DNA methylation in bull spermatozoa: evolutionary impacts, interindividual variability, and contribution to the embryo

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INVITED REVIEW

DNA methylation in bull spermatozoa: evolutionary impacts, interindividual variability, and contribution to the embryo[1]

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Abstract: The DNA methylome of spermatozoa results from a unique epigenetic reprogramming crucial for chromatin compaction and the protection of the paternal genetic heritage. Although bull semen is widely used for artificial insemination (AI), little is known about the sperm epigenome in cattle. The purpose of this review is to synthetize recent work on the bull sperm methylome in light of the knowledge accumulated in humans and model species. We will address sperm-specific DNA methylation features and their potential evolutionary impacts, with particular emphasis on hypomethylated regions and repetitive elements. We will review recent examples of interindividual variability and intra-individual plasticity of the bull sperm methylome as related to fertility and age, respectively. Finally, we will address paternal methylome reprogramming after fertilization, as well as the mechanisms potentially involved in epigenetic inheritance, and provide some examples of disturbances that alter the dynamics of reprogramming in cattle. Because the selection of AI bulls is closely based on their genotypes, we will also discuss the complex interplay between sequence polymorphism and DNA methylation, which represents both a difficulty in addressing the role of DNA methylation in shaping phenotypes and an opportunity to better understand genome plasticity.

Key words: sperm, bull, DNA methylation, epigenetics, embryo.

Résumé : Le méthylome spermatique joue un rôle essentiel dans la compaction de la chromatine et la protection du patrimoine génétique paternel. Alors que la semence bovine est un produit largement diffusé sur le marché de l'insémination artificielle (IA), il existe peu de données sur l'épigénome spermatique bovin. Cette revue a pour but de synthétiser des travaux récents sur le méthylome du spermatozoïde bovin à la lumière des connaissances obtenues chez l'Homme et les espèces modèles. Nous aborderons les spécificités du méthylome spermatique et leur potentiel impact sur l'évolution des génomes, avec un focus sur les séquences hypométhylées et les éléments répétés. Nous évoquerons ensuite quelques exemples de variabilité interindividuelle et de plasticité intra-individuelle en relation avec la fertilité et l'âge, respectivement. Enfin, nous aborderons la reprogrammation du méthylome paternel après la fécondation ainsi que les mécanismes potentiellement en jeu dans la transmission épigénétique intergénérationnelle, et apporterons quelques exemples d'altération de la dynamique de reprogrammation chez le bovin. Les taureaux d'IA étant sélectionnés sur la base de leurs génotypes, nous discuterons des interactions entre polymorphismes de séquence et méthylation, qui constituent un facteur de variation de l'épigénome mais aussi une opportunité de mieux comprendre le fonctionnement et la plasticité du génome.

Mots-clés : spermatozoïdes, taureaux, méthylation de l'ADN, épigénétique, embryon.

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Introduction

Epigenetics refers to the molecular mechanisms which alter gene regulation in a DNA sequence-independent fashion and are transmitted to the daughter cells through cell divisions. More specifically, combinations of histone modifications and DNA methylation states can be used to delineate active enhancers, active promoters, and transcribed genes in a given cell type; transmission of these epigenetic features to daughter cells constitutes the memory of tissue-specific regulations (Bird 2007). This definition of epigenetics does not apply to spermatozoa, which are transcriptionally inactive and represent the ultimate form of cell differentiation, destined to give rise to a new individual after fertilization of an oocyte and not to daughter cells with a similar phenotype. The differentiation of male germ cells into functional spermatozoa requires a unique epigenetic reprogramming involving large-scale DNA methylation changes, the replacement of most histones by protamines, and an accumulation of specific noncoding RNAs. Even though transcription is barely detectable in mature sperm cells, the male germline differentiation programme is orchestrated by a dynamic sequence of transcriptional regulations that are directly reliant on epigenetic reprogramming (Seisenberger et al. 2012; Hammoud et al. 2015; Stewart et al. 2016; Hill et al. 2018). Another important function of epigenetic reprogramming is that it leads to an unsurpassed compaction of chromatin at the end of spermatogenesis. Sperm cells need to survive during the journey through the female genital tract and are devoid of antioxidant mechanisms because most of the cytoplasm has been lost during spermiogenesis. Chromatin compaction is, therefore, crucial to protecting the paternal genome against oxidative stress, and also contributes to reducing nuclear volume and to the hydrodynamic shape of spermatozoa (Carrell 2012). Moreover, the epigenetic features of sperm carry essential information for genomic imprinting, postfertilisation reprogramming, the establishment of totipotency, and embryo development; recent reports have also suggested that they may influence long-term phenotypes of the offspring through epigenetic inheritance (Chavatte-Palmer et al. 2016; Champroux et al. 2018).

Thanks to the development of technologies offering quantitative and base-level resolution methylome maps, DNA methylation at specific CpG dinucleotides has been associated with a variety of diseases or phenotypes in humans (Teschendorff and Relton 2018). Likewise, a large proportion of the epigenetic studies performed on sperm cells has been focused on DNA methylation at all (whole genome bisulfite sequencing; WGBS) or sampled CpG sites of the genome (reduced representation bisulfite sequencing, RRBS; or Illumina Infinium BeadChips for humans) to identify biomarkers for fertility or exposure to environmental disturbances that could be transmitted to the next generation. The methylation patterns associated with the epiblast origin of primordial germ cells (PGCs) are erased while PGCs migrate and colonise the gonad, and global DNA methylation reaches a minimum at E13.5 in the mouse (Popp et al. 2010; Seisenberger et al. 2012; von Meyenn and Reik 2015; Tang et al. 2016). Gametic DNA methylation is then progressively established through the action of the de novo methyltransferases DNMT3A, DNMT3B, and their cofactor DNMT3L. At birth, an important part of DNA methylation is in place (Kato et al. 2007; Seisenberger et al. 2013) and must be maintained by DNMT1 throughout adulthood across the different stages of spermatogenesis. The reprogramming of DNA methylation is highly sensitive to environmental factors (Ly et al. 2015; Wu et al. 2015; Donkin and Barres 2018). The maternal environment during gestation affects DNA methylation erasure and de novo DNA methylation, whereas the environment during spermatogenesis may influence the maintenance of DNA methylation in crosstalk with other epigenetic marks, throughout mitosis, meiosis, and histone-to-protamine transition. Any exposure to a deleterious environment may change DNA methylation patterns in male germ cells and interfere with differentiation into functional sperm cells, ultimately impairing male fertility. In line with this, numerous studies in humans and model species have reported changes to sperm DNA methylation in the context of spermatogenesis defects, male infertility, and exposure to toxins or nutritional challenges (Boissonnas et al. 2013; Schagdarsurengin and Steger 2016; McSwiggin and O'Doherty 2018).

By contrast, and although cattle are an important agronomic species, little is known about the bull sperm epigenome, despite the fact that bull semen is widely employed for artificial insemination (AI). Unsuccessful AI can result in direct and indirect economic losses, as well as a waste of resources through extended calving intervals, increased culling rates and lower rates of genetic gain. The success of AI is, therefore, a crucial issue for both breeders and the AI industry, but it is difficult to predict accurately because many factors, including epigenetic features, may contribute to variation in reproductive efficiency (Fair and Lonergan 2018). The selection of AI bulls is based on their genetic merit, and they are usually obtained from the crossbreeding of high breeding value sires and high-producing dairy cows. During periconception period and foetal life they are, therefore, more likely to be exposed to an adverse in utero environment. Indeed, the negative energy balance experienced by the lactating mother at the start of gestation affects the oocyte epigenome (O'Doherty et al. 2014); and altered DNA methylation patterns have been reported in bovine embryos exposed to various metabolic stresses (Desmet et al. 2016; Laskowski et al. 2018; Tremblay et al. 2018). It has also been demonstrated that milk production is reduced in the female offspring of high genetic merit cows when conception was coincident to lactation (Gonzalez-Recio et al. 2012), suggesting long-term effects of maternal metabolism on the

performances of next generation. Although this issue has not been addressed in the male offspring, epigenetic changes in spermatozoa in response to maternal metabolism have been reported in mice and humans; suggesting that a similar phenomenon may occur in the gametes of bulls conceived from lactating elite cows (Chavatte-Palmer et al. 2016). Furthermore, current selection and breeding practices tend to reduce the generation interval to accelerate genetic gain. Depending on local regulations, these practices may involve hormonal treatments of the mothers, embryo manipulations, the hastened growth and puberty of male calves, and the early collection of semen to produce the next generation of AI bulls, and some of these factors may have a long-term impact on sperm epigenome (Urrego et al. 2014). Finally, bull semen undergoes several technological treatments before its use for AI, including dilution with an extender, packaging into straws, cryopreservation, and thawing. According to studies conducted in other species, these steps may also affect the chromatin structure (Aurich et al. 2016).

In the current context of environmental instability and changes to practices in the cattle industry, more information on the epigenetic features transferred to the embryo alongside the paternal genetic heritage is necessary to prevent adverse effects on the offspring. The purpose of this review is, therefore, to synthesize recent work on the bull sperm methylome in light of knowledge accumulated in other species. We will address sperm-specific DNA methylation features relative to other cell types, interindividual variability and intra-individual plasticity, as well as potential transmission to the embryo. We will not address the reprogramming of DNA methylation in the male germline because to our knowledge, no data are available in the literature for the bovine species.

Hypomethylated Genomic Features in Spermatozoa: Species Peculiarities and Evolutionary Impacts

Genome-wide studies of DNA methylation have long centred on genes or imprinted loci because of the availability of microarray technologies such as methylated DNA immunoprecipitation (MeDIP) followed by hybridisation on a DNA chip (Weber et al. 2007), Illumina Infinium BeadChips for humans (Krausz et al. 2012), and the EmbryoGENE DNA methylation analysis (EDMA) platform for bovine species (Shojaei Saadi et al. 2014). Given the unique structure of the sperm chromatin, to which nongenic regions make a considerable contribution, and the absence of detectable transcriptional activity, knowledge on the sperm methylome has recently evolved in parallel with the development of technologies interrogating CpG sites without any a priori. We have, therefore, summarised the studies on the DNA methylation status of promoters and genes in Table 1 to mainly focus this section on repetitive elements, a substantial part of which exhibit sperm-specific DNA methylation status. The overall differences in DNA methylation content between sperm and somatic cells at different functional elements of the genome and according to CpG density are summarised in Figs. 1A and 1B and are detailed below.

Using WGBS, Molaro et al. (2011) were the first to establish the genome-wide undermethylation of pericentromeric satellites in human and chimp sperm cells compared with human embryonic stem (ES) cells. According to a recent study, the degree of satellite undermethylation in sperm differs in the mouse and human, and it may depend on the different satellite subfamilies present in each species and their relative abundance (Li et al. 2018). Retrotransposons display variable methylation attributes in sperm according to the subfamily to which they belong and the density of CpGs and the species, and hypomethylated promoters are found in larger numbers in sperm than in ES cells (Molaro et al. 2011). This is consistent with the findings of another study which reports a lower methylation percentage among promoters in sperm compared with ES cells (Popp et al. 2010). More frequent and wider hypomethylated regions (HMRs) have been observed in the sperm than in the somatic cells of seven species (humans and three non-human primates, mouse, rat, and dog) (Qu et al. 2018). Furthermore, in all seven species, sperm HMRs overlap promoters, transposons, and pericentromeric satellites more frequently than HMRs from other cells. Although the hypomethylation of promoters in sperm is conserved between species (although differences are noted in the size of overlapping HMRs), an important divergence is observed for HMRs located outside the promoters. Ancestral HMRs reconstructed using a phylo-epigenetic model appear to be wider than more recently acquired HMRs. This means that DNA methylation tends to be lost at the periphery of sperm HMRs, suggesting an evolutionary expansion of hypomethylated sperm loci in mammalian genomes (Qu et al. 2018).

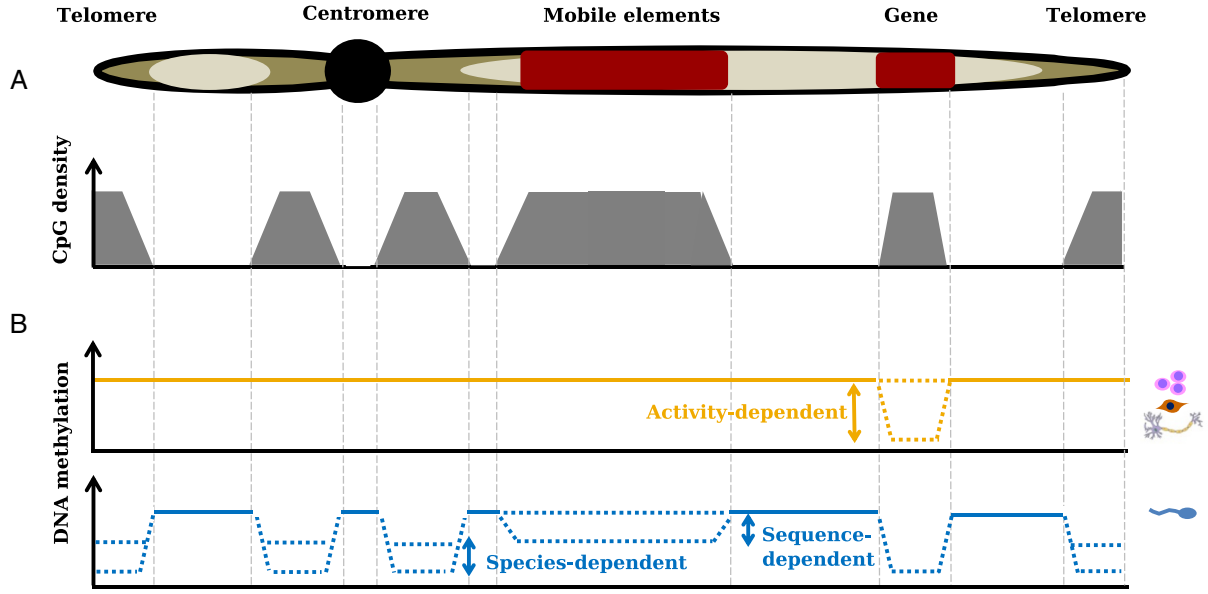
Using RRBS in cattle, we reported that compared with somatic cells, bull spermatozoa are both hypomethylated at satellites and also at rDNA repeats encoding ribosomal RNAs. Strikingly, in a luminometric DNA methylation assay, bull sperm cells displayed the lowest global DNA methylation at CCGG sites when compared with the sperm of rams, goats, boars, stallions, mice, and humans. In parallel, the bovine genome contains the highest proportion of CCGG sites located in satellites, suggesting that the lower global DNA methylation of bull sperm is due to both the abundance of satellites in the genome and their hypomethylated status in sperm (Perrier et al. 2018). At a genome-wide scale, this supports the conclusions of an earlier report which stated that most satellites are largely undermethylated in the sperm of cattle, whereas they are only slightly undermethylated in mouse sperm when compared with somatic tissues (Adams et al. 1983). Using WGBS in

Table 1. DNA methylation status and functions of gene elements in sperm cells from different species.

Species	Genome-wide DNA methylation assay	Definition of DNA methylation status	Enriched genomic or chromatin features	Enriched functions	References
Human	MeDIP and microarray hybridisation	Promoters undermethylated in sperm compared with somatic cells	CpG-rich	Germline-specific genes	Weber et al. 2007
Human and chimp	WGBS	Promoters with sperm-specific HMRs, as compared with ES cells	Nucleosomes and H3K4me3	Homophilic cell adhesion, sexual reproduction, biological adhesion, piRNA metabolic process, meiosis	Molaro et al. 2011; Nucleosome-enriched regions from Hammoud et al. (2009)
Human	Infinium 450K	Genes with hypomethylated CpGs (<20%)	Nucleosomes	Metabolic and biosynthetic processes, developmental genes, gamete generation and piRNA pathway	Krausz et al. 2012; Nucleosome-enriched regions from Hammoud et al. (2009)
Mouse	MethylC-Seq (modified WGBS)	Promoters undermethylated in sperm compared with oocytes or embryos	NA	Spermatogenesis, chromosome organization and meiosis, gene silencing	Wang et al. 2014
Human	MethylC-Seq (modified WGBS)	Promoters undermethylated in sperm compared with oocytes or 6 wk embryos, respectively	NA	Nervous system and embryonic development; or gamete development, meiosis and piRNA metabolism, respectively	Li et al. 2018
Human, chimp, gorilla, rhesus, dog, rat and mouse	WGBS	Promoters with conserved HMRs in sperm	H3K4me3, H3K27me3	Developmental process, signalling, regulation of multicellular organismal process	Qu et al. 2018; Nucleosome-enriched regions from Lesch et al. (2016)
Cattle	WGBS	Genes with sperm-specific HMRs, as compared with somatic cells	Nucleosomes	DNA methylation involved in gamete generation, piRNA metabolic process, gene silencing by RNA, male meiosis	Zhou et al. 2018; Nucleosome-enriched regions from Samans et al. (2014)
Cattle	MeDIP and microarray hybridization, RRBS	Promoters and genes undermethylated in sperm compared with somatic cells	NA	Sexual reproduction, fertilisation and RNA transport (MeDIP); sexual reproduction, cell adhesion, the regulation of signalling and cell migration (RRBS)	Perrier et al. 2018
Cattle	RRBS	Genes with differential methylation between sperm and oocytes	NA	Developmental growth, signalling pathways, cell fate commitment, regulation of cell shape, regulation of transcription	Jiang et al. 2018

Note: NA, not assessed; HMR, hypomethylated region; MeDIP, methylated DNA immunoprecipitation; WBGS, whole genome bisulfite sequencing; RRBS, reduced representation bisulfite sequencing; ES, embryonic stem; piRNA, PIWI-interacting RNA.

Fig. 1. Relationships between genomic location, CpG density, and DNA methylation in somatic cells and sperm cells. (A) Genome-wide CpG density and (B) DNA methylation for different genomic features are represented schematically along a single chromosome. In (B), the full lines illustrate global hypermethylation of the genome, which appears to be relatively conserved between cell types and species, whereas DNA methylation variations are shown as dashed lines [in yellow (top), somatic cells; in blue (bottom), sperm cells]. The representation of DNA methylation at subtelomeric regions in sperm, on which we did not find any reports in our survey of the literature, is an extrapolation from the data at pericentromeric regions.[Colour online.]



different cattle cell types, another team reported that sperm-specific HMRs are enriched for satellites (and especially putative pericentromeric satellites depending on their density and genomic localisation); they also contain young transposable elements from the short interspersed element (SINE) family, suggesting individual transposons that escaped PIWI-mediated silencing in the bovine male germ cells (Zhou et al. 2018).

The silencing of pericentromeric satellites by the formation of constitutive heterochromatin is essential to maintaining genome stability and preventing both recombination and inappropriate chromosome segregation. In somatic cells, pericentromeric heterochromatin formation is primarily achieved through DNA methylation and the recruitment of heterochromatin protein HP1 to H3K9me3 histone modification (Nishibuchi and Dejardin 2017). In sperm cells, histone retention appears to particularly affect CpGs that lack methylation in humans (Krausz et al. 2012) and the mouse (Erkek et al. 2013); however, the composition of the pericentromeric chromatin has been a matter of debate because of the different methodologies used to extract histones from the tightly compacted chromatin (Saitou and Kurimoto 2014) and mapping artefacts due to repetitive elements that are potentially generated during sequence analysis (Dansranjavin and Schagdarsurengin 2016; Royo et al. 2016). Using a novel method to prepare sperm chromatin, the association of satellites with nucleosomes bearing the H3K9me3 modification was recently confirmed in mouse sperm. In addition, histones are detected in distal intergenic regions and CpG-rich promoters and those of developmentally important genes associated with H3K4me3 (Yamaguchi et al. 2018). This approach has not yet been applied to bull sperm; two studies have reported partially concordant results regarding the genome-wide localisation of nucleosomes which appear to overlap the two types of repetitive elements that we found to be heavily hypomethylated in bull sperm: satellites (Samans et al. 2014; Sillaste et al. 2017) and rDNA repeats (Sillaste et al. 2017).

Data obtained in the mouse suggest that DNA methylation remains relatively stable in the male germline during adulthood (Oakes et al. 2007; Hammoud et al. 2014). The sperm methylome, therefore, grossly reflects DNA methylation in the germline during spermatogenesis when meiotic recombination takes place and neomutations transmitted to the next generations are more likely to occur. In this way, the DNA methylation landscape in spermatozoa can be regarded as a driver of genetic variation across evolution and individuals. Indeed, methylcytosines (5mC) are prone to spontaneous deamination to thymine, resulting in a CpG-to-TpG mutation rate that is 14-fold higher than seen in other dinucleotides (Hodgkinson and Eyre-Walker 2011). During evolution, the mutagenic nature of methylcytosines has contributed to sequence divergence across primate genomes (Hernando-Herraez et al. 2015). In sperm cells in particular, the divergence of DNA methylation is associated with sequence divergence in seven species (humans and three non-human primates, mouse, rat,

and dog), indicating a potential coevolution of the genome and sperm methylome (Qu et al. 2018). In humans, variations affecting the germline methylome are correlated with sequence variations (Mugal and Ellegren 2011), suggesting a link between DNA methylation in sperm and interindividual genetic diversity in a given species. Furthermore, structural variations and human-specific evolutionary rearrangements are enriched in HMRs of human sperm (Li et al. 2012). Another interesting observation in humans is that the meiotic recombination rate is reduced between regulatory elements and their target genes; these are, therefore, transmitted as single units of inheritance. These "recombination rate valleys" are highly methylated in germ cells, pointing to DNA methylation at the crossing-over stage as a potential mechanism in reducing recombination events (Liu et al. 2017). In the male germline of the mouse, sex-biased recombination hotspots are marked by distinct DNA methylation patterns according to their preferential usage in males or females, which could thus modulate the affinity for its binding site of the determinant of hotspot localisation, PRDM9. Interestingly, sex-biased usage tends to disappear in *Dnmt3l*−/− mutants, thus demonstrating the role of DNA methylation in mediating differences between males and females in meiotic recombination (Brick et al. 2018). Strikingly, in cattle, higher recombination rates are observed in males than in females (Kadri et al. 2016), which echoes the undermethylation of bull sperm relative to other species on which we have reported. In light of the data obtained in other species, it would, therefore, be interesting to investigate the relationships between hypomethylated sequences and meiotic recombination in the cattle male germline, as well as between germline DNA methylation and sequence variation in the offspring.

Inter- and Intra-individual Variations in the Sperm DNA Methylome of Cattle

In humans, numerous studies have reported altered DNA methylation patterns in the context of decreased sperm count and motility and morphological defects (Boissonnas et al. 2010; Laqqan et al. 2017), in vitro fertilisation (IVF) outcomes (Aston et al. 2015; Camprubi et al. 2016) or unexplained infertility (Urdinguio et al. 2015). Given the negative impact that subfertile AI bulls can have on the sustainability of the dairy sector, it is not surprising that work on interindividual variations affecting the sperm DNA methylome in cattle has been undertaken to pinpoint the methylation changes associated with altered semen parameters, decreased field fertility, or IVF outcomes. Although most of these studies have focused on individual candidate loci (germline genes or imprinted loci; Table 2), two of them described alterations at the whole genome scale. Based on the conception rate after more than 50 inseminations, and the percentage of IVF embryos that developed up to the blastocyst stage, both high fertility and subfertile buffalo bulls were selected ($n = 3$ per group), and their sperm DNA methylation patterns were compared using MeDIP and hybridisation on a custom-made buffalo-specific CpG island/promoter microarray (2967 unique genes represented). The fertility-related differentially methylated regions (DMRs) targeted genes involved in regulating transcription and vesicle-mediated transport, as well as 13 genes important to spermatogenesis (including *CYP26B1* that metabolises retinoic acid, a key player in spermatogenesis), sperm maturation and capacitation, and embryo development (such as the pluripotency gene *POU5F1*) (Verma et al. 2014). The second study compared the sperm DNA methylome of bulls with contrasted sire conception rates after more than 300 AI records (three pools of two fertile bulls vs. three pools of two subfertile bulls), using a methyl-binding domain capture assay combined with next generation sequencing (MBD sequencing). Most of the 76 fertility-related DMRs identified were less methylated in subfertile bulls and located in genes with functional roles in spermatogenesis and fertilisation; i.e., *MMP2* and *KCK4* (encoding, respectively, a matrix metalloprotease and a potassium channel and both localized on the acrosome) and *CTCF* (which encodes a DNA-binding protein with functions in the delineation of active/inactive chromatin loops). Although these semen samples were identical in terms of IVF outcomes, the blastocysts obtained using fertile and subfertile groups exhibited differences in their transcriptomes; however, no correlation with DNA methylation defects was described (Kropp et al. 2017). The authors also reported that their findings did not overlap with those of Verma et al. (2014) or with studies conducted in humans. The results of this study suggest that the sperm methylome might impact reproductive potential by decreasing the developmental competence of early embryos at the molecular level, leading to potential adverse effects on the development at later stages.

Variations affecting the sperm DNA methylome as a function of age have been demonstrated in men and hypothesised as contributing to the reduced fecundity associated with advanced paternal age and an increased incidence of neuropsychiatric disorders in offspring (Jenkins et al. 2018). In the mouse, sperm cells from elderly fathers display DNA methylation signatures of age in genes relevant to senescence, some of which are transmitted to the offspring and potentially contribute to their shorter lifespan and exacerbate aging-associated pathologies (Xie et al. 2018). In the dairy industry, the use of semen collected from very young bulls enables both reduced rearing costs and increased genetic gain, but this practice is not without significance because intra-individual variations affecting DNA methylation in semen collected at different ages have recently been reported (Takeda et al. 2017, 2019; Lambert et al. 2018). In the earlier study (Takeda et al. 2017), semen samples displaying contrasted quality parameters, and IVF

Table 2. DNA methylation changes in sperm associated with altered bull fertility at individual loci.

Locus	Phenotypes related to fertility	Experimental design	DNA methylation assay	Results	Reference
IGF2-H19 imprinted locus	CR	5 high CR vs. 5 low CR (crossbred Karan-Fries bulls)	Bisulfite, PCR, cloning, sequencing	Lower DNA methylation at one CTCF-binding site in high CR group	Jena et al. 2014
SPEF2 gene promoter	SQ: sperm motility and concentration, abnormal sperm percentage	2 high SQ vs. 2 low SQ (full-sib Holstein bulls)	Bisulfite, PCR, cloning, sequencing	No difference	Guo et al. 2014*a*
HIBADH gene promoter	SQ: sperm motility	3 high SQ vs. 3 low SQ (Holstein bulls)	Bisulfite, PCR, cloning, sequencing	No difference	Zhang et al. 2015
DAZL gene promoter	SQ: sperm motility	5 high SQ vs. 5 medium SQ vs. 5 low SQ (crossbred Frieswal bulls)	Bisulfite, PCR, cloning, sequencing	Tendency to be slightly more methylated in low SQ group	Sarova et al. 2018
Bvh gene promoter	SQ: sperm motility	5 high SQ vs. 5 low SQ (crossbred Frieswal bulls)	Bisulfite, PCR, cloning, sequencing	Tendency to be slightly more methylated in low SQ group	Ahlawat et al. 2019
GNAS and *XIST* imprinted loci	SQ: sperm motility and kinetics, sperm DNA fragmentation; IVF outcomes: cleavage and eight-cell embryo rates	Sperm samples exposed to various concentrations of CPF	Bisulfite, pyrosequencing	Increased DNA methylation at *XIST* in the presence of highest dose of CPF; no difference for *GNAS*	Pallotta et al. 2019

Note: CR, conception rate; SQ, semen quality; IVF, in vitro fertilization; CPF, chlorpyrifos; PCR, polymerase chain reaction.

success rates were obtained from three sexually mature Japanese Black bulls of various ages and one Holstein bull during the peripubertal period (10–10.5 mo) and after puberty (15–25 mo). Sperm methylomes were generated using the Illumina Infinium HM450 BeadChip for humans, together with methylomes from cattle somatic tissues. Although only 37 224 individual CpGs could be used (representing 7.7% of all CpGs covered by the human microarray), this was sufficient to clearly distinguish somatic tissues from sperm, thereby confirming the importance of epigenetic marks to specifying the germline and soma. The peripubertal semen samples clustered apart from the others, suggesting that at the 37 224 CpGs analysed the difference between peripubertal and mature semen samples was more pronounced than any differences related to the breed or IVF outcomes. Although no conclusions could be reached regarding IVF, one CpG located in the mitochondrial glutamate carrier 1 gene displayed a marked increase in DNA methylation with age. Interestingly, the methylation status of this individual CpG does not stabilise after puberty but continues to increase in older bulls. In a recent study using semen samples collected between 10 and 162 mo, the same team has identified seven additional CpGs showing a similar gain of DNA methylation with age, which were demethylated to the same extent after fertilisation independently of the paternal age (Takeda et al. 2019). The continuous increase in DNA methylation at these few CpGs contrasted with the results of another study which demonstrated important plasticity of the sperm DNA methylome, essentially during the peripubertal period (Lambert et al. 2018). Indeed, semen samples collected from the same bulls at 10, 12, and 16 mo of age and analysed using the EDMA platform did not display any significant differences in DNA methylation between 12 and 16 mo, whereas many DMRs can distinguish the 10 and 16 mo stages (Lambert et al. 2018). DMRs target genes involved in PKA signalling, sperm motility, calcium signalling, protein G signalling, and androgen signalling, all pathways that are relevant to sperm functions. Interestingly, the majority of these DMRs are more methylated in semen samples collected at 16 mo, which may reflect the undermethylation of genomic regions associated with incomplete DNA packaging at 10 mo. Overall, the results reported in cattle are consistent with those of human studies demonstrating a global gain of DNA methylation in the sperm of elderly patients, contrasting with the global age-related loss observed in somatic tissues (Jenkins et al. 2018).

Although the physiological impact of age seems to consistently affect the sperm DNA methylome in several species, DNA methylation signatures of fertility seem to have varied considerably between studies. Differences in the technologies used to obtain DNA methylation profiles may partly account for these variations. For instance, the MeDIP-chip used by Verma et al. (2014) and MBD sequencing used by Kropp et al. (2017) differ in terms of genome coverage, resolution, and data analysis. Another important limitation that may explain some inconsistencies between findings is the small number of individuals in each fertility group (in our survey of the literature, there were six at most for cattle). Fertility-related traits are markedly influenced by the metabolic and physiological status of the animal, as well as by the surrounding environment. It is, therefore, possible that for each animal or study, the DNA methylation signatures reported actually reflected quite heterogeneous situations. The final point is that AI bulls are primarily selected based on their genotype. Definitely, sperm methylome is not entirely controlled by genetic mechanisms, as clearly demonstrated by DNA methylation differences in the sperm of monozygotic twin bulls using the EDMA platform (Shojaei Saadi et al. 2017). Indeed, 580 probes were commonly divergent between the monozygotic twins for four pairs of bulls. Because genetic factors could be excluded as sources of epigenetic variability, the authors propose that these divergent DNA methylation patterns were underlain by both environmental and stochastic factors. However, genetic diversity between bulls probably accounts for a part of the interindividual variations observed in DNA methylation patterns. Indeed, the methylome is shaped by the underlying genomic sequence in two different ways: (*i*) CpG sites can be directly disrupted by single-nucleotide polymorphisms (SNPs) targeting either the C or G; (*ii*) the methylation status of individual or clustered CpG sites may be influenced genetically by quantitative trait loci (methylation QTLs), as demonstrated in humans (Do et al. 2017). In our view, taking account of these genetic–epigenetic interactions is crucial when studying complex traits such as male fertility. On the one hand, genetically driven epigenetic variations can be regarded as a confounding factor that complicates the interpretation of fertility-related methylation marks. On the other hand, genetic factors also contribute to male-fertility-related traits in cattle (Taylor et al. 2018), and SNPs in *DNMT* genes have been associated with idiopathic male infertility, as well as abnormal semen parameters, in humans (Tang et al. 2017). Taking account of synergistic interactions between sequence polymorphism and DNA methylation may, therefore, shed light on the elaboration of this multifactorial phenotype.

Postfertilisation Reprogramming of the Sperm DNA Methylome

We now turn to the reprogramming dynamics of the repetitive elements, which we found to be hypomethylated in bull sperm. Satellites remain hypomethylated after fertilisation in cattle blastocysts (Kaneda et al. 2011) and throughout mouse preimplantation development (Yamagata et al. 2007). By contrast, embryos resulting from somatic cell nuclear transfer and having impaired development potential display somatic-like

hypermethylated satellites in the mouse (Yamagata et al. 2007) and cattle (Kang et al. 2005; Yamanaka et al. 2011*a*, 2011*b*). The significance of satellite hypomethylation in sperm may be related to the transcriptional burst that arises from paternal satellites in early mouse development, which is necessary for normal heterochromatin formation in embryos and to ensure developmental progression (Probst et al. 2010). In line with this hypothesis, targeted DNA methylation at major satellites using epigenome editing in mouse embryos has been shown to affect heterochromatin formation at the two-cell stage but does not hamper chromosome segregation (Yamazaki et al. 2017). As for rDNA repeats, the hypomethylation that we reported in bull sperm was also observed in mouse sperm, as well as in mouse oocytes and embryos. The progressive establishment of DNA methylation at rDNA repeats then occur post implantation in the different cell lineages, with the exception of PGCs where they seem to escape genome-wide remethylation (Furuta and Nakamura 2017). The average methylation of rDNA in mouse sperm is highly variable across individuals. The level of methylation was shown not to be related to dietary variations but correlated to the rDNA copy number in each mouse sire, and it was transmitted to the somatic cells of offspring (Shea et al. 2015). This observation suggests that DNA methylation patterns underpinned by sequence variations are inherited by the next generation independently of reprogramming events.

Most studies on the reprogramming dynamics of the sperm DNA methylome tended to focus on sequences that are highly methylated in sperm. For instance, a kinetic study of postfertilisation DNA methylation reprogramming in the mouse using RRBS revealed that most regions displaying differential methylation between sperm and oocytes were more methylated in sperm and largely demethylated as early as the zygotic stage (Smith et al. 2012), in line with the findings of immunochemical labelling studies of single-cell embryos which suggested active demethylation of the paternal pronucleus (Mayer et al. 2000; Santos et al. 2002). These regions are enriched in retrotransposons such as long interspersed elements (LINEs), most long terminal repeat elements (LTRs), and to a lesser extent, SINEs (which are only partially methylated in sperm). Intracisternal A-particles are an exception in that they are highly methylated in mouse sperm and resist demethylation in the embryo (Smith et al. 2012). MethylC-seq, a technique very similar to WGBS, highlighted the fact that 6.8% of CpG sites in the mouse genome display an unchanged DNA methylation status in the sperm, inner cell mass (ICM), and E7.5 embryos. Although most of these sites are hypomethylated (<20% methylation) across all three stages, some CpGs with stable DNA methylation (mainly located in intergenic regions, LINE and LTR repeats), are hypermethylated (>80%), thus demonstrating the resistance of these sequences to reprogramming (Wang et al. 2014). These two studies, therefore, highlighted the heterogeneous behaviour of retrotransposons, with specific subfamilies escaping demethylation, but the bulk of retrotransposons that are methylated in sperm undergoing extensive reprogramming.

In humans, the kinetics of reprogramming are similar to those described in the mouse, but differences have been observed in the methylation percentages at each developmental stage and in the DNA methylation patterns of individual transposable elements. Indeed, evolutionarily young transposable elements and LTRs that contain variable number tandem repeats were seen to retain a higher degree of remnant methylation (Guo et al. 2014*b*; Okae et al. 2014). Moreover, a postbisulfite adapter tagging approach interrogating about 10 million CpGs at the single-cell level revealed a complex pattern of reprogramming, involving successive waves of demethylation and de novo methylation in human preimplantation embryos. The first wave of demethylation principally targets intragenic and enhancer elements of the paternal genome and occurs during the first 12 h after fertilisation. Subsequent waves of demethylation (late zygote to two-cell stages and eight-cell to morula stages) affect introns and SINEs and evolutionarily young transposable elements. The paternal genome is remethylated from the early to mid-pronuclear stages, whereas the second wave of remethylation occurs from the four-cell to the eight-cell stages. De novo DNA methylation focuses on SINEs, LINEs, and LTRs and particularly on evolutionarily younger subfamilies, suggesting its essential function in repressing the mobilisation of these elements (Zhu et al. 2018). One possible explanation for these successive waves is that proper embryo development requires a subtle balance between methylation erasure, the expression of endogenous retroviruses and the activation of specific regulatory elements on the one hand, and de novo DNA methylation and epigenetic silencing of these elements on the other.

In cattle, methylomes from sperm cells and blastocysts were compared using the EDMA platform, and this work revealed more abundant DNA methylation in sperm regarding most genomic features covered by the platform. High-density CpG islands and low complexity repetitive elements displayed the opposite behaviour, tending to be more methylated in blastocysts (Shojaei Saadi et al. 2014). A recent study on the reprogramming dynamics of gamete methylomes throughout preimplantation development confirms the hypomethylated status of the blastocyst stage using RRBS. The successive waves of demethylation and de novo methylation seen in human preimplantation embryos has also been observed in cattle and coincides with the timing of developmental events such as minor embryonic genome activation (EGA) between zygote and two-cell stages, major EGA at the eight-cell stage, and the differentiation of trophectoderm and ICM between the morula and blastocyst stages.

These events are associated with reductions in DNA methylation levels and the expression of specific genes, including those encoding de novo DNA methyltransferases; this may explain why they are followed by waves of remethylation (Jiang et al. 2018). It should be noted that during this study the global level of methylation in sperm determined by RRBS was grossly similar to that of in vivo matured oocytes (~40%), which differed from findings in the mouse (Smith et al. 2012), but was consistent with the lower global methylation level of bull sperm we reported when compared with sperm from other species (Perrier et al. 2018).

The reprogramming dynamics of loci with contrasted DNA methylation patterns in sperm and oocytes has also attracted attention in several species. Loci hypermethylated in oocytes (>75%–80%) and hypomethylated in sperm (<20%–25%) are rich in CpG sites and mostly span intragenic regions, whereas loci that are hypermethylated in sperm and hypomethylated in oocytes are less dense in CpG sites and comprise more intergenic regions (Smallwood et al. 2011; Kobayashi et al. 2012) and tissue-specific enhancers (Guo et al. 2014*b*; Zhu et al. 2018). Interestingly, among these loci with contrasted DNA methylation patterns in gametes, those still methylated at the blastocyst stage are mostly hypermethylated in oocytes (Smallwood et al. 2011; Kobayashi et al. 2012; Guo et al. 2014*b*). In cattle, regions that are more methylated in sperm than in oocytes are enriched in LTR elements and quickly lose their methylation by the two-cell stage; in contrast, regions that are more methylated in oocytes than in sperm preferentially overlap exons and CGIs and gradually demethylate across cleavage stages (Jiang et al. 2018). In the human species, single-cell methylome analyses have confirmed the lower methylation level of the paternal genome throughout preimplantation development from the late zygote stage (Zhu et al. 2018).

Taken together, studies on postfertilisation reprogramming in several mammals (including cattle) have suggested a limited inheritance of hypermethylated features in the paternal genome, whereas hypomethylated features seem to be transmitted at least until the blastocyst stage. Global reprogramming of the paternal methylome may result from a dynamic interplay between (*i*) active processes involving 10–11 translocation (TET) dioxygenases to convert 5mC into oxidised derivatives (5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine; 5hmC, 5fC, and 5caC) that are diluted during replication or removed through DNA repair mechanisms; (*ii*) the passive and replication-dependent dilution of 5mC, through a low level of expression and the exclusion of DNMT1 from nuclei; and (*iii*) de novo DNA methylation catalysed by DNA methyltransferases inherited from the oocyte (DNMT3A) or expressed after fertilisation (DNMT3B) (Seah and Messerschmidt 2018). Although many studies have reported the essential role of the Stella/PGC7/DPPA3 maternal factor in protecting the maternal pronucleus from active demethylation, including in cattle embryos (Bakhtari and Ross 2014), a 5hmC signal has been observed in both pronuclei by the immunochemical labelling of mouse zygotes. However, the dynamics governing the onset of the 5hmC signal are not fully correlated with a reduction in 5mC and are dependent upon de novo methylation, thus supporting additional functions for hydroxymethylation in embryos, as well as active demethylation (Salvaing et al. 2012; Amouroux et al. 2016). Active demethylation targeting both parental genomes is also suggested by the presence of 5hmC, 5fC, and 5caC in the paternal and maternal genomes of mouse two-cell embryos (Wang et al. 2014), and it has recently been reported in bovine zygotes (Wyck et al. 2018). Taken together, these studies support the hypothesis that both active and passive demethylation and de novo DNA methylation may also account for reprogramming of the maternal methylome, although the magnitude and dynamics differ from those seen in the paternal methylome. It has been proposed that Mettl23 (an arginine methyltransferase that catalyses histone modification H3R17me2a) drives the TET enzyme towards specific regions of the paternal genome in the mouse. Mettl23 and H3R17me2a are both necessary for H3.3 deposition and de novo nucleosome assembly after the removal of protamines from the paternal pronucleus. Mettl23 tethers a complex containing TET3, where H3R17me2a is located on the paternal genome, driving active demethylation at sites triggered by the replacement of protamines with H3.3. Although Mettl23 and H3R17me2a are symmetrically distributed in both the paternal and maternal pronuclei, the presence of Stella/PGC7/DPPA3 in the maternal pronucleus presumably protects the maternal genome from this process (Hatanaka et al. 2017). A question that remains unanswered is the fate of the nucleosomes inherited from sperm. Are they subjected to replacement by maternal histones, and the associated DNA triggered by the complex containing Mettl23 and TET? An alternative hypothesis would be that these genome regions remain attached to paternal histones during the early stages of embryo development, thereby escaping the Mettl23/TET-driven active demethylation.

Interestingly, the exposure of sperm cells to oxidative stress alters the dynamics of postfertilisation reprogramming in cattle. Sperm cells treated with hydrogen peroxide under controlled conditions carry DNA damage and display a marked reduction of motility, although they are still able to fertilise oocytes. However, the blastocyst rate is dramatically reduced, with major developmental arrest occurring at the two- to four-cell stages. The base excision repair machinery is recruited to the paternal pronucleus, and achievement of the first cell cycle indicates that at least some of the oxidative lesions have been successfully repaired. Because zygotes obtained with treated sperm retain more DNA methylation at the paternal pronucleus, the authors of the study

proposed that oxidation-induced DNA damage and active DNA demethylation through the replacement of 5mC or derivatives with unmodified cytosine competed with each other for the same DNA repair mechanism (Wyck et al. 2018). Another adverse environmental factor that has been shown to interfere with reprogramming of the paternal methylome in cattle embryos is the exposure of male germ cells to heat stress during spermatogenesis. Spermatozoa from heat-stressed bulls also exhibit defects in the replacement of histones by protamines, altered chromatin conformation and reduced fertilisation rates (Rahman et al. 2018).

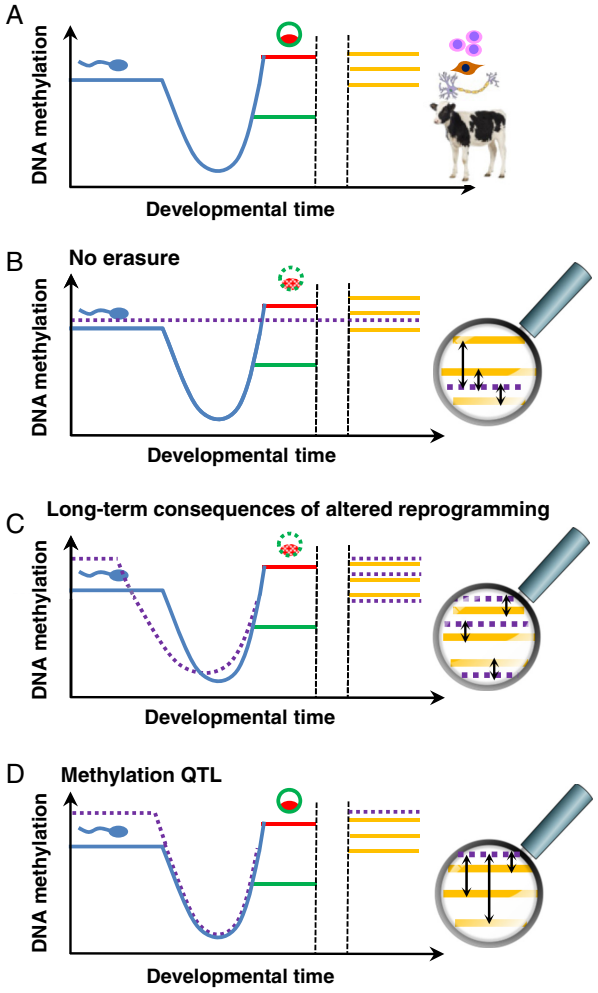
Is the Sperm DNA Methylome a Molecular Support for the Intergenerational Transmission of Phenotypes?

Individual loci in the paternal genome are specifically targeted by the DNA methylation maintenance machinery and are faithfully maintained throughout postfertilisation reprogramming. This phenomenon has been described extensively with respect to imprinted loci. Residual DNMT1 is attracted at these loci by the KRAB domain-interacting protein TRIM28, which is indirectly recruited to the methylated allele through its interaction with ZFP57, a methylated DNA-binding KRAB zinc finger protein (Seah and Messerschmidt 2018). Mechanisms involving TRIM28 and KRAB zinc finger proteins have also been suggested for the epigenetic silencing of endogenous retroviruses during reprogramming, which may explain why subfamilies of transposable elements quickly regain methylation after a demethylation wave. As well as paternally imprinted loci and endogenous retroviruses, individual genes in the paternal genome resist demethylation; for example, the Y chromosome gene *Rbmy1a1* in the mouse. The zygotic expression of this gene is repressed by sperm-inherited DNA methylation of its promoter, which has been demonstrated by the loss of methylation, ectopic expression, and periimplantation lethality of male embryos in the absence of maternal TRIM28 (Sampath Kumar et al. 2017). In the near future, the catalogue of regions resistant to reprogramming will probably expand with development of the miniaturisation of sequencing technologies and growth in the number of studies on the DNA methylation landscape of embryos. These regions are postulated to provide support for the intergenerational transmission of nongenetic information and may impact the long-term phenotype of offspring in response to environmental changes affecting the epigenome of paternal germ cells (Champroux et al. 2018; Donkin and Barres 2018). To the best of our knowledge, epigenetic inheritance via the paternal route has not so far been reported in cattle, but this would be of considerable interest in the context of animal selection, as suggested previously (Goddard and Whitelaw 2014; Ibeagha-Awemu and Zhao 2015). Beyond epigenetic inheritance, we believe that other molecular mechanisms involving specific DNA methylation signatures in sperm may modulate the phenotype of offspring (Figs. 2A–2D). For instance, aberrant DNA methylation patterns in sperm may alter the dynamics of postfertilisation reprogramming and affect the timing of the activation of developmentally regulated genes. The resulting embryo may carry subtle molecular, morphological, or metabolic defects that drive long-term effects on the phenotype, in line with the theory regarding the developmental origin of health and diseases. Likewise, some DNA methylation signatures in sperm may be inherited by offspring because they are controlled by genetic mechanisms (methylation QTLs). In humans, and depending on the studies, 13.9%–65.7% of DNA methylation variability has been attributed to DNA sequence polymorphism; this genetically controlled variability may represent a confounding factor in epigenome-wide association studies (Lappalainen and Greally 2017). Because both DNA methylation controlled by methylation QTLs and DNA methylation that has not been erased during postfertilisation epigenetic reprogramming are inherited by the next generation, a clearer understanding of the proportion of DNA methylation under genetic control is essential if we are to disentangle these two effects and produce an initial estimate of intergenerational epigenetic inheritance in cattle and its impact on phenotypes.

Summary and Conclusions

The first studies on DNA methylation in sperm cells were performed to describe the dynamics of epigenetic reprogramming, and they reported more methylation in sperm than in oocytes, early embryos, germ cells, and placenta. Their conclusions have sometimes been misinterpreted, leading to the idea that sperm cells are more methylated than any other cell type, which is not true relative to adult somatic cells. Low levels of DNA methylation affecting most gene promoters appear to be a general feature of sperm cells, which has given rise to the hypothesis that promoters in sperm are already epigenetically reprogrammed and resemble those in pluripotent cells (Farthing et al. 2008). By contrast, a broad range of behaviours is observed in nongenic sequences, and especially repetitive elements, which display variable methylation depending on the species and repeat subfamily (Figs. 1A and 1B). These species-specific DNA methylation patterns may be underpinned by variations in the genomic DNA sequence and different epigenetic reprogramming strategies during differentiation of the germline. Conversely, the DNA methylation landscape of the male germline may also be a driver of genetic diversity between both species and individuals. In cattle, humans, and mice, age-dependent intra-individual variations in the sperm DNA methylome have been reported consistently, suggesting an unexpected plasticity of the sperm DNA methylome during adulthood. This raises the possibility that intra-individual variations in semen quality across ejaculates (a problem frequently

Fig. 2. Hypothetical mechanisms for modulation of the offspring methylome and phenotype by specific DNA methylation signatures in sperm. (A) Following the genome-wide erasure of paternal methylation marks (in blue), DNA methylation is differentially established in embryonic (red) and extraembryonic (green) lineages, and subsequently in the different cell types contributing to the whole organism (yellow). (B–D) The dashed violet line indicates the behaviour of specific DNA methylation signatures that escape the global scheme depicted in (A). The magnifying glasses represent adjustments to the offspring methylomes as compared with the situation in (A). (B) Specific DNA methylation signatures resisting reprogramming may be conserved in the offspring and lead to methylation differences of varying magnitude depending on the cell type. The phenotype of each cell type would, therefore, be differentially impacted as a result of these methylation differences. (C) Instead of resisting reprogramming, aberrant DNA methylation signatures in sperm may be reprogrammed according to different dynamics, potentially affecting the embryo and driving long-term effects on the phenotype. Small adjustments to the methylome may appear secondarily to physiological adaptations in many cell types. (D) Some DNA methylation signatures in sperm may be driven by a methylation quantitative trait locus (QTL). Unless the QTL specifically targets the molecular actors of reprogramming (which probably would not be compatible with normal development) these genetically driven methylation signatures should be reprogrammed and return to the genetically controlled state at later stages, this having varying but not necessarily adverse effects on the phenotype.[Colour online.]



encountered by breeding companies) are similarly affected by differences in DNA methylation, appealing for the development of routine quality control procedures based on epigenetic marks. Because DNA methylation is reliant on the genomic sequence, and AI bulls are highly selected on the basis of their genotypes, we believe that taking account of genetic diversity is a prerequisite to exploring the contribution of DNA methylation to inter-individual phenotypic differences. In particular, integrated approaches that combine both genetic and epigenetic information may help to understand the architecture of male fertility. Finally, although a considerable mass of data in the literature has described the nongenetic transmission of traits or diseases via the paternal route, evidence that some regions escape reprogramming of the paternal methylome and mediate this phenomenon is still scarce but does exist in humans and some model species. Evaluating the degree to which this intergenerational epigenetic inheritance may affect the development and long-term

phenotype of progeny remains a challenge in cattle. However, because the DNA methylation signatures of sperm may contribute to shaping the phenotype of offspring through mechanisms beyond epigenetic inheritance (Figs. 2A–2D), we propose that it is now time to integrate epigenetic information in the evaluation of AI bulls. This could be done by a systematic control of sperm DNA methylation through the design of an affordable epigenotyping tool.

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