Human Molecular Genetics, 2016, Vol. 25, No. 22 4996–5005

doi: 10.1093/hmg/ddw328
Advance Access Publication Date: 23 September 2016
Original Article

ORIGINAL ARTICLE

Paternal age effects on sperm *FOXK1* and *KCNA7* methylation and transmission into the next generation

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Abstract

Children of older fathers carry an increased risk for developing autism and other disorders. To elucidate the underlying mechanisms, we investigated the correlation of sperm DNA methylation with paternal age and its impact on the epigenome of the offspring. Methylation levels of nine candidate genes and *LINE-1* repeats were quantified by bisulfite pyrosequencing in sperm DNA of 162 donors and 191 cord blood samples of resulting children (conceived by IVF/ICSI with the same sperm samples). Four genes showed a significant negative correlation between sperm methylation and paternal age. For *FOXK1* and *KCNA7*, the age effect on the sperm epigenome was replicated in an independent cohort of 188 sperm samples. For *FOXK1*, paternal age also significantly correlated with foetal cord blood (FCB) methylation. Deep bisulfite sequencing and allele-specific pyrosequencing allowed us to distinguish between maternal and paternal alleles in FCB samples with an informative SNP. FCB methylation of the paternal *FOXK1* allele was negatively correlated with paternal age, whereas maternal allele was unaffected by maternal age. Since *FOXK1* duplication has been associated with autism, we studied blood *FOXK1* methylation in 74 children with autism and 41 age-matched controls. The *FOXK1* promoter showed a trend for accelerated demethylation in the autism group. Dual luciferase reporter assay revealed that *FOXK1* methylation influences gene expression. Collectively, our study demonstrates that age-related DNA methylation changes in sperm can be transmitted to the next generation and may contribute to the increased disease risk in offspring of older fathers.

[†]The authors wish it to be known that, in their opinion, the first three authors should be regarded as co-first authors.
Received: July 26, 2016. **Revised:** September 16, 2016. **Accepted:** September 21, 2016

Introduction

A growing trend for delayed parenthood is evident in Western countries where many couples postpone their wish for children up to their late thirties. Fathers older than 35 years accounted for 25% of birth in 1993 and for 40% in 2003 (1). Both prospective parents and physicians are mainly concerned about advanced maternal age and increasing oocyte aneuploidy rates which cause fertility problems, spontaneous abortions and children with Down syndrome (2). Because of life-long spermatogenesis and potential male fertility, the influence of paternal age on reproduction and offspring health is usually underestimated.

Accumulating evidence from animal and human epidemiological studies also suggests effects of paternal factors, most importantly age, on the development of the offspring (3). It has long been recognized that male age at conception is associated with an increased risk for *de novo* mutations causing achondroplasia and other rare monogenetic disorders (4). More recently, there has been considerable interest in paternal age as a risk factor for neurodevelopmental disorders including autism, bipolar disorder, and schizophrenia (5–9). Paternal age also has an effect on learning and cognition in children (10). One widely accepted explanation for these paternal age effects is that the rate of *de novo* mutations is increasing with age. Spermatogenesis is a life-long process and the number of spermatogonial cell divisions prior to spermiogenesis increase from 35 at puberty to >800 at 50 years. During each replication cycle (every 2–3 weeks) and cell division, genetic mutations occur, continuously increasing the mutational load in the sperm of older males (11). However, genetic *de novo* mutations explain only part of the increased disease risk in children of older fathers. Despite enormous research efforts (GWAS), neuropsychiatric and other complex disorders with paternal age effect display missing heritability. During each cell division not only the DNA itself, but also the epigenetic modifications must be copied to the daughter cells. Since biochemical copying of the epigenetic information is much more error-prone than DNA replication (4,12), it is plausible to assume that aging male germ cells accumulate many more epigenetic than DNA sequence changes. Epigenetic changes, mainly in DNA methylation, during aging have been extensively studied and associated with age-related diseases (13–15). Specific DNA methylation alterations have been found in the brain of individuals with neurodevelopmental diseases and linked to the developmental trajectories exhibiting dynamic methylation changes during foetal brain development (16–18).

Elegant mouse studies provide a link between age-associated sperm methylation alterations and changes in brain gene expression, methylation, and behaviour in the offspring of older mice (19,20). These findings are consistent with the view that sperm epigenetic marks can transmit paternal effects into the next generation, influencing the offspring's disease susceptibility (21). Recently, Jenkins *et al.* (22) studied DNA methylation in sperm of 17 fertile donors collected 9–19 years apart and identified 139 regions which became significantly hypomethylated and 8 which became hypermethylated with paternal age. Twenty-one of these sperm differentially methylated regions (DMRs) were confirmed by bisulfite sequencing.

For this study, we selected a subset of nine genes with validated sperm DMRs that have been associated with neuropsychiatric disorders and other diseases. To study the possible transmission of paternal age effects to the next generation, we performed an in depth methylation analysis of IVF/ICSI sperm samples and foetal cord bloods (FCBs) of the resulting children.

Results

Genes with paternal age-related methylation changes in sperm

A previous study (22) identified several genes which showed a correlation between paternal age and sperm methylation. We selected 9 previously reported age-related genes, *DRD4, NCOR2, NKX2-5, KCNA7, GET4, DMPK, PDE4C, TNXB*, and *FOXK1*, because of their association with neuropsychiatric and other disorders (Supplementary Material, Table S5). None of the studied genes, apart from *PDE4C*, was previously reported to exhibit age-associated methylation changes in blood cells or any other somatic tissue. Repetitive *LINE-1* elements, which comprise approximately 17% of the human genome, were used as a surrogate marker for global methylation (23). Bisulfite pyrosequencing was used to quantify average methylation levels of these 10 loci in 162 IVF/ICSI sperm samples (cohort 1) which had led to a pregnancy. Four genes, *DMPK, FOXK1, KCNA7*, and *NCOR2*, showed significant negative correlation with paternal age (Table 1; Fig. 1, upper panel). *FOXK1* and *KCNA7* revealed the strongest hypomethylation with correlation coefficients of −0.35 and −0.34, respectively. The remaining five genes and *LINE-1* elements did not show a significant correlation between average methylation (of the entire target region) and paternal age, although a few individual CpG sites, for example CpG2 ($P = 0.05$) in the *LINE-1* assay, were borderline significant. We then extended our analysis by using multivariate regression models, to adjust for the cofactor sperm concentration. This analysis also revealed a strong negative correlation for *FOXK1, KCNA7*, and *NCOR2* but not for *DMPK* (Supplementary Material, Table S6). Before studying single sperm methylation of *FOXK1* and *KCNA7*, bisulfite pyrosequencing was performed in 188 independent sperm samples (cohort 2) that did not lead to pregnancy after IVF/ICSI. Consistent with the results of cohort 1, both *FOXK1* (−0.25; 0.001) and *KCNA7* (−0.37, $P < 0.001$) methylation negatively correlated with paternal age.

For single allele DBS analysis, we selected 13 sperm samples of younger (25–35 years) and 13 of older (40–55 years) donors from cohort 1. Figure 2 presents the distribution of single allele methylation patterns for *FOXK1* and *KCNA7*. Sperm with 0 methylated CpGs are fully demethylated; sperm with five methylated CpGs in *FOXK1* and 12 in *KCNA7*, respectively, are fully methylated. For both genes, sperm of younger donors display an enormous (0–100%) methylation variation with most alleles (reads) exhibiting mixed methylation patterns (50–90%). In the sperm of older donors methylation variation is drastically reduced and shows a trend towards hypomethylation (0–20% for *FOXK1* and 10–40% for *KCNA7*). Consistent with bisulfite pyrosequencing (Table 1), average methylations levels (of all sequence reads of a sample) correlated significantly with paternal age for both *FOXK1* (−0.63; $P = 5.78E-04$) and *KCNA7* (−0.77; $P = 1.05E-05$).

Transmission of *FOXK1* and *KCNA7* sperm epigenetic signatures to the next generation

In cohort 1, sperm methylation of *DMPK, FOXK1, KCNA7*, and *NCOR2* showed a negative correlation with age of the donor. To study possible transmission of sperm epigenetic signatures to the next generation, we first performed bisulfite pyrosequencing on foetal cord bloods of >190 children conceived by IVF/ICSI. Although pyrosequencing can only determine the average methylation level of a mixture of millions of paternal and maternal DNA molecules in a genomic DNA sample, FCB

Table 1. Spearman correlation of paternal age with DNA methylation of sperm and foetal cord blood (of resulting children), respectively. P-values were corrected for multiple testing by the Benjamini and Hochberg method. Data are represented as mean ± standard deviation (SD) of percent methylation values.

Gene/Repeat	Sperm			Foetal cord blood		
	Correlation coefficient	Adjusted P-value	Mean ± SD	Correlation coefficient	Adjusted P-value	Mean ± SD
DMPK	−0.24	0.01	9.7 ± 8.3	−0.07	0.57	85.2 ± 4.6
DRD4	−0.04	0.55	24.3 ± 14.7	n.d.	n.d.	n.d.
FOXK1	−0.35	<0.001	33.6 ± 12.8	−0.20	0.04	21.1 ± 3.7
GET4	−0.09	0.37	9.9 ± 7.4	n.d.	n.d.	n.d.
KCNA7	−0.34	<0.001	28.7 ± 12	−0.15	0.15	68.8 ± 3.3
NCOR2	−0.24	0.01	38.0 ± 16.1	−0.09	0.57	11.9 ± 3.3
NKX2	−0.06	0.45	1.4 ± 2.0	n.d.	n.d.	n.d.
PDE4C	−0.06	0.45	4.8 ± 6.7	n.d.	n.d.	n.d.
TNXB	−0.15	0.15	36.80 ±13.2	n.d.	n.d.	n.d.
LINE1	0.12	0.19	69.8 ± 6.6	n.d.	n.d	n.d.

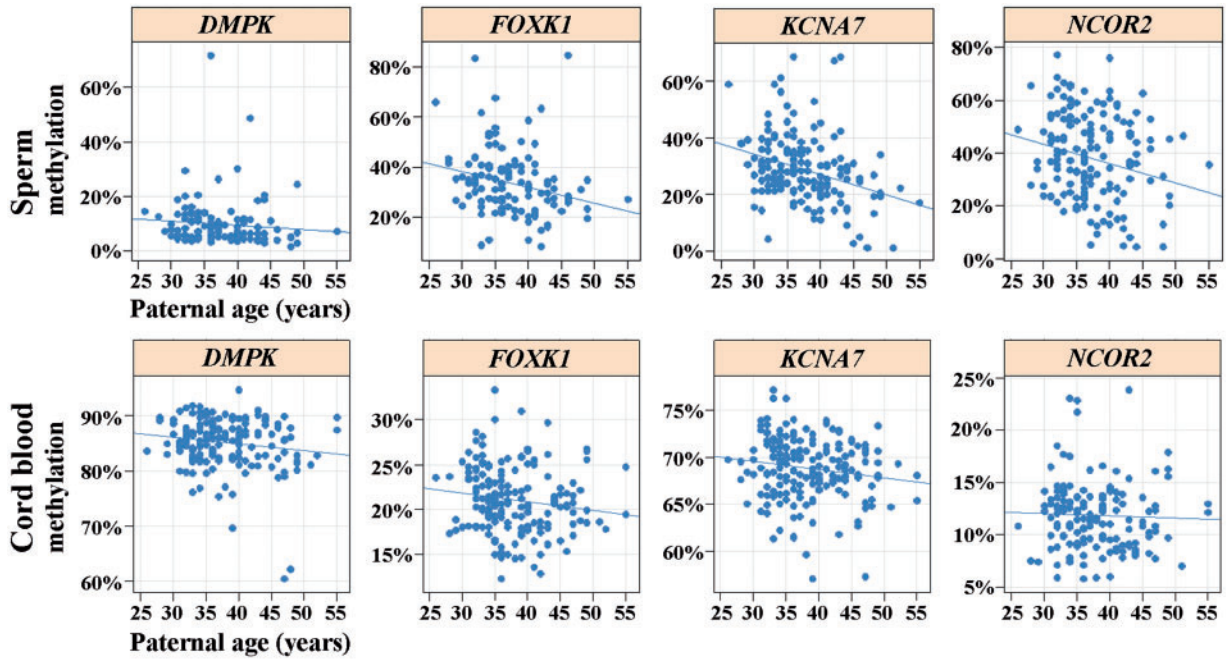

Figure 1. Correlation between *DMPK*, *FOXK1*, *KCNA7*, and *NCOR2* methylation and paternal age in sperm cohort 1 (upper panel) and cord blood (lower panel). Each dot represents average methylation of several target CpGs in an individual sperm or cord blood sample, measured by bisulfite pyrosequencing. Regression lines suggest a negative correlation with paternal age.

methylation of *FOXK1* was significantly (−0.20; FDR-adjusted P = 0.04) correlated with paternal age and *KCNA7* showed at least a trend (−0.15; FDR-adjusted P = 0.15) (Table 1; Fig. 1, lower panel). There was no indication for a paternal age effect on FCB methylation of *DMPK1* and *NCOR2*. A multivariate regression model correcting for gender and birth-weight as cofactors revealed a similar significant correlation for both *FOXK1* (−0.127, P = 0.012) and *KCNA7* (−0.105, P = 0.015) (Supplementary Material, Table S7).

For a more thorough analysis, it is necessary to distinguish between paternal and maternal alleles in the offspring's epigenome. To this end, we searched for SNPs with high heterozygosity rate in the *FOXK1* and *KCNA7* target regions. rs9791644 (G/A) in the *FOXK1* promoter has a heterozygosity rate of 26.4% in European populations. Unfortunately, the only SNP in close proximity to the *KCNA7* target, rs117676231 (A/G), has a heterozygosity rate of only 7%. Two other SNPs with higher minor allele frequencies, rs35622785 and rs12971902, are located more than 500 bp downstream of our *KCNA7* amplicon. However, when we measured sperm methylation of this downstream region, it was not correlated with paternal age (data not shown).

We first developed genotyping pyrosequencing assays for bisulfite-treated DNA which can determine the SNP sequence and at the same time quantify methylation of several individual CpGs in the target region. Both the *FOXK1* and the *KCNA7* assay showed significant correlation between FCB methylation and paternal age (data not shown). For *FOXK1*, we identified 21 informative duos which were heterozygous for rs9791644 in the offspring (FCB) and homozygous in the father (sperm). For *KCNA7* (rs117676231), only nine such sperm-FCB duos were found. DBS

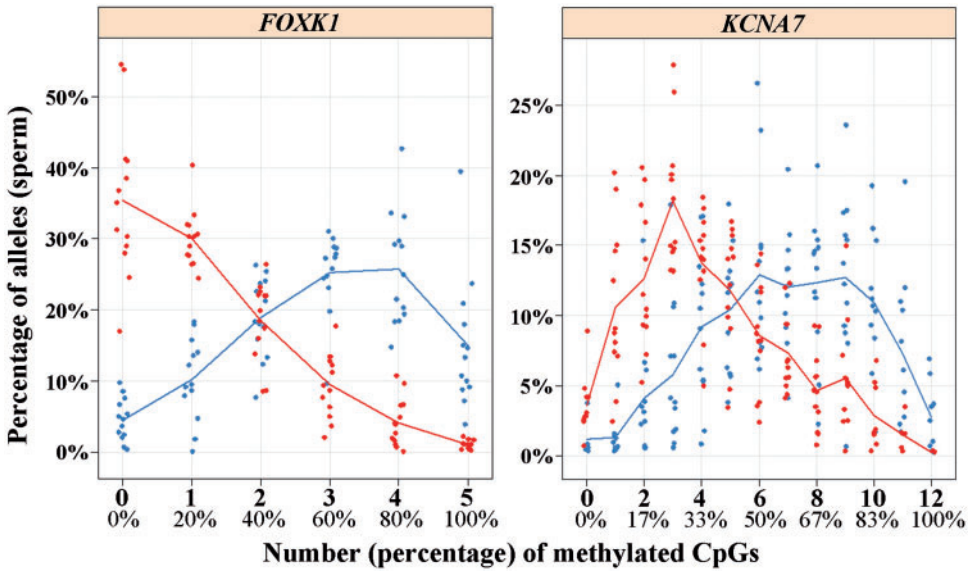

Figure 2. Distribution of single sperm (allele) methylation of *FOXK1* (left diagram) and *KCNA7* (right). Red dots represent sperm samples of older (40–55 years) and blue dots of younger (25–35 years) donors. The *FOXK1* assay targets 5 and the *KCNA7* assay 12 CpG sites. The Y axis indicates the percentage of reads (individual sperm) with 0, 1, 2, 3, 4 or 5 methylated CpGs for *FOXK1* and 0–12 methylated CpGs for *KCNA7*, respectively. Methylation measurements were performed using deep bisulfite sequencing. Average methylation values for the measured CpG sites are provided in Supplementary Material, Table S10.

yielded approximately 538 reads for *FOXK1* and 921 reads for *KCNA7* from informative FCBs. Reads were classified with the SNP into paternal and maternal alleles. For *KCNA7*, average methylation of the paternal FCB allele did not correlate with paternal age, which may be due to the low sample size ($n = 9$). For *FOXK1* ($n = 21$), methylation of the paternal allele displayed a significant (-0.43; $P = 0.049$) correlation with paternal age, whereas maternal allele methylation did not correlate (-0.267, $P = 0.23$) with maternal age. To confirm the DBS results, allele-specific *FOXK1* bisulfite pyrosequencing assays were developed. Sequencing primers were specific for rs9791644 (G/A) with one containing an A and the other a G at the 3' end (Supplementary Material, Table S8). Consistent with DBS, allele-specific pyrosequencing revealed a significant correlation (-0.442; $P = 0.04$) of the paternal *FOXK1* allele with paternal age, but no correlation (-0.198; $P = 0.38$) of the maternal allele with maternal age.

FOXK1 blood methylation in subjects with autism

Because *FOXK1* copy number variations (CNVs) have been associated with autism, we measured *FOXK1* methylation in blood samples of 74 males with ASD and 41 age and sex-matched controls (24). Average methylation levels did not differ significantly between ASD and control group. However, we noted that in both groups *FOXK1* methylation significantly decreased with age. Therefore, we used a linear regression model with age and status as factors to test whether ASD patients display different *FOXK1* methylation trajectories after birth. The results indicate that in both groups *FOXK1* methylation significantly ($P = 2.23E-08$) decreased with age. This analysis additionally revealed a trend ($P = 0.07$) for lower *FOXK1* methylation in ASD patients (Fig. 3).

FOXK1 promoter methylation affects gene expression

The dual-luciferase reporter assay was used to test the effect of *FOXK1* promoter methylation on gene regulation. A 711 bp DNA segment containing the *FOXK1* DMR and known to function as a promoter (ENSR00000156049) was cloned into the CpG-free vector *pCpGL*. The reporter vector with either the methylated or the unmethylated insert was co-transfected with an internal control (Renilla vector) into the human neuroblastoma cell line SH-SY5Y. After normalization of luciferase activity against the internal control, luciferase expression of the unmethylated *pCpGL* vector containing the unmethylated *FOXK1* DMR was significantly ($P = 0.002$) higher than that with the methylated insert (Fig. 4). However, due to the low transfection efficiency of SH-SY5Y cells the firefly/renilla ration was relatively low, compared to negative controls (empty *pCpGL* vector and non-transfected cells). Therefore, we repeated the assay in human osteosarcoma and cervical carcinoma cells. In both U2OS ($P = 0.005$) and HELA ($P = 0.001$) there was a significantly increased luciferase expression using the unmethylated reporter (Fig. 4, Supplementary Material, Table S9).

Discussion

It becomes increasingly clear that the sperm contributes more to the embryo than just the paternal genome. Sperm DNA methylation and chromatin packaging are thought to epigenetically mark paternal genes that may play an essential role for early embryonic development (25). In addition, sperm RNA may contribute to embryo development (26). In the male germ line, DNA methylation marks are established after prenatal mitotic arrest and completed postnatally during meiosis (27,28). Exposure to adverse paternal factors during this genome-wide reprogramming phase may influence sperm methylation patterns. The most thoroughly studied paternal factor that has been associated with changes in sperm DNA methylation and histone-protamine configuration is male subfertility (29–31). Clinically it is well known that male fertility including sperm production, motility and genome integrity decline with paternal age (32) and, therefore, it is not unexpected that paternal age is also associated with sperm methylation alterations (22). The sperm transmits epigenetic signatures of paternal age and other factors into the early embryo, where a second reprogramming

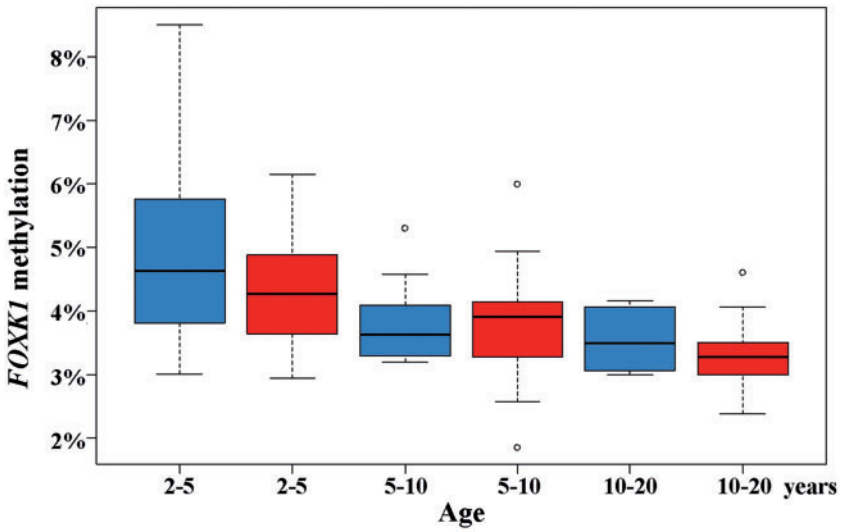

Figure 3. Boxplots showing the distribution of *FOXK1* methylation in 74 male ASD patients (red boxes) and 41 age and sex-matched controls (blue boxes) in three different age groups (2–5, 5–10, and 10–20 years). The median is represented by a horizontal black line. The bottom of the box indicates the 25th, the top the 75th percentile. Outliers are shown as open circles.

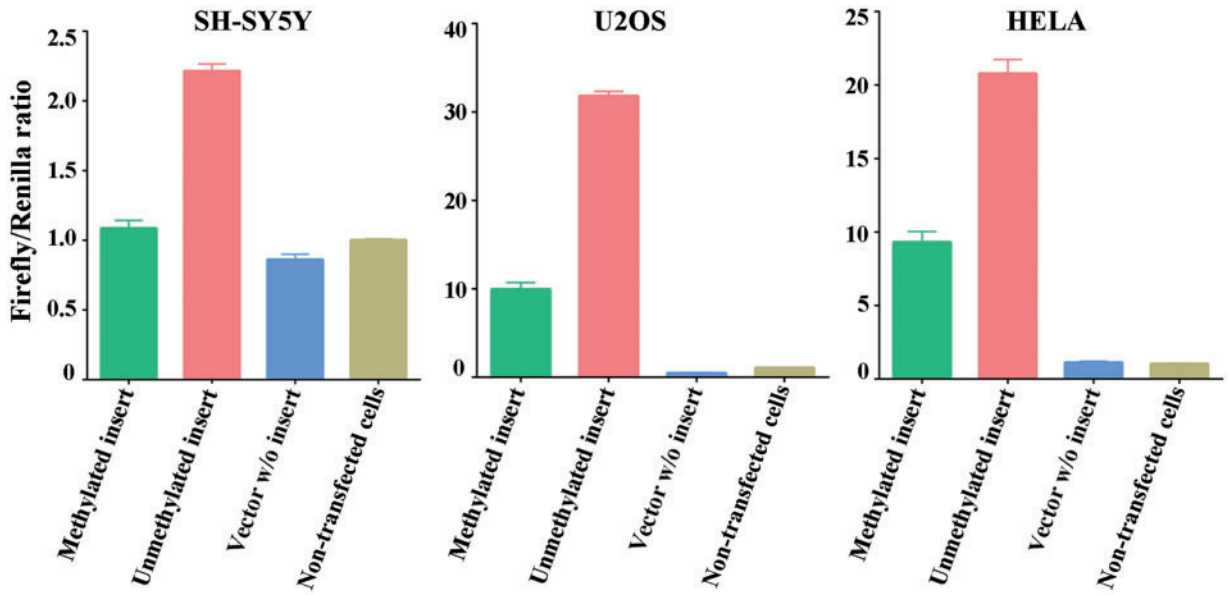

Figure 4. Luciferase activity of *pCpGL* vector containing either methylated or unmethylated *FOXK1* promoter, normalized to activity of an internal control vector (Firefly/Renilla ratio). *pCpGL* and control vector were co-transfected in a 500:1 ratio in three different cell lines, SH-SY5Y, U2OS, and HELA. Empty vector and non-transfected cells served as negative controls. All measurements were done in duplicates.

phase occurs. Most DNA methylation patterns that have been established in the male germ line are erased and replaced by somatic patterns which are identical on both parental chromosomes and mark genes for somatic development (33). The question how sperm epigenetic signatures can influence further development and have long-lasting effects on the health of the individual remains to be elucidated. A relatively small number of 100–200 imprinted genes escape reprogramming after fertilization and, thus, sperm DNA alterations persist during ontogeny. Therefore, imprinted genes are frequently used as a model to study the epigenetic effects of adverse parental factors and assisted reproductive technologies (30,34). However, it is plausible to assume that non-imprinted genes are also involved in transgenerational epigenetic inheritance. Our results suggest that postzygotic reprogramming is incomplete and sperm epigenetic alterations can persist in differentiated somatic cells (at least at the cell population/tissue level) of the next generation. Previously, we observed an association between sperm hypomethylation of *ALU* repetitive elements and pregnancy loss (35). Rodent studies revealed that paternal aging affects brain expression and behaviour in the offspring (19,20).

Our study shows that increased paternal age is associated with reduced sperm methylation of the transcriptional regulator *FOXK1* and the potassium channel *KCNA7*. We exclusively used IVF/ICSI sperm samples (with a wide range of semen parameters) and, therefore, cannot exclude assisted reproduction

as a confounding factor. However, the same age-related effect was observed in a previous study on sperm of fertile donors (22). Interestingly, for both genes sperm samples of young donors showed considerably higher methylation variation with individual sperm methylation ranging from 0 to 100%. Sperm samples of older fathers exhibited reduced variation with the majority of sperm being demethylated. Assuming that sperm samples of younger fathers have a higher potential to achieve a pregnancy and live-birth, sperm methylation variation may have an evolutionary advantage at the population level. For some genes, there may be no optimum methylation status for an individual sperm. Depending on the environmental conditions at fertilization (i.e. pre- or postovulatory age of the oocyte), (epi)genetic background of the mating partner, etc., either methylated or unmethylated sperm (or sperm with methylation values in between) may have the best developmental potential or confer an advantage to the resulting individual. The reduced methylation variation in sperm of old fathers is consistent with 'selfish spermatogonial selection' (36,37). *De novo* alterations that confer a proliferative advantage to the spermatogonial stem cell would lead to the expansion of this cell clone (and consequently sperm carrying this mutation or epimutation).

ASD is a neurodevelopmental disorder characterized by stereotypical behavior, deficits in social interactions and communication (38). Usually, symptoms start to develop in the second year of life and may aggravate during the following years. Epidemiological studies provide evidence for an increased autism risk in children of older fathers (12,39,40). One plausible explanation for this association is transmission of an adverse paternal factor through sperm to the offspring. Although whole genome sequencing clearly showed that the rate of *de novo* mutations in offspring increases with paternal age (41), age-related genetic mutations cannot explain much of the increased risk for ASD and other psychiatric disorders (42). In our opinion, age-related epigenetic alterations may also contribute to the increased disease risk. ASD and other complex psychiatric disorders manifest when the sum of adverse genetic, epigenetic and/or environmental factors exceeds a critical threshold (43). By two independent methods, DBS and allele-specific pyrosequencing we showed that paternal age is not only associated with *FOXK1* hypomethylation in sperm but also with reduced methylation of the paternal allele in somatic cells (FCB) of the resulting offspring. In contrast, methylation of the maternal allele was not correlated with maternal age. Luciferase assays suggest that this age-related promoter hypomethylation leads to increased gene expression.

At the single CpG level, an individual sperm (and also the oocyte) can either transmit an unmethylated or a fully methylated state to the embryo. Therefore, one might expect a 0, 50, or 100% methylation pattern in the zygote and also in the offspring if epigenetic inheritance occurs. However, it is usually the density of methylated CpGs in a cis-regulatory region rather than an individual CpG that turns a gene on or off (44,45). Therefore, all our assays including DBS (for single allele analysis) measured the average methylation of several neighboring CpGs in the target region. As shown in Fig. 1, individual sperm do not only display 0 or 100% methylation at a particular locus but also anything in between. Since older males have a higher percentage of sperm with low methylation values, the fertilized eggs are more likely endowed with a hypomethylated paternal allele, compared to younger males. Irrespective of the methylation status of the fertilizing sperm, the transmitted paternal allele is influenced by genome-wide epigenetic reprogramming after fertilization, developmental and differentiation processes. Nevertheless, at a population level when comparing cord blood methylation in children conceived by old versus young fathers we can still detect an epigenetic signature of paternal age during ontogeny. Children from old fathers who more often developed from a zygote with a hypomethylated sperm display a slightly reduced methylation in the paternal *FOXK1* allele. However, in this context, it is important to emphasize that both children from old and young fathers display enormous methylation variation and the distributions largely overlap. The methylation change due to paternal age is much smaller than the variation between individuals. Thus, age effects can only be detected at the population level.

FOXK1 (forkhead box K1) encodes a transcription factor of the forkhead family which has a known role in the activation of myogenic progenitor cells and skeletal muscle regeneration (46). The more than 40 members of the forkhead family are involved in the regulation of gene expression in embryonic development, metabolism, cell-cycle control, and cancer. *Foxk1* and *Foxk2* regulate autophagy and atrophy genes in muscle cells and fibroblasts (47). *Foxk1* knockout mice are viable, but runted, and suffer from skeletal muscle atrophy and impaired satellite cell function (48). On the other hand, FOX genes such as *FOXP1* and *FOXP2* have been associated with autism and specific language impairment (49–51). A CNV array study (24) demonstrated microduplications of the *FOXK1* region in 2 out of 23 ASD patients. As expected for an ASD gene (18,52), *FOXK1* methylation is highly dynamic during foetal brain development. We found that *FOXK1* FCB demethylation is accelerated in autistic individuals during the first years of life (2–5 years). However, due to the relatively small sample size and the high methylation dynamics after birth, there is only a trend ($P = 0.07$) difference between ASD subjects and controls. Larger follow-up studies are necessary to validate this effect. A recent 450K methylation array study on adult cortex identified several CpG sites, including 5 in the *FOXK1* gene body, which displayed significant methylation differences between autistic and control subjects (17). This further strengthens the possible role of *FOXK1* methylation in the development of autism. Considering its function in myogenic progenitor and muscle cells, *FOXK1* deregulation may also be associated with clumsiness and hypotonia, which is often observed in young children with autism (38).

The *KCNA7* (potassium voltage-gated channel subfamily A member 7) gene has been implicated in cardiac disorders and sperm volume regulation (53,54). Voltage gated potassium channels are crucial for the electrical excitability of nerve and muscle fibres, and have been associated with ASD (55–57). We identified a DMR in the CTCF binding site (ENSR00000641867) close to the *KCNA7* promoter which was hypomethylated in sperm and resulting offspring of older males. Unfortunately, DBS analysis of the paternal allele in FCB did not yield a significant age effect due to the low number of informative samples with a heterozygous SNP.

Conclusion

Here, we provide evidence that paternal age is associated with reduced methylation and variation of the transcriptional regulator *FOXK1* and the potassium channel *KCNA7* in sperm. These age-related sperm epigenetic signatures can be transmitted to the next generation. Methylation of the paternal *FOXK1* allele in FCB of the offspring is inversely correlated with paternal age. *FOXK1* methylation is highly dynamic during foetal brain development and, to the extent it could be studied in FCB, also after birth. Previously, it has been shown that the *FOXK1* gene is

differentially methylated in adult cortex of autistic individuals. We propose that accelerated methylation dynamics during development persists into the adult brain and may contribute to an increased ASD risk of children from older fathers. In our opinion, transmission of age-related epigenetic changes through the male germ line is an underestimated mechanism underlying the paternal age effect of neuropsychiatric and other complex disease.

Materials and Methods

Ethics statement

The study of human sperm and FCB samples was approved (no. 111/13) by the Ethics Committee at the Medical Faculty of Würzburg University and informed consent was acquired from all participating couples.

Samples

Following IVF/ICSI, the left-over swim-up fractions of sperm samples were collected at the Fertility Center Wiesbaden and frozen at −80 °C until further use. Cohort 1 consisted of 162 samples which had led to the birth of a child (Age Range: 26–55 years Median = 37) and cohort 2 of 188 samples without pregnancy (Cohort 2: Age Range 25-66 y Median = 39). Following live-birth, 191 FCBs (including some twins) were obtained from collaborating obstetric clinics throughout Germany. Relevant clinical parameters of sperm and FCB samples are listed in Supplementary Material, Table S1.

Blood DNA samples of 75 male individuals (up to 20 years) with autism spectrum disorder (ASD) were obtained from the Centre de Référence déficiences intellectuelles de causes rares and the 'Centre diagnostic autism' Pitié-Salpêtrière Hospital (Paris, France). Index cases were evaluated by specialized geneticists and paediatric neurologists and/or child psychiatrists. Patients were assessed with the Autism Diagnostic Interview-Revised (ADI-R). Informed consent was obtained and studies were approved by local ethics committees.

For sperm DNA isolation with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), the swim-up sperm fraction was first purified using Silane-coated silica density gradients PureSperm 40/80 (Nidacon, Mölndal, Sweden). Briefly, the purified sperm was resuspended in 300 µl buffer. The stock consisted of 2 ml 0.5 EDTA (pH 8), 5 ml 5 M NaCl, 5 ml 1 M Tris-HCl (pH 8), 5 ml 10% SDS (pH 7.2), 1 ml 100% β-mercaptoethanol, and 33 ml dH_2O. After addition of 100 µl proteinase K (20 mg/ml), the samples were incubated at 56 °C with slight shaking. After 2 h, additional 20 µl proteinase K was added and samples incubated for another 2 h. Subsequent steps were performed according to the DNeasy Blood and Tissue Kit protocol. Blood DNA was isolated using the FlexiGene Kit (Qiagen, Hilden, Germany) following manufacturer's recommendations. The amount and quality of the DNA were determined with the NanoDrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA). Sperm and blood DNA (1 µg) were bisulfite converted using the EpiTect Fast 96 Bisulfite Kit (Qiagen, Hilden, Germany) and bisulfite converted DNA was stored at -20 °C.

Bisulfite pyrosequencing

The PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany) was used for primer design (Supplementary Tables S2 and S3). Assays were established using standard DNAs with 0, 25, 50, 75, and 100% methylation. PCR was performed in 25 µl reactions consisting of 2.5 µl 10x PCR buffer with $MgCl_2$, 0.5 µl dNTPs, 1.25 µl (10 pmol/ml) of each forward and reverse primer, 0.2 µl FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 1 µl (~25 ng) bisulfite converted DNA. Pyrosequencing was performed on a PyroMark Q96 MD system using the PyroMark Gold Q96 CDT reagent kit and Pyro Q-CpG software (Qiagen, Hilden, Germany). In our experience, the average methylation difference between technical replicates (including bisulfite conversion, PCR, and pyrosequencing) is approximately 1-2 percentage points. Artificially methylated and unmethylated DNA standards (Qiagen, Hilden, Germany) were included as controls in each pyrosequencing run.

Deep bisulfite sequencing

Deep bisulfite sequencing (DBS) is an amplicon-based next generation sequencing technique to determine the methylation patterns of hundreds to thousands of individual molecules of a target gene per sample. The *KCNA7* and *FOXK1* DMRs were PCR amplified from bisulfite-treated sperm or cord blood DNA. The *FOXK1* amplicon included 5 and *KCNA7* 12 CpG sites. Region-specific primers with a universal adapter were used in the first-round PCR (Supplementary Material, Table S4). The resulting PCR product was then amplified with sample-specific multiplex identifiers (MIDs), 454 Titanium A and B sequences, and key (TCAG) sequences. The second-round products were purified with Agencourt AMPure XP Beads (Beckman Coulter, Krefeld, Germany) to remove remaining nucleotides and primers. DNA quality was checked on the Bioanalyzer with the DNA 7500 LabChip kit. Amplicons were quantified using the NanoDrop 2000c spectrophotometer, pooled, and diluted to 1x 10^9 molecules/µl. The bisulfite amplicon libraries (generated by emulsion PCR) were sequenced on a Roche/454 GS Junior system following the manufacturer's protocol. The sequence reads were processed using the Roche Genome Sequencer software and SFF files were further analysed using the Amplikyzer software (58). Target regions included a single nucleotide polymorphism (SNP) to distinguish between parental alleles. Each DBS run included DNA standards with 0, 50 and 100% methylation. Processing of standard flowgram files (SFF) has been performed using the Amplikyzer pipeline which directly aligns the intensity sequences from SFF files to amplicon reference sequences and provides a detailed nucleotide-level analysis including the calculation of CpG methylation rates. The direct use of SFF information, without prior conversion to FASTQ format avoids information loss by rounding flow intensities. For downstream processing of Amplikyzer output files in-house R-Scripts have been used and subsequent statistical analyses of methylation rates has been performed in R (Version 3.2.2) as described below.

Luciferase reporter assay

Functional analysis of *FOXK1* promoter methylation was performed with a dual-luciferase reporter assay. A 711 bp fragment (Supplementary Material, Fig. S1) was amplified using forward primer 5'-GGGGGATCCAGGACAGTGGGAGGGTGTT-3' and reverse primer 5'-CCAAGCTTCCAAAATGCACACCTTTCAGC-3'. The forward primer contained a BamH*I* and the reverse primer a Hind*III* recognition site. The *pCpGL* vector completely lacks CpG dinucleotides in its backbone which could influence the activity of the luciferase reporter gene (59). Following double

digestion of *pCpGL* vector and *FOXK1* insert with BamH*I* and Hind*III* (New England Biolabs, Frankfurt, Germany), the insert was ligated into the multiple cloning site upstream of the luciferase gene. The vector was transformed into one-shot competent PIR1 bacterial cells (Thermo Fisher Scientific). Colony PCR was performed to check whether the insert is in the right orientation using primers F1 (AAACCACTGATTTTTGTTTATGTGA) and R1 (AGAAAGTGGCTCCAGAGGAA) or F2 (ACCTCAAG GTCTGTTGATCAG) and R2 (GACCAGGGCATACCTCTTCA), respectively. Plasmid DNA was purified using a Midi or Maxi Prep kit (Zymo Research; Irvine, CA). The *pCpGL* vector with *FOXK1* insert was *in vitro* methylated using Sss*I*, Hha*I*, and Hpa*II* methylases (New England Biolabs, Frankfurt, Germany). *In vitro* methylation was confirmed with the methylation sensitive and insensitive restriction enzymes Hpa*II* and Msp*I* (New England Biolabs, Frankfurt, Germany). Finally, either the methylated or the unmethylated *pCpGL* vector was co-transfected with Renilla control vector (in a 500:1 ratio) in three different cell lines, SH-SY5Y (derived from bone marrow neuroblastoma), U2OS (bone marrow osteosarcoma), and HELA (cervical carcinoma), using Lipofectamine 3000. Luciferase activity was measured with a Berthold Tristar microplate luminometer. The *pCpGL* reporter (firefly fluorescence) was normalized against the activity of the Renilla control vector. All transfections and dual luciferase reporter assay measurements were done in duplicates. *pCpGL* with a cytomegalovirus promoter insert served as positive, empty *pCpGL* vector and non-transfected cells as negative controls.

Statistical testing

Statistical analyses have been performed with the statistical software package R (Version 3.2.2). To obtain a first overview of the bisulfite pyrosequencing data the Spearman correlation between the DNA methylation percentage (averaged over CpG sites) and the father's age have been calculated for each gene. Multiple testing adjustment has been performed using the method of Benjamini and Hochberg (60). To adjust for potential confounding factors these analyses have subsequently been extended using multivariate linear regression models. The regression coefficients of paternal age have been adjusted for birth weight (numerical) and gender in the FCB model and for sperm concentration (numerical) in the sperm model. Potential confounders were chosen based on previously reported associations with DNA methylation changes. Analogously, bisulfite pyrosequencing data of autism patients have been analysed using a linear model including age (as categorical variable with three age classes, 2–5, 5–10 and 10–20 years). To control for varying variance across observations a heteroscedasticity-corrected covariance matrix ('hc3') has been used as described in Long and Ervin (61) and implemented in the car package (62).

For DBS, processing of standard flowgram files (SFF) has been performed using the Amplikyzer pipeline (58) which directly aligns the intensity sequences from SFF files to amplicon reference sequences and provides a detailed nucleotide-level analysis including the calculation of CpG methylation rates. The direct use of SFF information, without prior conversion to FASTQ format avoids information loss by rounding flow intensities. For downstream processing of Amplikyzer output files in-house R-Scripts have been used and subsequent statistical analyses of methylation rates have been performed in R (Version 3.2.2) as previously described.

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgements

The authors thank Dr. Michael Rehli for providing the pCpGL vector, Dr. Douglas Carrell and Dr. Timothy Jenkins for unpublished information on sperm DMRs, and Professor Almut Schulze for SH-SY5Y cells. We sincerely thank all IVF/ICSI couples who participated in this study. R.P. was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.

Conflict of Interest statement. None declared.

Funding

This work was supported by the German Research Foundation (grant EL 742/1-1 to N.E.H.) and the European Union (ERAofART). Funding to pay the Open Access publication charges for this article was provided by revenues of the Würzburg Institute of Human Genetics.

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