and embryos will be included below.

Global DNA methylation and hydroxymethylation in growing oocytes

Immunostaining of 5-mC revealed a strong staining in the three classes of analysed GO that overlapped with DNA staining (Fig. 4), suggesting that DNA methylation is mostly established in the oocyte at 70 μM diameter and is widely distributed in the nucleus.

A weaker, but clearly visible 5-hmC signal was also observed during the whole analysed timeframe of growth. The hydroxymethylation signal is not uniformly distributed throughout the chromatin, but it seems to cluster in restricted areas (Fig. 5).

### Global DNA methylation and hydroxymethylation during meiotic progression

High levels of DNA methylation were detected by immunofluorescence analysis in GV and MII stage oocytes (Fig. 6). DNA hydroxymethylation signal was evident, but weaker compared to 5-mC, and it still seems to be clustered in areas throughout the oocyte chromatin (Fig. 7).

Global DNA methylation and hydroxymethylation during early embryonic development

Staining of 5-mC in embryos at the zygote stage revealed considerable levels of DNA methylation in both pronuclei with an apparently weaker signal in male PN compared to the female one (Fig. 8). Conversely, 5-hmC signal is restricted to the paternal PN, while only a faint signal can be recognized in the maternal one (Fig. 9). The signal in paternal PN maintains the peculiar scattered distribution, but is stronger compared to what was observed in oocytes and embryos at further stages. In 2- and 4-cell embryos, in fact, the intensity of 5-hmC signal decreases, while the methylation level seems to maintain a considerable strength (Fig. 9). A change in 5-mC pattern was observed between 2-cell and 4-cell stage, with a more evenly distributed signal in younger embryos and the presence of clusters with a stronger intensity in the older ones (Fig. 8).

Discussion

Three main novel findings emerged from our study. First, we report temporal methylation dynamics during ovine oocyte growth in terms of global methylation, hydroxymethylation and expression of the enzymes involved in their remodelling. Second, we show differences in methylation and hydroxymethylation
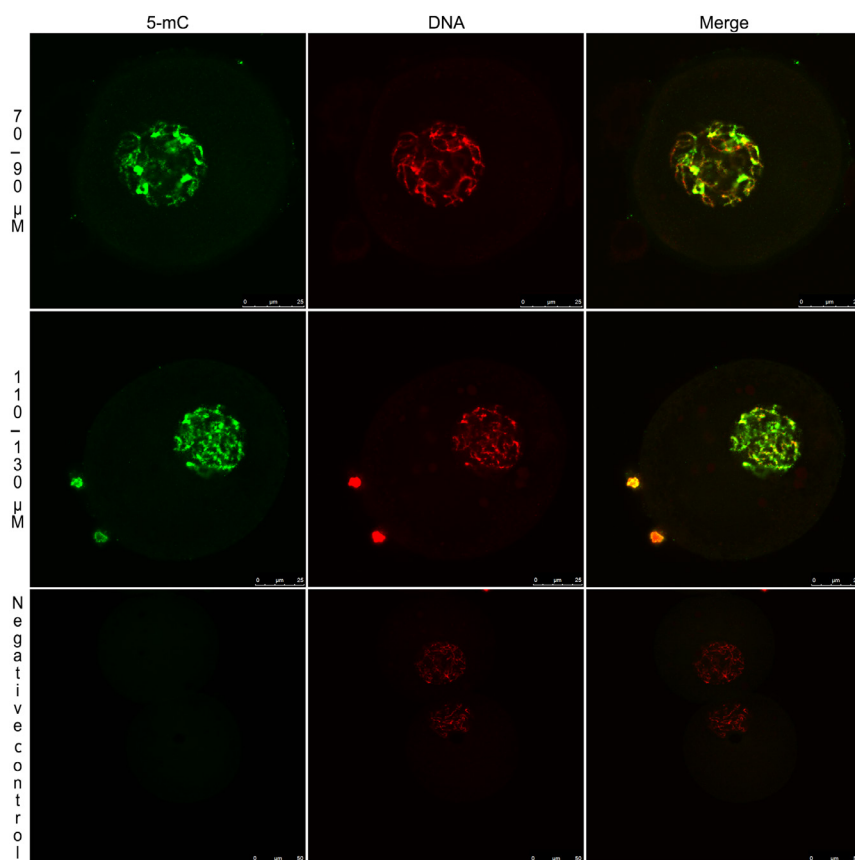
Figure 4 Immunofluorescent localization of 5-methylcytosine (5-mC) and negative control in freshly collected growing oocytes at different diameter (70/90 μm and 110/130 μm) derived from pre-pubertal animals. DNA was stained with propidium iodide and is shown in red. The scale bar represents 25 μm.
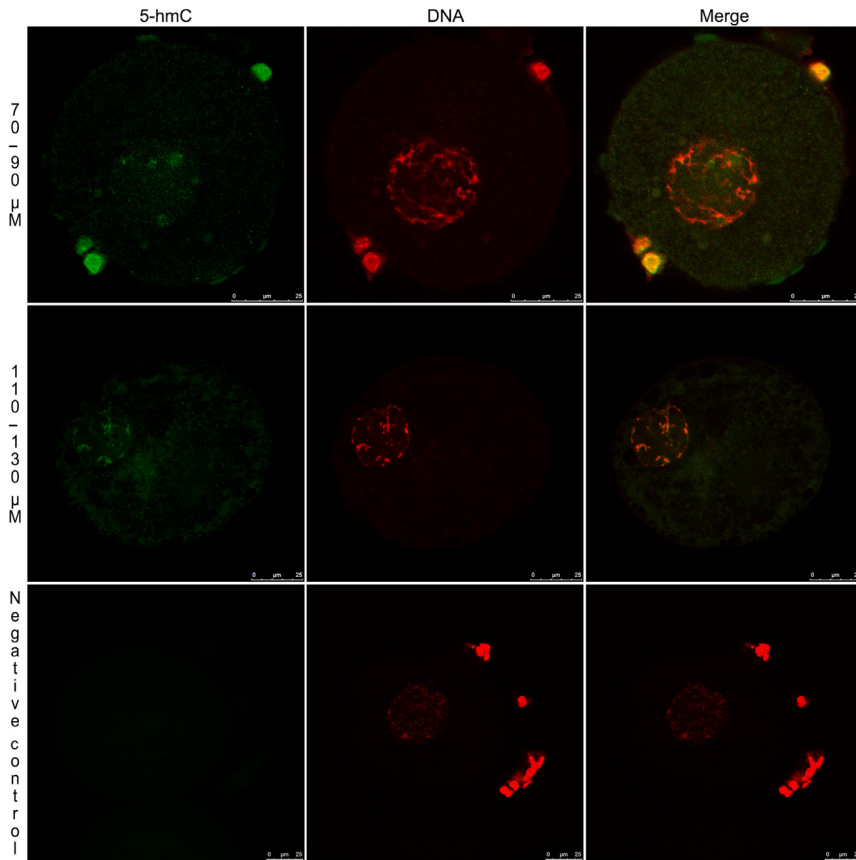
Figure 5 Immunofluorescent localization of 5-hydroxylmethylcytosine (5-hmC) and negative control in freshly collected growing oocytes at different diameter (70/90 µm and 110/130 µm) derived from pre-pubertal animals. DNA was stained with propidium iodide and is shown in red. The scale bar represents 25 µm.
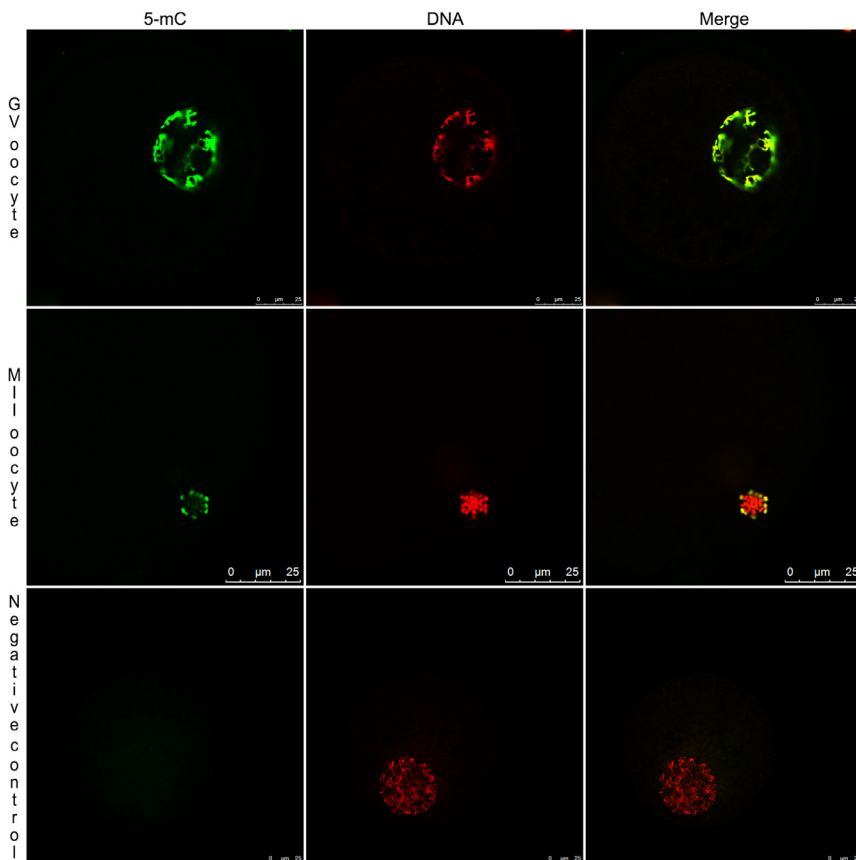
Figure 6 Immunofluorescent localization of 5-methylcytosine (5-mC) and negative control in freshly collected GV and MII oocytes derived from adult animals. DNA was stained with propidium iodide and is shown in red. The scale bar represents 25 µm.
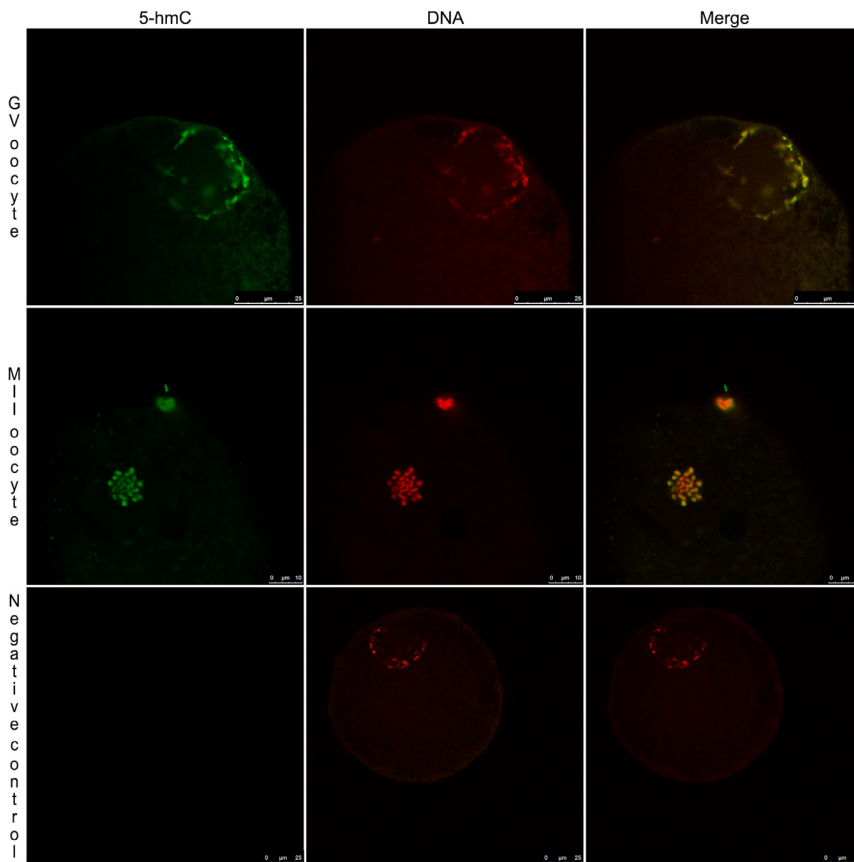
Figure 7 Immunofluorescent localization of 5-hydroxylmethylcytosine (5-hmC) and negative control in freshly collected GV and MII oocytes derived from adult animals. DNA was stained with propidium iodide and is shown in red. The scale bar represents 25 μm.

patterns between male and female pronucleus (PN) that suggest an active demethylation of male PN also in sheep. Third, we identified altered expression of *TET1*, *TET2* and *TET3*, and *MBD1* and *MBD3* in low quality oocytes and embryos respectively, in a differential model of developmental competence.

To investigate DNA methylation dynamics during folliculogenesis, oocyte growth was divided into three main time intervals based on the gamete's largest diameter (70–90, 90–110 and 110–130 μm). Oocytes derived from four weeks old lambs were used for the analysis in order to minimize any possible variation of the oocyte epigenetic status due to potential changes in the environment (i.e. nutrition, pathologies or accidental exposition to various contaminants; Cortessis *et al.* 2012).

Analysis of DNA methylation during oocyte growth showed high methylation at all analysed stages that overlapped with DNA staining (Fig. 4), suggesting that *de novo* DNA methylation is mostly concluded when the oocyte reaches 70 μm diameter and is widely distributed in the nucleus. Our results are in partial agreement with a previous work (Russo *et al.* 2007) reporting 5-mC immunopositivity in growing ovine oocytes with a diameter of at least 90 μm; the difference related to oocytes with 70–90 μm diameter could be explained by the improved affinity of the antibodies available

nowadays or to the different immunostaining protocol used.

Analysis of hydroxymethylation, not previously reported in ovine growing oocytes, revealed a weaker, but clearly visible signal. Interestingly, the signal is not uniformly distributed throughout the chromatin, but seems to cluster in restricted areas (dot-like; Fig. 5) as described in growing oocytes in mouse (Yamaguchi *et al.* 2013, Sakashita *et al.* 2014).

The molecules that will support oocyte maturation, fertilization and early embryo development are stored during oocyte growth, as these events happen in the absence of *de novo* transcription. In addition, post-fertilization epigenetic reprogramming occurs during transcriptional quiescence therefore it relies on the enzymes and co-factors deposited in the gamete during its growth. Messenger RNAs and proteins are stored in a temporally regulated manner during folliculogenesis. Our analysis allowed to discern during which stages of oogenesis the stocks of the molecules involved in methylation reprogramming are formed. Expression analysis of the methylating enzymes *DNMT1* and *DNMT3B* showed a significant increase during oocyte growth in relation to its diameter, highlighting an ongoing transcription (Fig. 1) in accordance with reports in mouse and bovine (Ratnam *et al.* 2002, Lucifero *et al.* 2007, Bessa *et al.* 2013). Conversely, *DNMT3A* abundance
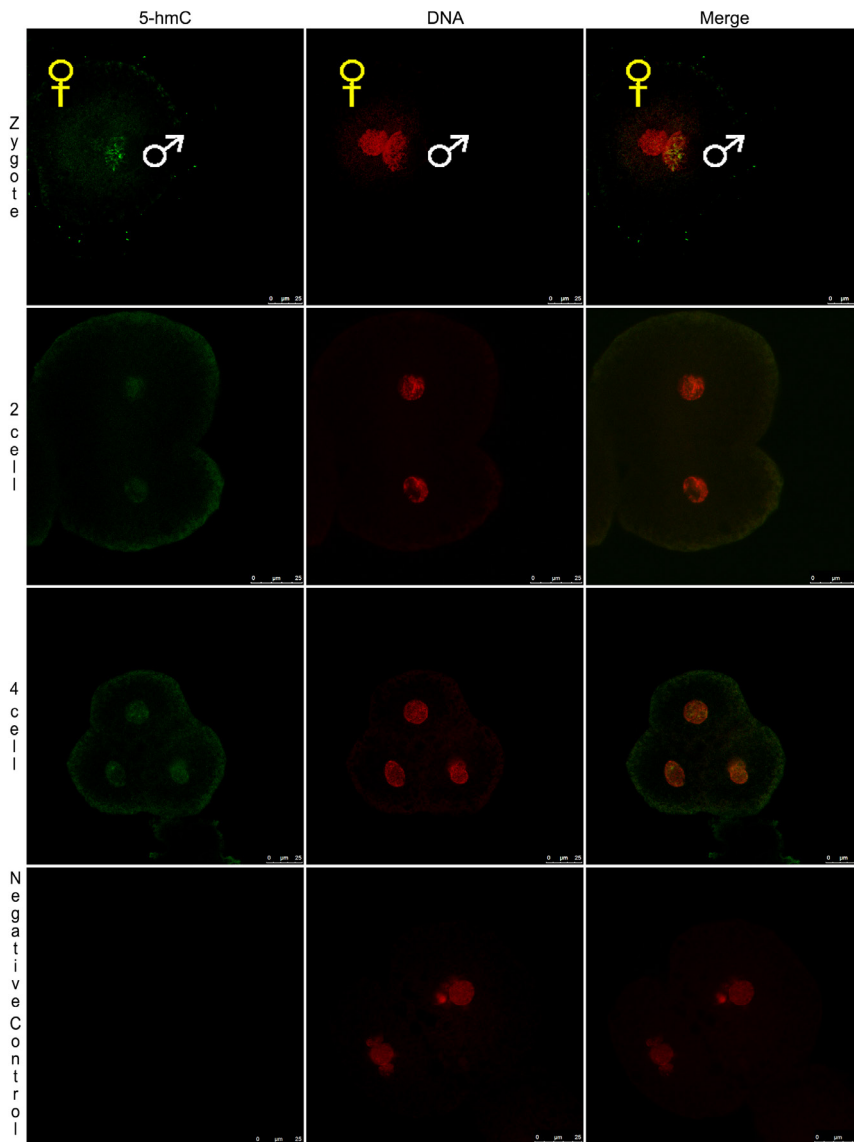
614 *L Masala and others*
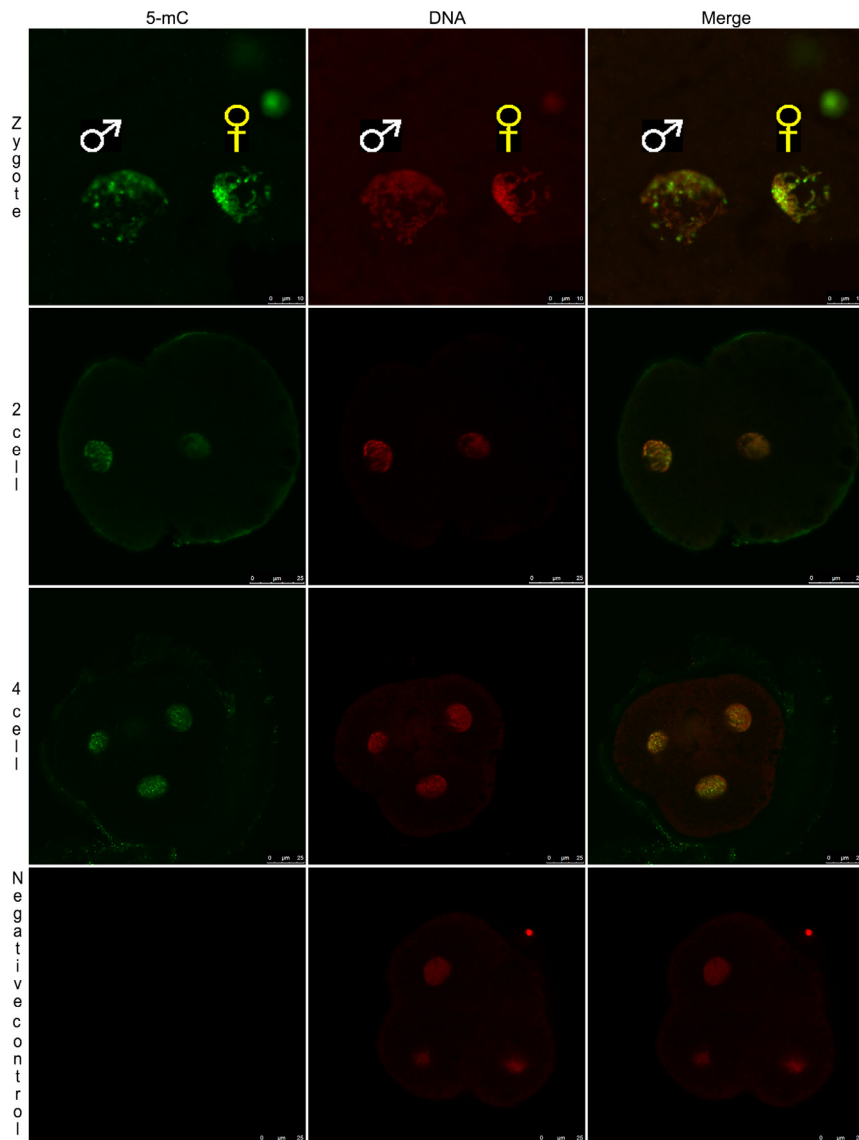
Figure 8 Immunofluorescent localization of 5-methylcytosine (5-mC) and negative control in *in vitro* matured and fertilized zygotes, 2-cell and 4-cell embryos. DNA was stained with propidium iodide and is shown in red. The scale bar represents 25 μm.

did not vary significantly, suggesting that the transcript accumulation may be already completed because of its early need during gamete growth. DNMT3A is in fact responsible of establishing new methylation patterns in the oocytes and was seen to be essential for methylation of both maternally and paternally imprinted genes (Kaneda *et al*. 2004). Its activity is most probably responsible for the high level of methylation already observed in oocytes with 70 μm diameter.

Expression analysis of the three known dioxygenases TET1, TET2 and TET3 showed a significant increase in *TET1* and *TET3* abundance during the first analysed phase (up to about 100 μm), while *TET2* transcript did not vary significantly throughout the analysed time frame. Studies in mouse, bovine, rabbit and pig reported different and sometimes contradictory patterns of *TET1*, *TET2* and *TET3* expressions during oogenesis and early development. A consensus regards Tet3 very

high expression in the oocyte and zygote in all species (Iqbal *et al*. 2011, Wossidlo *et al*. 2011, Lee *et al*. 2014, Page-Lariviere & Sirard 2014, Sakashita *et al*. 2014, Salvaing *et al*. 2016), while data on *Tet1* and *Tet2* are inconsistent. Sakashita and coworkers (Sakashita *et al*. 2014) reported a significant increase in *Tet3* expression during mouse oocyte growth, but failed to report the expression of *Tet1* or *Tet2* in growing or GV oocytes. Similarly, Iqbal and coworkers (Iqbal *et al*. 2011) observed *Tet3*, but not *Tet1* or *Tet2*, high expression in mouse oocytes and zygotes that was drastically down regulated at the two-cell stage; however, they detected *Tet1* at low levels in the two- and four-cell stage embryo. On the other hand, different works succeeded in observing *Tet1* and *Tet2* expressions in mouse oocytes and zygotes, even if low or moderate (Ito *et al*. 2010, Wossidlo *et al*. 2011). In bovine oocytes, TET2 expression was reported at very low levels and TET1 expression could not be detected

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Figure 9 Immunofluorescent localization 5-hydroxylmethylcytosine (5-hmC) and negative control in *in vitro* matured and fertilized zygotes, 2-cell and 4-cell embryos. DNA was stained with propidium iodide and is shown in red. The scale bar represents 25 µm.

(Page-Lariviere & Sirard 2014). Low *TET1* mRNA abundance was described in oocytes and early embryos in both pig and rabbit (Lee *et al.* 2014, Salvaing *et al.* 2016). Conversely, *TET2* was seen to be moderately expressed during oocyte maturation and early embryo pre-implantation in pig, but its higher abundance was reported in rabbit oocytes and 1- to 4-cell stage embryos (Salvaing *et al.* 2016). We first observed the presence of *TET1–3* transcripts in the growing oocyte, which persisted during oocyte maturation and in the early embryo (2- and 4-cell stage). While no absolute quantification was performed, the relative quantification with similar amplification efficiency ($R^2 > 0.99$) and the same amount of cDNA template indicate the highest abundance for *TET3*, a lower expression for *TET2* (about 25% of *TET3* expression) and the lowest level for *TET1* (about 4% of *TET3* expression) in GV oocytes, confirming the trend observed in the other species

(Fig. 10). The longer persistence of the transcripts we observed in ovine early embryos compared to mouse are most probably due to the higher number of cell cycles required in sheep for embryo genome activation (EGA; Fig. 3). Indeed, the activation of the genome at the 8- to 16-cell stage expands the time frame available for the regulation of gene expression and allows an easier observation (Ledda *et al.* 2012).

The distinct expression pattern of *TET1–3* we observed during oogenesis supports the hypothesis of non-overlapping biological functions that vary in a developmentally regulated and tissue-specific manner (Wu & Zhang 2011); however, the specific functions are yet to be clarified, as evidenced by the most recent literature. Currently, the accepted model for active DNA demethylation identifies Tet3 as the enzyme mostly responsible for the global loss of 5-mC that takes place in the paternal genome shortly after fertilization
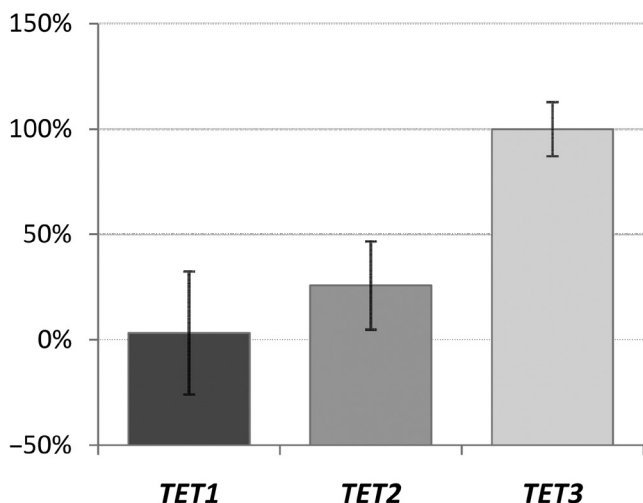
Figure 10 Relative mRNA abundance of *TET1*, *TET2* and *TET3* in GV oocytes as shown by RT-PCR analysis. Values are expressed in percentages; *TET3* abundance was set to 100% and *TET1* and *TET2* expressed relatively to *TET3*. The relative quantification was performed with similar amplification efficiencies ($R^2 > 0.99$) and same amount of cDNA template (0.5 embryo equivalent).

(Mayer *et al*. 2000). Recent data showed that Tet3 contributes to 5-mC oxidation also in the maternal genome (Shen *et al*. 2014), although to a lesser extent compared to the paternal DNA. However, the overall picture is continuously evolving and the latest results challenge Tet3 involvement in the initial loss of paternal PN 5-mC signal that may occur even in the absence of the enzyme hydroxylation activity (Dawlaty *et al*. 2013). TET1 and TET2 are also involved in hydroxymethylation, but information on their specific function is more limited. The expression of either enzyme was seen to compensate for deficiency of the other during development (Du *et al*. 2015); however, their different expression pattern in mouse oocyte (Wossidlo *et al*. 2011) and in the present work suggests the existence of individual functions for the two proteins that are yet to be identified.

Analysis of *MBD1*, *MBD3* and *MBD4* expressions showed no variation during the considered oocyte growth. Comparison with abundance at the GV stage suggests that the synthesis of the three mRNAs was completed during early oocyte growth and did not increase significantly in oocytes with diameter >70 μm. These observations support an early need for the co-factors during oocyte growth, in accordance with their involvement in the regulation and interpretation of DNA methylation and transcription (Du *et al*. 2015).

Analysis of gene expression during oocyte maturation showed no variation in mRNA abundance between GV and MII stages in oocytes derived from adult donors, in accordance with observations during meiotic progression of fully grown oocytes in mouse and pig (Lee *et al*. 2014, Sakashita *et al*. 2014). However, analysis of the transcripts in a model of differential

developmental competence revealed an association between TET enzyme expression and oocyte quality. We have adopted a previously developed model consisting of ovine oocytes deriving from adult (A) vs pre-pubertal (P) donors to study high vs low developmental competence respectively (reviewed in Ledda *et al*. 2012). The differential quality of the two types of gametes was proven in different species and refers to several aspects comprising resistance to cryopreservation (Leoni *et al*. 2006), transcript abundance (Leoni *et al*. 2007, Romar *et al*. 2011, Ledda *et al*. 2012), *in vitro* kinetic development (Leoni *et al*. 2006, 2015), mitochondrial distribution (Leoni *et al*. 2015) and the ability to give birth to live offspring (Armstrong *et al*. 1997, Ledda *et al*. 1999). Analysis of the transcripts in our model showed lower levels of the three *TET* transcripts in GV oocytes derived from young animals compared to GV oocytes derived from adult donors, but similar expression levels in P and A oocytes at the MII stage (Fig. 2). Such pattern suggests a delay in the synthesis and storage of *TET* transcripts in the pre-pubertal oocytes at the GV stage, which is recovered by the end of oocyte maturation, when differences disappear (Fig. 2). A transcriptional activity in GV has already been proposed in pre-pubertal mouse (Chouinard 1971), bovine (Fair *et al*. 1995) and sheep (Ptak *et al*. 2006). We have previously shown differences in mRNA abundance at GV stage between pre-pubertal and adult ovine oocytes (Leoni *et al*. 2007). The observations in the present work confirm the hypothesis that oocytes from juvenile animals have an incomplete molecular deposit (RNAs and proteins) that may affect competence acquisition, which involves deficiencies in the methylation machinery.

Immunofluorescence analysis of the global DNA methylation and hydroxymethylation during meiotic progression showed signal intensity and distribution similar to what was observed during oocyte growth (Figs 6 and 7). No variation depending on meiotic progression or donor origin was observed. However, the presence of subtle differences that are not detectable by immunostaining cannot be excluded.

Staining of 5-mC in embryos at the zygote stage revealed the presence of DNA methylation in both PN, with an apparently weaker signal in male PN compared to that of the female PN (Fig. 8). Conversely, hydroxymethylation signal is restricted to the paternal PN, while only a faint signal can be recognized in the maternal one (Fig. 9), in accordance with observations in mouse, bovine and rabbit zygotes (Wossidlo *et al*. 2011). The staining of paternal PN is stronger compared to what was observed in oocytes or embryos at further stages, but maintains the peculiar scattered distribution (Fig. 9). The observations of 5-mC and 5-hmC patterns in zygotes suggest for the first time that male pronucleus undergoes active demethylation also in sheep. Our results contrast with a previous report (Young & Beaujean 2004) that showed no loss of 5-mC staining in sheep paternal

genome within the first post-fertilization cell cycle and postulated sheep escape from the conventional model of early embryo epigenetic reprogramming observed in most mammalian species. The model proposes a strong active demethylation of the male PN soon after fertilization and a passive loss of methylation in the female PN through cell cycles (Mayer *et al.* 2000). The decrease in 5-mC that was observed in male PN in sheep is consistent with genome-wide DNA methylation data recently obtained in mouse and bovine (De Montera *et al.* 2013, Wang *et al.* 2014).

Immunofluorescence analysis in embryos at 2- and 4-cell stage evidenced immunopositivity for both 5-mC and 5-hmC antibodies; however, hydroxymethylation considerably decreased compared to the previous stages. The subnuclear localization pattern of 5-mC changed, with a restriction of the signal to limited foci that show stronger intensity in the 4-cell stage embryos (Fig. 8).

Expression analysis in embryos at 2- and 4-cell stage evidenced constant levels in mRNA abundance; however, low quality embryos in the differential model show a decrease in mRNA abundance for *MBD1* and *MBD3* (Fig. 3). Although identifying the reason for the observed reduction may be challenging before the specific functions of the two proteins in the early embryos are clarified, the patterns of expression suggest a different regulation of the two transcripts (increased translation or degradation) that may affect the embryo development.

The dynamics of hydroxymethylation we observed during late oogenesis and early embryo development suggest a role for 5-hmC other than just being a transient status during demethylation. The presence of 5-hmC during oocyte growth, together with stable levels of methylation, hints an additional biological role, as indicated by very recent evidence that show active demethylation of the paternal PN uncoupled from hydroxymethylation of the genome (Amouroux *et al.* 2016). In accordance, DNA methylation and hydroxymethylation were seen to co-exist in non-reciprocal patterns in human oocytes (Petrussa *et al.* 2016) and in both pronuclei of human (Petrussa *et al.* 2016) and mouse zygotes (Salvaing *et al.* 2012). In agreement with a potential role in the regulation of transcription (Kang *et al.* 2015), previous studies have established that hydroxymethylated and methylated DNA have different binding properties with co-factors involved in transcription regulation; for instance, the interaction of MBD proteins with 5-mC is abolished by hydroxymethylation (Valinluck *et al.* 2004). This implies that these two epigenetic modifications perform distinct biological functions. It is possible that the genomic content of 5-hmC could contribute to the regulation of expression of specific genes during development and differentiation by modulating the open or closed state of the chromatin (Ruzov *et al.* 2011).

The role of 5hmC in epigenetic reprogramming during female germ line cell development is not fully understood; yet, it is indeed probable that the areas with high 5-hmC are linked to substantial transcription and are less likely to demethylation in this stage of oocyte growth, when significant stores of mRNAs have to be accumulated and DNA methylation reestablishment is ongoing.

The results of the present study differ from a recent work (Fang *et al.* 2016) that investigated a restricted panel of enzymes involved in DNA methylation remodelling in relation to oocyte developmental capacity in sheep. The work reported lower levels of mRNA in GV, MII stage oocyte, zygotes and 2C embryos derived from pre-pubertal animals compared with the same stage of development derived from adult donors. However, the study was performed on oocytes and deriving embryos collected from hormonally treated animals (both young and adult donors), while in the present study the gametes were obtained from non-treated donors. Such crucial diversity, which is upstream of the entire experiment, is most likely responsible for the observed differences in gene expression, as repeatedly reported (Market-Velker *et al.* 2010, Chu *et al.* 2012, Urrego *et al.* 2014).

In conclusion, our work showed methylation dynamics during folliculogenesis and early embryo development in sheep. Our findings suggest an active DNA demethylation of the paternal pronucleus also in sheep, supporting the conservation of the mechanism in mammals and advancing the understanding of the reprogramming process that involves DNA methylation/demethylation.

The overall description of DNA methylation dynamics in growing oocytes could be useful to identify potential epigenetic alterations caused by assisted reproductive procedures, in order to optimize protocols and contribute to generate fully viable and healthy offspring.

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