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ORIGINAL ARTICLE

Sperm Biology

Obesity-induced sperm DNA methylation changes at satellite repeats are reprogrammed in rat offspring

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There is now strong evidence that the paternal contribution to offspring phenotype at fertilisation is more than just DNA. However, the identity and mechanisms of this nongenetic inheritance are poorly understood. One of the more important questions in this research area is: do changes in sperm DNA methylation have phenotypic consequences for offspring? We have previously reported that offspring of obese male rats have altered glucose metabolism compared with controls and that this effect was inherited through nongenetic means. Here, we describe investigations into sperm DNA methylation in a new cohort using the same protocol. Male rats on a high-fat diet were 30% heavier than control-fed males at the time of mating (16–19 weeks old, *n* = 14/14). A small (0.25%) increase in total 5-methyl-2′-deoxycytidine was detected in obese rat spermatozoa by liquid chromatography tandem mass spectrometry. Examination of the repetitive fraction of the genome with methyl-CpG binding domain protein-enriched genome sequencing (MBD-Seq) and pyrosequencing revealed that retrotransposon DNA methylation states in spermatozoa were not affected by obesity, but methylation at satellite repeats throughout the genome was increased. However, examination of muscle, liver, and spermatozoa from male 27-week-old offspring from obese and control fathers (both groups from *n* = 8 fathers) revealed that normal DNA methylation levels were restored during offspring development. Furthermore, no changes were found in three genomic imprints in obese rat spermatozoa. Our findings have implications for transgenerational epigenetic reprogramming. They suggest that postfertilization mechanisms exist for normalising some environmentally-induced DNA methylation changes in sperm cells.
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INTRODUCTION

It is accepted that the phenotype of an organism is a function of both its individual genetics and the environment that it experiences. There is increasing evidence for a third determinant of phenotype, nongenetic inheritance.[1–3] Nongenetic inheritance can be defined as a mechanism through which the genotype or environmental experience of previous generations influences the phenotype of an individual, even though they do not inherit the causative genotype, or experience the causative environment themselves. Nongenetic inheritance down the male line can be referred to as a paternal effect. The increase in the last decade of examples of paternal effects in mammals has led to considerable interest in identifying the nongenetic components of spermatozoa and seminal fluid that could influence offspring phenotype.[4–6] DNA methylation, which is the covalent attachment of a methyl group to the base cytosine, is considered as one of the better candidates for mediating paternal effects. This is due to its ability to repress gene transcription when present at gene promoters and enhancer regions. Furthermore, evidence for methylation states in the gametes being able to control gene expression throughout the lifetime of the next generation comes from the developmental process of genomic imprinting, where the methylation state of regulatory regions in spermatozoa (and oocytes) controls gene transcription in offspring.[7]

Like many other cell types, mature spermatozoa in humans and rodents have high methylation levels at most repetitive elements and intergenic regions that transcriptionally repress these regions. Gene-specific hyper- or hypo-methylation is found at promoters, with a general observation of decreasing methylation with increasing CpG density.[8–12] Furthermore, the promoters of genes involved in early development are more likely to be hypo-methylated, suggesting that they are primed for activation in offspring.[10,12]

Several studies have described alteration of normal sperm DNA methylation patterns, due to genotype, environmental exposure or disease. These include methylation changes associated with mutations in DNA methyltransferases,[13] reduced fertility,[14–17] toxin and drug exposure,[18–20] dietary alterations,[21,22] and stress exposure.[23,24] There is no sign of a "standard epigenetic response" to these insults, as increases and decreases in global and locus-specific DNA methylation have all been reported.

An obstacle to the persistence of sperm methylation states in offspring is the extensive demethylation of the paternally-inherited chromosomes after fertilisation in humans and rodents.[25,26] Therefore,

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sperm DNA methylation abnormalities could be erased very early in development and consequently would not alter offspring development. However, some regions such as genomic imprints, some repetitive element classes and some single-copy loci are resistant to this genome-wide DNA demethylation.[26–28] In keeping with this potential mechanism of nongenetic inheritance, there are some recent examples of environmentally-induced sperm DNA methylation changes that are also present in tissues of offspring and even grand-offspring.[18,21,23,24,28] In addition, a study on tissues from assisted reproductive technology found the same DNA methylation abnormalities in aborted human conceptuses as in the fathers' spermatozoa.[17]

An environmental insult that is increasingly being studied for its consequences on the next generation is obesity. The rate of obesity is increasing worldwide with 2013 estimates of 36·9% of men and 38·0% of women being overweight or obese.[29] The risk of an individual's developing obesity increases if their mother or father is also obese.[30] This increased risk is due in part to shared genetic and societal factors. There is now a large body of work describing nongenetic influences of maternal obesity on offspring phenotype, through mechanisms such as altered nutrition during gestation and lactation.[31] However, we and others have shown that nongenetic inheritance down the male line also contributes risk.[30,32–34] Obesity is known to have extensive effects on male fertility and spermatogenesis, so the potential nongenetic inheritance mechanisms are numerous, and they may prove to be a combination of factors such as DNA methylation, histone modifications, various RNA species, and seminal factors.[33]

In this study, we aimed first to identify DNA methylation changes in the spermatozoa of obese rats, and second to see if the changes persist in offspring tissues. These data are valuable for understanding how DNA methylation patterns in the spermatozoa are affected by environmental stimuli, and how these changes are affected by postfertilization epigenetic reprogramming processes.

MATERIALS AND METHODS

Animal procedures

All animal procedures were approved by the Animal Experimentation Ethics Committee of the University of New South Wales (project number 11/82B). F0 male Sprague-Dawley rats from the Animal Research Centre (ARC, Perth, Australia) were housed two per cage under a 12:12 h light/dark cycle. Three-week-old rats were split into two groups with equal average body weight (n = 14/14). Control rats were fed normal chow (energy: 11 kJ g^{-1}, 12% fat, 21% protein, 65% carbohydrate; Gordon's Stockfeeds, NSW, Australia) whilst the high-fat diet (HFD) group was provided with two commercial high-fat pelleted diets, SF03-020 (20 kJ g^{-1}, 43% fat, 17% protein, 40% carbohydrate; Specialty feeds, Glen Forest, WA, Australia) and SF01-025 (18.3 kJ g^{-1}, 44% fat, 17% protein, 39% carbohydrate; Specialty feeds), as well as normal chow. The F0 males were mated (16–19 weeks old) with females consuming control diet (12% energy as fat). The females and males only spent the daylight hours together, and then returned to their home cages to continue their assigned diet (to ensure that only the male was consuming the HFD). No difference in the initiation of pregnancy was observed between obese and control males. The F0 males were killed between 24 and 29 weeks of age. To minimise effects of litter size on pups weight gain, on postnatal day 1, offspring litters were adjusted to 8 to 12 pups per litter, by culling excess pups when litter size >12 or eliminating litters of <8 pups. F1 male offspring were weaned at PND21 on control diet and killed at 27 weeks of age. Animals were killed after anaesthesia induced by i.p. injection of 100 mg ketamine/kg body weight and 15 mg xylazine/kg body weight followed by decapitation. All rat phenotype data are expressed as the mean ± s.d.

Sperm isolation and DNA extraction

Mature spermatozoa were squeezed from the perforated cauda epididymis and vas deferens into 2 ml Dulbecco's Modified Eagle Medium (DMEM) in a Petri dish and mixed by pipetting. The suspension was transferred to a 12 ml Falcon tube and left upright for 20 min. Thereafter, the top 1.5 ml was transferred to two 1.5 ml microfuge tubes and stored on ice. Spermatozoa were pelleted by centrifugation for 1 min at 6000 *g*. Sperm cells were washed and re-pelleted once with phosphate-buffered saline. Any contaminating somatic cells were removed by resuspension in distilled water for osmotic lysis, then incubation for 20 min on ice in somatic cell lysis buffer (0.1% (*w/v*) SDS, 0.5% (*v/v*) Triton-X). Lysates were stored at −80°C until used. After a final repelleting, part of the pellet (5–10 mg) was extracted for DNA with DNeasy Blood and Tissue Kit (Qiagen Chadstone Centre VIC, Australia). The standard kit tissue extraction protocol was used with the exception of the addition of 16 µL 1mol l^{-1} DTT 5 min before addition of the buffer AL to aid in lysis of spermatozoa which are more compact than somatic tissues.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Absolute quantities of 5-methyl-2′-deoxycytidine (5mdC; global methylation) were determined by using LC-MS/MS as described previously.[35] Each individual rat sample was analyzed in triplicate. Results are expressed as the mean ± s.d. Control and HFD groups were compared using Student's *t*-test.

Sodium bisulfite mutagenesis and pyrosequencing

Genomic DNA was sodium bisulfite converted with EpiTect kits (Qiagen) according to the manufacturer's instructions. We investigated repetitive element DNA methylation in spermatozoa as DNA methylation at some classes of repeats is known to be resistant to transgenerational epigenetic reprogramming[26,27] and can thus facilitate epigenetic inheritance.[36,37] Regions of interest were amplified with the primers listed in Supplementary File 1, by using HotStarTaq (Qiagen) and the level of DNA methylation at individual CpG sites in the original sample was ascertained by pyrosequencing on a Pyromark Q96 ID (Qiagen). This technique determines the relative proportions of cytosine (indicating a methylated cytosine) and thymine (indicating an unmethylated, and consequently bisulfite converted cytosine) nucleotides at individual CpG sites in PCR product from regions of interest. Non-CpG C control dispensations were added in each pyrosequencing assay to confirm complete bisulfite conversion of unmethylated cytosine. Control and HFD groups were compared by using Student's *t*-test.

MBD-Seq

Methyl-CpG binding domain (MBD) protein-DNA enrichment and high-throughput sequencing (MBD-Seq) was used to profile DNA methylation genome-wide. Genomic DNA was fragmented in a water bath sonicator (Bioruptor, Diagenode, Denville, NJ, USA) to an average size of ~500 bp and methylated DNA was enriched using the MethylMiner kit (Invitrogen-Life Technologies, Scoresby, VIC, Australia) according to manufacturer's protocols. The MethylMiner kit contains a recombinant form of human methyl-CpG-binding domain protein 2 (MDB2) protein. Methylated DNA was enriched by overnight incubation with MBD2 beads at 4°C and eluted with 2 mol l^{-1} NaCl. MBD-bound (methylated) and unbound (unmethylated) DNA was

purified separately by ethanol precipitation. Successful enrichment of the methylated fraction was confirmed with qPCR of the H19-ICR, an imprinted region that is known to be highly methylated in spermatozoa. On average, the bound fraction had 38 times more H19-ICR DNA than the unbound fraction when equivalent amounts of DNA were used as a template for qPCR (data not shown), primers in Supplementary File 1.

The MBD-bound fractions were sequenced on an Illumina Hiseq2000 platform (50 base-pair single-end reads) after library generation with ChIP-Seq DNA Sample Prep Kit (Illumina, Scoresby, VIC, Australia). Postsequencing duplicated sequences were removed by using the picard tools and unique reads were mapped to the *Rattus norvegicus* rn5 genome assembly by BWA.

The BedTools software package[38] was employed to estimate the distribution of methylation. First all bam files that were generated from next generation sequencing data were converted into bed format with bedtools *bamToBed*. The command bedtools *slop* was then utilized to extend the end position of the 50 nt reads to a 500 nt genomic region to recapitulate the original ~500 bp of the original MBD2-captured DNA fragments. The *coverage* command of bedtools[38] was used with default options to calculate the number of times a repeat class (as described in the RepeatMasker Track of the UCSC Genome Browser, http://genome.ucsc.edu) overlapped with 500 bp regions from the MBD-Seq data set. The total number of 500 bp regions with homology to each repetitive element type was normalized by dividing the number of overlaps by the total number of unique sequence reads from the same sample. The genomic regions of the 12 SATI clusters were identified by a BLAT search on the UCSC genome browser (http://genome.ucsc.edu/) with the SATI consensus sequence (GenBank: V01570.1). The first 12 clusters in the BLAT results search were chosen for examination. Finally, bedtools *coverage* command was used to calculate the number of times each cluster was overlapped by 500 nt regions from the bed file. Control and HFD groups were compared by Student's *t*-test.

RESULTS

Effects of paternal diet on fathers and offspring

At mating, the average body weight of the obese male rat group was 638 ± 46 g (range: 580–726 g, $n = 14$) compared with 489 ± 59 g (range: 408–576 g, $n = 14$) in the control, chow-fed group, $P = 3.53 \times 10^{-8}$. At killing the difference in body weight between the groups had increased so that the control group averaged 535 ± 67 g (range: 451–631 g) and the HFD group 717 ± 67 g (range: 604–807 g), $P = 6.0 \times 10^{-8}$. **Supplementary Figure 1** shows weight gain.

Eight males in each group produced litters; there were no significant differences in litter size or sex ratio between groups. At birth, male offspring from obese fathers were lighter than those from controls. The mean male birth weights calculated per litter were 7.2 ± 0.9 and 6.5 ± 0.3 in litters from control and obese fathers, respectively ($n = 8$ and 8, $P = 0.045$). Litter size impacts the amount of milk available for each pup and consequently their weight gain. Therefore on postnatal day 1, offspring litter sizes were adjusted to 8 to 12 per litter to minimise effects of litter size on pups weight gain. Offspring were raised on control chow, and there was a difference in body weight at 6 months between the male offspring from obese 507 ± 31 g (range: 470–562 g), $n = 14$ versus offspring from control 564 ± 37 g (range: 308–611 g), $n = 13$, $P = 0.0003$ fathers. Both the HFD and control group 6-month-old male offspring that were used for the methylation analyses were from eight different fathers.

Global methylation of paternal spermatozoa

To investigate whether genome-wide DNA methylation changes could underlie the phenotypic changes induced by paternal obesity we initially measured the absolute levels of 5-methyl-2′-deoxycytidine in sperm DNA. Global DNA methylation in the spermatozoa from obese rats (4.70 ± 0.085) was higher than that in control rats (4.45 ± 0.07%) (difference 0.25%, $P = 0.03$) (**Figure 1**).

Methylation of different repetitive element classes in paternal spermatozoa and offspring tissues

We performed pyrosequencing on bisulfite-treated DNA for selected repetitive elements. Satellite repeats (SATI, ISAT and 91ES8) had increased methylation in the spermatozoa from obese rats compared with controls ($P = 0.03$, 0.01, and 0.04, respectively), while IAP LTRs and a type of LINE element showed no difference (**Figure 2**).

To investigate satellite repeats further, the methylated fraction of rat sperm DNA in four HFD and four control samples was isolated with MBD-Seq. High-throughput sequencing on the Illumina Hiseq2000 platform gave an average of 678 000 reads in the four control samples and 591 000 in the four HFD samples. The total number of sequence reads per sample did not allow examination of DNA methylation status of small single-copy regions of the genome. However, examination of common repetitive element classes and repetitive element clusters, such as those found at centromeres, was possible. Comparison of the proportion of sequence reads with homology to LINE, IAP, and SATI repeats confirmed the pyrosequencing data. There was no evidence for DNA methylation changes at LINE or IAP elements, but the data suggested that methylation of SATI repeats was increased in the sperm of obese rats (**Figure 3**, $P = 0.058$). Further, investigation of the normalized number of sequence reads that originated from 12 SATI repeat clusters showed a consistent relative increase in reads in spermatozoa from HFD rats compared with control rats (**Figure 3**). On average, there was a 17% increase in methylation in HFD rats, and when the results from all 12 clusters were combined there was a difference between the two groups ($P = 1.2 \times 10^{-6}$). This result supports the pyrosequencing-derived observation that satellite repeats have higher DNA methylation levels in the spermatozoa from obese than lean rats.

A major question for the investigation into the molecular mechanisms of paternal effects is, do sperm-borne epigenetic changes persist throughout offspring development, potentially to cause functional changes in offspring tissues? Therefore, we used the same pyrosequencing assay that was used in paternal spermatozoa to search for changes in offspring tissues.

However, examination of offspring from obese and control rats suggested that there was no difference in the DNA methylation levels of SATI satellites (**Figure 4**).

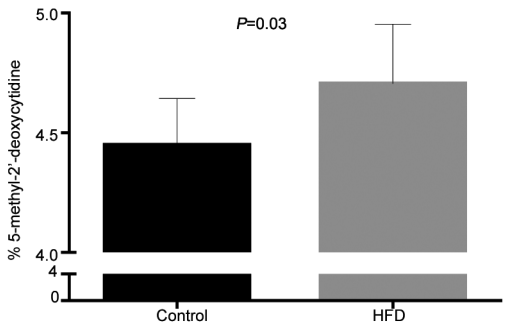

Figure 1: Global DNA methylation in obese rat spermatozoa. Absolute quantification of 5-methylcytosine by using liquid chromatography tandem mass spectrometry (LC-MS/MS) to examine methylation content in the DNA of spermatozoa from obese rats compared with that of control rats. Data are mean ± s.d., Control $n = 8$, HFD $n = 10$.

Methylation of imprinted regions in paternal spermatozoa

To investigate whether the setting of genomic imprints in sperm was affected by paternal obesity, we pyrosequenced three regions that have different methylation patterns in spermatozoa and oocytes - *H19*-ICR, *PEG3* DMR and *SNRPN* DMR.[39] These regions are referred to as differentially methylated regions (DMRs) or Imprint Control Regions (ICRs) and are known to control expression patterns of nearby and distally located genes in offspring. In normal spermatozoa *H19*-ICR is highly methylated, and the other two are unmethylated. This pattern was unaffected by male obesity (**Figure 5**).

DISCUSSION

The list of candidate molecules that may facilitate nongenetic inheritance from father to offspring is long.[4,6] Currently, there is evidence for sperm DNA methylation,[18,21,24,28,34] histone modifications,[9,10,19,21,40] noncoding RNAs,[41,42] reactive oxygen species,[43] and seminal fluid[44] being able to elicit a phenotype in offspring. It is important to identify all mechanisms for inherited phenotypes in order to understand nongenetic inheritance better and to design therapeutic interventions.

In this study, we used our established model for paternal obesity,[32,45,46] where male rats given a HFD are 30% heavier than control rats. The offspring of the obese rats have various phenotypic differences compared with the offspring of controls, including changes to glucose metabolism[45] and body weight.[46] While there is evidence for altered DNA methylation in the spermatozoa of obese males, few studies have tracked these changes into the next generation. We focused on repetitive element DNA methylation in spermatozoa, as repeats have been shown in mice to facilitate epigenetic inheritance.[36,37] Increased DNA methylation at satellite repeats was found in the spermatozoa of obese males but not in their offspring. Our findings provide an illustration of the effectiveness of the trans-generational epigenetic reprogramming mechanisms for normalising DNA methylation patterns. Importantly, we do not suggest that this correction of obesity-induced DNA methylation changes will apply to all

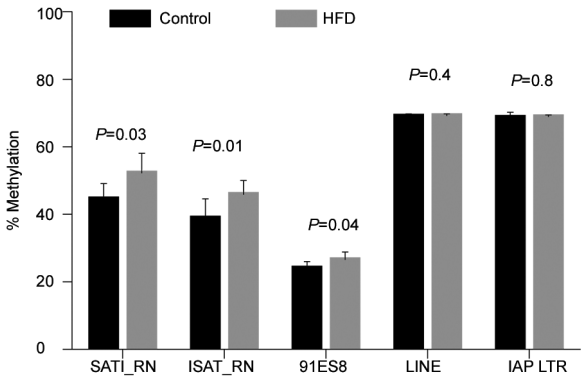

Figure 2: Pyrosequencing to evaluate repetitive-element class-specific methylation in rat spermatozoa. Pyrosequencing of CpG sites in three types of satellite repeats, LINE elements and IAP retrotransposon LTRs revealed the DNA methylation state of each in the spermatozoa of obese and control rats. Percentages displayed for each repetitive element type are from one CpG site in SATI, ISAT and 91ES8, an average of 7 CpG sites in LINE elements and an average of 3 CpG sites in the LTR of an IAP element. Data are mean ± s.d. SATI and 91ES8 Control $n = 7$, HFD $n = 9$; ISAT Control $n = 8$, HFD $n = 8$; LINE Control n = 8, HFD $n = 9$. IAP LTR Control $n = 8$, HFD $n = 8$.

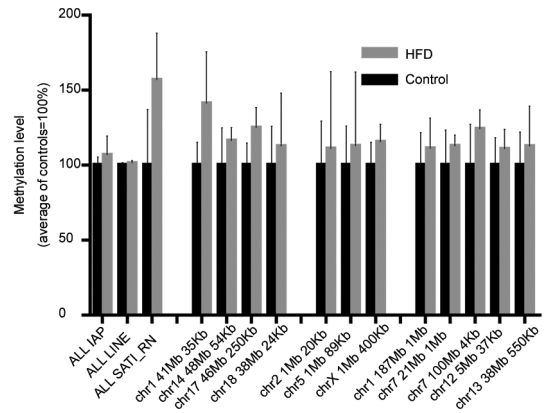

Figure 3: MBD-Seq confirms that SATI satellite repeats at centromeres and noncentromeric regions have increased methylation in obese rat spermatozoa. A completely different methylation assay (MBD-Seq) confirmed the pyrosequencing observations, of increased methylation at SATI sequences but not LINE or IAP elements. Furthermore MBD-Seq read counts at every SATI cluster investigated were higher in obese rat spermatozoa than those from control rats (after normalization to total reads per sample). From left to right, the first three comparisons are total genome-wide MBD-Seq sequence regions with IAP, LINE, and SATI homology. The next four comparisons are at SATI clusters at centromeres of metacentric chromosomes, the next three are clusters are at the centromeres of telocentric chromosomes and the last five are clusters at noncentromeric regions. Data are mean ± s.d. (4 rats per group). The X-axis indicates the chromosome number, location to the nearest Mb and cluster size.

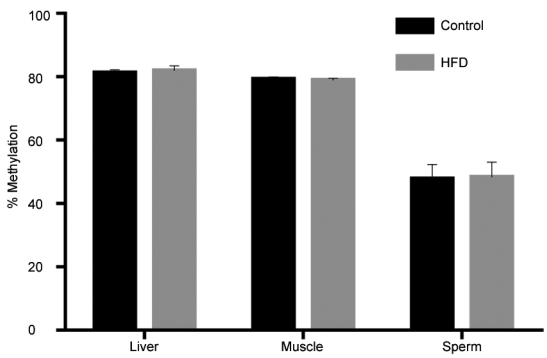

Figure 4: Results of pyrosequencing to investigate DNA methylation at SATI repeats in 6-month-old offspring of obese and control rats. Data are mean ± s.d., liver and spermatozoa control $n = 10$, HFD $n = 10$, muscle control $n = 6$, HFD $n = 8$. Percentages displayed are from one CpG site in SATI. All tissues $P > 0.2$.

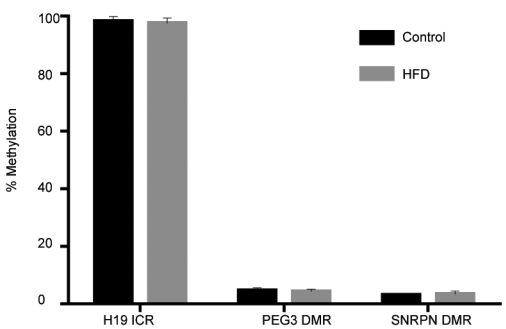

Figure 5: Results of pyrosequencing to investigate DNA methylation at genomic imprints in spermatozoa from obese and control rats. Data are mean ± s.d. H19-ICR is average of three CpG sites, Control $n = 5$, HFD $n = 8$. PEG3 DMR is average of four CpG sites, Control $n = 8$, HFD $n = 8$. SNRPN DMR is average of three CpG sites, Control $n = 8$, HFD $n = 7$. All imprints $P > 0.4$.

environmentally-induced epigenetic changes to sperm. Indeed, there are a growing number of examples where the abnormal methylation state in spermatozoa is detectable in offspring tissues.[24,28] In these studies, the methylation changes were at single-copy loci which were not assessable in our datasets owing to an insufficient read count in the MBD-Seq. Nonetheless, to understand the mechanisms and role of DNA methylation in the transgenerational epigenetic inheritance of phenotypes fully, it is important to report instances of methylation erasure as well as an inheritance.

The increase in global sperm methylation level in obese males compared with lean males is small (0.25%). However, even small changes at the global level have been shown to have large developmental consequences. An increase of 0.6% in global DNA methylation by *Dnmt1* overexpression with a transgene in ES cells is enough to induce embryonic lethality.[47] Our observation of an increase in the total amount of 5-methylcytosine in the spermatozoa from obese rats contrasts with other studies of paternal effects induced by the nutritional intervention of males. A murine model of paternal obesity displayed decreased DNA methylation in testes and spermatozoa when a semi-quantitative immunofluorescence technique was used.[34] Animal models of low-protein diet[48] revealed no DNA methylation changes with MeDIP-Seq in spermatozoa, but locus-specific increases and decreases in the liver. The reasons for these differences could be related to comparisons between quantitative and semi-quantitative methods, differences in global and site-specific methylation changes, or could indicate that the effects of diet on global sperm methylation levels may be species- or diet-specific. With regard to technical differences, HPLC-based techniques such as LC-MS/MS give the most accurate representation of global 5-methylcytosine levels.[49]

A frequently proposed explanation for paternal effects in mammals is an alteration of genomic imprints in spermatozoa. Recent studies in humans[50,51] have associated paternal obesity with alterations in imprint methylation in the offspring's umbilical cord blood leukocytes. The authors proposed that the changes were due to abnormal imprint programming during gametogenesis or early development. We investigated the former possibility by examining imprint methylation in spermatozoa at regions that are normally methylated (paternal imprints) or unmethylated (maternal imprints). The *H19*-ICR and *PEG3* DMR were among the imprints that were altered in human newborn offspring leukocytes.[50,51] We found no alteration in normal imprint states, suggesting that in our rat model there is no alteration in imprinted regions that could have induced offspring developmental changes and consequently the paternal effects. However, we are unable to exclude the possibility of altered imprinting at other regions.

Sequences with homology to repetitive elements make up roughly half of the mammalian genome.[52–54] Their ability to make copies of themselves (transposable elements) or expand (microsatellite repeats) can have deleterious effects on gene function, such as insertion mutagenesis.[55,56] To limit these processes, repetitive sequences usually bear a variety of epigenetic modifications, including DNA methylation that represses transcription and prevents microsatellite instability through replication errors.[57] However, the level of DNA methylation differs between types of repetitive element in response to a variety of processes, such as gametogenesis,[58–60] genome-wide demethylation after fertilisation,[26,27] and deletion or knock-down of epigenetic modifiers.[60,61] This variability among types of repetitive elements has been proposed to provide a mechanism for transgenerational epigenetic inheritance and phenotypic plasticity.[62,63] The main candidate for these hypotheses is the rodent-specific IAP retrotransposon. In the mouse, IAPs have been shown to mediate transgenerational epigenetic inheritance[36,37] and in certain instances the gamete methylation state reflects the offspring adult methylation state.[37,64] However, we did not detect an obesity-induced alteration in DNA methylation in rat IAP LTRs.

Satellite DNA or satellite repeats are sequences of variable length that are repeated throughout the genome, often in clusters. They are common at centromeres and telomeres in mammals though the particular sequences are species-specific. SATI, ISAT, and 91ES8 repeats are units of 370 bp, 395 bp and 203 bp, respectively, that are present throughout the rat genome but that are particularly enriched at centromeres and segmental duplications.[65–67] The absence in offspring tissues of increased DNA methylation in satellites argues against the paternal effects on offspring phenotype being caused by a long-term persistence of global DNA methylation changes between generations. Rather, the data suggest that the increases in DNA methylation in spermatozoa are reprogrammed during development, either in early preimplantation stages or during differentiation of liver, muscle and sperm cells. The most likely explanation is that the active demethylation of paternal chromosomes in the zygote is the process that normalizes the DNA methylation levels.[25,26] This "normalization" of the abnormal epigenetic state is an example of canalisation, a developmental mechanism that suppresses or buffers variation despite genetic or environmental differences.

However, the normalization of methylation levels in offspring in our model does not exclude the possibility that the global DNA methylation increase in obese fathers' spermatozoa indirectly contributes to the paternal effects on offspring phenotype. Before it is reprogrammed, the global methylation increase may influence developmental, metabolic or epigenetic mechanisms that ultimately cause the phenotype. Finally, as stated above, our study does not rule out small but functional locus-specific DNA methylation changes from initiating the inherited effects of paternal obesity.

AUTHOR CONTRIBUTIONS

MJM, VL and CAM designed the format of the rat cohort. VL and CAM carried out the animal procedures. NAY performed sperm isolation, DNA extraction, sodium bisulphite experiments, wet-lab steps of MBD-Seq and chose methylation analyses. JL and LH performed the LC-MS/MS. LH also provided technical advice on the wet-lab steps of the MBD-Seq. LK performed quality control and mapping of next-generation sequencing reads. PL and FL performed the bioinformatics analyses on repetitive elements. All authors contributed to the writing of the manuscript. All authors have read and approved the final version of the manuscript and agree with the order of presentation of the authors.

COMPETING INTERESTS

The authors have no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported. None of the authors declare competing financial interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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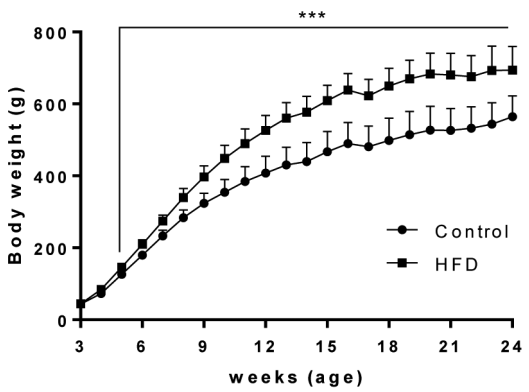

Supplementary Figure 1: Mean body weight changes over time in high-fat diet and control diet fathers. Data are mean ± s.d. Control $n = 14$, HFD $n = 14$, $^{***}P < 0.001$ ANOVA repeated measure (within subject factor = age in weeks; between subject factor = diet).

PRIMERS USED IN SPERM METHYLATION PAPER

Repetitive element pyrosequencing

IAP LTR designed on Repbase RNIAP1aLTR
rIAP BisF GGTAATTTTTTTATTTAAAGGGATA
rIAP BisR Biotin TCCTACTACAAAAAAACTTTAATAC
rIAP pyroseq ATTTTTTTATTTAAAGGGAT
ISAT designed on Repbase ISAT_RN
(Accession number M11460)
ISAT_RN Bis F GGAATTTAAGTATTATGGTGAATTTAGTAG
BTN ISAT_RN Bis R ACTCACCATATTTTAATAAATCTTTAAAAA
ISAT pyroseq TAATAAGTTTGTTATTTAAGATA
SATI designed on reverse strand of Repbase SATI_RN
(Accession number J00784)
SATI_RN Pyro F TTAGTAGTTTGTTTTTGTAATATGTGTATT
SATI_RN Pyro R biotin ATTTTATTAAAAATTTATTAAATTC
SATI pyroseq AGTAGTAATAAGTGGATTGTT
Sat91ES8 designed on Repbase R91ES8_RN
(Accession number X80155)
Sat91ES8 Bis F GTTTTAGGTTGGAAAGGTTTGTAG
BTN Sat91ES8 Bis R CCAAAAAATAAAATATTAAAACTAAAAAAA
Sat91ES8 pyro seq GTAAAAAGTATATTTTTTTATATAG

Line1 primers from Hamm *et al.*, PLos One Dec 2009, e8340
rLINE(F) TTGGTGAGTTTGGGATAT
rLINE(R) biotin AAATCTAAAAACAAAAAACTACTAC
rLINEseq – TAGATTTTTTTAGGAT
LINE pyrosequencing PCR product sequence (Marcelo Soares, personal communication. Product has homology to Repbase RN_HAL1 and L1_RN) TTGGTGAGTTTGGGATATATAGAGGTAG
AATTTTTTTAGGATCGGGTACGTTTTGTGTTTATCGGAAGTT
TTATATTCGCGGATTTCGGTTCGTAGTAGTTTTTTGTTTTTA
GATTTGGTGAGAGAGAGATTTAAT

Genomic imprint pyrosequencing

H19 ICR Bis F BTN GTAATTTGTTTTAGTAGGGATG
H19 ICR Bis R ATAAATACCCCAAATTCAATACCTC
H19 pyro seq GTAAAAACCAAACCTAC
H19 ICR PCR region
GCAATCTGTTTCAGCAGGGATGCGATGTACGCGACT
TCACTGCCGCCACGCGGCAGGCCTGGTTTTTACGCG
CAAAGGATTCTTCGCCGATAGTATGCCGAACTCGTTGATTTC
GGTGTCTGAGACCGCCACGGACCAGCCTAGAAATGCATGT
GTCCTGCCCTCCTAGTGAAGTTTGAGACCCTCTGAGG
TACTGAACTTGGGGTACCCAC
rPEG3 DMR F TTTTGTAGAGGATTTTGATAAGGAG
rPEG3 DMR R Biotin CAATCTAATACACCCACACTAAACC
rPEG3 pyroseq GATGTTTATTTTGGGTT
PEG3 DMR PCR region
CTCTGCAGAGGACCCTGACAAGGAGGTGTCCCGCAGCCCT
TGCTGCAGACGCTGGGGAGTCAAGAGTCGCGGGAGGACGAG
CATCGGAGGAGAAGCGGAGAGATGTCCAC CCTGGGCTGGTG
GCGCCGCTGGGCGCCCGGTTCAGTGTGGGTGCACTAGACTG
rSNRPN DMR Bis F TGAAGTTTAAAGGTTATTTAGTAAT
rSNRPN DMR Bis R AATTATAAAATCCAAAATATAAAAACTATATT
rSNRPN pyroseq TTAAAATATTTGAATTTATAGG
SNRPN DMR PCR region
T G A A G C C C A A A G G C C A T T C A G T A A C C G T T C
C A A A A T A C C T G A A T C T A C A G G T C G C G G C A A T A C A
GCTCGAACATCCAAAGATTCCACCG GCCGATACAAAAGAGC
T C T C G G A T C C C G G A G C C C A G A G G C T G C G G C
AACATAGCTCCTACATCCTGGATTTCACAATC

qPCR primers for amplification of H19 ICR from MBD-enriched DNA

H19 ICR F GGTGGCAGCAAAAATCACTT
H19 ICR R GCCGATAGTATGCCGAACTC