Maternal peri-conceptual undernourishment perturbs offspring sperm methylome

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Abstract

The genotype of an organism is stable throughout its life; however, its epigenome is dynamic and can be altered in response to environmental factors, such as diet. Inheritance of acquired epigenetic modifications by the next generation occurs through the germline, although the precise mechanisms remain to be elucidated. Here, we used a sheep model to evaluate if modification of the maternal diet (CTR; control, UND: undernutrition; FA: undernutrition and folic acid supplementation) during the peri-conceptual period affects the genome-wide methylation status of the gametes of male offspring. Sperm DNA methylation, measured by Reduced Representation Bisulfite Sequencing (RRBS), identified Differentially Methylated Regions (DMR) in offspring that experienced *in utero* undernutrition, both in UND (244) and FA (240), compared with CTR. Gene ontology (GO) analysis identified DMRs in categories related to sperm function, therefore we investigated whether the fertilizing capacity of the semen from the three groups differed in an *in vitro* fertilization assay. Spermatozoa from the undernourished groups showed lower motility and sperm chromatin structure abnormalities, represented by a higher percentage of DNA fragmentation and an increased number of immature cells, compared with CTR. While good quality blastocysts were obtained from all three groups, the proportion of embryos reaching the blastocyst stage was reduced in the UND vs CTR, an effect partially rescued by the FA treatment. The data reported here show that nutritional stress during early pregnancy leads to epigenetic modifications in the semen of the resulting offspring, the effects of which in next generation remain to be elucidated.

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Introduction

The relationship between foetal exposure to malnutrition and onset of diseases in adult life has been hypothesized since the 1980s (Barker hypothesis; [Barker et al. 1993](#)). First described in human epidemiological studies on the effects of maternal undernutrition on progeny during the Dutch Hunger Winter, the phenomenon has subsequently been demonstrated in several animal models ([Langley & Jackson 1994](#), [Langley-Evans et al. 1998](#), [Rae et al. 2002](#), [Zambrano et al. 2005](#)). Undernutrition or malnutrition during pregnancy not only affects the health of the exposed individuals and their offspring, but in some cases the phenotype induced by environmental stressors may be inherited across generations ([Patti 2013](#)). It has been proposed that transmission of such environmentally acquired phenotypes may be mediated by epigenetic

modification, which is described as transgenerational epigenetic inheritance ([Gluckman et al. 2007](#)).

Studies of the molecular mechanisms underlying epigenetic inheritance have focused on three classes of potential epigenetic information carriers: cytosine methylation, chromatin state, and small RNAs ([Rando 2012](#)). Retained histone modifications and abnormal populations of small noncoding RNAs in sperm have been implicated in the transgenerational inheritance of epigenetically controlled phenotypes that vary following male exposure to stress or malnutrition ([Rodgers et al. 2015](#), [Siklenka et al. 2015](#)). These epigenetic mechanisms of inheritance have been extensively studied in flies ([Seong et al. 2011](#)) and worms ([Greer et al. 2010](#), [Ashe et al. 2012](#)). However, embryo development in these species is different from mammals. Cytosine methylation is the best characterized epigenetic

mechanism regulating the expression of the mammalian genome, but it is less stable than previously thought, with dynamic changes during embryo development and gametogenesis (Hackett & Surani 2013). Nevertheless, methylation of DNA appears to play a primary role in the transmission of specific phenotypes to the next generations via epigenetic modifications in the germline (Daxinger & Whitelaw 2012, Radford *et al.* 2014, Wei *et al.* 2015, Huypens *et al.* 2016).

Nutritional stress experienced during early development alters DNA methylation patterns in the offspring via epigenetic changes in the gametes, although there is no clear link between the kind of stress and the specific epigenetic modification that result (Martínez *et al.* 2014, Radford *et al.* 2014). Most of the genome demethylation data has been obtained from mouse studies. Mouse primordial germ cells (PGCs) appear around day (E) 6.25 of embryonic development and undergo extensive DNA demethylation, which is essential for their transition from PGCs to gonocyte (12.5dpc) (Seisenberger *et al.* 2013, Hill *et al.* 2018). Following erasure, the genomic methylation patterns that are acquired in the germline differ markedly for male and female gametes, especially at imprinted loci that have allele-specific gene expression in the offspring. The second DNA demethylation wave takes place during early embryonic development and is crucial for establishing pluripotency (Hackett & Surani 2013, Messerschmidt *et al.* 2014). It is, therefore, unclear how the epigenetic information is passed to the progeny. There is less information on the timing of epigenetic reprogramming in sheep PGCs, but at day 28 sheep PGCs are largely localized in the genital ridges, suggesting that by this time methylation marks have been mostly erased (Ledda *et al.* 2010, Van Soom *et al.* 2013). The genome epi-modification occurring during these two developmental phases are particularly sensitive to environmental factors (Faulk & Dolinoy 2013). Exposure of a pregnant female to environmental insults during this crucial time-dependent developmental window can lead to alteration of the epigenetic profile of both PGC and the zygote (Bernal & Jirtle 2010).

The present study was carried out in sheep, for which the nutritional requirements and the reproductive physiology are well characterized and that are commonly used as a model for human foetal development (Sinclair *et al.* 2010). We evaluated whether maternal diet restriction during early pregnancy affects methylation of spermatozoa and if this has developmental effects on embryos following *in vitro* fertilization. Furthermore, we investigated if folic acid, a B complex vitamin critical for one carbon (C1) metabolism that acts as methyl donor for DNA methylation (Cetin *et al.* 2010, Anderson *et al.* 2012), may counteract the effects of restricted nutrition during

Figure 1 Schematic representation of the nutritional treatment of the three groups of sheep (CTR, UND, and FA) during the peri-conceptual period (–14/+28 days) in order to evaluate the effect on epigenetic patterns in the gametes of the male offspring.

the critical developmental window characterized by progressive DNA re-methylation.

Materials and methods

All experimental procedures involving animals were conducted in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609. All animal experiments were performed under the authority of the local ethical committee approval (CEISA-comitato etico interistituzionale per la sperimentazione animale N° PROT.UNCHD12-222/2014). All chemicals were obtained from Sigma Aldrich unless otherwise stated. The experimental design of the study is shown in Fig. 1.

Animal nutrition and breeding management

In a commercial flock, 27 multiparous Sardinian sheep were randomly assigned into three groups of nine ewes and fed one of the three diets during the periconceptual period, from 14 days before mating (oocyte maturation) until 28 days after mating (early organogenesis), which corresponds to Carnegie stage 19 (Butler & Juurlink 1987): 42 days in total.

Ewes in the control group (CTR) were fed *ad libitum* with a balanced diet satisfying nutritional requirements of pregnant ewes (300 g/animal/day). Ewes of the undernutrition group (UND) were fed a restricted diet (50% of the requirements: 150 g/animal/day) of the same diet. Ewes in the folic acid group (FA) were fed the same as the UND group, but were supplemented with folic acid (15 mg; Folina; Teofarma) injected i.m. every 24 h for during the 42 days of the nutritional treatment. The nutritional stress imposed on the UND animals was sufficient to induce a gross caloric restriction without affecting the health and well-being. All animals had a full clinical assessment each week. The Table 1 provides the details of the nutritional regimes used in the three groups.

To optimise the pregnancy rate, oestrus was synchronized by treating all ewes with intravaginal progesterone sponges (25-mg Chronogest; Intervet) for 14 days. At the time of sponge removal, a ram of proven fertility was used to mate the three nutritional treatment groups, temporarily hosted in a common paddock. From the time of mating onwards, ewes were group housed in pens under natural day length conditions with *ad libitum* access to water. At day 28 of gestation, all ewes

Table 1 Diet composition (complementary feed and pelleted alfalfa) in normal (100%: 300 g/animal) and restricted (half daily ratio-50%: 150 g/animal) experimental groups.

Ingredients	Amount (%)
Complementary feed OV3 (#05.0003; Europa mangimi; Italy)	
Crude protein	16.70
Crude cellulose	12.90
Crude ashes	8.80
Crude oil and fats	3.20
Sodium	0.20
Pelleted Alfalfa (#964; Mangimi Ariston srl; Italy)	
Crude Protein	14
Crude cellulose	32
Crude ashes	11

were returned to an *ad libitum* diet, to meet daily energy and nutritional needs and to ensure that all animals had regained sufficient fat reserves to maintain the pregnancy for the 149.7 days average gestation period for the *Sarda* sheep breed and for the onset and maintenance of lactation. Following parturition, all animals were conventionally managed, and all male lambs ($n=11$; 4 CTR, 3 UND; 4 FA) were weighed at seven time points from birth to day 30 (day 0, 1, 2, 5, 10, 20, and 30). For subsequent analysis, we excluded lambs born from twin pregnancies in order to avoid any confounding factors. A non-parametric Kruskal–Wallis test was used to assess the effect of nutritional treatment on lamb body weight using Graph Pad Prism software. Data are presented as mean $\pm$ S.E.M., with a P value <0.05 indicating statistically significant differences between groups.

Semen collection

The ram lambs were trained for semen collection with an artificial vagina after puberty (around 9 months in *Sarda* breed). Immediately after collection, semen volume and motility was evaluated under a stereomicroscope and concentration was assessed using a Burkner chamber. Part of each ejaculate was processed for molecular analysis and the rest cryopreserved.

Methylome analysis

Sperm preparation for RRBS analysis

After collection, the ejaculate was diluted in 3 mL of basic medium (BM), composed of 300 mM TRIS base, 105 mM citric acid, 82 mM fructose, 150,000 IU penicillin G, 2 mM streptomycin in (67.20 mL) double-distilled water (ddH₂O) (pH was adjusted to 6.7/6.8) and centrifuged at 461 g for 10 min to remove seminal plasma. Then, 1 mL of supernatant was discarded and replaced with 1 mL of clean BM, mixed and centrifuged again at 101 g for 5 min. Next, tubes were gently placed in a humidified atmosphere at 38.5°C and 5% CO₂, angled at 45°, for 45 to 60 min, to allow the motile spermatozoa to swim up. After incubation, supernatant was collected from the top of the liquid phase in 200 μ L aliquots. From each collection, 5 μ L were examined under the microscope, to exclude aliquots containing somatic cells. Once a minimum of 500 μ L of supernatant free of somatic cells had been collected, sperm concentration was assessed and tubes centrifuged at

10,000 g for 10 min. Finally, the pellet was resuspended at a concentration of 25×10^6 spermatozoa per 100 μ L in BM and stored at -80°C until processed.

Reduced Representational Bisulfite (RRBS) Library preparation and sequencing

Genomic DNA from each sperm sample was isolated using the NucleoSpin® Tissue kit (Macherey–Nagel, Düren, Germany), following the manufacturer's instructions. DNA concentration was estimated by PicoGreen® (Thermo Fisher Scientific). One μ g of DNA was digested with MspI (New England Biolabs, UK) by overnight incubation at 37°C, following the manufacturer's instruction. Libraries were generated using the TruSeq® DNA PCR-Free Library Preparation kit (Illumina). After adapter ligation, samples were converted using an EpiTect Bisulfite Kit (Qiagen) and finally PCR amplified with KAPA HiFi Uracil+ (Kapa Biosystems). RRBS libraries were sequenced on an Illumina HiSeq 3000 (San Diego, CA, United States) to generate 150-base paired-end reads.

Methyl-seq library preparation and sequencing for RRBS validation

For RRBS validation, methyl-seq libraries were prepared from genomic DNA isolated from each sperm sample. DNA was sonicated to produce fragments of about 350 bp. MethylMiner™ kit (Invitrogen) was used to enrich highly methylated DNA fragments in the sample. The enriched methylated fragments were then converted to libraries with TruSeq® Nano Library Preparation Kit (Illumina, San Diego, CA, USA). Methyl-seq libraries were sequenced on an Illumina HiSeq 3000 (San Diego, CA, United States) to generate 150-base paired-end reads.

Bioinformatic analysis

The preliminary quality control of raw sequence reads was carried out with FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Quality control and removal of adapters from the Illumina raw sequences was performed with Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Data are available in the Sequence Reads Archive (SRA), BioProject accession number, PRJNA491660.

Bismark software v.0.17.0 was used to align each read to a bisulfite-converted sheep genome (Oar_v3.1) with option -N 1. Methylation calls were extracted using the Bismark methylation_extractor function. Seqmonk software (version 1.36.0) (<http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>) was used for visualization and analysis of the Bismark output. To detect regions having sufficient sequence coverage, the reference genome was split into 400-bp sliding windows with a 200-bp offset, and methylation percentage was calculated for each window containing at least six cytosine with 5 $\times$ coverage. Only Methylated Regions (MRs) with sufficient sequence coverage in all nine samples were considered and analysed further. Differentially methylated regions (DMRs) were identified by comparing CTR vs UND, CTR vs FA, and UND vs FA. DMRs were calculated using the logistic regression filter in R (FDR <0.05 , absolute cut-off of

20%) (Ziller *et al.* 2015). Hierarchical analyses in Genesis software (Sturm *et al.* 2002) were used to illustrate sample clustering. To compute shared DMRs and DMR associated genes across comparisons, commonality analysis was run and visualized through Venn diagrams, using the Venn Diagrams software (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). DMR associated genes were classified according to classical GO categories, using the Cytoscape plug-in ClueGO which integrates GO (Bindea *et al.* 2009) and enhances biological interpretation of large lists of genes. In ClueGO, the *P* value was calculated with the Fisher Exact Test corrected using the Bonferroni step down method.

For validation, after quality assessment with FastQC, methyl-seq raw sequences were trimmed with Trimmomatic and subsequently mapped to sheep reference genome (Oar_v3.1). BAM files were imported into Seqmonk software (version 1.36.0) and DMRs were quantitated by calculating the normalized coverage for each region.

In vitro semen analysis

Sperm cryopreservation

Sperm was frozen as previously described (Anzalone *et al.* 2016). Briefly, Basic Medium (BM, see previous section for composition) used for the preparation of two other media, Medium A (67.20% of BM+20% of egg yolk+12.8% of ddH₂O) and Medium B (67.20% BM+20% of egg yolk+12.80% of glycerol). Medium A and Medium B were added in equal volumes to the ejaculate to reach a final concentration of 400×10^6 spermatozoa/mL. Medium A was added to the ejaculate first and transferred to 4°C and cooled for 2 h at a controlled rate ($\sim -0.2^\circ\text{C}/\text{min}$). Medium B was then added to the suspension and left for further 2 h at 4°C. Next, 250 μL straws were filled, sealed with polyvinyl alcohol (PVA) and placed onto a metallic grid, and stabilized at 4°C for 2 h. Finally, the straws were exposed to liquid nitrogen (LN) vapour (-80°C) in a Dewar flask for 6 min before being plunged into LN, where they were stored until use.

Computer-assisted semen analysis (CASA)

Sperm kinetic parameters were assessed using a CASA (Computer-Assisted Semen Analysis) system (ISAS® v1, Spain) as described in previous work (Capra *et al.* 2017). Samples were diluted to $\sim 30 \times 10^6$ cells/mL with EasyBuffer B® (IMV Technologies, L'Aigle, France). A pre-warmed (37°C) Makler counting chamber (10 μm depth) was loaded with 10 μL of each sample, and at least three fields were acquired. Two technical replicates per sample were performed. Using a 10 $\times$ objective in phase contrast, the image was relayed, digitized, and analyzed by the ISAS® software with user-defined settings as follows: frames acquired, 25; frame rate, 20 Hz; minimum particles area, 20 μm^2 ; maximum particles area, 70 μm^2 ; and progressivity of the threshold straightness, 70%. Spermatozoa speed was assigned to three broad categories: rapid (50 $\mu\text{m}/\text{s}$), medium (25 $\mu\text{m}/\text{s}$), and slow (10 $\mu\text{m}/\text{s}$). CASA kinetics parameters were follows: total motility (MOT TOT, %), progressive motility (PRG, %), curvilinear velocity (VCL, $\mu\text{m}/\text{s}$), straight-line velocity (VSL, $\mu\text{m}/\text{s}$), average path velocity (VAP,

$\mu\text{m}/\text{s}$), linearity coefficient (LIN, $\% = \text{VSL}/\text{VCL} \times 100$), amplitude of lateral head displacement (ALH, μm), straightness coefficient (STR, $\% = \text{VSL}/\text{VAP} \times 100$), wobble coefficient (WOB, $\% = \text{VAP}/\text{VCL} \times 100$), and beat cross frequency (BCF, Hz).

Flow cytometry analysis

Measurements were recorded using a Guava easyCyte™ 5HT microcapillary flow cytometer with cytoSoft and IMV easySoft software (Merck KGaA, Darmstadt, Germany; distributed by IMV Technologies). The fluorescent probes were excited by a 20 mW argon ion laser (488 nm). Forward Scatter (FSC) v. side-scatter plots were used to separate sperm cells from debris. Non-sperm events were excluded from further analysis. Fluorescence detection was set with three photomultiplier tubes: detector FL-1 (green: 525/30 nm), detector FL-2 (yellow/orange: 583/26 nm), and detector FL-3 (red: 655/50 nm). A total of 5000 sperm events per sample were analyzed at a flow rate 200 cells/s.

Nuclear chromatin was analyzed as previously described (Gliozzi *et al.* 2017) using acridine orange, a fluorochrome that turns from red to green depending on the degree of chromatin compaction.

The technique is based on the susceptibility of sperm DNA to acid-induced denaturation, as low-pH treatment causes partial DNA denaturation in sperm with altered chromatin structure. Acridine orange is a planar molecule which intercalates into dsDNA but stacks on ssDNA causing a metachromatic shift from green (dsDNA) to red fluorescence (ssDNA), when exposed to the 488nm laser of the flow cytometer.

The assessment was performed using the sperm chromatin structure assay (Evenson & Jost 2000): 3.0×10^5 cells were diluted in 200 μL of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4) and added to 400 μL of an acidic solution (Triton X-100 0.1%, 0.15 M NaCl, 0.08 M HCl; pH 1.2). After 30 s, cells were stained with 1.2 mL of acridine orange solution (6 $\mu\text{g}/\text{mL}$ in 0.1 M citric acid, 0.2 M Na_2HPO_4 , 1 mM EDTA, 0.15 M NaCl; pH 6). After 2.5 min, two replicates per sample were read on a flow cytometer (Guava easyCyte™ 5HT microcapillary flow cytometer). The fluorescent probes were excited by a 20 mW argon ion laser (488 nm). Forward scatter (FSC) v. side-scatter plots were used to separate sperm cells from debris. Non-sperm events were excluded from further analysis. Fluorescence detection was set with three photomultiplier tubes: detector FL-1 (green: 525/30 nm), detector FL-2 (yellow/orange: 583/26 nm), and detector FL-3 (red: 655/50 nm). Calibration was carried out using standard beads (Guava® Easy Check Kit; Merck Millipore). Data were analyzed using cytoSoft and IMV easySoft software (Merck KGaA, Darmstadt, Germany; distributed by IMV Technologies), with the following parameters: Alpha-T that is indicative of the shift from green to red fluorescence and is expressed as the ratio of red to total fluorescence intensity ($\text{red}/(\text{red} + \text{green})$), which quantifies the degree of abnormal chromatin structure that has an increased susceptibility to acid-induced denaturation; s.d. of Alpha-T (ATSD) that shows the extent of abnormality in chromatin structure within a population; %DFI that indicates the percentage of sperm with fragmented DNA; and percentage of sperm with high green (%HG) fluorescence that is representative of the percentage

of immature cells with reduced nuclear condensation (incomplete histone–protamine exchange).

Statistical analysis

Data obtained from CASA and flow cytometry measurements were analysed using the SASTM package v 9.4 (SAS Institute Inc., Cary, NC, USA). The General Linear Model procedure (PROC GLM) was used to evaluate the effect of the experimental groups (CTR, UND, FA) on semen quality parameters. Results are given as adjusted least squares means (LSM) $\pm$ S.E.M.

In vitro sheep embryo production

Oocyte collection and in vitro maturation (IVM)

Sheep oocytes were retrieved from ovaries collected from a local slaughterhouse. Oocytes were aspirated using 21 g needles into warm TCM-199 medium (Gibco, Life Technologies) buffered with 0.47% HEPES buffer and supplemented with 0.005% (w:v) heparin. Only oocytes with 2–3 layers of compact cumulus cells were selected for IVM and incubated for 24 h in a humidified atmosphere at 38.5°C and 5% CO₂ in IVM medium. The latter was composed of bicarbonate-buffered TCM-199 (Gibco) containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 μ M cysteamine, 10% foetal bovine serum (FBS) (Gibco), 5 μ g/mL follicle stimulating hormone (FSH) (Ovagen, ICP, Auckland, New Zealand), 5 μ g/mL luteinizing hormone (LH), and 1 μ g/mL β -estradiol. Following IVM, mature oocytes (MII) showed expanded cumulus and extruded first polar body.

In vitro fertilization (IVF)

In vitro fertilization was performed as previously described (Anzalone et al. 2016). Briefly, frozen semen was fast-thawed in a waterbath at 35°C for 10 s and centrifuged in SOF-containing 0.4% BSA (w/v), at 180 g for 5 min. Matured oocytes were gently pipetted in 300 U/mL hyaluronidase solution (dissolved in H199) to partially remove granulosa cell, then placed into 50 μ L drops of IVF medium covered by mineral oil, and incubated with sperm (5×10^6 spermatozoa/mL) overnight, in a humidified atmosphere at 38.5°C, 5% CO₂, and 7% O₂. The day after, putative zygotes were pipetted in SOF-medium to remove most of the spermatozoa attached to the zona pellucida and cultured as described.

Embryo culture

All putative zygotes from *in vitro* fertilized oocytes were cultured into 20- μ L drops of SOF-enriched with 2% (v:v) basal medium Eagle essential amino acids (EAA), 1% (v:v) minimum essential medium (MEM)-nonessential amino acids (NEAA) (Gibco), 1 mM glutamine, and 8 mg/mL fatty acid-free BSA, covered by mineral oil. The medium was renewed on day 3 (SOF supplemented with 0.27 mg/mL glucose (SOF+), 2% EAA, 1% NEAA), on day 5 (SOF+ with 10% of charcoal stripped FBS (cs-FBS), 2% EAA, 1% NEAA), and on day 6 (1:1 MEM/M199 enriched with 10% cs-FBS, 2.5 μ g/mL gentamicin and 1% sodium pyruvate), and then incubated until day 7–8. The *in vitro* development was evaluated on day 1 (2 cell-stage) and the 7/8th day (blastocyst stage). Fisher's exact test was used

to compare *in vitro* embryo developmental stages between the groups. Data were analyzed using PRISM software (version 6). GraphPad and the values were considered to be significantly different when $P < 0.05$.

Results

Lamb body weight

Body weight of male lambs from controls (CTR) and the two feed restricted groups (UND and FA) showed no significant difference (Fig. 2) at birth (CTR: 3.53 ± 0.50 kg; UND: 3.96 ± 0.28 kg; FA: 4.20 ± 0.35 kg) and up to day 30 post-partum (7.90 ± 0.70 kg; UND: 10.30 ± 0.37 kg; FA: 9.70 ± 1.90 kg).

Sperm methylation

The average number of sequence reads per sperm sample was 28.1 M (ranging from 10.9 M to 56.6 M). Reduced representation bisulphite sequencing (RRBS) showed a high-level cytosine methylation in the context of CpG, which was, on average, 67.3% across all sperm samples (Supplementary file 1, see section on [supplementary materials](#) given at the end of this article). A total of 86,944 MRs were detected across all samples by SeqMonk software. Considering differential cytosine methylation, where variation was greater than 20%, the pairwise comparison of sperm methylation between experimental groups identified 244 differentially methylated regions (DMRs) in CTR vs UND, 240 DMRs in CTR vs FA, and 106 DMRs in UND vs FA. Annotation of the 244, 240, and 106 DMRs identified 75, 69, and 38 DMR associated genes (Supplementary file 2). The number of DMRs from the comparison of offspring from undernourished mothers (UND) with undernourished mothers supplemented with folic acid (FA) was considerably lower than that

Figure 2 Weights of male lamb for the three groups of sheep. Animals were weighed at birth and at five subsequent time points (day 2, 5, 10, 20, and 30) in both treated (UND and FA) and CTR groups.

observed in the other two comparisons (CTR vs UND and CTR vs FA). DMR associated genes shared by CTR vs UND and CTR vs FA comparisons were 19.7% (87 out of 442) and 22.3% (29 out of 130), respectively, of the total DMRs and DMR associated genes (Fig. 3). Hierarchical clustering based on DMRs obtained from different contrasts (CTR vs UND, CTR vs FA, and UND vs FA) highlighted some common patterns. In particular, all CTR animals consistently clustered together, as did UND1 and UND2. UND3 consistently clustered with FA3 and behaved as belonging to the FA rather than to the UND group. CTR vs UND DMRs separated controls from underfed animals, with the exception of FA2 that clustered with the CTR group (Fig. 4A). CTR vs FA DMRs were even more effective in distinguishing underfed from CTR animals (Fig. 4B). Both sets of DMRs clustered undernourished animals receiving or not receiving FA supplementation separately (with exception of UND3). UND vs FA DMRs showed a closer relationship between CTR and FA compared to CTR and UND (with the exception of UND3), suggesting a possible role of FA supplementation in restoring the epigenetic asset (Fig. 4C).

GO analysis was performed on the GO classes that included at least two differentially methylated genes. DMR associated genes between CTR vs UND were involved in the regulation of I-Kappa B (I-KB) and NF-kappa B (NF-KB) signalling, regulation of chondroitin sulphate synthesis, potassium ion import, and response to parathyroid hormone. Whereas the CTR vs FA comparison identified genes within DMR related to biotin metabolism, response to forskolin, and regulation of mitochondrial function. Finally, comparing UND vs FA identified variation in the methylation of genes associated to neural development, response to glucagon, ISG15-protein conjugation, and activation of adenylate cyclase activity (Table 2).

Figure 3 Venn diagrams representing the number and percentage of differentially methylated regions (DMRs) and DMR associated genes shared and unique in comparisons of CTR vs UND, CTR vs FA, and UND vs FA groups.

Figure 4 The three heatmaps comparing the methylation status of sheep at DMRs detected in the three comparisons: (A) 244 differentially methylated regions (DMRs) in CTR vs UND, (B) 240 DMRs in CTR vs FA, and (C) 106 DMRs in UND vs FA. The list of DMRs is only partially overlapping across comparisons.

The 244, 240, and 106 DMRs found in CTR vs UND, CTR vs FA, and UND vs FA, respectively, were verified by comparing the average RRBS methylation percentage and the average of normalized count obtained by methyl-seq. The intersect between the subsets representing the 25th percentile of the HMRs for each condition (CTR, UND, and FA) obtained by RRBS and methyl-seq showed a higher percentage of shared regions than that due to chance (Supplementary file 3).

In vitro semen analysis

Computer-assisted semen analysis (CASA) sperm kinetic parameters, including total motility (MOT TOT) and progressive (PRG) motility, were significantly lower in spermatozoa collected from both undernourished groups (UND and FA) compared with controls (Table 3).

Differences were also observed in the extent of chromatin structure abnormality, with a significant increase of Alpha-T s.d. (ATSD) for the FA groups compared with CTR, although not in the UND group with respect to the CTR group (Table 3).

Embryo development

Following *in vitro* fertilization, significant differences in embryo development were seen between all groups (Table 4). At 24 h, the percentage of two cells embryos was 31.5% in CTR, 23.6% in UND (44/186), and 39% (46/118) in the FA, and these differences are all statistically significant ($P=0.006615$). The rate of embryos reaching blastocyst stage was 14.6% (26/178)

Table 2 Gene Ontology (GO) analysis of DMR associated genes obtained by comparing CTR vs UND, CTR vs FA and UND vs FA groups.

GOID	Associated Genes Found	GO Term	P Value	Corrected P value*
CTR-UND				
1903659	<i>IL10, IL11</i>	regulation of complement-dependent cytotoxicity	1.4E-04	2.5E-03
0071374	<i>HDAC4, PRKCA</i>	cellular response to parathyroid hormone stimulus	2.7E-04	4.3E-03
0030397	<i>LMNA, PRKCA</i>	membrane disassembly	1.1E-02	1.1E-02
0051081	<i>LMNA, PRKCA</i>	nuclear envelope disassembly	1.1E-02	1.1E-02
0043124	<i>FBXW11, IL10, TNIP1</i>	negative regulation of I-kappaB kinase/NF-kappaB signalling	8.9E-04	1.2E-02
0071107	<i>HDAC4, PRKCA</i>	response to parathyroid hormone	8.7E-04	1.3E-02
2000810	<i>IKBKB, PRKCA</i>	regulation of bicellular tight junction assembly	1.4E-03	1.9E-02
0072577	<i>IL10, IL11</i>	endothelial cell apoptotic process	1.0E-02	2.1E-02
0010882	<i>HDAC4, PRKCA</i>	regulation of cardiac muscle contraction by calcium ion signalling	2.2E-03	2.6E-02
0030206	<i>NCAN, XYLT1</i>	chondroitin sulphate biosynthetic process	2.6E-03	2.8E-02
0042347	<i>FBXW11, IL10</i>	negative regulation of NF-kappaB import into nucleus	2.6E-03	2.8E-02
0042345	<i>FBXW11, IL10</i>	regulation of NF-kappaB import into nucleus	1.0E-02	3.0E-02
0042348	<i>FBXW11, IL10</i>	NF-kappaB import into nucleus	1.0E-02	3.0E-02
0007077	<i>LMNA, PRKCA</i>	mitotic nuclear envelope disassembly	9.2E-03	3.7E-02
0042992	<i>FBXW11, IL10</i>	negative regulation of transcription factor import into nucleus	9.2E-03	3.7E-02
0050650	<i>NCAN, XYLT1</i>	chondroitin sulphate proteoglycan biosynthetic process	3.7E-03	3.7E-02
2000352	<i>IL10, IL11</i>	negative regulation of endothelial cell apoptotic process	3.7E-03	3.7E-02
0010107	<i>KCNJ12, KCNJ6</i>	potassium ion import	4.5E-03	4.1E-02
2000351	<i>IL10, IL11</i>	regulation of endothelial cell apoptotic process	8.5E-03	4.2E-02
0050654	<i>NCAN, XYLT1</i>	chondroitin sulphate proteoglycan metabolic process	8.1E-03	4.9E-02
1904036	<i>IL10, IL11</i>	negative regulation of epithelial cell apoptotic process	8.1E-03	4.9E-02
CTR-FA				
1904321	<i>ADCY5, EFNA5</i>	response to forskolin	2.7E-04	2.7E-03
1904322	<i>ADCY5, EFNA5</i>	cellular response to forskolin	2.7E-04	2.7E-03
0046928	<i>CACNA1A, PARK2, SEPT9</i>	regulation of neurotransmitter secretion	4.9E-04	4.4E-03
0050885	<i>ADCY5, CACNA1A, RBFOX1</i>	neuromuscular process controlling balance	6.6E-04	5.3E-03
0051588	<i>CACNA1A, PARK2, SEPT9</i>	regulation of neurotransmitter transport	9.1E-04	6.4E-03
0006768	<i>HLCS, PCCA</i>	biotin metabolic process	1.1E-03	6.9E-03
0010823	<i>LMNA, PARK2</i>	negative regulation of mitochondrion organization	8.6E-03	8.6E-03
0090201	<i>LMNA, PARK2</i>	negative regulation of release of cytochrome c from mitochondria	1.9E-03	9.4E-03
0043525	<i>HDAC4, PARK2</i>	positive regulation of neuron apoptotic process	8.3E-03	1.7E-02
0044003	<i>PARK2, PCCA</i>	modification by symbiont of host morphology or physiology	8.3E-03	1.7E-02
0045071	<i>MX1, PARK2</i>	negative regulation of viral genome replication	8.3E-03	1.7E-02
1902803	<i>PARK2, SEPT9</i>	regulation of synaptic vesicle transport	5.6E-03	2.2E-02
0043113	<i>CACNA1A, DLG2</i>	receptor clustering	8.0E-03	2.4E-02
UND-FA				
0021631	<i>EPHB2, GLI3</i>	optic nerve morphogenesis	3.6E-05	5.0E-04
0032020	<i>UBE2E2, UBE2L6</i>	ISG15-protein conjugation	3.6E-05	5.0E-04
0043535	<i>AMOTL1, MAP2K5, PRKCA</i>	regulation of blood vessel endothelial cell migration	8.2E-05	1.1E-03
0021554	<i>EPHB2, GLI3</i>	optic nerve development	1.8E-04	2.2E-03
0032330	<i>GLI3, RARB</i>	regulation of chondrocyte differentiation	2.5E-03	2.5E-03
0060831	<i>GLI3, PRKCA</i>	smoothened signalling pathway involved in dorsal/ventral neural tube patterning	2.5E-04	2.7E-03
0021532	<i>GLI3, PRKCA</i>	neural tube patterning	2.2E-03	4.3E-03
0071377	<i>ADCY5, PRKCA</i>	cellular response to glucagon stimulus	2.2E-03	4.3E-03
0007190	<i>ADCY5, PRKCA</i>	activation of adenylate cyclase activity	1.8E-03	5.4E-03
0021544	<i>GLI3, RARB</i>	subpallium development	5.4E-04	5.4E-03
0021602	<i>EPHB2, GLI3</i>	cranial nerve morphogenesis	8.8E-04	6.2E-03
0031641	<i>RARB, TG</i>	regulation of myelination	1.1E-03	6.5E-03
0021904	<i>GLI3, PRKCA</i>	dorsal/ventral neural tube patterning	8.2E-04	6.5E-03
0043536	<i>AMOTL1, PRKCA</i>	positive regulation of blood vessel endothelial cell migration	7.6E-04	6.8E-03
0045879	<i>GLI3, PRKCA</i>	negative regulation of smoothened signalling pathway	7.6E-04	6.8E-03
0003091	<i>ADCY5, PRKCA</i>	renal water homeostasis	1.7E-03	6.8E-03
0007212	<i>ADCY5, RGS9</i>	dopamine receptor signalling pathway	1.7E-03	6.8E-03
0048566	<i>GLI3, RARB</i>	embryonic digestive tract development	1.4E-03	6.9E-03

*P values were corrected with Bonferroni step down.

Table 3 Computer-assisted semen analysis and flow cytometry recorded semen quality parameters in the control (CTR) and two undernourished (UND and FA) groups. Results are given as adjusted least squares means (LSM) $\pm$ S.E.M.

Parameters	CTR (LSM $\pm$ S.E.M.)	UND (LSM $\pm$ S.E.M.)	FA (LSM $\pm$ S.E.M.)
CASA			
MOT TOT (%)*	72.6 $\pm$ 3.68 ^a	57.12 $\pm$ 3.68 ^a	54.75 $\pm$ 3.00 ^b
PRG (%)*	17.35 $\pm$ 1.49 ^a	12.56 $\pm$ 1.49 ^{ab}	11.22 $\pm$ 1.21 ^b
VCL (μ m/s)	26.57 $\pm$ 5.17	29.08 $\pm$ 5.17	21.96 $\pm$ 4.22
VSL (μ m/s)	77.41 $\pm$ 3.50	72.82 $\pm$ 3.50	69.66 $\pm$ 2.86
VAP (μ m/s)	40.17 $\pm$ 3.60	41.27 $\pm$ 3.60	33.85 $\pm$ 2.94
LIN (%)	34.32 $\pm$ 9.02	40.73 $\pm$ 9.02	32.10 $\pm$ 7.36
STR (%)	66.15 $\pm$ 7.23	69.13 $\pm$ 7.23	64.10 $\pm$ 5.90
WOB (%)	51.88 $\pm$ 7.46	57.31 $\pm$ 7.46	49.01 $\pm$ 6.09
ALH (μ m)	3.69 $\pm$ 0.26	3.42 $\pm$ 0.26	3.39 $\pm$ 0.21
BCF (Hz)	5.35 $\pm$ 0.94	5.83 $\pm$ 0.94	4.34 $\pm$ 0.76
Cytofluorimetric			
ALPHA-T	0.4095 $\pm$ 0.0027	0.4085 $\pm$ 0.0027	0.4085 $\pm$ 0.0022
ATSD*	0.0122 $\pm$ 0.0002 ^a	0.0127 $\pm$ 0.0002 ^a	0.0138 $\pm$ 0.0001 ^b
DFI (%)	2.1625 $\pm$ 0.2004	2.5275 $\pm$ 0.2004	2.565 $\pm$ 0.1636
HG (%)	3.2825 $\pm$ 0.4374	2.165 $\pm$ 0.4374	2.8816 $\pm$ 0.3571

ALH, amplitude of lateral head displacement; ALPHA-T, red/ (red+ green) fluorescence intensity; ATSD, Alpha-T standard deviation; BCF, beat cross frequency; DFI, fragmented DNA sperm; HG, high green fluorescence sperm; LIN, linearity coefficient (calculated as VSL/VCL $\times$ 100); MOT TOT, total motility; PRG, progressive motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble coefficient (calculated as VAP/VCL $\times$ 100). ^{a,b} Values within a row with different superscript letters indicates statistically significant differences.

in CTR, 5.4% (10/186) in UND, and 13.5% (16/118) in FA Group, respectively.

Discussion

Although maternal dietary restriction during early pregnancy did not result in any gross phenotypic difference in the progeny, including birth weight and growth rate up to 12 months of age, the data presented here show that nutritional differences cause epigenetic modifications in the spermatozoa of the offspring. This is consistent with studies that show nutritional stress in early pregnancy does not affect growth (Everitt 1964, Parr *et al.* 1986, Rae *et al.* 2002), whereas postnatal growth is compromised when nutritional restriction is applied from mid-gestation until term (Schinckel & Short 1961, Robinson & McDonald 1979), Ford *et al.* 2007. Semen collected from male progeny of the undernourished dams showed reduced total and progressive motility compared with controls. Likewise, there was a trend in higher abnormalities in the chromatin structure (ATSD value, see Table 3) in the progeny of nutritionally stressed dams, but was still below the subfertility threshold in

rams. The sperm chromatin parameters evaluated are correlated with DNA strand breaks (Sailer *et al.* 1995) and fertility (Evenson *et al.* 1980). A significant negative correlation between sperm chromatin index and classical semen quality parameters has been reported for rams (Peris *et al.* 2004) and others species including human (Evenson *et al.* 1999, Januskauskas *et al.* 2001, Saleh *et al.* 2002).

We also found that the early development of embryos produced by *in vitro* fertilization and embryo culture using the spermatozoa of lambs born to feed restricted ewes was reduced compared with controls; an effect partially compensated by folic acid supplementation. The supplementation with folic acid may have partially corrected the effects of nutrient restriction, increasing the availability of methyl groups that are broadly required in this developmental window for the acquisition of paternal imprinted regions and transposon silencing in male gonocyte (Trasler 2009). Although the overall level of global methylation was not altered by maternal dietary restriction, specific loci did show differential methylation between UND, FA, and CTR groups. Studies on mice have shown that postnatal feed restriction alters methylation across the genome in an unpredictable way, an effect detectable over two successive generations (Xue *et al.* 2016), even though the affected loci differ in each generation. Undernourishment *in utero*, however, affects the germline DNA methylome of F1 adult males in a locus-specific manner, rather than a genome-wide manner (Martínez *et al.* 2014, Radford *et al.* 2014). In the current study, we identified changes in DNA methylation in genes essential for sperm maturation and function, including laminin A (LMNA) (Alsheimer *et al.* 2004, Shen *et al.* 2014) and β -Transducin repeat-

Table 4 IVF outcomes: IVF was performed using sperm from lambs born from control (CTR), undernourished (UND) and Folic Acid supplemented (FA) ewes. Development time points were 2 cells and blastocyst stages.

	Oocytes (n)	2-Cells stage embryo (%)	Blastocysts (%)
CTR	178	56/178 (31.5)	26/178 (14.6)
UND	186	44/186 (23.6) ^b	10/186 (5.4) ^{a,b}
FA	118	46/118 (39)	16/118 (13.5)

^a $P < 0.005$ between UND and CTR, ^b $P < 0.05$ between UND and FA; Fisher's exact test.

containing protein (β -TRCP2; FBXW11) (Nakagawa *et al.* 2017) between the UND vs CTR groups. These results suggest a direct effect of the maternal nutritional restriction on the offspring sperm methylome, as there were genes related to sperm specific function among the DMRs detected.

Further investigations are needed to understand if the epigenetic changes identified are responsible for the reduced sperm quality, which may have been related to the lower embryo developmental rate in the UND group. Interestingly, the *in vitro* development of oocytes fertilized with spermatozoa of the FA group was similar to the CTR one, although there were many differences in DMRs between the FA and CTR groups. The undernourished animals supplemented with FA showed sperm methylation alterations in specific genes involved in the regulation of PI3K activation during spermatozoa capacitation, such as protein kinase C alpha (PRKCA) (Rotman *et al.* 2010) or spermatozoa motility, such as parkin (PARK2) (Borowska *et al.* 2018). Recently, PARK2 was proposed as candidate gene associated with quality variation in bull semen, including total sperm motility, amplitude of lateral head displacement, mitochondrial integrity, and ATP content (Borowska *et al.* 2018). This could explain the lower levels of sperm motility found in the FA group, notwithstanding the better developmental potential of IVF embryos obtained from these samples. Differential cytosine methylation has also been reported in these genes in primordial germ cells (PGCs) of mouse concepti that underwent intrauterine hyperglycaemic exposure (Ren *et al.* 2018).

In conclusion, we report here that maternal undernutrition during the pre- to post-fertilization period results in an alteration of the methylation profile in the spermatozoa of the resulting offspring. Embryos produced *in vitro* using spermatozoa from the low nutrition group had a reduced developmental potential compared with the controls. However, sperm from lambs of ewes supplemented with folic acid produced embryos with increased developmental potential over the nutrition restricted group, despite there being DMR between the CTR and FA groups. The relationship between these DMRs and development is yet to be explored.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0549>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J L W, P A M and P L conceived and planned the experiments; P L supervised the animal work and collected the samples; J L W, P A M and P L critically analysed the results and contributed to manuscript writing; P T contributed to sample collection, E C and P T ran experiments, B L, E C and P T analysed and interpreted the data; P T wrote the manuscript; A S and P A S provided advice; F P and F T performed *in vitro* semen analysis (CASA) and cytofluorimetric analysis; D A A, collected semen and performed *in vitro* fertilization studies. All authors read and approved the final manuscript.

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