Molecular Human Reproduction, Vol.18, No.7 pp. 333–340, 2012
Advanced Access publication on March 23, 2012 doi:10.1093/molehr/gas014

MHR Basic science of reproductive medicine

NEW RESEARCH HORIZON *Review*

Active DNA demethylation in mammalian preimplantation embryos: new insights and new perspectives

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Submitted on December 14, 2011; resubmitted on February 27, 2012; accepted on February 28, 2012

ABSTRACT: DNA methylation and demethylation are crucial for modulating gene expression and regulating cell differentiation. Functions and mechanisms of DNA methylation/demethylation in mammalian embryos are still far from being understood clearly. In this review we firstly describe new insights into DNA demethylation mechanisms, and secondly introduce the differences in active DNA methylation patterns in zygotes and early embryos in various mammalian species. Thirdly, we attempt to clarify the functions of DNA demethylation in early embryos. Most importantly we summarize the importance of active DNA demethylation and its possible relevance to human IVF clinics. Finally research perspectives regarding DNA demethylation are also discussed.

Key words: active DNA demethylation / zygote / preimplantation embryos / 5-methylcytosine / 5-hydroxymethylcytosine

Introduction

DNA methylation and demethylation are crucial for modulating gene expression and regulating cell differentiation. The process is based on the fifth carbon of cytosines at the CpG sites of mammalian genomic DNA that can be methylated to 5-methylcytosine (5-meC). The DNA methylation pattern is the main epigenetic memory of the cell and it also determines the cell's fate. The primary function of DNA methylation that had been proposed was limiting the transcription noise of background genes including transposons (Bird, 2002). DNA methylation not only represses the transcription of genes, but also results in gene imprinting and global X chromosome inactivation. Different cell types display unique DNA methylation patterns that can be passed to cells of the offspring (heritable), or it may be altered depending on the changes in the microenvironment.

During mammalian preimplantation embryo development, the DNA methylation patterns from the zygote through blastocyst stages undergo dynamic changes. In Waddington's epigenetic landscape model, which suggests that differentiation potency is analogous to a mountain (named Waddington's mountain), cells with higher potency occupy the higher places of the mountain, and vice versa (Hochedlinger and Plath, 2009). The totipotent zygote residing at the highest peak of Waddington's mountain tumbles from the peak to the first branch at the blastocyst stage, with inner and outer blastomeres differentiated into inner cell mass (ICM) cells and trophectoderm (TE) cells, respectively. The epigenetic landscape change during preimplantation embryo development mainly concerns DNA methylation and demethylation.

In the mouse, 5-meCs of the zygotic paternal pronucleus (PPN) is globally hydroxylated by methylcytosine dioxygenase TET3 protein (TET3) to 5-hydroxymethylcytosines (5-hmCs; Gu *et al.*, 2011; Wossidlo *et al.*, 2011). By using immunofluorescence detection method, 5-meCs signals were found to gradually diminish in PPN (Mayer *et al.*, 2000), whereas 5-hmCs signals became gradually apparent in PPN (Iqbal *et al.*, 2011; Ruzov *et al.*, 2011; Wossidlo *et al.*, 2011). During mouse preimplatation embryo development, both paternal and maternal genomes show passive and DNA replication-dependent demethylation (Rougier *et al.*, 1998; Inoue and Zhang, 2011). When mouse embryos enter the blastocyst stage, the genomes of ICM and TE cells start global *de novo* methylation (Santos *et al.*, 2002). By fluorescent tagging of 5-meC, it was shown that the 5-meC signal intensity of ICM cells was clearly stronger than that of TE cells (Dean *et al.*, 2001). However, the DNA methylation dynamics observed in mouse preimplantation embryos are not well conserved in other mammals.

As of now, the mechanisms of DNA methylation are still under investigation, as they are incompletely understood. In this review, we summarize recent findings concerning DNA demethylation mechanisms, active DNA methylation patterns in different mammalian species and in zygotes or zygote-like cells derived from different technologies. We attempt to clarify the potential functions of DNA demethylation in mammalian preimplatation embryos. The possible

relevance of DNA demethylation to human IVF clinics and related research perspective are also discussed.

New insight into active DNA demethylation

Methylation of DNA can be established *de novo* by DNA methyltransferase 3A (DNMT3A) and DNMT3b, and maintained during DNA replication by DNMT1. The methyl group on cytosine can be lost during DNA replication (DNA replication-dependent passive demethylation) at the newly synthesized DNA strand (Rougier *et al.*, 1998) or it may be removed independent of DNA replication (active demethylation) in primordial germ cells (Hajkova *et al.*, 2002; Lee *et al.*, 2002), zygotes (Mayer *et al.*, 2000; Oswald *et al.*, 2000) and even somatic cells (Kangaspeska *et al.*, 2008; Metivier *et al.*, 2008; Kim *et al.*, 2009; Hitoshi *et al.*, 2011). The detailed mechanisms and functions of active DNA demethylation are still under investigation. Based on reported evidence, four classes of proteins participate in active DNA demethylation (Table I).

DNMT3A and DNMT3B have been reported as *de novo* DNA methyltransferases. However, Kangaspeska *et al.* (2008) reported that DNMT3A and DNMT3B, as well as thymine–DNA glycosylase (TDG) could be recruited to the *pS2* gene promoter (Kangaspeska *et al.*, 2008). When recruiting of DNMT3A and DNMT3B was decreased by depletion of estrogen receptor (ERα) protein, demethylation of *pS2* promoter was repressed (Metivier *et al.*, 2008). These results show the dual functions of DNMT3A and DNMT3B on *de novo* DNA methylation and demethylation. By using the ChIP method, TDG, a DNA glycosylase recognizing and repairing the T:G DNA mismatch on DNA, was found to be recruited to the promoter region at the beginning of *pS2* gene activation. When the function of TDG was impaired, the expression of the *pS2* gene decreased ~2.5-fold (Metivier *et al.*, 2008). In another report by Kim *et al.* (2009), DNA demethylation on gene promoter was found impaired in the *Mbd4* gene (another T:G mismatch repair associated with the glycosylase gene) mutant mice. Taken together, all data indicate that DNA glycosylases (such as TDG and MBD4) participate in active DNA demethylation at specific DNA promoters in somatic cells.

Unlike DNMT3A and DNMT3B, the DNA demethylation mechanism of the TET family proteins (TET1, TET2 and TET3) is indirect. 5-meCs on CpG sites are oxidized to 5-hmC by TET proteins (Tahiliani *et al.*, 2009; Ito *et al.*, 2010). When maternal TET proteins were reduced in zygote by RNA interfering with the mRNAs in MII oocytes, both the decrease of 5-meC signals and the increase of 5-hmC signals in the PPN were weakened (Wossidlo *et al.*, 2011). In addition to 5-hmC, other intermediates like 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) were detected in most

Table I List of active DNA demethylation-associated proteins.

Mechanisms of DNA active demethylation	Associated proteins	Functional annotation of proteins in DNA demethylation	References
Base excision repair	Aicda, Apobec1	Both enzymes promote demethylation of 5-hmCs in mouse brain cells. However, Aicda and Apobec1 cannot be detected in PGC cells when PGC cells start active demethylation	Hajkova *et al.* (2010); Popp *et al.* (2010); Guo *et al.* (2011)
	Tdg, Mbd4 and Smug1	Tdg protects specific regions of genome from *de novo* methylation, and actively demethylate genome-specific regions. *In vitro*, Tdg can also excise 5-formylcytosine and 5-carboxylcytosine. Mbd4 was proved to participate in specific DNA demethylation in CYP27B1 gene promoter Smug1 protein can repair DNA base excisions including 5-hmU, Uracil, 5-hydroxyuracil and so on	Masaoka *et al.* (2003); An *et al.* (2005); Kim *et al.* (2009); Cortazar *et al.*, 2011); Cortellino *et al.* (2011); Maiti and Drohat (2011)
	Aprp1, Xrcc1 and Apex1	When the activity of either Aprp1 or Apex1 was inhibited, the active demethylation of PPN was weakened. Xrcc1, could form complex with Aprp1 and Apex1, only bounds to PPN DNA in zygote	Hajkova *et al.* (2010)
DNA methyltransferases mediated DNA demethylation	Dnmt3a and Dnmt3b	Promote DNA demethylation of specific promoter DNA	Kangaspeska *et al.* (2008); Metivier *et al.* (2008)
Indirect DNA demethylation	Tet1, Tet2 and Tet3	All three enzymes can hydroxylate 5-meCs to 5-hmCs, 5-formylcytosines and 5-carboxylcytosines. Tet3, but not Tet1 or Tet2, is highly expressed in zygote, and takes function on PPN active demthylation. Tet1 is highly expressed in mouse ICM cells but not in TE cells	Tahiliani *et al.* (2009); Gu *et al.* (2011); He *et al.* (2011); Ito *et al.* (2011); Iqbal *et al.* (2011); Wossidlo *et al.* (2011)
Other unclear mechanisms	Elp3	Impairing PPN active demethylation	Okada *et al.* (2010)
	Gcm1, Gcm2	Impairing DNA active demethylation	Hitoshi *et al.* (2011)
	PGC7	Protecting maternal pronuclear from global demethylation	Wossidlo *et al.* (2011)
	H2A.X-p (γH2A.X)	Phosphorylated H2A.X was a marker of DNA double-strand breaks, which particularly appears at PPN from PN3 through PN5 stage	Wossidlo *et al.* (2010)

tissues and cell lines (Ito *et al*., 2011). When *Tet* genes were overexpressed or knocked down, the contents of 5-fCs and 5-caCs increased or decreased, respectively (He *et al*., 2011; Ito *et al*., 2011). The 5-caCs produced by TET proteins but not 5-hmC could be excised by TDG and no 5-caC excision activity could be detected for MBD4, SMUG1 and UNG (uracil-DNA glycosylase; He *et al*., 2011). 5-hmU, which may be produced by deamination of the cytosine of 5-hmC by ACIDA (Fritz *et al*., 2010; Popp *et al*., 2010), was also an intermediate of TET proteins associated with the active DNA demethylation pathway. 5-hmUs cannot be detected in normal HEK293 cells, but could be detected when TET1 was induced (Guo *et al*., 2011). The 5-hmUs induced by TET1 could be removed when SMUG1 was expressed (Guo *et al*., 2011). The above evidence shows that TDG can complete active DNA demethylation by excising 5-caC produced by TET proteins and SMUG1 can excise 5-hmU induced by TET1 protein.

In addition to TDG, MBD4 and SMUG1, other base excision repair (BER) associated proteins such as ACIDA/APOBEC1 (Bhutani *et al*., 2009), APRP1, APEX1 and XRCC1 (Hajkova *et al*., 2010) were shown to participate in DNA demethylation. These results confirmed that DNA demethylation was dependent on the processes of BER. However, MBD4 deleted mice were able to survive (Millar *et al*., 2002), whereas TDG deleted mice were embryonic lethal at about E11.5 (Cortazar *et al*., 2011). APRP1 and APEX1 were shown to promote active DNA demethylation in mouse zygote and primordial germ cells (PGCs), whereas the expression level of ACIDA was extremely low in these two cell types (Hajkova *et al*., 2010). This indicates that the mechanisms of active DNA demethylation in different cell types or specific DNA regions are not conserved.

In addition, ELP3 (Okada *et al*., 2010), GCM1 and GCM2 (Hitoshi *et al*., 2011), PGC7 (Wossidlo *et al*., 2011) and γH2A.X (Wossidlo *et al*., 2010) are also considered to be DNA demethylation associated factors, but details concerning molecular functions of these proteins in DNA demethylation are still not clearly understood. In *PGC7* (also known as DPPA3 or stella) depleted zygotes, the maternal pronucleus could be actively demethylated and 5-meCs signals were lost. Correspondingly, the 5-meCs of the maternal pronucleus in *PGC7*-depleted zygotes could also be hydroxylated to 5-hmCs (Wossidlo *et al*., 2011).

DNA methylation/demethylation patterns in zygotes and preimplantation embryos of different mammalian species

Mouse zygotic PPN undergoes active demethylation; however, this phenomenon is not conserved in mammalian species. Furthermore, when blastomeres show differentiation at the blastocyst stage, the DNA methylation patterns in ICM and TE cells are also not conserved in mammalian species (Table II and Fig. 1).

The 5-meC signals are almost lost in the late zygotic PPN of human (Fulka *et al*., 2004), monkey (Yang *et al*., 2007), mouse (Mayer *et al*., 2000) and rat (Zaitseva *et al*., 2007). Whereas, the 5-meC signals in sheep PPN are maintained at the zygote stage (Beaujean *et al*., 2004a, b).

The active demethylation of PPN in cow, goat, pig and rabbit has faced controversy. In Dean's results, 5-meCs signals in zygotic PPNs of pig and cow were weaker than that in the maternal pronucleus (Dean *et al*., 2001). From Beaujean's and Abdalla's results, cow PPNs also partially lost 5-meC fluorescent signals (Beaujean *et al*., 2004a, b; Abdalla *et al*., 2009); however, the PPN of the cow zygote was able to remethylate after partial demethylation (Park *et al*., 2007). In addition, it was shown that active demethylation of goat occurred not only at zygotic PPN but also in 2-cell stage embryos (Park *et al*., 2007, 2010). Pig zygotic PPNs were nearly completely demethylated as Dean described in Fulka's report (Fulka *et al*., 2006), whereas Jeong's results (Jeong *et al*., 2007) showed that pig zygotic PPN demethylation was preserved. Zygotic PPN active

Table II Different DNA methylation patterns in mammalian preimplantation embryos.

5-meC fluorescent signals change in zygotic PPN (or one PN)	Species	5-meC fluorescent signals intensity in ICM and TE cells of early blastocyst and (/) fully expanded blastocyst[a]	References
Mostly lost 5-meC signal	Human Monkey Mouse Rat	ICM = TE/ICM < TE Blastocyst: ICM < TE Blastocyst: ICM > TE NA	Fulka *et al*. (2004) Yang *et al*. (2007) Mayer *et al*. (2000) Dean *et al*. (2001); Zaitseva *et al*. (2007)
Without obvious loss of 5-meC signal	Sheep	ICM = TE/ICM > TE	Beaujean *et al*. (2004a, b)
Under controversy (see text)	Cow Goat Pig Rabbit	Blastocyst: ICM > TE Blastocyst: ICM = TE ICM = TE/ICM > TE NA	Dean *et al*. (2001) Park *et al*. (2007, 2010) Fulka *et al*. (2006) Beaujean *et al*. (2004a, b); Lepikhov *et al*. (2008)

NA, not available.

[a]For monkey, mouse, cow and goat, the DNA methylation level of ICM cells is same as that of TE cells both in early blastocyst stage and expanding blastocyst stage. For human, DNA methylation level of ICM cells is the same as that of TE cells at the early blastocyst stage, but is weaker than that of TE cells at expanding blastocyst stage. For sheep and pig, the DNA methylation level of ICM cells is the same as that of TE cells at the early blastocyst stage, but is stronger than that of TE cells at expanding blastocyst stage.

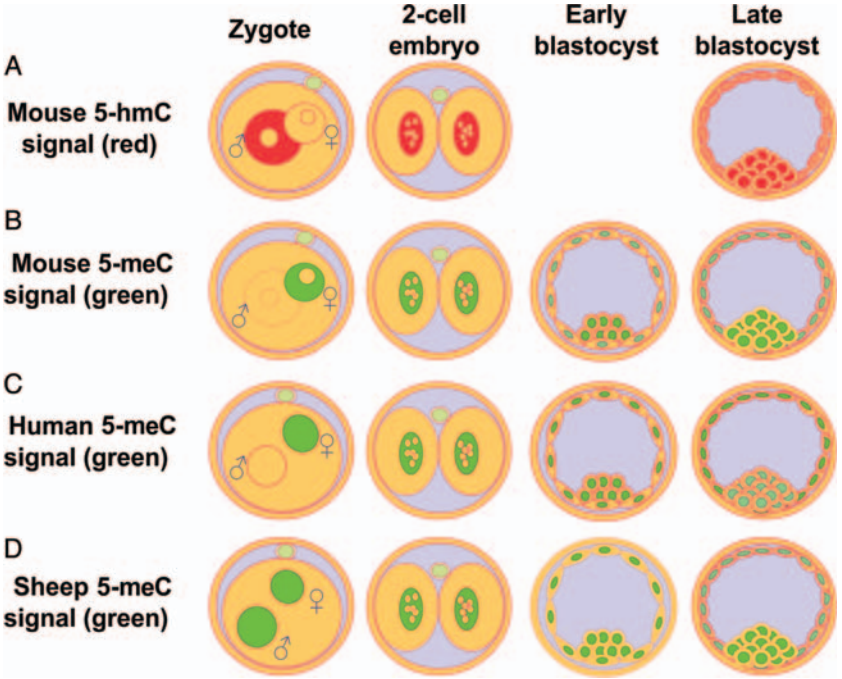

Figure 1 DNA methylation patterns in mammalian zygotes and preimplantation embryos. (**A**) 5-hydroxymethylcytosine (5-hmC) signals appear in the zygotic PPN (♂, red) of the mouse. From the 2-cell embryo through the blastocyst stage, 5-hmC signals can be detected in cell nuclei. However, at the late (expanding) blastocyst stage, the intensity of 5-hmC signals is weaker in TE cells (light red) than that in ICM cells (red). (**B**) 5-methylcytosine (5-meC) signals clearly disappear in the mouse PPN, while 5-meC signals are evident in the maternal pronucleus (MPN; ♀, green). At the blastocyst stage, the intensity of 5-meC signals is weaker in TE cells (light green) than that in ICM cells (green). (**C**) Human PPN DNA is actively demethylated while 5-meC signals are evident in the MPN (green). At the late blastocyst stage, the intensity of ICM 5-meC signals (light green) is weaker than that of TE cells (green). (**D**) The 5-meC signals of sheep PPN display no changes at the zygote stage. At the late blastocyst stage, the intensity of ICM 5-meC signals (green) is stronger than that of TE cells (light green).

demethylation in rabbit did not exist in Beaujean's report (Beaujean *et al.*, 2004a, b), while Lepikhov *et al.* (2008) showed clear DNA active demethylation in rabbit zygotic PPN.

At the early blastocyst stage, the *de novo* DNA methylation level (5-meC signals) in ICM cells is nearly the same as that in TE cells in human, sheep, goat and pig. It is still puzzling that the 5-meC signals of sheep and pig expanding blastocysts are decreased in TE cells (Beaujean *et al.*, 2004a; Fulka *et al.*, 2006), while the 5-meC signals display no obvious changes in goat blastocysts (Park *et al.*, 2010), but decreased in human ICM cells (Fulka *et al.*, 2004). Interestingly, the 5-meC signal intensity in ICM cells is stronger than that of TE cells at the whole blastocyst stage in mouse and cow (Dean *et al.*, 2001) but it is weaker than that of TE cells in monkey blastocysts (Yang *et al.*, 2007). This indicates that DNA demethylation (active or passive) may exist in the ICM cells of human and TE cells of sheep and pig.

The 5-hmC pattern in preimplantation embryos so far has only been described in the mouse. While 5-meC signals gradually decrease in PPN, 5-hmC signals gradually increase in PPN. From the 2-cell stage embryo through the blastocyst stage, signals of 5-meC and 5-hmC are mixed. At the blastocyst stage, the 5-hmC signal in ICM cells, like that of 5-meC, is stronger than that in TE cells (Fig. 1; Ruzov *et al.*, 2011). 5-hmCs could also be detected in bovine and rabbit PPNs and in nuclei of mouse one cell embryos derived from somatic cell nuclear transfer (Wossidlo *et al.*, 2011).

Active DNA demethylation in mouse zygotes and zygote-like cells

There are many approaches to generate zygotes or zygote-like cells, which are classified into three classes (Table III). True zygotes are formed bys fusion of haploid male and female gametes. In addition to normal *in vivo* fertilization, all technologies like IVF, ICSI and others which assist male gamete entry into the ooplasm, can produce a true zygote. Unlike true zygotes, zygote-like cells differ from zygotes in the origin or number of their genome (parthenogenesis, androgenesis, haploid, diploid gynogenesis, diploid androgenesis, polyploid and nuclear transfer induced zygote-like cells). Interspecies zygote-like cells allow us to determine the functional role of the oocyte on the sperm or the functional role of sperm on the oocyte regarding DNA demethylation.

The investigation of mouse DNA methylation reprogramming in different technologies-induced zygotes and zygote-like cells was mostly performed through 5-meC secondary antibody staining (Table III). In zygotes and zygote-like cells, only the pronucleus originating from the sperm was able to lose most 5-meC signals (Mayer *et al.*, 2000; Barton *et al.*, 2001; Santos, *et al.*, 2002; Beaujean *et al.*, 2004a, b; Fulka and Fulka, 2006; Peters *et al.*, 2009; Wossidlo *et al.*, 2011).

Table III DNA methylation reprogramming in mouse zygotes and zygote-like cells as revealed by 5-meC secondary antibody staining.

Zygote type	Fluorescent signal of 5-meC of PPN	References
True zygotes		
Normal fertilization	Signal lost	Mayer *et al.* (2000); Fulka and Fulka (2006)
IVF	Signal lost	Santos *et al.* (2002); Fulka and Fulka (2006)
Zona pellucida laser microdissection-facilitated IVF	Signal lost	Peters *et al.* (2009)
ICSI	Signal lost	Beaujean *et al.* (2004a, b); Fulka and Fulka (2006)
ROSI/elongating spermatid injection	Signals partially lost 6 h after injection, but resumed 10 h after injection	Ohta *et al.* (2009)
Zygote-like cells		
Parthenogenesis	Signal not lost	Barton *et al.* (2001)
Androgenesis	Signal lost	
Haploid, diploid gynogenetic, diploid androgenetic, triploid diandric and triploid digynic embryos	PPN lost, MPN not lost	
Nuclear transfer	Signal not lost	Wossidlo *et al.* (2011)
Interspecific zygote-like cells		
Mouse sperm/cow oocyte	Partially lost	Beaujean *et al.* (2004a, b)
Mouse sperm/sheep oocyte	Partially lost	
Mouse oocyte/cow sperm	Partially lost	
Mouse oocyte/sheep sperm	Partially lost	
Mouse oocyte/pig sperm	Partially lost	Barnetova *et al.* (2010)

All data above are derived from indirect immunofluorescence labeling of pronuclear 5-meCs. The data for 5-hmCs still await replenishing.

The DNA methylation reprogramming of round or elongating spermatids resembled that of cow sperm described by Park *et al.* (2007); Ohta *et al.* (2009). Based on these results we can speculate that the factors determining the loss of PPN 5-meC signals may exist in the sperm itself. But based on the results obtained from interspecies zygote-like cells (Beaujean *et al.*, 2004a, b; Barnetova *et al.*, 2010), the mouse ooplasm was also able to partially reduce the 5-meC signal intensity of PPN from sheep sperm, which failed to lose 5-meC signals in the sheep ooplasm (Beaujean *et al.*, 2004a, b). In addition, 5-hmC signals could also be detected in the nucleus of mouse somatic cell nuclear transfer (SCNT) zygote-like cells (Wossidlo *et al.*, 2011). Therefore, the ooplasm also possesses factors inducing PPN demethylation reprogramming.

Functions of DNA demethylation in mammalian preimplantation embryos

The distribution of 5-hmC on the genome was determined by using high throughput sequencing. The results showed that 5-hmCs were mainly enriched at gene start sites where histones were dually modified by trimethylation of H3K27, an inactive or poised promoter marker and trimethylation of H3K4, which was mainly distributed at the gene promoter and enhancer regions (Pastor *et al.*, 2011). These results suggest that the 5-meC hydroxylation to 5-hmC is associated with gene expression modulation (Ficz *et al.*, 2011; Wu *et al.*, 2011).

The functions of active DNA demethylation in preimplantation embryonic development are puzzling since different DNA methylation patterns are found in different species. Based on the results from the mouse, *Tdg* knockout caused death of the fetus at about E11.5, and gene promoters unmethylated in normal cells were found methylated (Cortazar *et al.*, 2011). *Tet3* mutation mice died at the neonatal stage, but oocyte conditional knockout of *Tet3* could repress 5-meC hydroxylation to 5-hmC in zygotes and impair the demethylation of paternal *Oct4* promoter (Gu *et al.*, 2011). Although *Tet1* is expressed only at a low level in zygotes, it is expressed at 2-cell stage embryos and highly expressed in embryonic stem cells (Iqbal *et al.*, 2011; Wossidlo *et al.*, 2011) and knockdown of *Tet1* induces blastomeres prone to differentiate into TE cells (Ito *et al.*, 2010), which was confirmed by Ruzov's results showing that 5-meC hydroxylation mainly occurs in ICM cells (Ruzov *et al.*, 2011). In addition, mostly PGC7 null mouse embryos became fragmented or abnormal at the 2–4 cell stage, and only 3% of them were able to develop to blastocyst stages (Nakamura *et al.*, 2007). Based on these results, we can conclude that active DNA demethylation may not be crucial in PPN, but that it is important for blastomere differentiation. On the other hand, maternal pronucleus demethylation may be harmful for preimplantation embryo development. Recent evidence showed that *Tet1* homozygous knockout mice were viable and fertile, but 75% of the mutant pups displayed a small body size (Dawlaty *et al.*, 2011),

indicating that functions of TET proteins may be redundant during development.

Clinical relevance of active PPN demethylation

In addition to data shown in Table III, recent studies suggested that both oocyte quality and sperm manipulation could impair the active PPN demethylation. For the mouse, it was found that *in vitro* development failure of IVF or superovulated embryos was correlated with an abnormal methylation pattern of 2-cell embryos (Shi and Haaf, 2002). For the pig, when oocytes matured *in vitro* from the germinal vesicle stage was used for IVF, active PPN demethylation occurred only in 40% of late PN stage zygotes. Whereas, if oocytes were *in vitro* matured from GVBD stage and were *in vitro* fertilized, active PPN demethylation occurred in 73% of zygotes. In human, a small number of 3 PN ICSI zygotes failed to show any DNA methylation staining difference among the three pronuclei (Xu *et al*., 2005). Similar demethylation abnormalities have also been observed in mice; 2–4% of normally fertilized zygotes exhibited no obvious PPN demethylation (Barton *et al*., 2001). It is possible that the abnormal methylation patterns are the result of dysfunctional cytoplasm in a small number of oocytes, which may affect embryonic viability (Gioia *et al*., 2005). No difference in active demethylation was observed when testicular or epididymal spermatozoa were used for ICSI in mice (Kishigami *et al*., 2006). For bovine, when freeze-thawed spermatozoa were used for IVF or ICSI, the relative methylation level (RM, male/female) of ~40% of zygotes was <0.6, and that of ~50% zygotes was between 0.6 and 1.0. Whereas when spermatozoa which were freeze dried and stored at 4°C or −196°C were used for ICSI, over 60% of zygotes showed RM <0.6 (Abdalla *et al*., 2009). Active demethylation of male pronucleus was delayed in embryos produced by ICSI with DNA-fragmented spermatozoa (Fernández-Gonzalez *et al*., 2008). These results may indicate that damage to sperm could alter active DNA methylation. Human spermatozoa injected into mouse oocytes were able to undergo normal epigenetic changes, and thus interspecies ICSI may be used as a tool to analyze the sperm's ability for active demethylation in human IVF clinics (Fulka *et al*., 2008). The above evidence shows that abnormalities of active PPN demethylation can be caused by either low oocyte quality or sperm damage, and thus may indicate possible developmental failure. But whether active DNA methylation itself correlates with developmental potential needs further clarification.

Strikingly, in contrast to ICSI-derived zygotes, round spermatid injection (ROSI)-derived zygotes possessed only slightly demethylated paternal DNA but both types of zygotes developed to term with similar rates in mice (Polanski *et al*., 2008). However, another group suggested a correlation between epigenetic abnormalities of the mouse paternal zygotic genome derived from microinsemination of round spermatids with poor developmental potential of embryos. Thus, selective paternal DNA demethylation may have an adverse impact on an embryo's later development. The long-term consequences of active DNA demethylation need further investigation.

Evidence showed that assisted reproduction technologies (ARTs) and suboptimal *in vitro* culture conditions were able to change the epigenetic inheritance (Fernández-Gonzalez *et al*., 2007; van Montfoort *et al*., 2012). No differences were observed in the DNA methylation pattern in mouse zygotes produced *in vivo*, *in vitro* or by ICSI (Fulka and Fulka, 2006). But the demethylation dynamics of PPN in rat zygotes were impaired when using routine protocols for *in vitro* embryo production such as IVF and ICSI (Yoshizawa *et al*., 2010). Although there is no data showing that abnormal epigenetic changes caused by ART are associated with active PPN demethylation, ART has the hypothetical potential for epigenetic effects and websites from the Centers for Disease Control and Prevention call for attention, as it is estimated that ART accounts for >1% of total births in the USA (http://www.cdc.gov/art/).

Perspectives

DNA methylation patterns are not only affected by intrinsic factors such as DNA methyltransferases, TET proteins and BER pathway proteins, but they are also affected by environmental factors such as ageing and diet (Jaenisch and Bird, 2003). Active DNA demethylation in preimplantation embryos is not regulated by a single pathway but by integrated biological processes, which have been reviewed by Saitou *et al*. (2011).

From data presented in Tables II and III, an interesting puzzle is apparent. Why are DNA methylation patterns different in mammalian species at the expanding blastocyst stage? Furthermore, the functions of the three TET proteins also need to be verified in other mammals to clarify the question why the zygotic PPN DNA methylation patterns are not conserved in mammals. Although the genome information for sheep has not yet been completed, we have obtained the TET protein corresponding genome regions in sheep from the UCSC BLAT program and found that all three TET proteins exist in sheep. If functional TET proteins of sheep are expressed in their zygotes, there must be other mechanisms protecting PPN from active demethylation.

High throughput 5-meCpG or 5-hmCpG sequencing of preimplantation embryos may be helpful for us to understand how active DNA demethylation affects blastomere differentiation. Bisulfite sequencing is not feasible for distinguishing 5-meC and 5-hmC (Huang *et al*., 2010), and chemical labeling of 5-hmC followed by the single molecule real-time DNA sequencing method cannot be used for trace sample sequencing (Song *et al*., 2011). Methods for blastomere genome 5-meC and 5-hmC sequencing need to be developed; only then may we be able to better understand the mechanisms and functions of DNA methylation and demethylation in early embryonic blastomeres.

Acknowledgements

We apologize to colleagues whose work could not be cited due to space limitation. We thank other members of the Sun laboratory for stimulating discussions during writing of the manuscript.

Authors' roles

J.Y.M. wrote most parts of the manuscript (MS); X.W.L. participated in the discussion of the MS; H.S. revised the MS; Q.Y.S. finished the outline of the MS, wrote part of the MS and revised the MS.

Funding

Q.Y.S. was supported by the Major Basic Research Program of China (2012CB944404 2011CB944501). This review did not receive any specific grant from any funding agency in the public commercial or not-for-profit sector.

Conflict of interest

None declared.

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