UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO IN

SCIENZE VETERINARIE

INDIRIZZO: **Riproduzione, Produzione e benessere animale (XXVIII CICLO)**

Study of DNA methylation dynamics in a model of differential developmental competence in the ovine species

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Anno Accademico 2014-2015

La presente tesi è stata prodotta nell'ambito della Scuola di Dottorato in "Scienze Veterinarie" dell'Università degli Studi di Sassari, a.a. 2014/2015 – XXVIII ciclo, con il supporto di una borsa di studio finanziata con le risorse del P.O.R. SARDEGNA F.S.E. 2007-2013 - Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l.3.1.

Ai miei genitori

La tenacia è spinta dalla passione

Tear Gas Rag

Old Southern Rag, Blind Blake

Tear Gas Tear Gas
Tear gas tore my throat
Can't say my mantra
Tear Gas got my goat

Tear Gas O Lord Tear Gas
I can't find my mind
Bombing North Vietnam
I'm stumbling around blind

Tear Gas in Boulder
Tear Gas in my heart
Frightened on college hill
Nixon's poison Fart

Tear Gas here Tear Gas there
Colorado and Saigon
They'll be droppin teargas
Everytime I get a hardon

Allen Ginsberg
May 10, 1972
Ft. Collins, Colorado

Table of contents

Chapter 1: Summary

Since 1956, when Conrad Waddington first used the term epigenetics, the understanding of epigenetic phenomena is progressively growing year by year. In less than 80 years the classical believe that everything was written in the DNA sequence has changed and now we know that epigenetic modifications interact with the DNA sequence and together they regulate the expression of genes in response to cell needs and environmental stimuli. Different kinds of epigenetic modifications have been identified and part of the regulating mechanisms are now clear, but at the same time several questions still need to be answered in order to have a complete picture of the complex epigenetic mechanisms and regulations. Sophisticated studies demonstrated that the epigenetic status undergoes dynamic changes that are physiologic during embryo development (Messerschmidt et al. 2014), but they could also be involved in the pathogenesis of certain diseases, such as cancer (Feinberg et al. 2002). The most studied epigenetic modification is DNA methylation, that implicates the addition of a methyl group (CH3) to the 5-carbon position of cytosine, frequently within a CpG dinucleotide (Razin and Szyf 1984). Such modification is involved in the two waves of epigenetic remodelling that take place during embryogenesis, one during early embryonic development and the second one during primordial germ cell specification. DNA methylation has been the focus of this thesis, as we studied the dynamics of this particular epigenetic modification in relation to oocyte quality and during early embryo development in the ovine species.

Chapter 2: Introduction

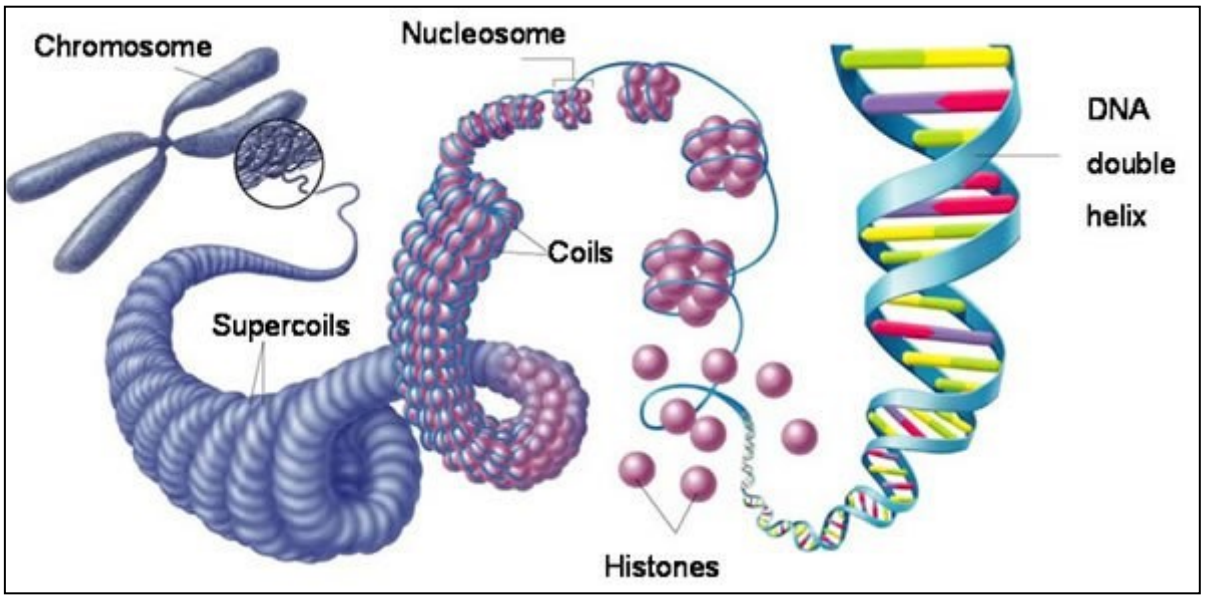
2.1. Epigenetic mechanisms and nuclear DNA packaging

Epigenetics is the study of mechanisms involved in the regulation of gene activity that are not determined by changes in DNA sequence. These mechanisms include the so called epigenetic modifications that have an effect on DNA structure and how it is organized in the nucleus rather than in the DNA sequence itself (Handy et al. 2011).

The nuclear DNA of eukaryotic cells is finely packaged in chromosomes. Each chromosome consists of one continuous thread-like molecule of DNA coiled around specialized proteins that bind to and fold the DNA. This organization of DNA and protein refers to a condensed structure called chromatin that is assembled in a precise and ordered manner. The first level of chromatin organization is the nucleosome, it is composed of an octameric core made up of different kind of assembled histone proteins that tightly wrap the DNA (Mariño-Ramírez et al. 2005). Individual nucleosomes are then linked together by stretches of linker DNA and coiled into chromatin fibers (Fig 2.1.), (Wu et al. 2007). Although this fine asset of DNA packaging, a large amount of genes must be actively transcribed during life. Thus, chromatin could be considered as a dynamic genome organization and, depending on cell demand, its condensation state could be modulated in order to make the DNA accessible to the transcription machinery in a time and tissue specific manner (Baker 2011). This general introduction has been necessary to explain how chromatin is organized, in order to give a background for the understanding of the interaction between epigenetic modification and

chromatin structure. However, this thesis will focus on DNA methylation, a particular kind of epigenetic modification that has been demonstrated to interact with the other epigenetic mark to control chromatin structure modification and gene expression (Weissmann and Lyko 2003).

Figure 2.1: chromosome structure



2.2. Epigenetics modifications

Three different epigenetic modifications are involved in the complex epigenetic machinery: Histone modification, Non coding RNAs, DNA methylation. Although the three modifications are diverse, achieved by different chemical reactions and set up by distinct enzymes, studies demonstrate that all of them interact with each other to regulate gene activity (Cedar and Bergman 2009; Portela and Esteller 2010).

2.2.1. Histone modifications

As discussed above, nucleosomes are the first level of chromatin organization consisting of different classes of histone proteins that wrap the DNA. All the histones have terminal amino

acid tails that protrude out from the nucleosome. The function of terminal tails is to regulate high–order chromatin by binding the DNA and also to regulate the interaction between neighbour nucleosomes (Iwasaki et al. 2013). Terminal tails are subjected to post-transcriptional modification at N-terminal ends, such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (Kouzarides 2007). Histone modifications have important roles in transcriptional regulation and chromosome condensation. The chromatin can be found in two main states: the actively transcribed euchromatin and the transcriptionally inactive heterochromatin. The first state presents high levels of acetylation and trimethylation of the tails, while the transcriptionally inactive state is characterized by high levels of methylation and low acetylation (Li et al. 2007).

2.2.2. Non coding RNA

Non coding RNAs (ncRNAs) are RNA molecules are not translated into proteins, but have a regulatory activity; in fact ncRNAs function is to regulate gene expression at both transcriptional and post-transcriptional level. The ncRNAs involved in epigenetic processes are the short ncRNAs [not longer than 30 nucleotides (nts)], such as microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs), and the long ncRNAs (longer than 200 nts) (Mattick and Makunin 2006). This particular class of RNAs plays a role in targeting histone modifying complexes, DNA methylation and gene silencing. For instance, it has been demonstrated that some ncRNAs interact with critical regions of CpG islands of imprinted genes to promote the recruitment of methyltransferase 1 (Peschansky and Wahlestedt 2014).

2.2.3. DNA methylation

DNA methylation is characterized by the covalent addition of a methyl group to the 5-carbon position of cytosine, frequently within a CpG dinucleotide (Razin and Szyf 1984). DNA methylation is usually associated with repression of gene transcription as this chemical modification modulates the condensed state of chromatin by the interaction with histones modifications (Cedar and Bergman 2009). CpGs dinucleotides are mostly distributed across the whole genome and are usually methylated (Larsen et al. 1992). However, specific areas of the genome are characterized by regions rich in CpG multiple repeats known as CpG islands; they are often located in gene promoters and are unmethylated in somatic cells, with the exceptions of imprinted genes and the inactive X chromosome in females (Deaton and Bird 2011).

2.3. Enzymes involved in DNA methylation remodelling

DNA methylation patterns undergo multiple rounds of dynamic changes during development and are maintained stable in adult somatic cells. Establishment, erasure, re- establishment and maintenance and of DNA methylation are coordinated by particular mechanisms that regulate both DNA methylation and demethylation.

2.3.1. DNA-methyltransferases (DNMTs) enzymes

The addition of methyl groups is controlled at different levels in cells and is carried out by a family of DNA-methyltransferase (DNMTs) enzymes. These enzymes are widely conserved among eukaryotes (Kim et al. 2009). Three major types are responsible of the regulation of DNA methylation: DNMT1, DNMT3A and DNMT3B. All of them have a catalytic domain that allows the association of the cytosine with the methyl donor, S-adenosyl-L-methionine

(SAM), and the formation of 5-methylcytosine (5-mc). Two main mechanisms are involved in DNA methylation: DNA methylation maintenance, performed by DNMT1, and *de novo* DNA methylation, carried out by DNMT3A and DNMT3B. Mutant mice lacking either the maintenance enzyme DNMT1 or both *de novo* methylases DNMT3A and DNMT3B exhibit demethylation in the genome and die at the mid-gestation stage, indicating that methylation is essential for embryogenesis (Okano et al. 1999)

2.3.1.1. DNMT1 and DNA methylation maintenance

DNMT1 was the first methyltransferase to be discovered (Bestor et al. 1988). It is responsible of the maintenance of DNA methylation during cells division. In fact, DNA replication implicates the loss of DNA methylation marks in the newly synthesized DNA strand. It has been demonstrated that DNMT1 is the enzyme responsible of the maintenance of DNA methylation, it has high affinity for hemi-methylated DNA (Yoder et al. 1997; Vilkaitis et al. 2005) and it interacts with the proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase δ in eukaryotic cells, essential for replication. The targeted mutation of the *DNMT1* gene results in embryonic lethality (Li et al. 1992) suggesting that it has a major role during embryo development.

2.3.1.2. DNMT3A and DNMT3B and *de novo* DNA methylation

Two enzymes are responsible of *de novo* DNA methylation: DNMT3A and DNMT3B (Okano et al. 1998a); they possess the ability to methylate a previously unmodified cytosine residue. *DNMT3*A transcript is provided as a maternal RNA by the oocyte and is important during early preimplantation development and for the establishment of the differential DNA methylation patterns at imprinting control regions (ICRs) in male and female gametes

(Kaneda et al. 2004; Kato et al. 2007). Conversely, DNMT3B is transcribed upon embryonic genomic activation and is strongly expressed by the blastocyst stage, at which time its presence is predominantly restricted to the epiblast lineage. As DNMT1, both DNMT3A and DNMT3B are essential during embryo development. Deletion of DNMT3B causes embryonic lethality, while DNMT3A knockouts are partially viable (Okano et al. 1999) Combined genetic deletion results in earlier embryonic lethality, indicating at least partial functional redundancy of the two enzymes (Okano et al. 1999).

2.3.2. DNA demethylation dynamics

In contrast to DNA methyltransferases enzymes, that are able to catalyse the addition of the methyl group into the cytosine, a family of enzymes capable to directly remove the methyl group does not exist or at list has not been yet identified. Two models of DNA demethylation have been proposed, involving a passive or an active mechanism.

2.3.2.1. Passive replication-dependent demethylation

Passive DNA demethylation does not involve any active enzymatic process that alters the 5-mc itself. It refers to the loss of it during successive rounds of replication, in the absence of functional DNA methylation maintenance, which results in a retention of the unmethylated state of the newly synthesized DNA strand. Passive replication-dependent demethylation occurs in maternal pronucleus (PN) during subsequent cleavage divisions (Mayer et al. 2000).

2.3.2.2. Ten-eleven translocation (*TET*) enzymes and active DNA demethylation

Active DNA demethylation involves an enzymatic process that modifies the methyl group. This active process is coordinated by Ten-eleven translocation (TET) enzymes. TET enzymes,

called respectively TET1, TET2 and TET3, mediate the oxidation of 5-mc to 5 hydroxymethylcytosine (5-hmc) and further to 5-formylcytosine (5-fc) and 5-carboxycytosine (5-cac) (Ito et al. 2011). All three TET enzymes have a CD (Cys-rich and DSBH regions) domain that uses O2 to decarboxylate α-ketoglutaric acid generating a high-valet iron oxide that converts 5-mc to 5-hmc (as well as 5-hmc to 5-fc and 5-fc to 5-cac). TET1 and TET2 are mainly implicated in primordial germ cell (PGCs) reprogramming, but further studies are needed to clarify the exact contribution of TET1 and TET2 in the PGC methylome reset. Recently, (Yamaguchi et al. 2012) reported significant reduction of female germ-cell number and fertility in TET1 deficient mice suggesting that TET1 is responsible of meiosis and meiotic gene activation in female germ cells. Conversely, TET3 is responsible of global hydroxilation of methyl groups in the male PN following fertilization. In 2011, Gu and colleagues reported that TET3-deficient zygotes obtained from conditional KO mice were unable to undergo male PN hydroxylation. Different ways of active DNA demethylation that involves the tree intermediates that originate from TETs oxidation have been proposed. 5fc and 5cac can be recognized and excised out of the DNA by the thymine DNA glycosylase enzyme (TDG) in a process involving the base excision repair pathway (Maiti and Drohat 2011). It has been demonstrated that depletion of TDG in mESCs leads to the accumulation of 5cac and 5fc (Raiber et al. 2012; He et al. 2011). 5-hmc modification could aid passive DNA demethylation even in the presence of DNMT1, which has low affinity for 5-hmc, so it is not able to maintain the DNA mark trough replication (Valinluck and Sowers 2007). Recent studies suggest a different role for DNA hydroxymethylation other than DNA 5-mc removal. Ruzov et al.,(2011) found a persistent and stable level of DNA hydroxymethylation during early embryo

development and suggest that 5-hmc may be an epigenetic modification with a specific biological role during cell self-renewal and differentiation other than being only a transient condition during active demethylation (Ruzov et al. 2011).

2.3.3. Others proteins and cofactors involved in DNA methylation remodelling

2.3.3.1. DNMT2

The role of DNMT2 protein in DNA methylation is controversial. DNMT2 protein was originally assigned to the DNA methyltransferase family, due to the strong similarity with DNMT1 and DNMT3B catalytic domain (Okano et al. 1999). Despite its high similarity with others DNMTs, it has been shown that DNMT2 has only a weak DNA methylation activity (Jeltsch et al. 2006). In fact, targeted deletion of the *DNMT2* gene in embryonic stem cells causes no detectable effect on global DNA methylation, suggesting that this enzyme has little involvement in setting DNA methylation patterns (Okano et al. 1998b). However in 2006 it has been shown that DNMT2 has an active role as RNA methyltransferase: it seems that DNMT2 specifically methylates cytosine 38 of transfer RNAAsp (Goll et al. 2006). Methylation of tRNAs has an impact on the folding and stability of the structure (Alexandrov et al. 2006). DNMT2 has been shown to methyl other tRNAs and a protective function was hypothesized (Schaefer et al. 2010). Researchers are currently examining whether DNMT2 acts on other small RNAs.

2.3.3.2. DNMT3L

The DNA methyltransferase 3-like (DNMT3L) protein is a crucial cofactor for *de novo* methyltransferase DNMT3A. DNMT3L retains a carboxy-terminal domain, structurally similar

to the methyltransferases, which dimerises with the de novo enzyme DNMT3A (Jia et al. 2007). DNMT3A and DNMT3L, are largely involved in establishing maternal DNA methylation imprints in mice and cow (Kaneda et al. 2004; Bourc'his et al. 2001). Conversely in human *DNMT3L* mRNA expression was undetected until postfertilization (Huntriss et al. 2004), while in rhesus monkey *the* transcript was barely detectable (Vassena et al. 2005); this suggests a later role for DNMT3L in de novo methylation during early embryonic development and not in the establishment of maternal imprints.

2.3.3.3. Methyl-CpG-binding proteins (MBDs)

Methyl-CpG-binding proteins (MBDs) are a family of proteins that consists in five members: MeCP2, MBD1, MBD2, MBD3 and MBD4 (Berger and Bird, 2005). Each of these proteins, with the exception of MBD3, are characterized by a binding domain which preferentially binds to methylated CpG(s) (Hendrich and Bird 1998). MBD proteins possess also a CxxC domain that helps the interaction with DNMTs and a transcription repression domain (TRD) that acts to silence the gene transcriptional machinery. MBD interaction with methylated DNA is crucial as it prevents binding of transcription factors and RNA polymerase and thereby represses transcription (Hendrich and Tweedie 2003).

2.3.3.3.1. Methyl-CpG-binding domain protein 1 (*MBD1*)

MBD1 has high affinity for related to DNA methylation and chromatin modifiers such as the Suv39h1–HP1 complex, to enhance DNA methylation-mediated transcriptional repression (Fujita et al. 2003). The functional importance of MBD1 was demonstrated in human HeLa cells, where MBD1 was shown to associate with the H3K9 methyltransferase SETDB1 (Sarraf and Stancheva 2004). Its MBD domain recognizes and binds to methylated CpGs. The CXXC3

domain of MBD1 makes it a unique member of the MBD family due to its affinity to unmethylated DNA. This binding allows the protein to trigger methylation of H3K9 and results in transcriptional repression (Fujita et al. 2000).

2.3.3.3.2. Methyl-CpG-binding domain proteins 2 and 3 (*MBD2* and *MBD3*)

MBD2 and MBD3 are components of the NuRD (Mi-2) histone deacetylase and nucleosome remodelling complex (Tazi and Bird 1990; Zhang et al. 1999). MBD2 is required for transcriptional silencing of methylated promoters and for proper nurturing behaviour in mothers, while MBD3 is essential for successful embryogenesis. Both proteins were identified as potential methyl-CpG binding proteins, although in mammals only MBD2 specifically binds to methylated DNA both *in vitro* and *in vivo* (Hendrich and Bird 1998). Biochemical assays have shown that MBD3 cannot bind methylated DNA directly, but it is an essential subunit of the Mi-2/NuRD chromatin remodelling complex together with MBD2 (Saito and Ishikawa 2002). The MBD3 protein is 70% identical to MBD2, but lacks the 152 amino-acid N-terminal extension that is implicated in methylated DNA binding (Hendrich and Bird 1998; Hendrich et al. 1999). Both genes are activated in embryogenesis following implantation and are widely expressed in adult mice (Hendrich et al. 2001; Hendrich and Bird 1998).

2.3.3.3.3. Methyl-CpG-binding domain protein 4 (*MBD4*)

MBD4, as others *MBD* proteins, seems to acts as a transcriptional repressor and is also involved in DNA repair due to the presence of a glycosylase domain. The protein uses its N-terminal MBD to bind methylated CpG and its C-terminal glycosylase (which is homologous to base excision repair proteins) to mediate repair of the hypermutable CpG. Cells act

spontaneous hydrolytic deamination of methylated cytosine which causes mCpG-TpG transitions (Bird 1980), whereas non-methylated CpG mutates to UpG. MBD4 is able to excise and repair both mutated nucleotides (Hendrich et al. 1999). Its repair function is suggested by the two to three times higher number of mCpG-TpG transitions in *MBD4*-null mice, showing that MBD4 acts to reduce mCpG-TpG mutations (Millar et al. 2002; Wong et al. 2002). Despite its well characterized DNA repair activity , its role as a transcriptional repressor still needs to be confirmed. In fact, a study in zebrafish provided evidence for a role of MBD4 in active demethylation (Rai et al. 2008).

2.4. DNA methylation dynamics during development

In mammals, DNA methylation patterns are relatively stable in somatic cells, but dynamic changes occur during embryonic development and gametogenesis. DNA methylation remodelling is essential to regulate mechanisms that are crucial for normal development and the future health of the new born. (Saitou et al. 2012; Messerschmidt et al. 2014; Okae et al. 2014). During embryo development, different waves of DNA methylation reprogramming occur, specifically after fertilization and during germ-cell specification, Fig. 2.2.

2.4.1. DNA methylation reprogramming in the early embryo

During early embryogenesis a massive erasure of DNA methylation occurs to maintain a specific transcriptional state essential for pluripotency establishment (Paranjpe and Veenstra 2015). Soon after fertilization, the genome of the zygote undergoes massive demethylation. The zygote is characterized by the presence of two PNs one originated from the sperm and one form the egg. Both PNs undergo DNA demethylation (Mayer et al. 2000; Oswald et al. 2000; Santos et al. 2002; Messerschmidt et al. 2014), but two different kinetics has been

proposed for the two PNs. The paternal genome, which is high methylated in the sperm, is actively demethylated in the zygote. Recently, some authors strongly evidenced that the active demethylation of the male PN is mediated by TET3 enzyme by hydroxylation of the 5-mc (Wossidlo et al. 2011; Guo et al. 2014) as previously mentioned (section 2.3.2.2). Conversely, the maternal genome undergoes subsequent passive demethylation via DNA replication during cleavage. It seems that the maternal genome is protected from Tet3 oxidation by an interaction between STELLA and H3K9me2, which is mostly present in female PN. This interaction alters chromatin configuration and protects the DNA from TET3 hydroxylation (Nakamura et al. 2012).

Both active and passive demethylation continue through cell divisions, in fact genome-wide methylation analysis demonstrated that the newly formed embryo reaches the lowest methylation levels at the blastocyst stage (Messerschmidt et al. 2014).

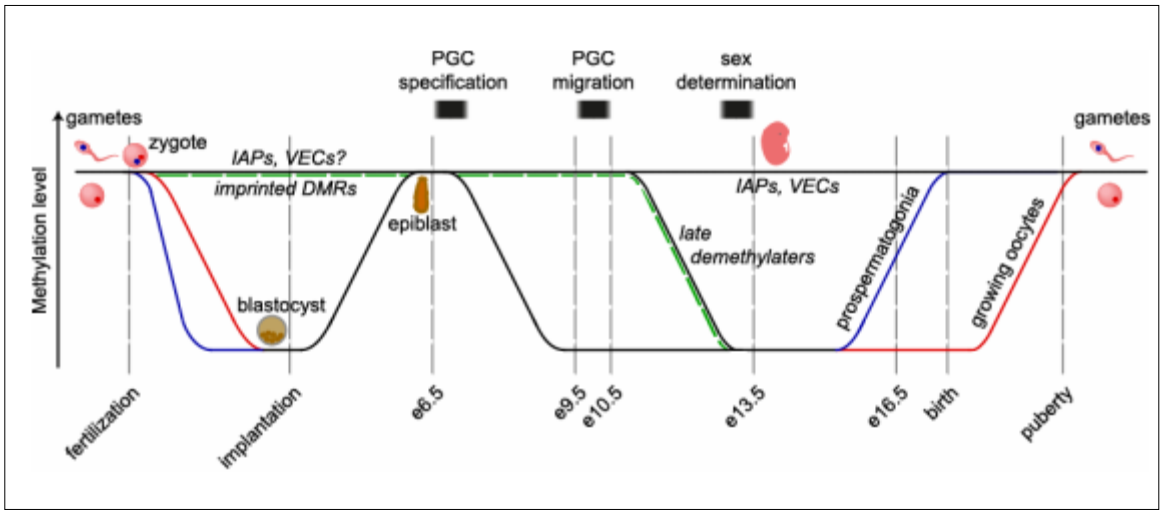
Some sequences are resistant to the wave of global DNA demethylation : repetitive DNA (Lane et al. 2003; Guibert et al. 2012), a subset of single-copy genes (Guibert et al. 2012; Borgel et al. 2010) and imprinted loci (Nakamura et al. 2007). The mechanisms by which these particular DNA sequences escape massive DNA methylation erasure is not known, but it has been demonstrated that both maintenance and *de novo* methylation enzymes are dispensable for the maintenance of imprinting (Hirasawa et al. 2008). Following blastocyst implantation , the epiblast rapidly adopts a somatic epigenetic re-methylation of the genome. This process is initiated via *de novo* methylation (Popp et al. 2010; Borgel et al. 2010). In particular, pluripotency genes (i.e., Oct4 *and* Nanog) and CGI-associated germline-

specific genes are tightly repressed by DNA methylation, to prevent ectopically gene activation (Borgel et al. 2010; Maatouk et al. 2006; Seisenberger et al. 2012).

2.4.2. DNA methylation reprogramming during germ-cell specification

In post-implantation mammalian embryos, a population of pluripotent cells in the epiblast gives rise to the PGCs precursors. The PGCs precursors then proliferate and migrate across the embryo to reach the genital ridges. PGCs continue to proliferate until when they enter into meiotic prophase in female gonads and mitotic arrest in male gonads (Saitou and Yamaji 2012). When PGC precursors initiate to differentiate in the epiblast, they undergo global DNA demethylation, which is mostly concluded when the cells colonize the genital ridge. During their migration, some sequences escape demethylation*;* these sequences include, but are not limited to, the imprinted differentially methylated regions (DMRs) (Leitch et al. 2013). In fact, depending on the sex of the organism, specific imprints are reset during the development of germ cells. The methylation patterns in male germ cells are then fully established at birth and are maintained throughout many cycles of mitotic divisions before the cells enter meiosis (Davis et al. 2000; Henckel et al. 2009). On the contrary, in female germ cells re-methylation is not complete at birth, but continues during the oocyte growth phase, while oocytes are arrested in prophase of meiosis I (Kota and Feil 2010).

Figure 2.2: Dynamic changes of DNA methylation during embryonic development
After fertilization, the paternal genome (blue line) is rapidly demethylated by active mechanisms, whereas the maternal genome (red line) is passively demethylated. Differentially methylated regions (DMRs) associated with imprinted genes are protected from this erasure (dashed green line). De novo methylation occurs after implantation (black line), but primordial germ cells (PGCs) are not specified until the epiblast stage (shading at the top of the figure). This methylation must be reset in PGCs. The figure shows the methylation dynamics after the epiblast formation for germline cells only . After migration most sequences are demethylated in PGCs. But some sequences are subject to late demethylation and are not reprogrammed until after PGC sex determination. Following sex determination, the germ cells undergo de novo methylation, but the dynamics are sex-specific. Methylation is completed in the prospermatogonia before birth, whereas methylation in the oocytes is established during postnatal growth (Cowley and Oakey 2012).



2.5. Particular genome regions controlled by DNA methylation

2.5.1. X Chromosome inactivation

In mammals, females receive two copies of the X chromosome while males receive one copy. To prevent female cells from having twice as many gene products from the X chromosomes as males, one copy of the X chromosome in each female cell is inactivated (XCI). The necessity of dosage compensation by XCI is likely to start at the onset of genome activation (Huynh and Lee 2003). This process is regulated by a region on the X chromosome called the X chromosome inactivation centre (Brown et al. 1991). This region maps the *Xist* gene and its

expression seems to be upregulated in the future inactive X chromosome in order to trigger the establishment of chromosome-wide silencing (Brockdorff 2002; Penny et al. 1996). DNA methylation is involved in the transcriptional regulation of this gene. After *Xist* has been upregulated and XCI triggered on one X chromosome in female cells, *Xist* repression of the second allele of *Xist* (on the active X chromosome) is maintained by DNA methylation of its promoter (Norris et al. 1994).

2.5.2. Genomic imprinting

Genomic imprinting is a unique regulated molecular process for controlling parental-origin-specific gene expression, depending on the DNA methylation status of the differentially methylated region (DMR) of each imprinted gene (Li et al. 1993). By this mechanism, genes are expressed in a mono-allelic manner rather than from both chromosome homologues (bi-allelic expression) as it happens for most genes. It means that if the DMR is methylated in the allele derived from the sperm (paternal imprinting), the gene is maternally expressed and vice versa. Imprinted genes are normally found in clusters and are controlled within DMRs, which are CpG-rich sequences located within specific regions called imprinting control regions (ICs). In the germ-line, parental-specific DNA methylation is erased during the differentiation of primordial germ cells, is re-established during spermatogenesis or oogenesis and subsequently maintained throughout development (Delaval and Feil 2004). Imprinted genes have important roles in normal growth and foetal development (DeChiara et al. 1991; Salas et al. 2004).

2.5.3. Repetitive elements

A large portion of the genome is composed by repetitive elements, consisting of interspersed repeats derived from transposable elements (Weisenberger et al. 2005) and tandem repeats of simple (satellite DNA) or complex sequences. Repetitive elements comprise ∼45% of the human genome. Those include 1 million alu sequences occupying ∼10% of the genome, and LINE-1 elements that also represent a large genomic portion (Ehrlich 2002). These interspersed repeated DNA sequences contain numerous CpG dinucleotides. Therefore, the methylation status of these sequences represents the most global DNA methylation patterns that have been found in the genome (Yang et al. 2004) and have an active role in a variety of human diseases (Pogribny and Beland 2009). Loss of DNA methylation in these sequences represents the global hypomethylation state that characterizes many types of cancers (Ross et al. 2010).

2.6. Role of DNA methylation in disease

2.6.1. Epigenetic and assisted reproductive technologies (ART)

Assisted reproductive technologies (ARTs) refer to the methods aimed at overcoming infertility problems (Pinborg et al. 2015). ART treatments involve several *in vitro* manipulation steps in order to achieve pregnancy by artificial or partially artificial procedures. Different procedures used for ARTs consist of: hormonal stimulation, used to induce superovulation; *in vitro* oocytes maturation prior to fertilization; intracytoplasmic sperm injection (CSI), *in vitro* culture of preimplantation embryos and cryopreservation of either gametes or embryos (Iliadou et al. 2011; Wright et al. 2003), that based on the infertility problems could be used alone or in combination. The use of ART has become a

widely accepted and implemented therapy for some forms of infertility. However, it has been noticed that the use of ART is related with an increased risk of pregnancy disorders and birth defects (Hansen et al. 2005). An association between ART and epigenetic defects has also been proposed. This hypothesis is supported by experimental studies involving ART in mouse and ruminants and by the increased incidence of imprinting disorders, such as Beckwith-Wiedemann, Angleman and Prader-Willi syndromes, of individuals conceived with the use of ART (Niemitz and Feinberg 2004). As discussed above, during embryonic development and germ cells specification and maturation, a crucial reset and rearrangement of epigenetics marks takes place; thereby, the manipulation of germ cells and/or embryos could influence and disturb the process of methylation and⁄ or demethylation and lead to embryo defects. Studies have shown that the culture media used for embryo culture have a role in the deregulation of developmentally important genes (Khosla et al. 2001). A study published in 2000, comparing two different embryo culture media to determine the potential effect on imprinted genes, showed that *H19* expression and methylation were adversely affected by culture media composition (Doherty et al. 2000). Although results from animal studies support the association between ART and epigenetic alternations, it has also to be mentioned that most children conceived through ARTs are developing normally (Lu et al. 2013). It has to be kept in mind that ART patients often have reproductive problems that may be mainly responsible for the birth defects associated with ARTs

2.6.2. Disorders due to loss of imprinting

Imprinted genes are a particular class of genes expressed in a mono-allelic manner depending upon parental-origin allele specific methylation at specific DMR located at

imprinting control regions (ICRs). Imprinted genes have important roles in normal growth and foetal development (DeChiara et al. 1991; Salas et al. 2004), A number of human diseases, identified as imprinting disorders, have been found to be caused by incorrect establishment of DNA methylation at DMR during embryo development(Cassidy and Schwartz 1998). The first identified imprinting disorders are Prader-Willi (PWS) and Angelman (AS) syndromes. Both disorders arise from an imprinting defect in the chromosome 15, but the abnormality is on the paternally derived chromosome 15 for PWS and on the maternally derived 15 for AS. The involved chromosomal region contains a differentially methylated region (*SNRPN/SNURF* ICR), which regulates the expression of imprinted genes (Kalsner and Chamberlain 2015). The loss of maternally expressed genes controlled by the *SNRPN* ICR results in AS that involves a cognitive and neurologic impairment, while PWS results in behavioural and endocrine disorders derived from the loss of those genes which are expressed from the paternal allele (Cassidy and Schwartz 1998; Cassidy et al. 2000). Beckwith-Weidemann Syndrome (BWS) and Silver-Russell Syndrome (SRS) are also due to imprinting defects. Both of them are caused by changes in the level of methylation of at ICR which controls the expression of both *H19* and *IGF2.* BWS syndrome is caused by a hypermethylation of the *H19* ICR that results in decrease of *H19* and increase of *IGF2* expression, giving rise to a phenotype of overgrowth (Ko 2013). By contrast, SRS is caused by a hypomethylation of the *H19* ICR that results in decreased *IGF2* and increased *H19* expression, giving rise to a phenotype with growth problems (Searle and Johnson 2015).

2.6.3. The role of DNA methylation in cancer

As this thesis is focused on DNA methylation dynamics that physiologically occur during embryo development, a brief overview of the pathological involvement of DNA methylation in cancer will be described. During the last decade, numerous studies have shown that defects in the epigenetics mechanisms could contribute to the neoplastic phenotype. Regarding DNA methylation, tumours are usually characterized by low level of CpG methylation in comparison with their normal-tissue counterparts, indicating that cancer cells have a specific epigenome (Ehrlich 2009). Loss of methylation could be related to the faster growth rate that is characteristic of cancer cells, and may be also related to reactivation of transposable elements that are usually suppressed by DNA methylation (Ross et al. 2010). Reactivation of transposable elements can promote their integration in to the genome, leading o gene activity disruption. Nevertheless, some important regions that control the expression of tumour suppressor genes have been found to be hypermethylated in cancer cells. This results in the silencing of genes possibly involved in cell cycle control and DNA repair (Lahtz and Pfeifer 2011; Kulis and Esteller 2010).

2.7. General introduction to *in vitro* embryo production technique

In vitro embryo production (IVP) is currently one of the most important biotechnologies in ruminant breeding and husbandry. This technique allows to better control animal reproduction, helping genetic improvement in livestock, but it is also an important research tool for developmental biology studies in mammals (McEvoy et al. 2000b; Paramio and Izquierdo 2014; de Souza-Fabjan et al. 2014). IVP involves four main steps: oocyte collection,

oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture of the resulting embryos (IVC).

2.7.1. Oocyte collection

The oocyte is a high specialized type of cell that, together with the spermatozoon, allows the generation of a new individual. Oocytes reside within the follicles, that constitute the functional unit of the ovaries, since when they migrate into the germinal ridge during embryonic development (Tingen et al. 2009). From foetal life until primordial follicle activation, oocytes remain arrested at diplotene stage of meiosis into the primordial follicle until luteinizing hormone surge, at puberty (Grondahl et al. 2013; Celik et al. 2015). In a normal oestrous cycle, only one or maximum two follicles mature and ovulate the oocyte. For the set-up of the IVF technique, large quantities of material are obtained by ovaries collected at the abattoir. The transport of ovaries to the laboratory is a critical step, in fact it has been demonstrated that low temperatures and/or long period of transportation slow down or completely arrest metabolic activities (Naoi et al. 2007). The oocytes are then collected by ovary aspiration or slicing, with an average collection of 5 to 10 oocytes from each bovine ovary (Mermillod and Marchal 1999) and one to two oocytes from sheep or goat ovaries (Cognie 1999; Paramio and Izquierdo 2014). Thereafter, the recovered oocytes are screened under a stereomicroscope to be selected for IVM.

The oocytes are surrounded by several layers of somatic cells (granulosa cells). These cells are very important for oocyte growth and maturation as they establish contacts with the oocyte through gap junctions to transfer small metabolites and regulatory molecules into the oocyte (Van Soom et al. 2002). The efficient recovery of the cumulus-oocyte complexes

(COC) is one of the most important factors that influences a successful IVM (Merton et al. 2003; Dadashpour Davachi et al. 2014; Lonergan et al. 2003). Therefore, only COC with complete and compact cumulus should be selected for IVF (Leibfried and First 1979; Stangl et al. 1999).

2.7.2. Oocyte maturation

As mentioned above, since foetal life the oocytes are arrested in the ovaries at the diplotene stage of meiotic prophase I. Several key factors, such as molecular and morphological events, are needed to allow the oocyte to fully acquire developmental competence and maturation. (Von Stetina and Orr-Weaver 2011). The developmental competence refers to oocyte ability to mature, be fertilized and give rise to an embryo, up to the birth of a live offspring The maturation events involved in oocyte growth and acquisition of developmental competence include proliferation and differentiation of cytoplasmic organelles, synthesis and storage of mRNA and proteins required to initial early embryogenesis, resumption and completion of meiosis (Fair 2010; Eppig et al. 1994) and acquisition of epigenetic modifications (O'Doherty et al. 2012; Hanna and Kelsey 2014). For example, maternal effect genes, that are transcriptional regulators required only after fertilization, are transcribed and stored during oocyte growth (Li et al. 2010; Fair 2010; Bebbere et al. 2014).

Oocytes collected from ovaries for IVP are blocked at the prophase stage of meiosis; as soon as they are retrieved from the follicular inhibitory environment, meiotic resumption occurs spontaneously and oocytes progress to metaphase II (MII) (Sirard 2001). *In vitro* maturation is one of the most critical steps of IVP. Ruminant oocytes are usually *in vitro* matured at 39° C under a 5% CO2 in a humidified atmosphere. The most commonly used medium is TCM-199

medium supplemented with bicarbonate, minerals, carbon and energy sources (glucose, glutamine) vitamins, amino acids , hormones and growth factors (foetal calf serum, serum of female in oestrus, follicular fluid). The optimal maturation time is 22-24 hours (hrs). The first visual sign that indicates oocyte maturation is the expansion of cumulus cells. As mentioned above, before IVM granulosa cells in the COC are compacted in different layers around the oocyte, and expand upon oocyte maturation (Van Soom et al. 2002). Usually, when performing IVP, it is necessary to remove cumulus cells after IVM to allow the visualization of cytoplasm vacuolization and granulation and of the first polar body extrusion, as they are indicative of oocyte maturation (Van Soom et al. 2002).

2.7.3. Fertilization

In vivo, soon after the spermatozoa reach the female tract, the capacitation process takes place. By the capacitation, the spermatozoa acquire fertilization ability by membrane modifications (Ghersevich et al. 2015; Cognie et al. 2004) in order to penetrate the oocyte membrane. Once a spermatozoa reach the oocyte, it takes contact with the oocyte's coat known as zona pellucida (ZP) and fuses with it through the acrosome reaction. The fusion of the two membranes stimulates the oocyte to complete the second meiotic division, in order to reduce the maternal genome to a haploid state by the extrusion of the second polar body (Azzarello et al. 2012). At this stage the fused oocyte is denoted zygote and the maternal and paternal PNs migrate toward each other and start to replicate their DNA. During *in vitro* fertilization, oocytes and spermatozoa are incubated for 18-24 hrs at 39° C under a 5% CO2 in a humidified atmosphere. The medium is commonly auditioned with capacitating agents (Parrish et *al*. 1986). Usually frozen sperm is used for *in vitro* fertilization, at a concentration

that ranges between 0.5 and $2x10^6$ spermatozoa per ml. Before spermatozoa and oocyte incubation, the swim-up or sperm centrifugation in a discontinuous density gradient (Percoll) techniques are used to collect the most motile sperms. In ruminants, such as cow or sheep, it is not possible to identify the fertilized oocytes because the zygotes are not transparent due to the high lipid content; therefore, after 24 hrs from IVF, all presumptive zygotes are transferred to a new media that contains specific compounds needed for embryo development.

2.7.4. Preimplantation Embryonic development

Preimplantation embryo development initiates with fertilization and concludes with formation of the blastocyst (Rossant, J 2009). As mentioned above, after fertilization the two PNs start to migrate toward each other and replicate their DNA. The zygote enters then the first mitotic division and two identical embryonic cells are formed. The embryo continues with cell divisions, this process is called cleavage. The fourth cleavage cycle results in a 16 cell embryo, then the embryo starts to compact into a morula. At this stage the cells are called blastomers and a series of inter- and -intra organization events occurs, such as polarization and formation of particular junctions and cytoskeletal adherences between blastomers. Soon after morula formation, a blastocelic cavity starts to develop, defining the transition from morula to blastocyst stage. At the same time, the differentiation between the inner cell mass (ICM) and traphectoderm (TE) occurs; the ICM will differentiate in the three primordial germ layers that will later develop into the foetus, while the TE will be responsible for uterine wall invasion and placenta formation. Just after fertilization, the transcriptome of the embryo consists only of maternal RNAs ,that has been deposited during oocyte growth (Seydoux

1996), and will later be specifically degraded and replaced by transcripts produced by the embryo. This gradual transition, termed embryo genome activation (EGA), is a crucial step during embryonic ontogeny and coincides with a developmental block observed within mammalian embryo culture *in vitro*. The developmental stage at which EGA occurs in mammalian species varies considerably: at the 1- to 2-cell stage in mice, at the 4- to 8-cell stage in cows and humans, and at the 8- to 16-cell stage in sheep and rabbits (Schultz and Heyner 1992). For *in vitro* embryo culture, after 24 hrs incubation in IVF media, presumptive zygotes are checked and only cells that show cell division are transferred to a new culture media. The remaining oocytes, not showing any cell division after 24 hrs from IVF, are maintained in the incubator in IVF media for further 8 hrs (until 32 hrs) because a delayed fertilization may occur. After 32 hrs, presumptive zygotes are checked again and late 2 cells embryo are transferred to a new culture medium specific for IVC. Two different methods are used for IVC. One provides the coculture of the embryos in a somatic cell support, while the other one involves the use of semi-defined media. The development usually takes place at 39° C in droplets of culture medium overlaid with oil for 7 to 9 days. A high density of embryos per volume seems beneficial to development (Ferry *et al*. 1994). When embryos are cultured without cell support, the oxygen concentration in atmosphere should be reduced to 5% to avoid induction of oxidative stress on the embryos (Olson and Seidel, 2000; Voelkel and Hu, 1992). The rate of cleavage is usually high (over 80% of the total oocytes in sheep and bovine), when appropriate bull/ram and fertilization conditions are used. However, the rate of development to blastocyst stage plateaus at around 40% of the total oocytes. It has been clearly established that oocyte quality (intrinsic quality of primary oocyte coupled to

maturation conditions used) determines the rate of blastocyst production, whereas developmental environment is determinant for embryo viability (Rizos et al. 2002).

2.8. Developmental competence

2.8.1. Oocyte quality

It is widely accepted that the earliest stages of life set the basis for the offspring future health (Barker 1997). Well-developed embryos originate from high quality gametes. The developmental competence of the oocyte is the final result of a complex biological process that is hormonally regulated and driven by the follicular environment. Cumulus cells play a pivotal role in this process, affecting the oocyte growth and maturation through a biochemical/molecular exchange with the oocyte. This bidirectional communication has been demonstrated and extensively studied (Moor et al. 1980; Gilchrist et al. 2006; Hussein et al. 2006) .Large scale gene expression analysis showed that this physiological interaction is crucial for the uptake of molecules involved in the attainment of oocyte developmental competence (Regassa et al. 2011). Recently, it has also been hypothesized that the acquisition of epigenetic modifications could affect the gaining of developmental competence during oocyte growth (Ptak et al. 2006; O'Doherty et al. 2012). During the final phase of oocyte development, global transcriptional activity decreases, allowing the oocytes to progress through meiosis (Brevini-Gandolfi et al. 1999) and bout 30% of the total mRNA is degraded (Su et al. 2007; Bestor et al. 1988; Yuan et al. 2011) Either maintenance of transcription or aberrant degradation during oocyte maturation could also be deleterious to oocyte quality. Evidences indicate that transcriptional silencing process could be mediated by epigenetic mechanisms, including DNA methylation (Curradi et al. 2002). This complex

system translates into specific molecular mechanism, whose deeper understanding is fundamental for optimizing the safety and efficiency of in vitro reproductive technologies.

2.8.2. Sheep model of differential developmental competence

Several models have been proposed in order to understand the mechanisms underlying developmental competence. We have developed a model consisting of ovine oocytes deriving from adult versus prepubertal donors to study high versus low developmental competence, respectively. The oocytes derived from adult animals are fully grown and contain all the molecules necessary for maturation, fertilization and the first phases of pre-implantation development. Conversely, oocytes deriving from prepubertal donors have not completed their growth however, they can be fertilized, develop and, with a reduce percentage, give rise to a live offspring, as observed in several species (Armstrong et al. 1992; Armstrong et al. 1997; Ledda et al. 1999). This ability indicates that at least some prepubertal oocytes contain a subset of molecules sufficient to support complete development, and may be helpful to analyse the molecular pathways involved in oocyte developmental plasticity. We have previously shown that prepubertal and adult ovine oocytes show differences in mRNA abundance at germinal vesicle (GV) stage (Leoni et al. 2007), in accordance with studies in cattle (Romar et al. 2011). These differences confirm the hypothesis that prepubertal oocytes have not finished preparing the molecular machinery (RNAs and proteins) involved in meiotic progression, fertilization and early development. We hypothesized that oocytes from adult and prepubertal animals undergo the completion of meiosis and the following stages of development employing differently the molecular machinery involved. Due to the recent hypothesis that the acquisition of epigenetic

modifications could affect the gaining of developmental competence during oocyte growth, we decided to analyse DNA methylation dynamics through this model of developmental competence.

Chapter 3: Aim

The aims of this work were :

- To contribute to the knowledge on DNA methylation dynamics during oocyte growth, meiotic progression and early embryo development in a large animal model

- To evaluate DNA methylation dynamics in relation to oocyte quality in the ovine species, in order to understand whether the acquisition of epigenetic modifications affects oocyte developmental competence

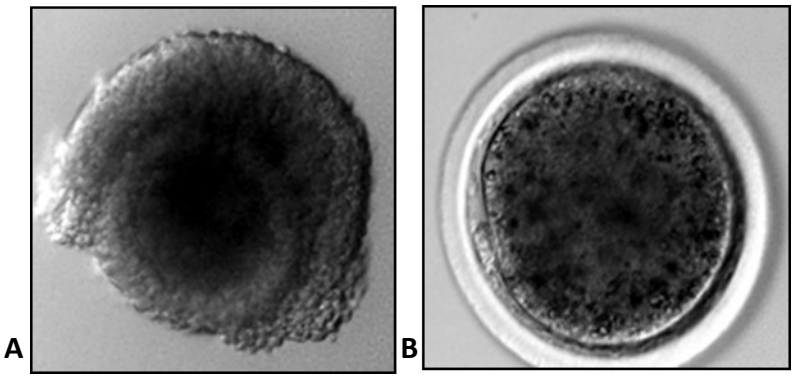
Chapter 4: Materials and Methods

4.1 Sample preparation

4.1.1 Oocyte recovery and In Vitro Maturation

Oocytes were recovered from prepubertal (P) (30–40 days of age, body weight 6–10 kg) and adult (A) ovine ovaries collected at a local slaughterhouse and transported to the laboratory within 1 hour (hr) 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 TC-199 medium (TCM-199) (with Earle's salts and bicarbonate) supplemented with 25 mmol Hydroxyethyl piperazine ethane sulfonic acid *(*HEPES), penicillin, streptomycin, and 0.1% (w/v) polyvinyl alcohol. The COC 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), 0.1 IU/ml follicle-stimulating hormone (FSH) and 0.1 IU/ml luteinizing hormone (LH) (Pergonal, Serono Italy) and 100 µM cysteamine Then, 30–35 COCs were cultured for 24 hours (hrs) in 5% CO2 in air at 38.5° C in four-well Petri dishes (Nunclon; Nalge Nunc, Roskilde, 100 Denmark) with 600 µl maturation medium, layered with 300 µl mineral oil. After IVM, oocytes were vigorously pipetted and only oocytes presenting compact cytoplasm and polar body were selected.

Figure 4.1.: Sheep oocytes: **A)** Germinal Vesicle (GV) and **B)** metaphase II (MII) stage



4.1.2. Semen processing and swim-up technique

Frozen–thawed spermatozoa (stored in liquid nitrogen in pellets of 250 µl), were used following swim up procedure for all in vitro embryo production experiments. Two pellets per *in vitro* fertilization (IVF) were thawed at 37° C for 30 seconds in a water bath. Afterwards an aliquot of 200 µl of semen was deposited at the bottom of a glass vial under 1 ml of Synthetic Oviductal Fluid (SOF) medium and incubated at 38.5° C for 15 min. After incubation 6 drops of 100 µl of supernatant containing the most viable sperms were gently collected and placed on a warm Petri dish. Only the drops containing the highest number of motile spermatozoa and less debris were used for IVF.

4.1.3. In vitro fertilization and embryo development

Selected spermatozoa and IVM oocytes were incubated in fertilization medium (SOF medium with 2% OSS, 1 µg/ml heparin and 1 µg/ml hypotaurine) for 22 hrs at 38.5° C in an atmosphere of 5% CO2 and 5% O2 in N2 in four-well Petri dishes (Tervit et al. 1972). Thereafter, presumptive zygotes were transferred and cultured for 8 days in four-well Petri dishes containing SOF + 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% CO2, 5% O2 and 90% N2 at 38.5° C (Bogliolo et al. 2011). The first cleavage was recorded between 24 and 26 hrs after fertilization.

4.1.4. Sheep and bovine tissue collection

Sheep liver, oesophagus and ovaries and bovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 1 hr in PBS. Subsequent tissues were snap frozen in liquid nitrogen and stored at -80° C until required for further analysis.

4.1.5. Sperm collection

Ejaculates were collected from healthy adult (2-6 years old) Sarda rams (Genetic Centre of AGRIS, Agenzia Regionale per la Ricerca in Agricoltura, Bonassai, Italy). The animals were of proven fertility and trained to service an artificial vagina. After collection the semen was immediately submitted to swim up technique following the protocol described in section 3.1.2. and immediately used for DNA extraction.

4.1.6. Blood collection

Blood was collected from 3 healthy Sarda sheep from the jugular vein. The wool on both sides of the neck was removed and the area was carefully disinfected to avoid contaminations. Blood samples (5 ml) were collected using Vacutainer®vials (BD, Becton Dickinson and company) containing Ethylenediaminetetraacetic acid (EDTA) to prevent blood coagulation.

4.2. Gene expression analysis

4.2.1. Samples collection for gene expression analysis

Gene expression analysis was performed on pools of growing oocytes (GO) at different diameter (70/90 µm, 90/110 µm, 110/130 µm) derived from P animals, GV, IVM MII oocytes, IVP (*in vitro* produced) embryos at two (2C) and four (4C) cell stage and blastocysts derived from P and A donors were added to 30 µl RLT buffer [RNeasy Micro Kit (Qiagen, Hilden, Germany)], snap frozen in liquid nitrogen, and stored at – 80° C until RNA isolation was performed. As a control for IVM procedure, a pool of oocytes in each IVM run was cultured until the blastocyst stage to assess the developmental competence of the fertilized eggs.

4.2.2. RNA Isolation

Total RNA was isolated from pools of oocytes, embryos and blastocyst with the RNeasy Micro Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Five pg of luciferase mRNA (Promega) were added to each group prior to RNA extraction to account for RNA loss during the isolation process. Importantly, during the procedure, RNA was treated with DNase I (Qiagen, Hilden, Germany) to exclude any potential genomic DNA contamination. Isolated RNA was eluted in 12 µL NUCLEASE-free water (NF-H_2O (Sigma-Aldrich)). Following extraction, RNA was immediately transferred to the -80^o C freezer were it was stored until reverse transcription–polymerase chain reaction (RT-PCR) was performed.

4.2.3 Reverse transcription

The reverse transcription allows the synthesis of DNA from an RNA template. This technique produces complementary DNA (cDNA) by a Reverse transcriptase (RT) that uses an RNA

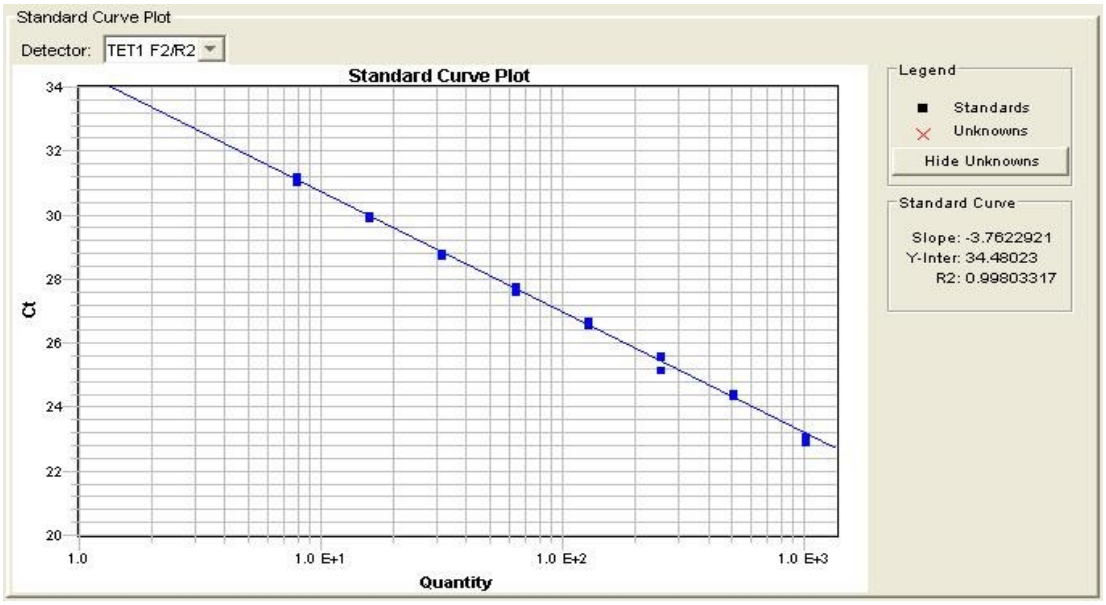
template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR). The conversion of RNA into cDNA is fundamental because RNA is chemically unstable and easily degraded by RNAses, that are ubiquitous in nature besides, PCR amplification requires DNA as template. RT was performed in a final volume of 20 µL, consisting of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 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 Corporation, Carlsbad, CA). The reaction tubes were incubated at 25° C for 10 min, then at 42° C for 1 hr 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 rate, 5 pg of luciferase mRNA (not subjected to RNA isolation) were subjected to cDNA synthesis as well.

4.2.4. Primers design and validation

Primers were designed by Primer3, a free online tool to design and analyse primers for PCR and real time PCR experiments (http://primer3.ut.ee/). All primers were designed in order to amplify an intron-spanning region. To rule any potential genome DNA contamination. In fact, intron-spanning primers allow to discriminate between genomic DNA and cDNA, since any product amplified from genomic DNA would be larger than the one amplified from intron-less cDNA. For each primer pair, the efficiency of the PCR reaction was determined by Real Time PCR analysis. A standard curve with serial dilutions of a known amount of template, was built, covering at least three orders of magnitude, so that the standard curve's linear interval

included the interval above and below the abundance of the targets. Only primers achieving an efficiency of reaction between 90 and 110% (3.6 > slope > 3.1) and a coefficient of determination r2 > 0.99 were used for the analysis (Fig 4.2).

Figure 4.2: Example of a Standard curve to asses Primers efficiency (3.6 > slope > 3.1 and a coefficient of determination r2 > 0.99).



4.2.5. Agarose gel electrophoresis

To further confirm the size of the amplicons, 3 µl of each PCR products were mixed with 5 µl of Gel Loading Solution (Sigma Aldrich) before being subject to electrophoresis on a 2% agarose (Sigma Aldrich) gel prepared using 0.5X TBE buffer. Such buffer from made using a 10X concentrated stock which consisted of 890 mM Tris-base, 20 mM EDTA (Sigma-Aldrich,), 890 mM Boric acid. Samples were run alongside TrackIt™ ΦX174RF DNA/Hae III Fragments. Agarose gels stained with 20 µl syber safe (Invitrogen, Paisley, U.K) were imaged by Safe Imager™ 2.0 Blue Light Transilluminator.

4.2.6. PCR products sequencing

After gel checking PCR product were purification with MinElute PCR purification kit (Qiagen, Hilden, Germany). 10 ng of purified PCR products and 3.2 pmol of each primer were put together in a PCR tube and let dry at 65° C for no more than 5 min to allow water evaporation to obtain a pellet. Subsequently the PCR products sequenced by singer sequencing (Model 3130 xl Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) by BMR genomics s.r.l a company in DNA sequencing technology. After identities were confirmed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Information on the primers used for real-time PCR and sequences of PCR products are shown in Tab. 4.1. and 4.2.

Table 4.1: Primers designed for real-time PCR

Gene	Primer	Sequence (5'-3')	TA	Size
DNMT1	FW	CAGCTCTCGTACATCCACAG	60°C	158bps
	RV	AATCTCGCGTAGTCTTGGTC		
DNMT3A	FW	GTGATGATTGATGCCAAAGA	60°C	165bps
	RV	GGTCCTCACTTTGCTGAACT		
DNMT3B	FW	ATTGCAACAGGGTACTTGGT	60°C	122bps
	RV	ATATTTGATGTTGCCCTCGT		
TET1	FW	CAGTATGCTCCAGCTGCTTA	60°C	166bps
	RV	TGCTCCCATTATTCATGTTG		
TET2	FW	TACAAGAAACTCGCACCTGA	60°C	159bps
	RV	CTGCATGTTCTGCAAGTCTC		
TET3	FW	TGGAGCATGTACTTCAATGG	60°C	173bps
	RV	GGTCACCTGGTTCTGATAGG		
MBD1	FW	GAAGTGTCAGGTTGGACCTC	60°C	209bps
	RV	GATTGAAAGCGATCCTCTGT		
MBD3	FW	ACATCAGGAAGCAGGAAGAG	60°C	203bps
	RV	GCTCTAGACGTGCTCCATTT		
MBD4	FW	AACAGACGACAGCTCACAAA	60°C	204bps
	RV	TCCTGGACGAGATTAAAAGG		

Forward PCR primer (FW); reverse PCR primer (RV); Annealing temperature (TA)

Table 4.2: Sequence of each region amplified for gene expression analysis

Gene	Sequence
DNMT1	CAGCTCTCGTACATCCACAGCAAGGTGCAGGTCATCTATAAGGCCCCCTCGGAGAACTGGGCC TTGGAGGGAGGCGTGGACCCCGAGGCCCTGATGTCGCAGGACGACGGGAAGACCTACTTCTA TCAGCTGTGGTACGACCAAGACTACGCGAGATT
DNMT3A	GTTGATTGATGCCAAAGAAGTGTCAGCTGCGCACAGGGCCCGCTACTTCTGGGGGAACCTTCC TGGTATGAACAGGCCATTGGCATCCACTGTGAATGATAAGCTGGAGCTGCAGGAGTGTCTGGA GCACGGCCGAATAGCCAAGTTCAGCAAAGTGAGGACC
DNMT3B	ATTGCAACAGGGTACTTGGTCCTCAAAGAACTGGGCATCAAAGTGGAGAAATACGTGGCCTCT GAAGTGTGGAAGAGTCCATTGCTGTTGGCACCGTTAAGCACGAGGGCAACATCAAATAT
TET1	TTTTAGTATGCTCCAGCTGCTTATCAAAACCAGGTGGCGCTTGAACATATTGCCCGAGAATGTC GGCTTGGGAAGAAAGAAGGTCGTCCTTTCTCTGGGGTCACTGCTTGCCTAGACTTCtgTGCCCAt CCCCACAGGGACATTCACAACATGAATAATGGGAGCA
TET2	TACAAGAAACTCGCACCTGATGCATACAATAATCAGATTGAATATGAACACAGAGCACCTGAGT GCCGTCTGGGTCTGAAGGAAGGCCGCCCATTCTCAGGGGTCACCGCATGTTTGGACTTCTGTG CGCATGCCCACAGAGACTTGCAGAACATGCAG
TET3	TGGAGCATGTACTTCAATGGCTGCAAATATGCTCGGAGCAAGACTCCTCGCAAGTTCCGCCTCG CAGGGGACAACCCCAAAGAGGAAGAAGTGCTCCGGAAGAGTTTCCAGGACCTGGCCACCGAA GTTGCTCCCCTGTATAAGCGGCTGGCGCCCCAGGCCTATCAGAACCAGGTGACC
MBD1	GAAGTGTCAGGTTGGACCTCAGAAGAGTGAAGTCAGGAAGGAGGCACCAAGGGATGATACCA AGGCTGACACTGACACAGTCCCAGCTTCACTTCCTGCCCCTGGGTGCTGTGAGAACTGTGGAAT CAGCTTTTCAGGAGATGGCACCCGAAGGCAGCGGCTCAAGACTTTGTGCAAGGACTGCCGAGC ACAGAGGATCGCTTTCAATC
MBD3	ACATCAGGAAGCAGGAAGAGCTGGTGCAGCAAGTCCGCAAGCGGCTGGAGGAGGCGCTGAT GGCCGACATGCTGGCCCACGTGGAGGAGCTGGCCCGGGACGGTGAGGCACCGTTGGACAGG GCGGGCGCTGATGAGGAGGAAGATGAGGACGAGGAGGAGGAGGAGCCCGACCAGGACCCA GAAATGGAGCACGTCTAGAGC
MBD4	AACAGACGACAGCTCACAAACAGAGAAAAACCCtTACGTCTGCGAAAGTATCTCAAGAAGACA CCGTCCCACGAACACAAATAGAAAAAAGGAAAACAAGCCTGTATTTTTCCAGCAAATACAACA AAGAAGCTCTTAGCCCCCCACGACGCAAGGCCTTTAAGAAGTGGACACCTCCGAGGTCACCTTT TAATCTCGTCCAGGA

4.2.7. Real time-polymerase chain reaction

Relative quantification of transcripts was performed by Real-Time polymerase chain reaction (RT-PCR) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR was performed in a 15 µL reaction volume containing 7.5 µL 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of each primer and cDNA equivalent to 0.25 oocytes or embryos. The PCR protocol consisted in two incubation steps (50° C for 5 min and 95° C for 2 min), followed by 40 cycles of amplification program 95° C for 15 s, 60° C for 30 s and 72° C for 30 s, a melting curve programme 65–95° C, starting fluorescence acquisition at 65° C and taking measurements at 10-s intervals until the temperature reached 95°C and finally a cooling step to 4° C. Fluorescence data were acquired during the 72° C extension steps. To minimise handling variation, all samples to be compared were run on the same plate using a PCR master mix containing all reaction components apart from the sample. The PCR products were analysed by generating a melting curve to check the specificity and identity of the amplification product. The relative quantification of all transcripts was performed after normalization normalised on the basis of Luciferase (Luc) mRNA levels.

4.3. Immuno detection

4.3.1. TET3 antibody validation

For the immunodetection of TET3 protein, an antibody specific for sheep is not commercially available. Due to this inconvenient, a rabbit polyclonal antibody against Human, Bovine, Mouse and Xenopus laevis TET3 protein has been used (GeneTex GTX 121453).

4.3.1.1. Western blotting

Western blot was performed on sheep oesophagus, sheep ovaries and bovine ovaries (positive control) to ensure the specificity of TET3 antibody on sheep samples (Kurien et al. 2011).

Ten mg of tissue were put into a glass tube with 500 µl of RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) and homogenised by TissueRuptor (Qiagen, Hilden, Germany) until it was well dissolved into the buffer The sample was kept on ice for 30 min and occasionally vortexed. Then Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris-HCl) was, then, added to each sample and heated at 95° C for 20 min. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein lysate, separated by 10% SDS/PAGE gel, were transferred to a nitrocellulose membrane . The membranes were, then, washed in TBS-Tween (10 mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4) and then blocked in a solution containing 4% non-fat milk extract in TBS-T for 1 hr. Membranes were further incubated at 4° C overnight in a solution containing primary TET3 antibody (1:5000 dilution; GeneTex) in 10 ml PBS/Tween-20. After the incubation membranes underwent two washed in PBS/Tween-20 for 15 minutes to remove the excess of primary antibody. After washing the membranes were incubated with an peroxidase conjugated anti-rabbit IgG secondary antibody diluted 1:100,000 in PBS/Tween.

The immunoreactive bands were detected incubating for 10 minutes the membranes in a chemiluminescence solution obtained mixing reagent A with reagent B (1:50); the related chemiluminescence developed was fixed on autoradiograms.

4.3.2. TET3 whole mount immunostaining

TET3 immunostaining using TET3 (GeneTex GTX 121453) antibody was performed in GO at different diameter (70/110 µm and 110/130 µm) derived from P animals, in GV oocytes stages and in IVP 2C and 4C embryos derived from P and adult donors. Oocytes and embryos were briefly washed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. After fixation, samples were blocked for non-specific binding in 30% goat serum containing 0.05% Tween 20 in PBS. After extensive washing, samples were incubated at 4° C overnight with TET3 Rabbit antibody (1:200 dilution; GeneTex) followed with a fluorescein anti-rabbit IgG secondary antibody (1:250; Alexa Fluor) at room temperature for 1 h. Samples undergoing the same procedure but with the omission of the primary antibody served as negative controls. Samples were put on glass slide through a glass pipette. Then, after removing the excess of liquid, samples were mounted on glass slide using a home-made mounting medium containing glycerol and 1:1000 4’,6-diamidino-2-phenylindole (DAPI) for nuclear counterstain. At least 10 oocytes/embryo were processed for each class of oocytes and embryos analysed, and the experiments were replicated 3 times.

4.3.3. Methylation and hydroxymethylation whole mount immunostaining

Methylation and hydroxymethylation immunostaining was performed on GO at different diameter (70/90 µm, 110/130 µm) derived from P animals, GV and MII oocytes stages, PN stage zygotes and IVP 2C and 4C embryos derived from P and A donors obtained as described in section 4.1.1. and 4.1.2., using Anti-5mc Mouse (Calbiochem 163 33 D3) anti-5-hmc Rabbit antibody (Active Motif 39769). Oocytes and embryos were briefly washed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. After fixation, samples were treated

with 4M HCl for 1 hr at room temperature, neutralized in 100 mM Tris HCl buffer (pH 8.5) for 10 min and incubated with propidium iodide (P.I.) (50 µg/ml) in PBS for nuclear and chromosome counterstain. Subsequently, oocytes and embryos were blocked for non-specific binding in 30% goat serum containing 0.05% Tween 20 in PBS. After extensive washing, samples were incubated at 4 °C overnight with anti-5mc (1: 200 dilution; EMD Millipore's) or anti-5-hmc (1: 200 dilution; Active Motif) followed with a fluorescein (Alexa Fluor 350 or 488-566)-conjugated anti- mouse or anti-rabbit IgG secondary antibody (1: 250; Alexa Fluor) at room temperature for 1 h. Samples undergoing the same procedure but not treated with the primary antibody were used as negative controls. Samples were put on glass slide through a glass pipette. Then, after removing the excess of liquid, samples were mounted on glass slide using a home-made mounting medium containing glycerol. At least 10 oocytes/embryo were processed for each class of oocytes and embryos analysed, and the experiments were replicated 3 times

4.3.4. Confocal Analysis

Immunofluorescence stain was evaluated using a TCS SP5 confocal laser scanning microscope (Leica) and images were captured with the LAS AF lite application software (Leica). After image acquisition, a semiquantitative grading system was apply for quantify the antibodies signals. In particular, each sample was scored considering the intensity of signals and the quantity/distribution of specific stain. As a result, each sample were included in the following categories: 0= absence of signal, 1 low, 2 moderate, 3 high.

4.4. Methylation analysis

4.4.1. DNA isolation

Different methods of DNA extraction were used based on sample characteristics.

4.4.1.1. DNA isolation from liver

The liver was collected and stored at -80° C according to section 4.1.4. After thaw the tissue was placed in a glass tube with 5 ml of lysis buffer (50 mM Tris; pH8, 0.1 M EDTA, 0.5% SDS; all from Sigma-Aldrich) and homogenised by a tissue ruptor (Qiagen, Hilden, Germany) until it was well dissolved into the buffer. 600 µl of the aqueous solution of homogenised tissue was transferred into 1.5 ml tubes supplemented with PK (Roche, West Sussex, UK) at a final concentration of 0.2 mg/mL and incubated at 55° C overnight for protein digestion. The sample was then subjected to three subsequent extraction by a solution of phenol:chloroform:isoamyl alcohol (PCIA) in a ratio of 25:24:1 at pH 8.0 (Sigma-Aldrich). 500 µl of PCIA solution were added to the sample, it was vortexed for about 15 seconds and then centrifuged at 13200 rpm for 10 min. The centrifugation allowed the formation of two different phases, the lower organic phase and the upper aqueous phase that contains nucleic acids. The upper aqueous layer was then gently collected and put into a new tube and the second extraction was repeated as described above. Then upper aqueous layer obtained from the second extraction was collected again and the last extraction was performed. Following the last collection of the upper layer the sample was incubated for 1 hr at -20° C with 1 ml of precipitation mix containing 1 M ammonium acetate in 90% ethanol (Sigma-Aldrich), and 1 µl of glycogen (Invitrogen) to allow DNA precipitation. After the incubation the sample was centrifuged for 10 min at 13200 rpm. At this stage the supernatant was

removed and the pellet was washed with 1 ml of 80% ethanol by pipetting up and down. The ethanol was then remove and the tube was placed on a 65° C heat block for no more than 5 min in order to remove any ethanol excess; DNA was then resuspended in 300 µl of NF-H20, and stored at -80° C.

4.4.1.2. DNA isolation from sperm

Sperm was collected according to section 3.1.5. The same protocol for DNA isolation from liver was applied, except for the composition of Lysis buffer is slightly different. Lysis step involved the resuspension of sperm in 400 µl of sperm lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 140 mM β-mercaptoethanol, 2% SDS, all from Sigma-Aldrich), with 0.2 mg/ml Proteinase K (PK) (Roche, West Sussex, UK). Then the same protocol described in section 3.4.1.1. was used.

4.4.1.3. DNA extraction from blood

Blood was collected as described in section 3.1.6. and immediately used for DNA extraction by Salting out methodology. Two point five mL of blood was placed in a tube and 22.5 ml of Lysis buffer (10 mM Tris; pH 7.5, 5 mM $MgCl_2$, 1% Triton, 0.35 M Sucrose and NF-H_2O) were added, mixed by inversion and incubated on ice for 15 min. Then, the sample was centrifuged at 3000 rpm for 10 min at 4° C. The supernatant was then removed and 22.5 mL of Fisio buffer (EDTA 25 mM, $NaCl_2$ 75 mM, NF-N_2O) were added to the pellet and centrifuged again at 3000 rpm for 10 min at 4° C. After centrifugation the pellet was resuspended by pipette up and down for 30 sec in 1.5 ml of Buffer A (10 mM Tris-HCl; pH 8, 2 mM EDTA and H2O). One hundred and twelve microliters of a fresh made solution of 20 mg/µl PK in 10%SDS were added and the sample was incubated at 65° C for 1 hr for protein

digestion. After incubation 0.5 ml of saturated sodium chloride (6M NaCl in NF-H_20) were added to inhibit the enzyme activity and the sample was centrifuged at 3000 rpm for 15 min at 4° C. At this stage the upper aqueous layer was collected, transferred into a new tube, and added to one Isopropanol. The tube was then incubate at -20° C for at least 1 hr. Following the incubation, samples underwent centrifugation for 10 min at 13200 rpm. The supernatant was removed and the pellet was washed once with freshly prepared 70% ethanol. Excess ethanol was eliminated by incubating the samples on a 65° C heat block for no more than 5 min; DNA was then resuspended in 500 µl of NF-H20 and kept at -80° C until required.

4.4.1.4. DNA from oocytes

DNA extraction from oocytes was not needed because DNA Methylation-Direct Kit (Zymo Research) has been used for bisulfite conversion. These product feature reliable and complete DNA bisulfite conversion directly from cells after a PK incubation without the prerequisite of DNA purification.

Pools of zona free (ZF) GV and IVM MII oocytes derived from A and from P donors were used for Bisulfite conversion. The ZP of the oocytes was removed by pronase treatment. ZF-oocytes were used in order to be sure that all contaminant cumulus cells were removed. Oocytes were vigorously pipetted, repeatedly washed in TCM-199 and treated with 0.5% pronase (Sigma-Aldrich) in TCM-199 by gentle pipetting. Oocytes with expanded and deformed ZP were then transferred to TCM-199 with Foetal Calf Serum (FCS) and incubated for 10 min at 39° C under 5% CO2. Only ZF-oocytes who recovered a normal morphology and intact cytoplasm were repeatedly washed in TCM-199, snap frozen in liquid nitrogen, and finally stored at – 80° C until bisulfite conversion. Before bisulfite conversion, ZF-oocytes

where digested at 50° C for 16 hrs in a final volume of 20 µl containing 0.5 U of PK and M-Digestion Buffer (2X) provided from EZ DNA Methylation-Direct Kit (Zymo Research).

4.4.1.5. Generation of in vitro methylated genomic DNAs

To evaluate a possible primer bias (Warnecke et al. 1997; Wojdacz and Dobrovic 2007) and to verify bisulfite conversion procedure, in vitro methylated DNA (controls) at different percentage of methylation were in vitro prepared (DNA 0%, 25%, 50%, 75% and 100% methylated). For this purpose, a negative/unmethlyated control and a positive/methylated control DNA were specifically created as follows: DNA 0% Methylated (Negative/unmethylated control) was obtained by treating 10 ng of DNA isolated from blood with REPLI-g mini kit (Qiagen, Hilden, Germany). That provides highly uniform amplification across the entire genome based on MDA technology (Dean et al. 2002) After amplification, DNA was purified by genomic DNA Clean& concentrator (Zymo Research, Irvine, CA, USA) and DNA quantity and purity were monitored spectroscopically by Nanodrop Lite spectrophotometer (Thermo Scientific). In order to obtain a 100% methylated control DNA, 500 ng of purified 0% methylated DNA were treated with M.SssI CpG Methylase (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. Afterwards, 0% and 100% methylated DNAs were mixed to obtain 25%, 50% and 75% methylated DNAs. To determine the integrity of DNA from controls (liver, sperm, blood and vitro differentially methylated genomic DNA , 3 µl of DNA from each sample were run on a 1% agarose gel and visualised by exposure to blue light. To examine purity of the DNA, spectrophotometry readings (range; 260 nm-280 nm) were taken using the Nanodrop Lite spectrophotometer Thermo Scientific.

4.4.2. Bisulfite conversion

Pools of 40 ZF-oocytes after PK digestion and 500 ng of DNA from sperm, liver (controls) were bisulfite converted using EZ DNA Methylation-Direct Kit (Zymo Research) following manufacture instructions, with minor modifications. Oocyte samples and DNA from sperm, liver and controls were incubated with 130 µl of conversion reagent for 5 hrs and 30 min at 64°C , after bisulfite modification each sample was transferred to a column pre-loaded with binding buffer and centrifuged for 30 sec at 13000 rpm. The flow-throughs were discarded and the columns were washed with washing buffer. Two hundred microliters of M-desulphonation buffer were added to each column and let it stand at room temperature for 25 min; after two washes, samples were eluted in 42 µl of elution buffer which was preheated to 50° C.

4.4.3. Pyrosequencing

4.4.3.1. Generation of PCR products for Pyrosequencing

Specific primers for bisulfite converted DNA (BC-DNA) were used in the amplification of presumptive H19 DMR DNA. The primers were obtained from Colosimo et al. 2009 (Colosimo et al. 2009) and are listed in Tab. 3.3. PCR amplification was carried out using the PyroMark PCR kit (Qiagen, Hilden, Germany) in a final volume of 25 µl containing 12.5 µl of master mix, 2.5 µl of coral, 0.5 µM of each primer, NF-H20. 2 µl and 2 µl of BC DNA from sperm, liver and controls and 5 µl of BC DNA from oocytes were amplified. PCR was carried out using the 2720 thermal cycler (Applied Biosystems) and conditions were: an initial denaturation step for 15 min at 95^{o} C followed by 45 cycles at 94^{o} C for 30 sec, 56° C for 30 sec, 72° C for 30 sec and a final elongation step at 72° C for 10 min. Then 2 µl of PCR products were runner in a 2%

agarose gel to check the PCR purity and DNA integrity. Then PCR product were used fresh as template for the pyrosequencing reaction.

Table 4.3: Pre-designed pyrosequencing assays used for PyroMark PCR kit amplification from Colosimo et al. 2009

Gene	Primer	Modification (5'-3')	Oligo sequence	Size
H19	FW	GTATTTTAAATAGGGTTGAGAGGTTGT	5' BIOTIN	202bp
	RV	AACTAAACAAACATCCCATCTCCCTAATAA	-	
	SEQ	CCCTATAAACCATTATAATATCC	-	

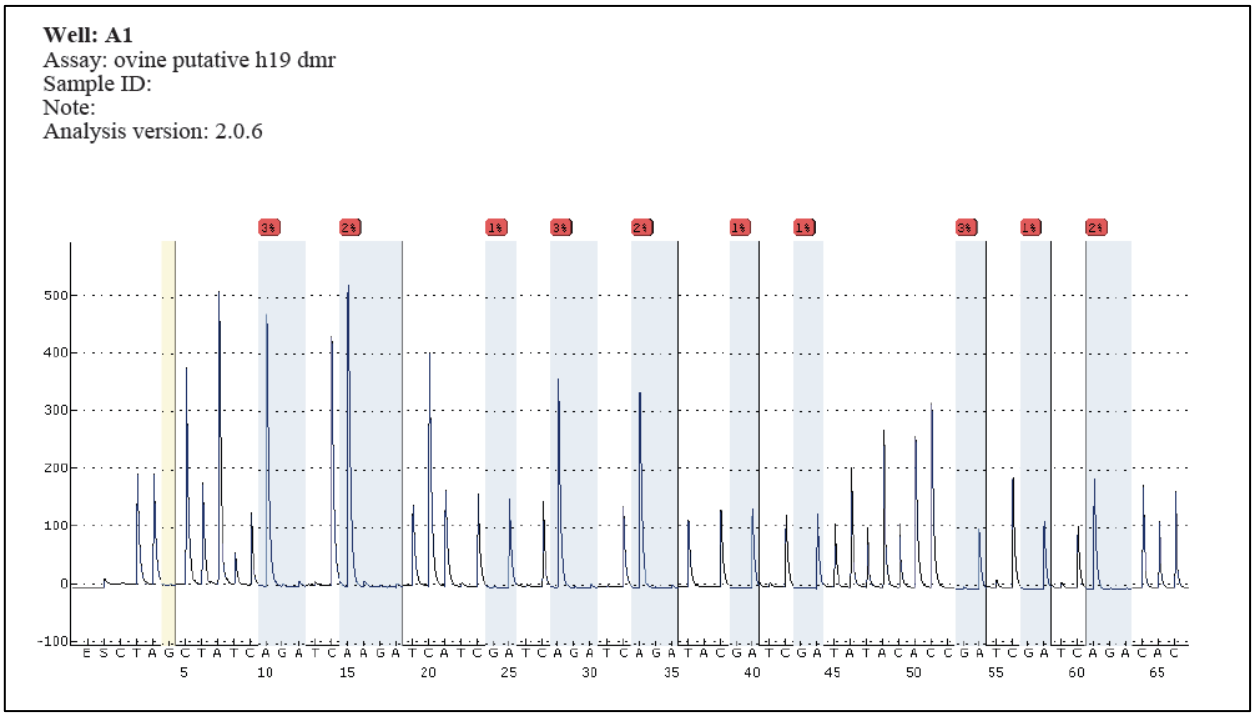
Forward pyrosequencing PCR primer (FW); reverse pyrosequencing PCR primer (RV); pyrosequencing sequencing primer (Seq)

4.4.3.2. Pyrosequencing

PCR product, were used for DNA methylation analysis on the Pyromark™ Q24 pyrosequencer (Qiagen, Hilden, Germany). PCRs were transferred to specific pyro tubes and 60 µl of mix 1 (2 µl of streptavidin sepharose beads (Healthcare *Bio-Sciences* AB, Sweden), 40 µl binding buffer (Qiagen, Hilden, Germany) and 18 µl NF-H_20) were added to each sample. Samples were then shaken for 5 min at 1400 rpm; to allow the attachment of the beads to the PCR product. Within the PyroMark™ Q24 workstation, sepharose bead bound PCR were washed with 70% ethanol for 5 sec, denaturation solution 5 sec (Qiagen, Hilden, Germany) and 1X wash buffer for 10 sec (Qiagen, Hilden, Germany). Subsequent to this, the PCR products were then released onto a PyroMark™ Q24 plate (Qiagen, Hilden, Germany) containing 0.3 µM sequencing primer in 25 µl annealing buffer (Qiagen, Hilden, Germany). The PyroMark™ Q24 plate were then incubated at 80° C for 2 min in a heat block and then the sample were let

cool at room temperature for minimum 5 min in order to ensure the annealing of the sequencing primer. Lastly, samples were run on the Pyromark™Q24 pyrosequencer and results were viewed using the associated PyroMark™ Q24software (Qiagen, Hilden, Germany) Fig 4.3.

Figure 4.3: Example of a pyrogram obtained by the analysis of the pyrosequencing run for H19 DMR by PyroMark™ Q24software



4.5. Statistical analysis

4.5.1. Gene expression 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 tests, respectively, transcript data were analysed with

analysis of variance (ANOVA). Student t-test was conducted to evaluate difference between the expression levels of A vs P oocytes. Differences were considered significant when $P < 0.05$.

Chapter 5: Results

5.1. Gene expression analysis

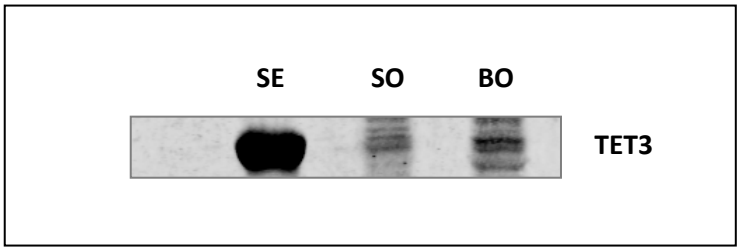
Gene expression was quantified for a panel of nine genes involved in DNA methylation machinery: DNA methyltransferases (*DNMTs*) (*DNMT1*, *DNMT3A* and *DNMT3B*), Ten-eleven Translocation (*TETs*) Enzymes (*TET1, TET2* and *TET3*) and Methyl-CpG-Binding proteins (*MBDs*) (*MBD1*, *MBD3* and *MBD4*). The transcript relative quantity was normalised on the basis of Luciferase (Luc) mRNA levels. Transcripts of all the analysed genes were detected in oocytes and in embryos at different stages of development and in obtained from both P and A animals.

5.2 Immunodetection

5.2.1 TET3 antibody specificity validation

TET3 polyclonal antibody used in the present work reacts with has Human, Bovine and Xenopus laevis species. For this reason, an antibody validation by western blotting was performed to ensure the specificity of TET3 antibody in sheep. Western blotting analysis was carried out on protein extracts from sheep oesophagus, sheep and bovine ovary. The analysis confirmed the specificity of TET3 antibody; as shown in Fig. 5.1, a single band at the expected molecular weight (180 kDa) resulted from sheep oesophagus, while additional bands, probably due to an alternative isoform of the protein, as document in Humans (http://www.uniprot.org/uniprot/O43151), resulted from ovary samples.

Figure 5.1: Western blot analysis to test the specificity of TET3 antibody on sheep, using protein extracts from sheep oesophagus (SE), sheep ovary (SO) and bovine ovary (BO).

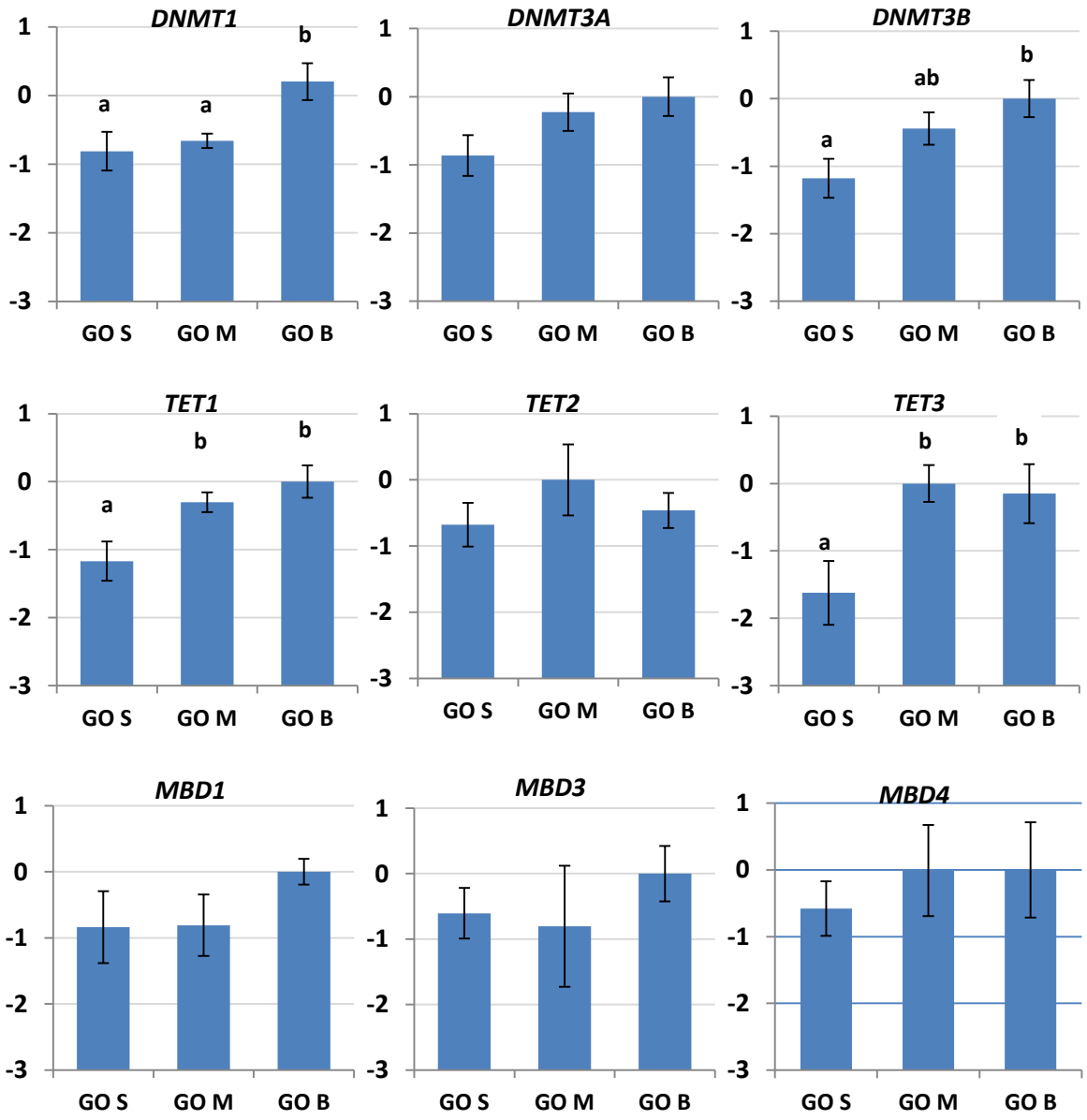


5.3. Methylation dynamics in growing oocytes

5.3.1. Gene expression analysis in growing oocytes

The expression of the selected genes was analysed in five pools of 30 ovine GO of different diameters (Small (GOS) 70/90 µm, Medium (GOM) 98/110 µm, Big (GOB) 110/130 µm) derived from P animals. Transcripts of all the analysed genes were detected in the experimental groups (Fig. 5.2). The analysis of *DNMTs* mRNA abundance showed no significant differences for *DNMT3A* in the three analysed classes. However, *DNMT1* and *DNMT3B* expression increased significantly from small to big GO ($P < 0.05$). Among the family of *TETs* enzymes, *TET1* and *TET3* expression resulted significantly lower in small oocytes compared to the other two classes ($P < 0.05$). No significant differences were found for mRNA expression of Methyl-CpG-Binding proteins in GO.

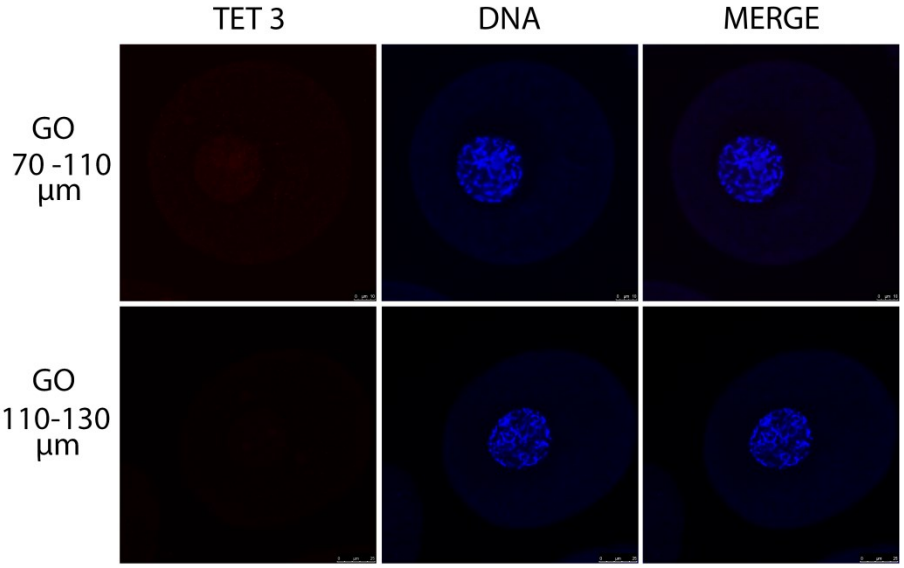
Figure 5.2: Relative expression of *DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, MBD1*, *MBD3*, and *MBD4* in ovine GO at different diameter (GOS 70/90 µm, GOM 90/110 µm, GOB 110/130 µm) derived from P animals. Relative abundance values are expressed as ΔCq and show the mean value ± s.e.m. of five replicates 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.



5.3.2. TET3 protein was not detected in GO

The presence of TET3 was investigated by whole-mount immunostaining in GO at different diameter (70/110 µm and 110/130 µm) derived from P animals. TET3 protein was not detected in GO (Fig. 5.3).

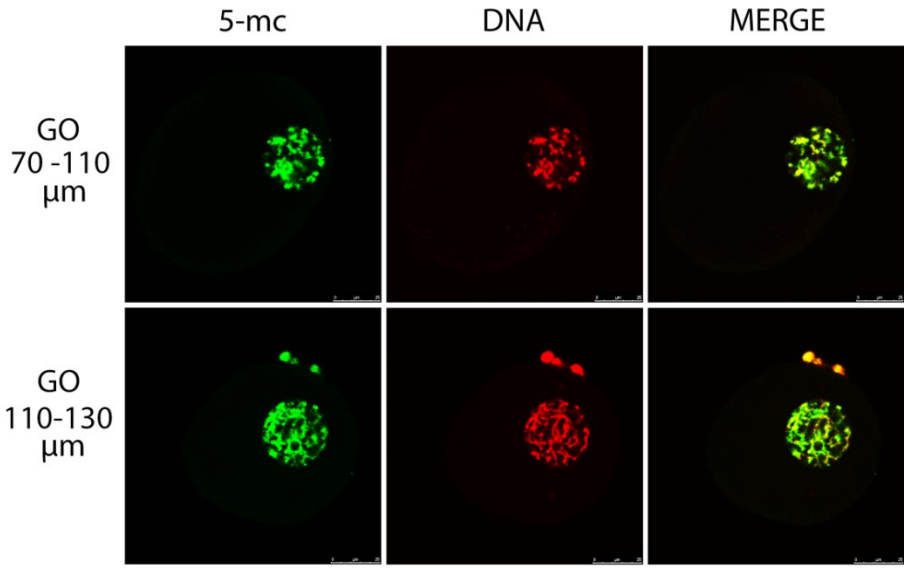
Figure 5.3: Immunofluorescent localization of TET3 in GO at different diameter (70/110 µm and 110/130 µm) derived from P animals. Confocal representation of oocytes stained in red with anti-TET3 antibody and in blue with DAPI to visualize the DNA. The scale bar represents 25 µm.



5.3.3. Global DNA Methylation analysis in GO

The global level of DNA methylation was investigated by whole-mount immunostaining in GO derived from P donors. The semiquantitative grading of intensity of signals and the quantity/distribution of 5-mc stain of each sample revealed high levels of DNA methylation (Fig. 5.14). Fig. 5.4 show the immunofluorescent localization observed in GO .

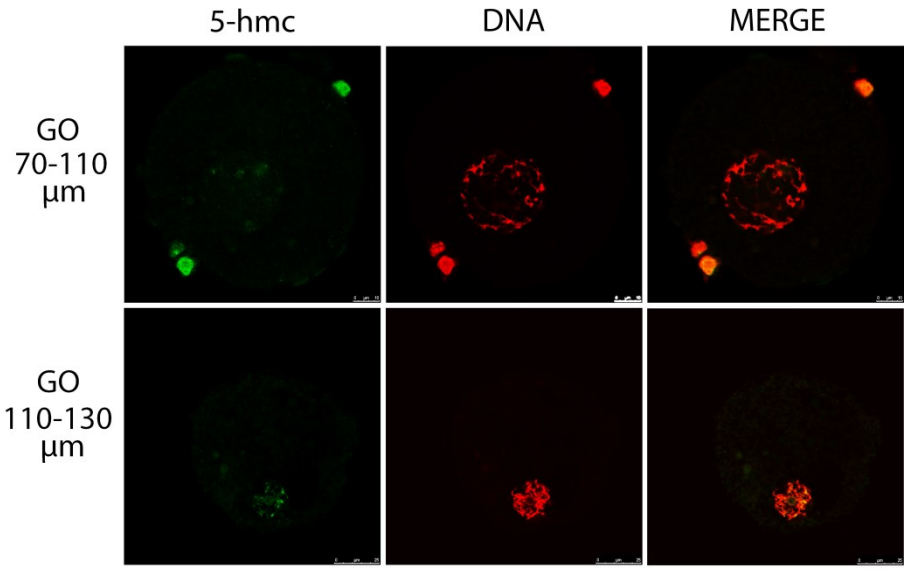
Figure 5.4: Immunofluorescent localization of 5-mc in GO at different diameter (70/110 µm and 110/130 µm) derived from P animals. Confocal representation of oocytes stained in green with anti-5-mc antibody and in red with propidium iodide to visualize the DNA. The scale bar represents 25 µm.



5.3.4. Global DNA Hydroxymethylation analysis in GO

The global level of DNA hydroxymethylation was investigated by whole-mount immunostaining in GO. Immunopositivity was scattered evident throughout the oocytes chromatin (Fig. 5.5). Fig. 5.15 reports graphical representation of the mean of the semiquantitative grading of intensity of signals and the quantity/distribution of 5-hmc stain of each sample.

Figure 5.5: Immunofluorescent localization of hydroxyl-methyl-cytosine (5-hmc) in GO at different diameter (70/110 µm and 110/130 µm) derived from P animals. Confocal representation of oocytes stained in green with anti-5-mc antibody and in red with propidium iodide to visualize the DNA. The scale bar represents 25 µm.

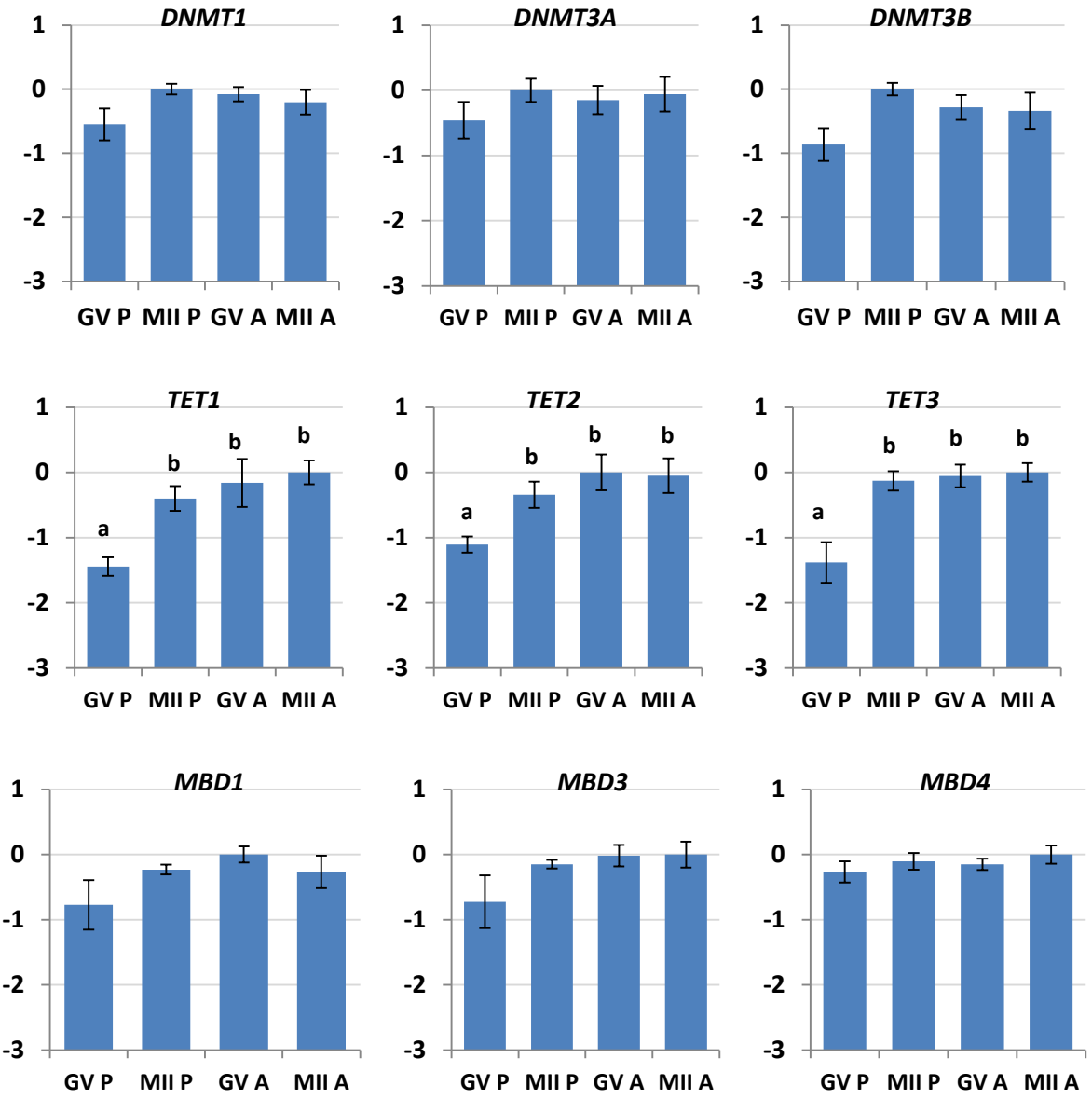


5.4. Methylation dynamics during meiotic progression

5.4.1. Gene expression analysis in GV and IVM MII oocytes derived from P and A donors

Four pools of 10 ovine oocytes at GV and IVM MII stage derived from P and A donors were used to detect the transcript abundance of all studied genes. Messenger RNA of all evaluated genes were detected in the experimental groups and only the abundance *TETs* genes were found significantly different among the analysed classes (Fig. 5.6). *TET1, TET2* and *TET3* expression resulted significantly lower in oocytes at the GV stage derived from P animals compared to As animals ($P < 0.05$).

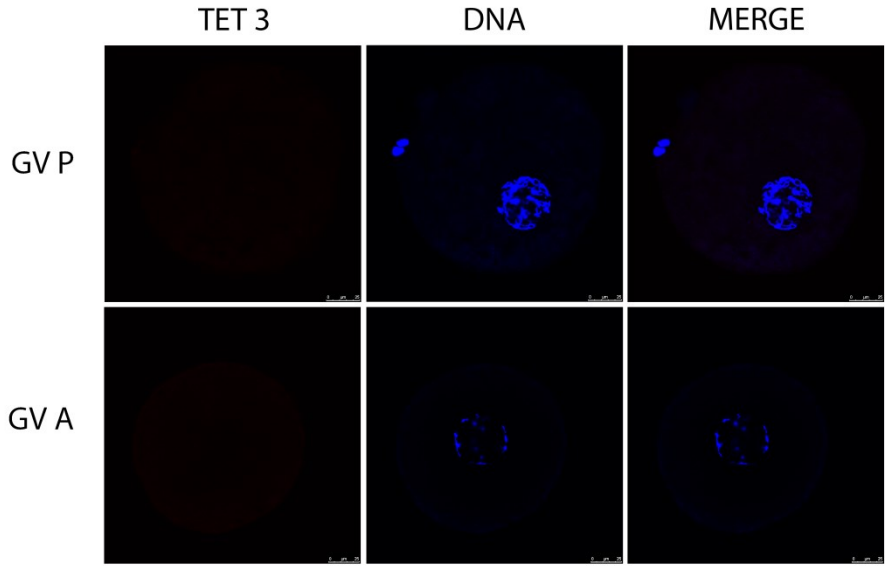
Figure 5.6: Relative expression of *DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, MBD1, MBD3*, and *MBD4* in ovine immature GV and IVM MII oocytes derived from P and A donors. Relative abundance values are expressed as ΔCq and show the mean value ± s.e.m. of four replicates for each stage of development (each replicate = pool of 10 oocytes). Different letters indicate a significant difference in relative mRNA abundance (ANOVA, P <0.05) between developmental stages and between donors age (P vs A).



5.4.2. TET3 protein was not detected at GV stage

The presence of TET3 was investigated by whole-mount immunostaining in GV stage oocytes from P and A donnors. TET3 protein was not detected in GV stage oocytes (Fig. 5.7).

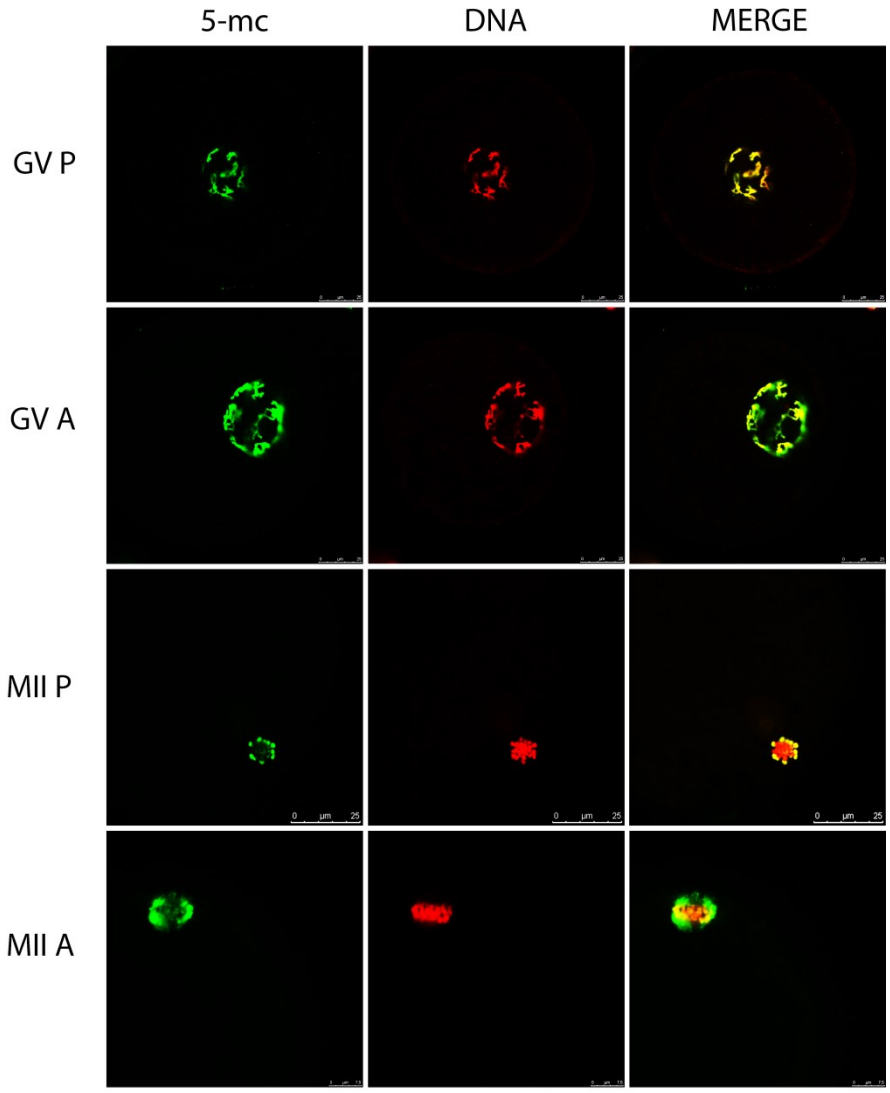
Figure 5.7: Immunofluorescent localization of TET3 in oocytes at GV stage derived from P and A (A) animals. Confocal representation of oocytes stained in red with anti-TET3 antibody and in blue with DAPI to visualize the DNA. The scale bar represents 25 µm.



5.4.3. Global DNA Methylation analysis during meiosis progression

The global level of DNA methylation was investigated by whole-mount immunostaining in GV and MII stage oocytes derived from P and A donors. The semiquantitative grading of intensity of signals and the quantity/distribution of 5-mc stain of each sample revealed high levels of DNA methylation (Fig. 5.14). Fig. 5.8 show the immunofluorescent localization observed during meiotic maturation in oocytes derived from P and A animals.

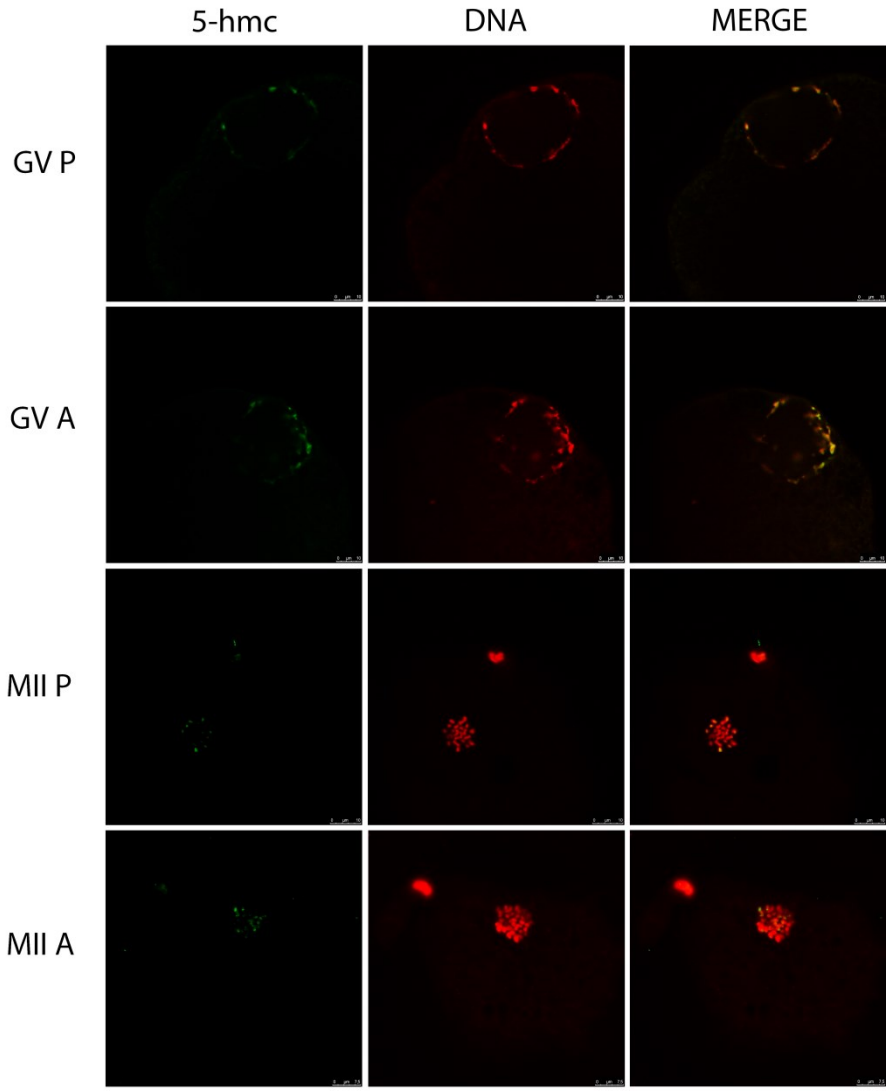
Figure 5.8: Immunofluorescent localization of 5-mc in oocytes at GV and MII stages derived from P and A animals. Confocal representation of oocytes stained in green with anti-5-mc antibody and in red with propidium iodide to visualize the DNA. The scale bar represents 25 µm.



5.4.4. Global DNA Hydroxymethylation analysis during meiosis progression

The global level of DNA hydroxymethylation was investigated by whole-mount immunostaining in GV and MII stage oocytes derived from P and A donors. Immunopositivity was scattered evident throughout the oocytes chromatin (Fig. 5.9). Fig. 5.15 reports graphical representation of the mean of the semiquantitative grading of intensity of signals and the quantity/distribution of 5-hmc stain of each sample.

Figure 5.9: Immunofluorescent localization of hydroxyl-methyl-cytosine (5-hmc) in oocytes at GV and MII stages derived from P and A animals. Confocal representation of oocytes stained in green with anti-5-mc antibody and in red with propidium iodide to visualize the DNA. Magnification. The scale bar represents 25 µm.

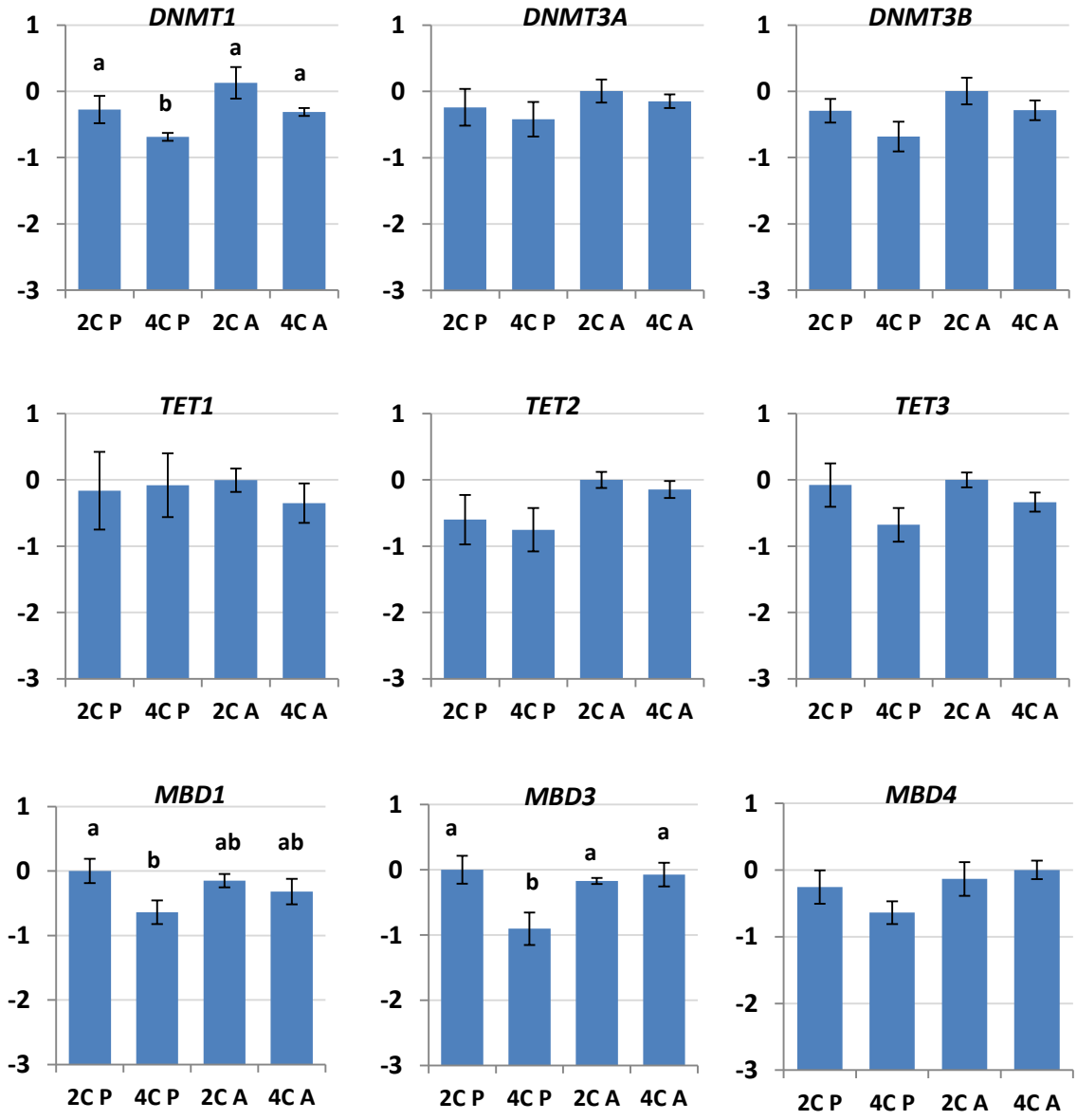


5.5. Methylation dynamics during early embryonic development

5.5.1. Gene expression analysis in IVC embryos at 2C and 4C stage

Three pools of 10 ovine IVC embryos at 2C and 4C stage derived from P and A donors were selected to study the gene expression of the nine genes involved in DNA methylation machinery. Transcripts of all the analysed genes were detected in the experimental group. (Fig. 5.10). The expression of *DNMT1* resulted lower in 4C embryos compared to the 2C embryos from lamb and 4 cell embryo from As animals, while no differences resulted from the comparison between two developmental stages derived from A oocytes nor between embryos at 2C stage from P and A animals. No significant differences were found for TETs genes between the two embryonic developmental stage or the different donor age. 4C embryos from P animals presented a lower abundance of *MBD1* transcripts when compared to 2C embryos from young animals, in accordance the expression level of *MBD3* was lower for this developmental stage compared to all the other analysed classes.

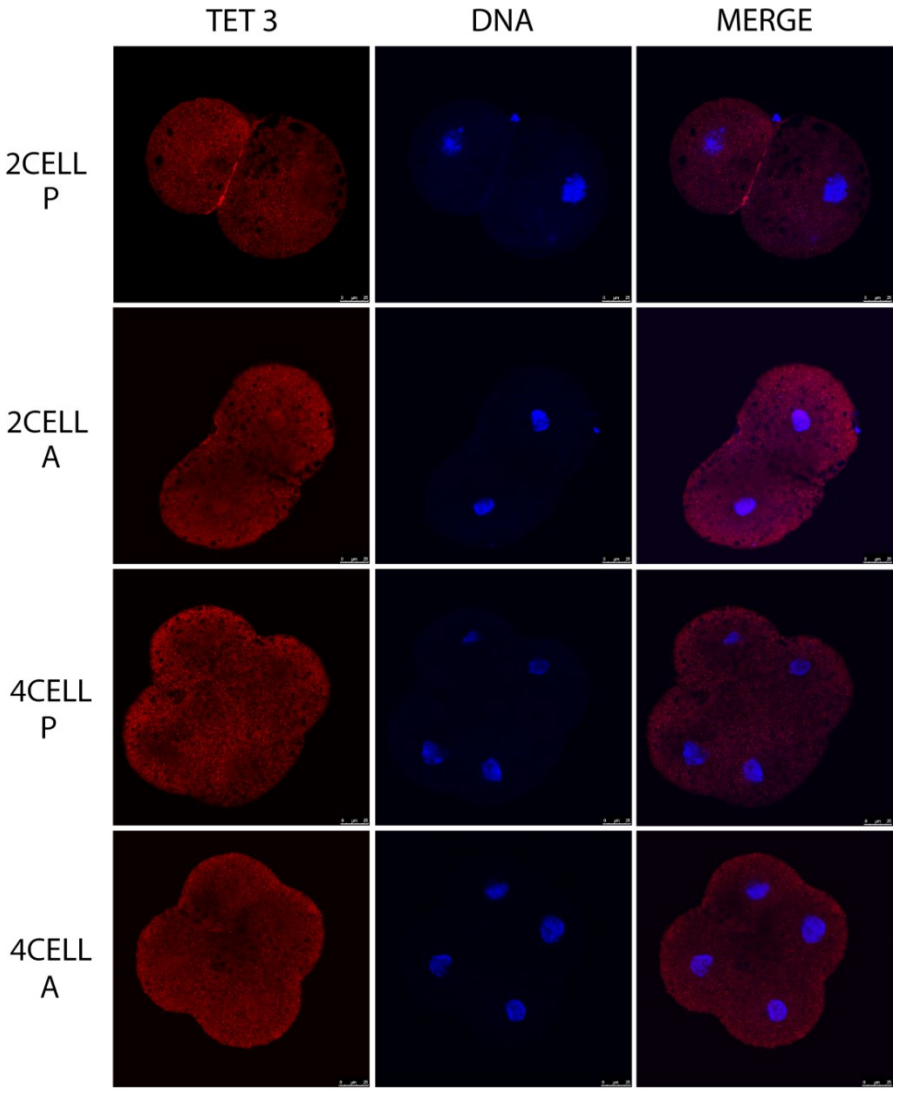
Figure 5.10: Relative expression of *DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, MBD1, MBD3*, and *MBD4* in ovine IVC two- (2C), four-cell (4C), embryos derived from P and A donors. Relative abundance values are expressed as ΔCq and show the mean value ± s.e.m. of three replicates for each stage of development (each replicate = pool of 10 embryos). Different letters indicate a significant difference in relative mRNA abundance (ANOVA, P <0.05) between developmental stages and between donors age (P vs A).



5.5.2. TET3 protein was detected only in embryos

The presence of TET3 was investigated by whole-mount immunostaining IVC embryos 2C and 4C stage derived from P and A donors. TET3 protein was not detected in oocytes at any studied stages as previous described (Fig. 5.3 and 5.7) while the immunofluorescence revealed the cytoplasm staining of TET3 protein in all the embryonic stage (Fig. 5.11).

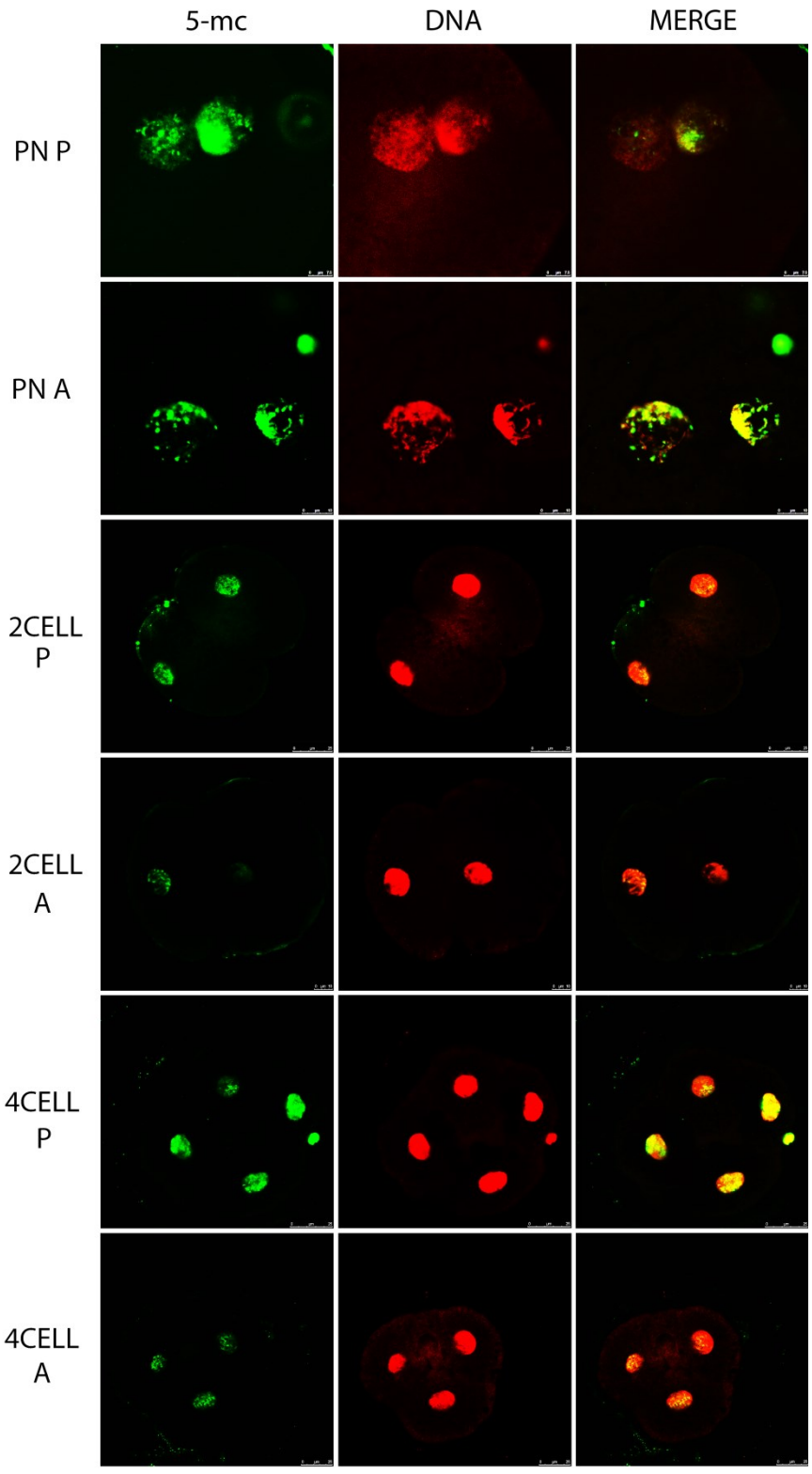
Figure 5.11: Immunofluorescent localization of TET3 in early embryos 2 and 4 cell stage derived from P and A animals. Confocal representation of oocytes stained in red with anti-TET3 antibody and in blue with DAPI to visualize the DNA. The scale bar represents 25 µm.



5.5.3. Global DNA Methylation analysis during early embryonic development

The global level of DNA methylation was investigated by whole-mount immunostaining in IVF PN stage zygotes and IVC embryos at 2C and 4C stage derived from P and A donors. The semiquantitative grading of intensity of signals and the quantity/distribution of 5-mc stain of each sample revealed high levels of DNA methylation (Fig. 5.14). Fig. Fig. 5.8 show the immunofluorescent localization observed in IVF PN stage zygotes and IVC 2C and 4C embryos derived from P and A donors. Interestingly, PN stage zygotes displayed a decrease in 5-mc signal in male PN compared to maternal PN in zygotes from both young and A animals(Fig. 5.12).

Figure 5.12: Immunofluorescent localization of 5-mc PN stages zygotes and early embryos at 2 and 4 cell stage derived from P and A animals. Confocal representation of oocytes stained in green with anti-5-mc antibody and in red with propidium iodide. to visualize the DNA. The scale bar represents 25 µm.



Laura Masala, *Study of DNA methylation dynamics in a model of differential developmental competence in the ovine species*, Tesi di Dottorato in Scienze Veterinarie XXVII ciclo, Indirizzo in Riproduzione, Produzione e Benessere Animale, Università degli Studi di Sassari

5.5.4. DNA Hydroxymethylation analysis during early embryonic development

The global level of DNA hydroxymethylation was investigated by whole-mount immunostaining PN stage zygotes and IVC embryos at 2C and 4C stage derived from P and A donors. Immunopositivity was scattered evident throughout the chromatin all the embryonic stage analysed. In PN stage zygotes Immunopositivity was observed only in male PN, and not in female PN (Fig. 5.13). Fig. 5.15 reports graphical representation of the mean of the semiquantitative grading of intensity of signals and the quantity/distribution of 5-hmc stain of each sample.

Figure 5.13: Immunofluorescent localization of hydroxyl-methyl-cytosine (5-hmc) in PN stages zygotes and early embryos at 2C and 4C stage derived from P and A animals**.** Confocal representation of oocytes stained in green with anti-5-mc antibody and in red with propidium iodide to visualize the DNA. The scale bar represents 25 µm.

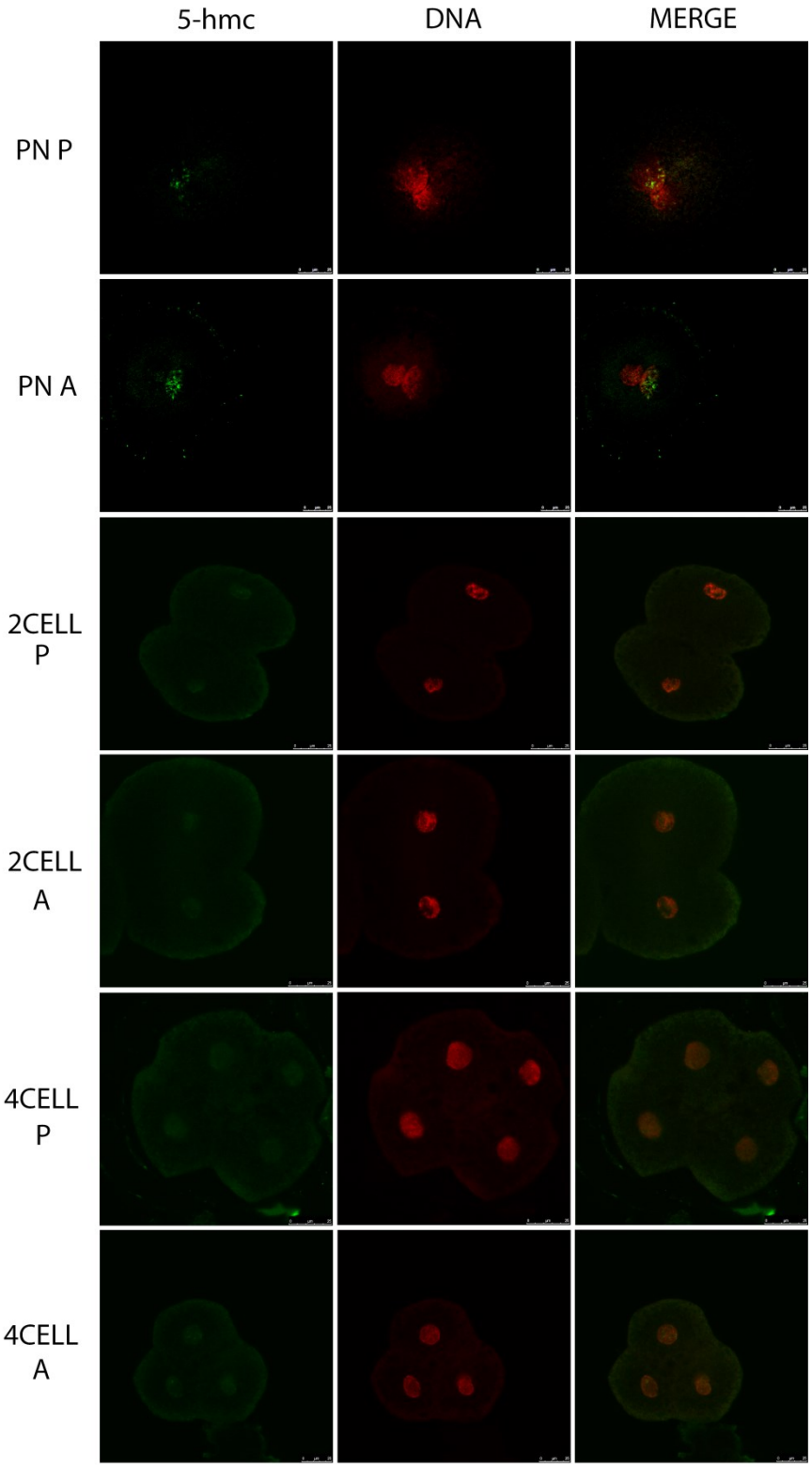


Figure 5.14: Graphical representation of the median of the semiquantitative grading of intensity of signals and the quantity/distribution of 5-mc stain for GO at different diameter (70/90 µm, 110/130 µm) derived from P animals, GV and MII oocyte stages, PN stage zygotes (male PN (m)and female PN (f)) and IVP 2C and 4C embryos derived from P and A donors. For the semiquantitative grading each sample was included in the following categories: 0= absence of signal, 1 low, 2 moderate, 3 high.

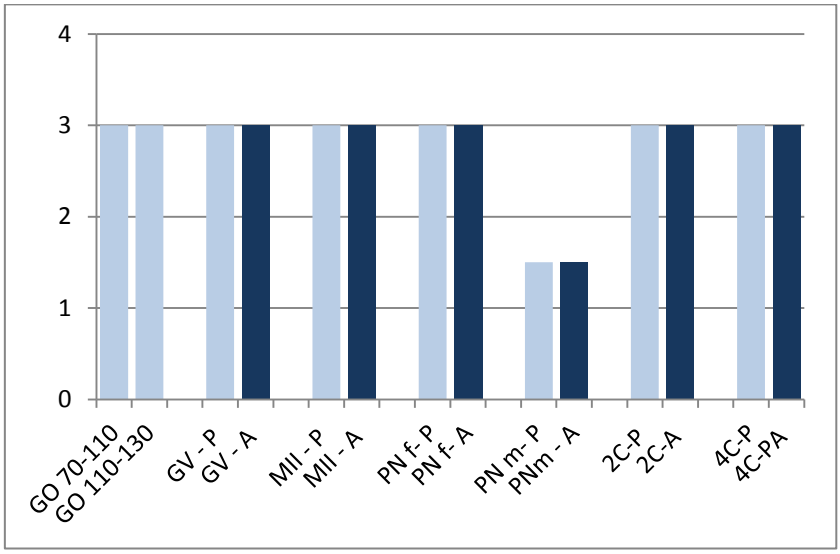
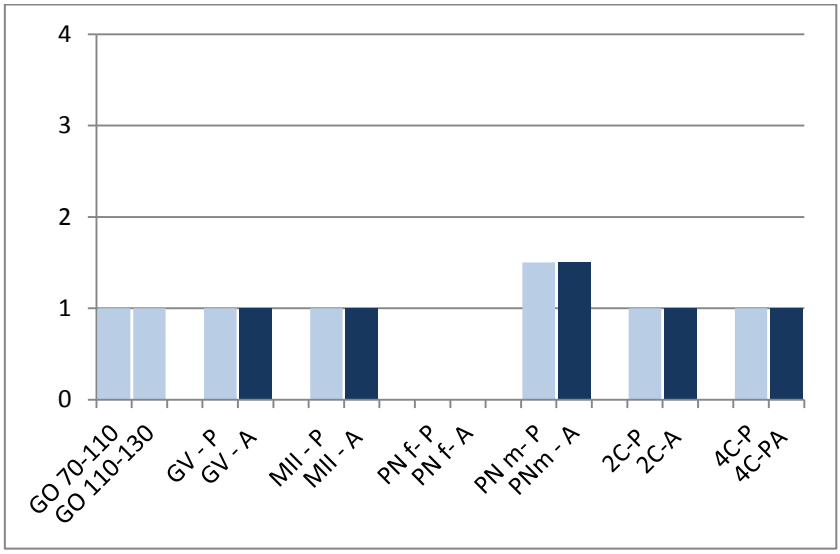


Figure 5.15: Graphical representation of the median of the semiquantitative grading of intensity of signals and the quantity/distribution of 5-hmc stain for GO at different diameter (70/90 µm, 110/130 µm) derived from P animals, GV and MII oocyte stages, PN stage zygotes (male PN (m)and female PN (f)) and IVP 2C and 4C embryos derived from P and A donors. For the semiquantitative grading each sample was included in the following categories: 0= absence of signal, 1 low, 2 moderate, 3 high.



5.6. Pyrosequencing

5.6.1. *H19* Assay validation with sperm and liver bisulfite converted DNA

Pyrosequencing analysis was employed to validate the pyroassay for presumptive DMR at *H19* locus(Colosimo et al. 2009). The assay validation was conducted using DNA from sperm and liver were bisulfite converted (BC) together with *in vitro* methylated DNA standards. BC DNA from *in vitro* methylated DNA standards, sperm and liver were used as a template for PyroPCR and then each PCR was analysed by pyrosequencing. The pyrosequencer output is the pyrogram; it contains a series of peaks that refers to the fluorescence released during nucleotide incorporation and acquired by the machine. Pyrograms obtained from sequencing of the presumptive H19 DMR locus in both sperm and liver are shown in Fig. 5.16. For sperm, each CpG site covered by the assay shows high level of methylation, while the analysis of liver shows a medium level. The mean methylation level at each of the five CpG sites is also shown by a graphical representation in Fig 5.17. The results obtained by pyrosequencing revealed that 1) Bisulfite conversion procedure was successful, as demonstrated by the *in vitro* methylated standard DNA that showed a progressive increase (Fig. 5.16); 2) The assay covers a DMR within *H19,* as confirmed by the different levels of methylation in sperm (83%) and liver (27%).

Figure 5.16: Pyrograms obtained from the validation of the pyroassay for presumptive DMR at H19 in sperm and liver.

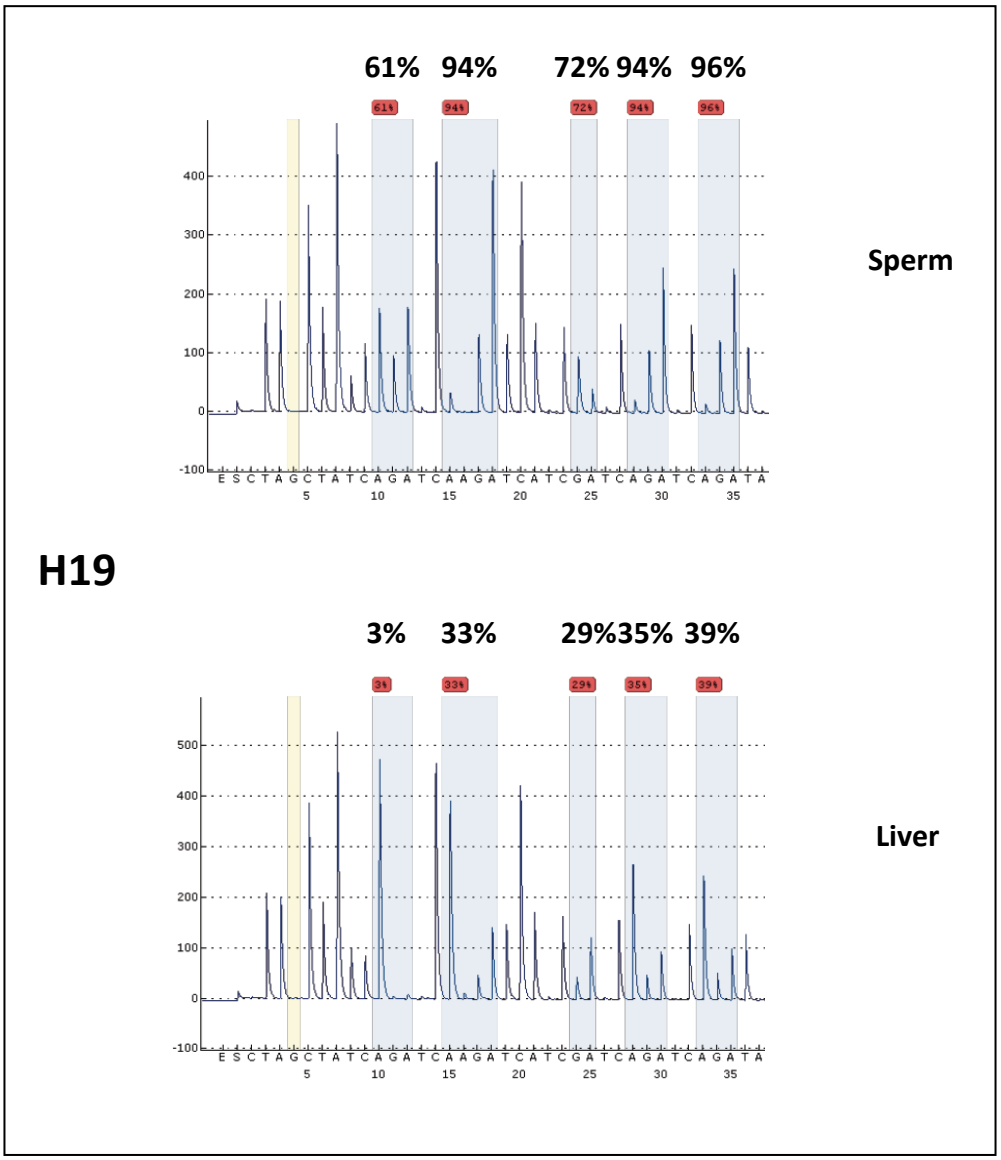
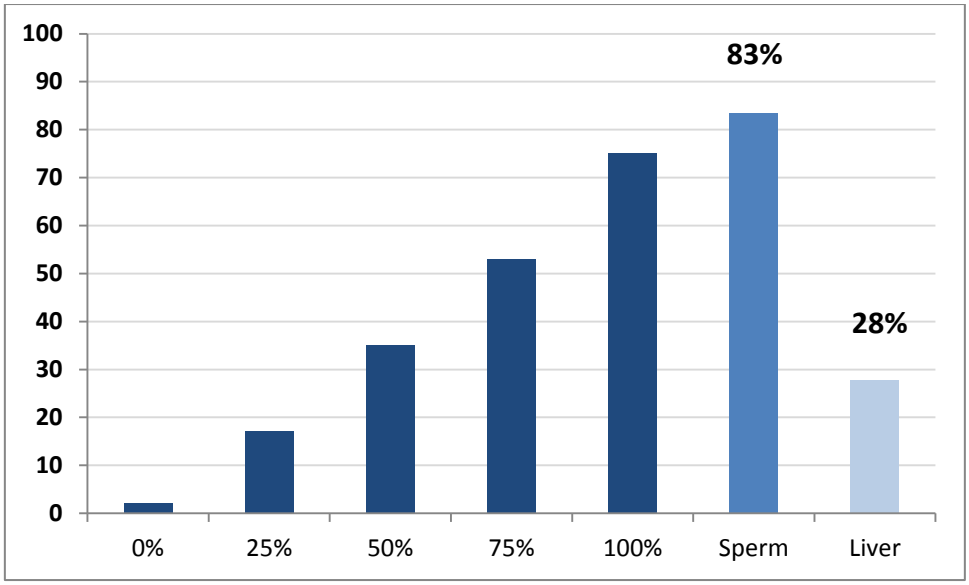


Figure 5.17: Graphical representation of the methylation level of *in vitro* methylated DNA standards (0%,25%, 50%, 75% and 100%), in sperm and liver obtained by pyrosequencing of presumptive H19 DMR region. The five CpG sites within the region of interest were averaged before to be plotted.



5.6.2. *H19* Assays validation with oocytes BC DNA from

The *H19* assay was performed on pools of forty oocytes. After the conversion, different volumes of DNA were tested to setup the oocyte specific pyro PCR; then the PCR products obtained amplifying the BC DNA equivalent to five oocytes were analysed by pyrosequencing, together with controls (standards, sperm and liver DNA) The obtained pyrogram revealed that all the five CpG sites covered by the assay have a low level of methylation, as expected for a paternally imprinted gene in the female gametes (Fig. 5.18, 5.19).

Figure 5.18: Pyrogram obtained from the pyrosequencing of presumptive DMR at H19 in oocytes.

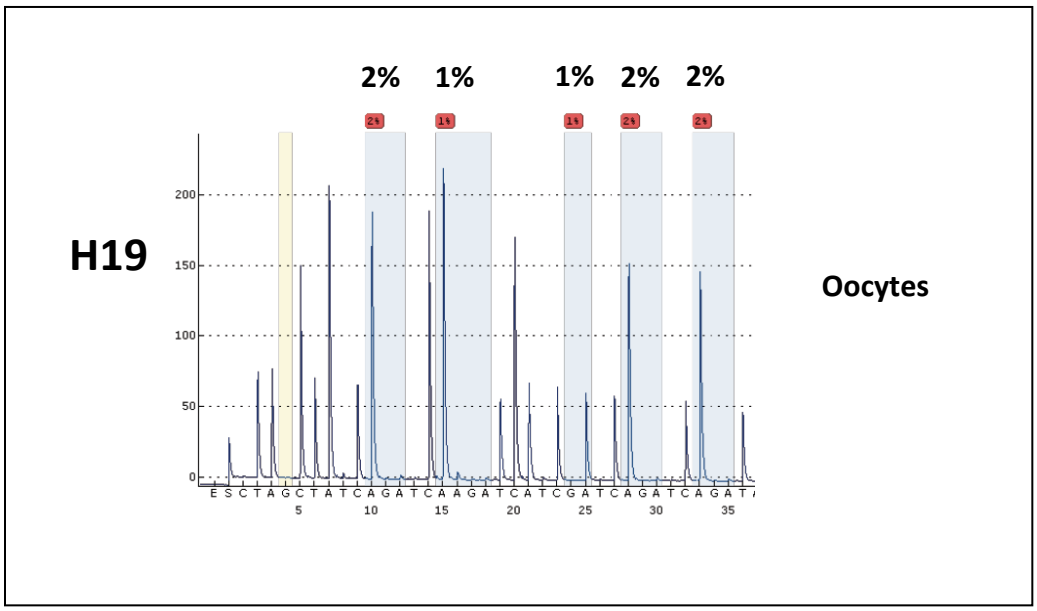
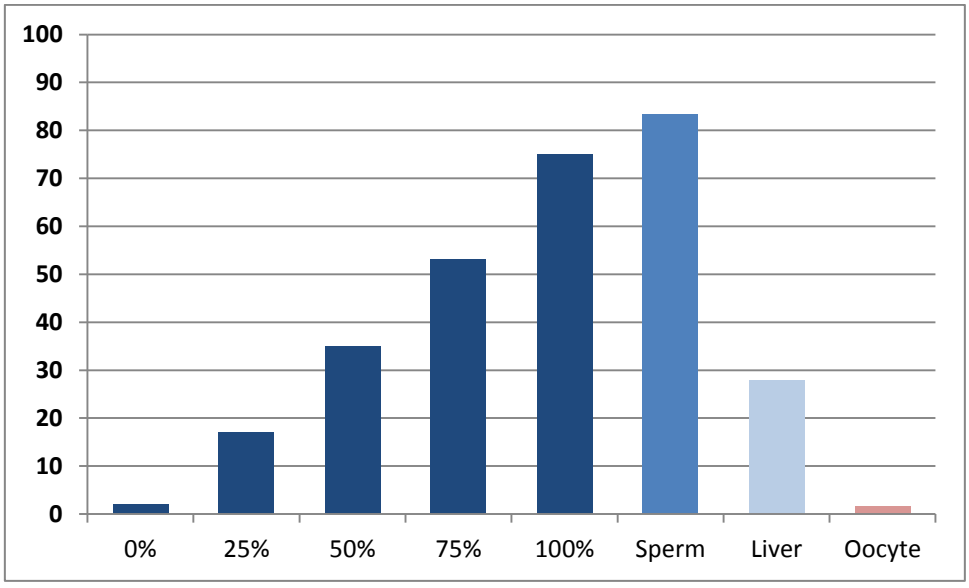


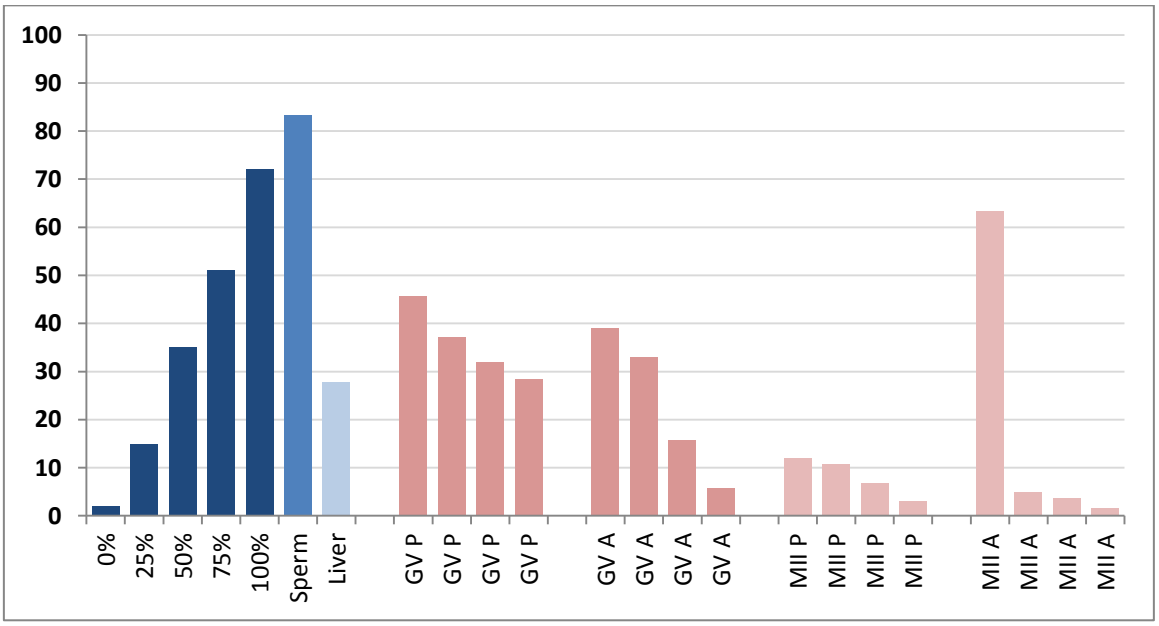
Figure 5.19: Graphical representation of the methylation level in vitro methylated DNA standards (0%,25%, 50%, 75% and 100%), in sperm , liver and Oocytes obtained by pyrosequencing of presumptive H19 DMR region. The five CpG sites within the region of interest were averaged before to be plotted.



5.6.3. Assessment of oocyte purity by H19 assay

Four pools of oocytes were analysed with H19 assay to test oocyte purity from cumulus cells. All pools were BC converted together with in vitro methylated standards and DNA from sperm and liver. The pyrosequencing analysis revealed that some pools were contaminated with cumulus cells, especially oocytes at the GV stage resulted more contaminated then MII stage oocytes. Fig. 5.20.

Figure 5.20: Graphical representation of the methylation level in vitro methylated DNA standards (0%,25%, 50%, 75% and 100%), in sperm , liver and pools of GV and MII oocytes from P and A animals (four pools for each class considered) obtained by pyrosequencing of presumptive H19 DMR region. The five CpG sites within the region of interest were averaged before to be plotted.



Chapter 6: Discussion

The present work analyses DNA methylation dynamics in oocytes and early embryos in the ovine species. Moreover, the study compares the DNA methylation machinery between adult and prepubertal animals in relation to oocyte developmental capacity. In recent years, it has been demonstrated that epigenetic mechanisms are fundamental for normal embryonic development and future health of the new born (Cutfield et al. 2007; Fedoriw et al. 2012). In fact epigenome is relatively stable in mammalian somatic tissues, however, during preimplantation embryogenesis and germ cell developmental processes, two waves of epigenetic reprogramming, that involve DNA methylation remodelling, occur to restore the genome to a pluripotent state and to address germ cells to specific sex differentiation (Morgan et al. 2005; Messerschmidt et al. 2014; Bernstein et al. 2007). Enzymes implicated in DNA methylation process are highly conserved (Kim et al. 2009), but at the same time the dynamics by which the whole process is regulated seems to differ between species (Young and Beaujean 2004; Vassena et al. 2005). Due to the interspecific differences, there is the need to study such modifications in large animal models, as most of the current knowledge on DNA methylation dynamics derives from mouse. Sheep could be a very good model due to its similarity with human reproduction characteristics (Baird 1983), but little is known about DNA modifications in ovine. Recently, it has been hypothesized that the acquisition of epigenetic modifications could affect the gaining of developmental competence during oocyte growth (Ptak et al. 2006; O'Doherty et al. 2012). Our model of oocyte differential

developmental competence confirmed differences in the epigenetic machinery between adult and prepubertal animals showing that also DNA methylation might be involved in oocyte competence acquisition.

Data presented in this thesis provide the first quantitative expression analysis of a panel of genes involved in the DNA methylation reprogramming in oocytes, early embryos and blastocysts in ovine species from both prepubertal and adult animals. The genes analysed are from the DNMTs family, responsible of DNA methylation, the TETs family, a particular class of enzymes involved in active DNA demethylation, and MBDs proteins, characterized by a methyl-CpG binding domain, that have a role in transcriptional regulation by coordinating the cooperation between DNA methylation and histone modifications (Messerschmidt et al. 2014). Our study showed differences in the expression of genes involved in DNA methylation and demethylation between oocytes from young and adult animals, allowing us to hypothesize that DNA methylation machinery of maternal genome is involved in the low developmental capacity of oocytes from young donors.

To identify the timing of synthesis of the transcripts during oocyte growth, we investigated the expression of these genes in GO. The analysis revealed that the abundance of *DNMT1, DNMT3B, TET1* and *TET3* transcripts increases in relation to oocyte diameter, showing that the synthesis of the transcripts starts when the oocyte has a diameter around 70/90 μm and is complete when the oocyte reaches 110/130 μm. Our results suggest that synthesis and storage occur during the final stage of oocyte growth and confirm what found in mouse and bovine (Lucifero et al. 2007; Ratnam et al. 2002; Bessa et al. 2013); in these species, the expression of *DNMT1* and *DNMT3B* increased in relation to the diameter of GO as well.

To have a global view of DNA methylation asset in GO, we investigated the global levels of cytosine methylation by immunostaining. We found a faint degree of global methylation in oocytes of 70 µm diameter that increased over the period of growth in relation to oocyte diameter increment. This result partially confirms what has been shown by Russo et al. (2007) in sheep; in fact they also noticed that 5-methyl-cytosine (5-mc) immunopositivity is directly correlated to oocyte diameter increase, but they did not report any 5-mc signal until the oocyte reached about 90 µm of diameter.

Analysis of gene expression during oocyte nuclear maturation showed lower mRNA abundance of *TETs* enzymes in GV oocytes from prepubertal animals, while no differences were found between the two stages of nuclear maturation in oocytes derived from adult donors. Our results confirm what found in adult mice and porcine oocytes, where no differences were found in the transcript levels of genes involved in DNA methylation machinery between GV and MII stages (Sakashita et al. 2014; Lee et al. 2014). Our results suggest that the lower expression of *TETs* genes observed in prepubertal GV could affect the developmental competence of oocytes from lamb. Recently, it has been hypothesized that the acquisition of epigenetic modifications may be involved in the gaining of oocyte competence during its growth (Ptak et al. 2006; O'Doherty et al. 2012). Our observations suggest a delay in the synthesis and storage of *TETs* transcripts in the prepubertal oocytes that results in a lower abundance at the GV stage recovered by the end of oocyte maturation. Accordingly, a transcriptional activity in GV has already been proposed in prepubertal mouse (Chouinard 1971) bovine (Fair et al. 1995; Fair et al. 1996) and sheep (Ptak et al. 2006).

We assessed the patterns of global methylation by immunofluorescence during meiotic progression. The immunostaining against 5-mc in GV and MII stage oocytes revealed high levels of DNA methylation in gametes from both prepubertal and adult animals. Conversely, Ptak et al (2006) showed the lack of incorporation of 5-mc antibody in GV oocytes from lambs. We can speculate that the differences in the results found in the present study compared to those reported by Ptak et al. (2006) might be related to the protocol and/or the affinity of the antibody to bind the methyl cytosine group (Calbiochem vs Eurogentec). Indeed, year by year companies are putting more effort into generating and characterizing more efficient antibodies and the antibodies available nowadays might result in more reliable binding than those used 10 years ago.

While immunostaining gives a global view of DNA status, a single base resolution analysis of DNA methylation (such us Whole-genome bisulfite sequencing and locus-specific approaches) are needed to asses possible site specific differences in DNA methylation in oocytes and embryos. It has to be noted, however, that oocytes are very complex samples for DNA methylation analysis, as they are characterized by a low amount of DNA compared to somatic cells (1n vs 2n) and are surrounded by several layers of cumulus cells strongly attached to the oocyte. The analysis of single base resolution DNA methylation in oocytes could be biased by the incomplete removal of cumulus cells (somatic cells). The complete removal of cumulus cells is indeed the first crucial step that has to be overcome prior to any methylation analysis in oocytes, as even the presence of a single cumulus cell could bias the correct interpretation of results. With our experimental work, we validated an assay for DMR

at *H19* locus by pyrosequencing that allowed us to assess cumulus cells contamination prior to any further analysis.

The immunostaining against TET3 did not reveal the protein in oocytes at the GV or MII stage, neither in adults nor in lambs. This finding supports our hypothesis of a delay in the synthesis and storage of *TET3* transcripts in the prepubertal GV, rather than a reduction of transcript abundance due to *TET3* mRNA translation. Conversely, we found that TET3 is localized in the cytoplasm of early embryos; the observations support the recent hypothesis of TET3 involvement in the wave of active demethylation that takes place after fertilization (Messerschmidt et al. 2014).

Ovine oocytes are characterized by a high cytoplasmic lipid content (McEvoy et al. 2000a); this particular characteristic confers a dark colour tone that does not allow to follow the progression of zygote pronuclear status during IVC. For this reason it has not been possible to analyse gene expression in the zygotes, as it was not possible to distinguish between zygotes and not fertilized oocytes.

The immunostaining of zygotes from adult and prepubertal animals did not detect any difference in the levels of global methylation or hydroxymethylation in the two considered ages (as in oocytes); however, we observed a decrease in 5-mc signal in male PN compared to maternal PN in zygotes from both young and adult animals. Our observations contrast with what reported by Young and Beaujean in 2004, that showed no loss of 5-mc staining in sheep paternal genome within the first postfertilization cell cycle. Results obtained by 5-mc immunolocalization in zygotes are controversial. The classical model of early embryo epigenetic reprogramming proposes a very 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; Oswald et al. 2000; Santos et al. 2002). Such model has been accepted from the scientific community based on the absence or on very low levels of 5-mC observed in the sperm derived PN compared to the maternally-inherited genome in mouse (Rougier et al. 1998; Mayer et al. 2000; Santos et al. 2002). The first conflicting result came in 2004 from Beaujean et al. that reported a different pattern of epigenetic reprogramming in sheep zygotes compared with mouse and cow. Soon after, more contradictory results from rabbit, cow and pig reported both loss and no loss of 5-mc staining in the male PN (Lepikhov et al. 2008; Park et al. 2007; Reis Silva et al. 2011; Griffin et al. 2006; Jeong et al. 2007). Those inconsistent reports could be due to experimental artefacts, as this immunostaining method involves a series of crucial steps. The first caution needs to be taken when HCl is used to denaturate the DNA for the epitope retrieval. Heras et al. (2014) using bovine zygotes demonstrated that HCl treatment is critical for antibody binding and it can influence the immunovisualization depending on the acid concentration and the timing of incubation. HCl treatment may also impair proper DNA staining that is essential to visualize 5-mc signal colocalization within the nucleus. When working with zygotes, it has to be kept in mind that chromatin configuration is different between PNs. In fact, sperm genome undergoes chromatin remodelling, as after fertilization the substitution of protamines with histone proteins takes place (Miller et al. 2010). The failure of 5-mc antibody binding to male PN might be due to these structural differences. The decrease in 5-mc we observed in male PN in sheep is consistent with genome wide DNA methylation data obtained in mouse and bovine (Wang et al. 2014; de Montera et al. 2013). Such studies confirmed an active

demethylation of male PN, but to a lower degree compared to what shown by a previous study performed by immunofluorescence (Young and Beaujean 2004). Different mechanisms had been proposed to explain active demethylation process soon after fertilization in male PN, and recently some authors strongly evidenced that TET-mediated oxidation of 5-mc is the main pathway involved in active hydroxylation in zygotes and early embryos (Wossidlo et al. 2011; Guo et al. 2014). Wossidlo et al. in 2011 showed immunopositivity of paternal PN when stained with 5-hmc, while maternal PN lack of 5-hmc incorporation. To address if TET3 is involved in the oxidation of the 5-mc to the 5-hmc in the male PN, they performed 5-hmc immunostaining in TET3 knockdown zygotes and found an increase in 5-mc and a decrease in 5-hmc signal in sperm derived PN. We also investigated the global level of DNA hydroxymethylation by immunostaining in zygotes; as expected, we found immunopositivity only in male PN, suggesting that also in sheep the decrease in 5-mc we observed could be due to active DNA demethylation by 5-mc hydroxylation. Taken together, these results indicate that TET3 pathway is responsible for the hydroxylation of 5-mc in male contribution during epigenetic reprogramming after fertilization.

The analysis of gene expression of embryos at two and 4C stage revealed a reduction of *DNMT1, MBD1* and *MBD3* transcripts during the transition from two to four cell embryonic stage in young animals. *DNMT1* expression level resulted lower also in 4C embryos from prepubertal animals compared with the same stage embryos from sheep. Conversely, expression analysis of *TETs* enzymes, whose abundance was lower in the prepubertal GV oocytes, did not shown any difference in embryos. This result suggests that TET-mediated oxidation is a fundamental step for embryo development; oocytes with reduced *TET3*

transcript might not be able to drive and sustain fertilisation or first cell divisions. This may indicate that the correct level of some mRNAs involved in DNA methylation remodelling is essential for the very first step of embryo development; on the contrary, other transcripts may be compatible with early embryo cleavage even at low levels.

We also examined global methylation and hydroxymethylation levels in early embryos at 2 and 4 cell stage and we registered immunopositivity for both 5-mc and 5-hmc antibodies. The intensity of the fluorescence did not show any perceptible variation neither for 5-mc nor for 5-hmc immunostaining in relation to developmental stage progression. The same results had been reported from another lab that performed the same analysis in mouse; they detected a 5-hmc signal of stable intensity throughout pre-implantation development up to the 8-cell stage , when the signal slightly decreased (Ruzov et al. 2011). These results suggest a different role for DNA hydroxymethylation other than DNA 5-mc removal. A persistent and stable level of DNA hydroxymethylation during early embryo development may be an epigenetic modification with a specific biological role during cell self-renewal and differentiation other than being only a transient condition during active demethylation. Previous studies have demonstrated that 5-mc and 5-hmc have different binding properties; for instance, MBD proteins can interact with 5-mc, but not with 5-hmc (Jin et al. 2010; Valinluck et al. 2004). Some MBD proteins have been demonstrated to play a role in gene silencing by coordinating the interaction between DNA methylation and histone modifications. Hydroxylated DNA could be needed to achieve a specific chromatin reorganization to allow the transcription of specific target genes that are essential for

embryonic development. However, further work is necessary to identify the exact function and role of 5-hmc and address which genes are affected by hydroxymethylation.

Further critical events during embryo epigenetic reprogramming need to be elucidated. For example, why maternal genome seems to be more resistant to active demethylation process compared to the paternal PN. Nakamura et al. (2012) proposed that STELLA (Developmental Pluripotency Associated) has a protective function from TET3 oxidation activity in female PN in mice. They suggested an interaction between STELLA and H3K9me2, which is mostly present in female PN, that alters chromatin configuration and protects the DNA from TET3 hydroxylation. Our observations of absence of hydroxylation in female PN is in accordance with the proposed theory.

Chapter 7: Conclusions

Oocyte quality is one of the most important factors that influences the success of embryo development. Our results demonstrate that oocytes and embryos derived from prepubertal animals have different expression levels of genes involved in DNA methylation remodelling in comparison with oocytes and embryos from adult animals. These patterns support the recent hypothesis that acquisition of epigenetic modifications may be involved in the gaining of oocyte competence during its growth. The analysis of global DNA methylation and hydroxymethylation suggest that also in sheep an active DNA demethylation occurs in the paternal pronucleus. Taken together these results indicate that the regulation of epigenetic mechanisms may affect oocyte quality and pave the way to better understanding methylation dynamics during sheep pre-implantation development. The overview on methylation dynamics in growing oocytes may be very useful to understand if environmental changes both *in vivo* and vitro affect the epigenetic status of oocyte maturation or embryo development. Potential changes in environment include nutrition, hormonal treatments or accidental exposition to varied contaminants; they could influence negatively the epigenetic status of the oocytes compromising their developmental competence and the resulting embryo *in vivo*. I*n vitro* reproductive technologies are routinely and successfully applied and their application is increasing steadily over recent years. Cryopreservation of ovarian tissue and in vitro folliculogenesis are valid methods to maintain fertility in young female cancer patients, but may also impact on the epigenetic dynamics. Our analysis of the DNA

methylation status of growing oocytes could help in identifying possible epigenetic alterations caused by cryopreservation and *in vitro* follicular culture. IVM and IVP are the most used biotechnologies in large animals and human and include several *in vitro* manipulation phases that could also affect the correct epigenetic pattern. Data derived from our experiment, when compared by additional experiments *in vivo*, could provide a set of information useful to optimize these ART protocols in order to have a fully viable and healthy offspring.

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Acknowledgments

Il primo ringraziamento va al Professor Sergio Ledda, la ringrazio per aver creduto in me dandomi la possibilità di perfezionare la mia formazione scientifica e di imparare molto. La ringrazio per avermi guidato duranti questi tre anni e per i tanti consigli ,anche se a volte non li ho seguiti proprio alla lettera. Il suo aiuto nell'analisi dei dati, soprattutto di immunofluorescenza è stato di fondamentale importanza per la stesura di questo lavoro.

Il secondo ma a pari merito con il primo ringraziamento è per la Dottoressa Daniela Bebbere. Grazie Dani per avermi trasmesso tutte le tue conoscenze, per i tanti consigli e per avere pazientemente corretto questa tesi.

Ringrazio Antonino, la più bella persona che ho conosciuto in tutta la mia vita e che fortunatamente si è innamorata di me e delle mie stramberie. Senza di te questi tre anni non riesco neanche a immaginarli.

Mi sento di ringraziare il Dottor Giovanni Burrai, Grazie Vanni per tutti i complimenti quotidiani e per avermi aiutato con gli esperimenti di immunofluorescenza.

Ringrazio inoltre tutti i colleghi della sezione di clinica ostetrica in particolare le dottoresse Federica Ariu, Luisella Bogliolo e Ombretta Murrone per la parte di biologia cellulare, per la cura e pazienza con la quale mi avete aiutato a preparare i campioni che ho usato per gli esperimenti. Sicuramente il mio dottorato non sarebbe stato lo stesso senza le "ragazze di Clinica Ostetrica" presenti e passate: Gina, Ombretta, Laura e Antonella. Vi ringrazio di cuore, in questi tre anni si è creato un bellissimo rapporto anche al di fuori del lavoro. Abbiamo trascorso dei bellissimi momenti insieme. Come non ricordare le risate del henné o il caipirinha party per dire addio a Letizia?

Ringrazio il Professor Colum Walsh dell'Università dell'Ulster in Irlanda del Nord per avermi dato la possibilità di frequentare il suo laboratorio e di collaborare con il suo team. In particolare la dottoressa Karla O'Neill che mi ha supervisionato e insegnato molto con tanta pazienza.

Il periodo in Irlanda è stato fondamentale per il mio dottorato. Mi ha proprio messo alla prova, e nonostante il freddo del clima e degli irlandesi mi è stato utile per crescere, ho imparato moltissimo sia dal punto di vista professionale che personale.

A Sassari, ai vicoli, i circoli e i bar del centro storico che mi hanno fatto capire che la malvasia di bosa è la migliore e soprattutto che gli intellettuali dovrebbero tenere in pugno la classe politica, ma chi sono oggi gli intellettuali? Non esistono più…

Ringrazio Ale e Isa per i 3 anni di convivialità diffusa, tra convivenze e ospitalità avete reso questi tre anni meno pesanti e molto molto divertenti.

In fine ringrazio i miei genitori perché se sono arrivata al massimo livello di formazione universitaria lo devo soprattutto a loro, che con grandi sacrifici mi hanno permesso di studiare. Mi avete appoggiato sempre in tutte le miei scelte, e ve ne sono infinitamente grata. Vi ringrazio perché con la vostra semplicità mi avete fatto diventare ciò che oggi sono: Lilly!!!