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# Sperm DNA methylation dynamics after chemotherapy: a longitudinal study of a patient with testicular germ cell tumor treatment

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## Abstract

**Background:** An important issue for young men affected by testicular germ cell tumor (TGCT) is how TGCT and its treatment will affect, transiently or permanently, their future reproductive health. Previous studies have reported that xenobiotics can induce changes on human sperm epigenome and have the potential to promote epigenetic alterations in the offspring.

**Objectives:** Here, we report the first longitudinal DNA methylation profiling of frozen sperm from a TGCT patient before and up to 2 years after a bleomycin, etoposide, and cisplatin (BEP) chemotherapy.

**Materials and methods:** A TGCT was diagnosed in a 30-year-old patient. A cryopreservation of spermatozoa was proposed before adjuvant BEP treatment. Semen samples were collected before and after chemotherapy at 6, 9, 12, and 24 months. The DNA methylation status was determined by RRBS to detect DNA differentially methylated regions (DMRs).

**Results:** The analysis revealed that among the 74 DMRs showing modified methylation status 6 months after therapy, 17 remained altered 24 months after treatment. We next associated DMRs with differentially methylated genes (DMGs), which were subsequently intersected with loci known to be important or expressed during early development.

**Discussion and conclusion:** The consequences of the cancer treatment on the sperm epigenome during the recovery periods are topical issues of increasing significance as epigenetic modifications to the paternal genome may have deleterious effects on the offspring. The altered methylated status of these DMGs important for early development might modify their expression pattern and thus affect their function

Anne-Sophie Neyroud and Antoine Dominique Rolland contributed equally to this work.

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during key stages of embryogenesis, potentially leading to developmental disorders or miscarriages.

#### KEYWORDS

bisulfite sequencing, chemotherapy, epigenome, methylation, sperm, testis cancer

## 1 | INTRODUCTION

Over the past quarter century and particularly in the last decade, the incidence of testicular cancer (TC) has been rising rapidly.<sup>1,2</sup> Although it is a proportionately rare disease which accounts for approximately 1% of all new male cancer cases, TC remains the most frequent cancer in male adolescents and young adults from 15 to 35 years in industrialized countries.<sup>3–5</sup> The TC incidence rate ranges between 5.7 and 13.4 per 100,000 in Europe.<sup>5,6</sup> In France, no national cancer registry exists, thus estimations of TC incidence are based on six specific cancer registries. Consequently, hypotheses on TC incidence tendencies are defined by a single standardized sperm bank network, the Centre d'Etude et de Conservation des Oeufs et du Sperme humain (CECOS), which provides precious data to indirectly estimate global TC incidence. This unique public network of sperm banks was established in 1973 and covers the whole country through 33 affiliated regional sperm banks. In the last decade, prognoses and survival rates have improved very markedly.<sup>7,8</sup> An important issue for these young men is how TC and its treatment will affect, transiently or permanently, their future reproductive health.<sup>9,10</sup> Semen banking is thus recommended before any treatment.<sup>11,12</sup>

Several late adverse effects of chemotherapy or radiotherapy have been described.<sup>13</sup> Post-treatment gonadic function could notably be drastically affected, and the intensity of spermatogenesis alterations is related to the type of drugs used, the cumulative doses of the drug(s), the radiation doses received by the testis, and the time between the last treatment and sperm analysis.<sup>14,15</sup> Other studies have also examined the impact on sperm DNA fragmentation and genome integrity<sup>16–18</sup> and DNA methylation alterations<sup>19,20</sup> after cancer treatment. Apart from the GAMATOX prospective study published by Rives and colleagues,<sup>21</sup> most of these debated studies are retrospective and consequently the dynamics of recovery are not precisely described. The consequences of cancer treatment on the sperm epigenome during the recovery periods are topical issues of significance as epigenetic modifications to the paternal genome may have deleterious effects on the pregnancy or the offspring.<sup>22–24</sup> One case report showed a drop on the methylation level in one patient following treatment by temozolomide for an anaplastic oligodendroglioma.<sup>20</sup> The other study reported modifications on DNA methylation regions in 18 sarcoma patients treated by cisplatin ± ifosfamide but the cross-sectional design of this study limited results interpretation.<sup>19</sup>

Here, we report a case study aiming at longitudinally evaluating the temporal evolution of the sperm DNA methylation status of a

testicular germ cell tumor (TGCT) patient before and 6, 9, 12, and 24 months after a bleomycin, etoposide, and cisplatin (BEP) chemotherapy.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient samples and ethical considerations

A TGCT was diagnosed in a 30-year-old patient. A preservation of fertility was proposed before treatment. Chemotherapy consisted of four cycles of BEP protocol: (i) etoposide (100 mg/m<sup>2</sup>/day) from days 1 to 5; (ii) cisplatin (20 mg/m<sup>2</sup>/day) from days 1 to 5; and, (iii) bleomycin (30 mg in 30 mn) at days 1, 8, and 15. After a local ethic committee approval with inclusion in GERMETHEQUE National Biobank (BB-0033-00081) and written informed consent (GERM1015012), sperm analyses were performed before and after his adjuvant treatment. Two semen samples were obtained before treatment (samples were named PreCT\_1 and PreCT\_2), while a single sample was obtained at each of four time points after treatment, that is, at 6, 9, 12, and 24 months (PostCT\_6 m, PostCT\_9 m, PostCT\_12m, PostCT\_24 m). Analysis of semen was performed in our laboratory according to standard World Health Organisation criteria (WHO, 2010). Sperm was collected in a sterile container by masturbation and analyzed after liquefaction according to WHO criteria. The parameters assessed included volume of ejaculate, sperm concentration, motility (forward movement), vitality, and morphology (David's criteria). The total sperm count was obtained by multiplying the sperm concentration by the volume of ejaculate. Throughout the study, the patient confirmed that he did not make any lifestyle changes (non-smoking, no alcohol or other drug consumption, no change in family or work situation) that could impact the sperm methylome.

### 2.2 | Sperm DNA extraction and purification

Sperm freezing straws were thawed and subjected to density gradient centrifugation (30 min at 830 g, 25°C) on a two-layer 80%/40% Percoll. Pellet (corresponding to the "motile" fraction) was washed and centrifuged twice for 15 min with 10 mL of Wash Buffer [NaCl 150 mM, Ethylenediaminetetraacetic acid (EDTA) 10 mM, pH 8] at 1600 g. Only the motile fraction was considered for the analysis. Cell lysis was performed in a low-binding tube and incubated in a thermomixer (Eppendorf) overnight at 55°C with 500 µL of Digestion Buffer (Tris

50 mM pH 8, NaCl 100 mM, EDTA 10 mM, SDS 1%) to which were added 21  $\mu$ L DTT 1 M, 20  $\mu$ L PK 20 mg/mL, and 2.5  $\mu$ L Triton X-100.

Cell debris was pelleted down by centrifugation at 14,000 $\times$ g, and the supernatant was subjected to a 10 mg/mL RNase A digest for 30 min at 37°C in a thermomixer. DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol followed by a second extraction with chloroform:isoamyl alcohol. The aqueous phase containing DNA was purified by a standard precipitation with NaCl 300 mM/2.5 V alcohol 100% and 1  $\mu$ L GlycoBlue (Thermo Fisher Scientific). The final DNA concentration was measured using DsDNA QuantiFluor Dye System (Promega).

### 2.3 | Reduced representation bisulfite sequencing libraries

The reduced representation bisulfite sequencing (RRBS) method<sup>25</sup> was performed using the Premium RRBS Kit according to the manufacturer's instructions (Diagenode). Briefly, 100 ng of DNA was digested using the methylation-sensitive restriction enzyme, MspI, followed by end-repair and A-tailing. Adaptor ligation was followed by a size selection (40–220 bp) with the AMPure XP Beads (Beckman Coulter). Quantification prior to sample pooling was evaluated by a q-PCR analysis. Then, DNA samples with adjusted quantity underwent bisulfite conversion followed by desulphonation and on-column purification. The converted DNA was amplified by polymerase chain reaction (PCR) and cleaned-up with the AMPure XP Beads. Finally, the quality of the RRBS libraries was evaluated using an Agilent 2100 bioanalyzer. The RRBS libraries were sequenced on an Illumina HiSeq 4000 sequencer as single-end 50 base reads following Illumina's instructions. Image analysis and base calling were performed with RTA 2.7.3 and bcl2fastq. Following the sequencing, quality controls were performed for each sample with FastQC. Briefly, we ensured that a percentage higher than 95% of bases above Q30 (always  $\geq$ Q27) was obtained for each sample. The proportion of each nucleotide along the read was verified, to ensure that reads start with CGG due to MspI digestion between cytosines at CCGG sites. Following a general tendency across samples, the nucleotide proportions on the first base of reads converge to a 50–50 ratio between cytosines and thymines (slightly higher for Cs). The cytosines correspond to bisulfite unconverted methylated cytosines, while thymines represent bisulfite converted unmethylated cytosines. This observation, paired with the high %G ( $\approx$ 95%) on the second and third bases of the reads, retranscribe MspI cutting sites.

### 2.4 | Data preprocessing and analysis

A four-step workflow was designed to preprocess RRBS data, to analyze the CpG methylation data and, finally to identify and annotate differentially methylated regions (DMRs) (Figure 1).

#### 2.4.1 | Read mapping

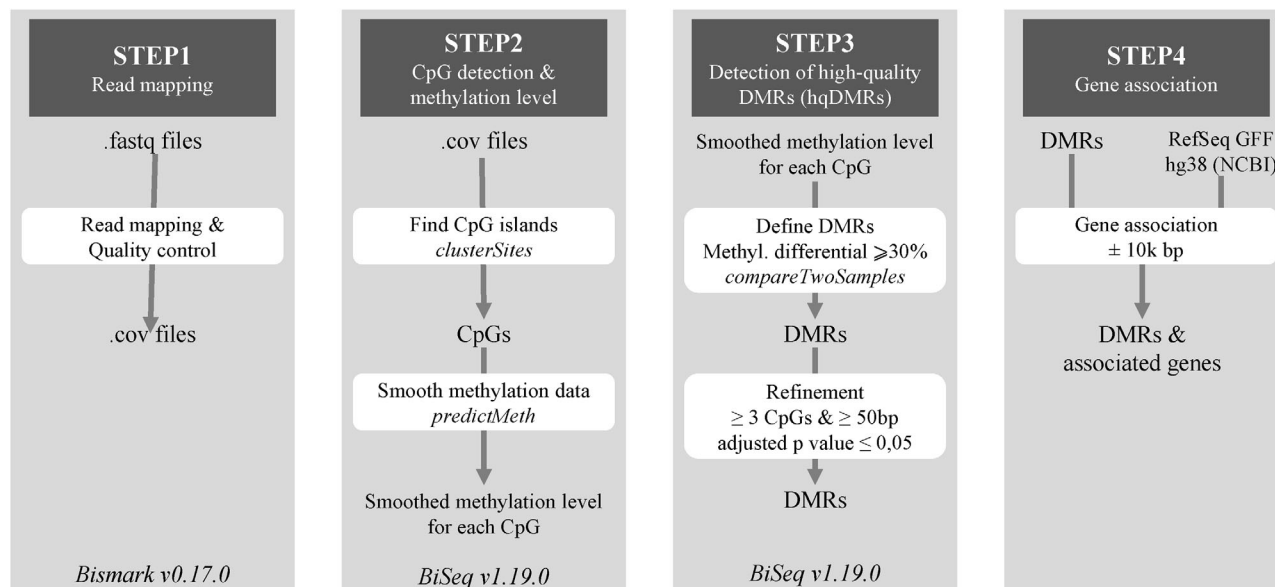
Reads were mapped on the human genome (hg38) with the Bismark tool (release v0.17.0).<sup>26</sup> After data quality control, methylation calls were extracted by Bismark and a CpG coverage file (.cov file) containing the average methylation level of each covered CpG site was generated for each sample.

#### 2.4.2 | CpG detection and methylation level

The *clusterSites* function implemented in BiSeq (v1.19.0) was used to identify CpG islands for each sample.<sup>27</sup> This function takes into account spatial correlation of the methylation of nearby CpG sites, constraining the analysis on CpG sites among CpG clusters. CpG islands were defined as regions containing at least 20 frequently covered CpG sites—that is, corresponding to those covered in 100% of the samples—with a maximum distance of 100 bp from each other. This is important to note that the frequently covered CpG sites were considered to define the CpG cluster boundaries only. For downstream analysis, all CpG methylation data within these CpG clusters were used. Since methylation levels show strong spatial correlation,<sup>28</sup> we smoothed the raw methylation to reduce the required sequencing coverage data, as previously suggested.<sup>29,30</sup> In addition, DMR detection methods without smoothing data often discard CpG sites of low coverage from further analyses. The smoothing of the methylation data was performed with the *predictMeth* function implemented in BiSeq (with a bandwidth of  $h = 80$  bp). Within each CpG cluster and for each sample, a smoothing function is modeled on the local raw methylation data. The resulting methylation levels range from 0 to 1.

#### 2.4.3 | Detection of differentially methylated regions

The detection of DMRs corresponds to the identification of genomic regions holding smoothed CpG methylation level differences between two experimental conditions. The *compareTwoSample* function implemented in BiSeq was used to identify DMRs for every pairwise comparison between samples. DMRs are assembled with CpGs closer than a maximum distance of 100 base pairs that have a methylation difference greater than 30%. DMRs extension is stopped if one of these rules is broken, establishing the DMR boundaries and thereby can be constituted of only one differentially methylated CpG. It is worthwhile to remember that one of the earliest studies of DNA methylation impacting transcription factor binding considered a single 6 bp region and the impact of one methylated cytosine within that region.<sup>31</sup> The resulting nonredundant DMRs are then aggregated together in a single DMR file. Each detected DMR is then quantified in each sample by calculating the median smoothed methylation level of all CpG sites within this genomic region.



**FIGURE 1** RRBS data preprocessing and analysis. The workflow includes four distinct steps: read mapping on the human genome and quality control (STEP1), CpG detection and methylation level measurement (STEP2), detection of differentially methylated regions (DMRs) (STEP3), and gene association (STEP4).

To further characterize chemotherapy-associated differential methylation of sperm DNA, we focused our analysis on a subset of DMRs (with a minimum length of 50 bp and composed of at least three CpG sites) showing changes between the two PreCT samples (PreCT\_1 & PreCT\_2) and each PostCT sample (i.e., at month 6, 9, 12, or 24 after treatment) (Figure S1, panels A–D; Table 3). We first applied a methylation difference cutoff of at least 30% for each of the eight pairwise comparisons (PreCT\_1 vs. each of the four PostCT samples and PreCT\_2 vs. each of the four PostCT samples). We next used the linear models for microarray data (LIMMA) package and a  $p$ -value cutoff of 0.05 adjusted with the Benjamini and Hochberg method<sup>32</sup> to compare in a single statistical test the two PreCT samples to each of the four PostCT samples.

#### 2.4.4 | Gene association

We first assembled a unique set of human reference transcripts. To address this issue, Ensembl<sup>33</sup> and RefSeq<sup>34,35</sup> transcript annotations from the hg38 release of the human genome were downloaded from the University of California Santa Cruz (UCSC) genome browser website<sup>36</sup> on November 11, 2017. Both transcript annotation files (GTF and GFF formats) were subsequently merged into a combined set of nonredundant human reference transcripts with Cuffcompare.<sup>37</sup> Based on this reference transcriptome, we further associated DMRs with their proximal adjacent human genes, that is, less than 10 kbp away. MethGO was also used to analyze the RRBS data at gene-centric levels based on the features described in the human reference transcriptome (promoters, genes, exons, introns, and intergenic regions).<sup>38</sup>

#### 2.5 | Functional analysis

The enrichment analysis module implemented in the AMEN suite<sup>39</sup> was used to identify gene ontology terms significantly over-represented in each gene group by calculating Fisher's exact probability using the Gaussian hypergeometric function ( $p$ -value  $\leq 0.01$  adjusted by the false discovery rate (FDR) method, number of genes in a given group associated with a given annotation term  $\geq 5$ ).

#### 2.6 | Genes of interest

##### 2.6.1 | Genes expressed during early embryo development

To identify genes expressed during the early embryo development process, we integrated microarray data (Affymetrix Human Genome U133 Plus 2.0 Array) published by Xie et al.<sup>40</sup> available on the NCBI Gene Expression Omnibus (GEO) repository under the accession number GSE18290. This dataset includes the transcriptome of 6 embryonic stages (18 samples): 1-, 2-, 4-, and 8-cell stages as well as morula and blastocyst. Raw data (cel files) were normalized and background corrected with the RMA method based on the Brainarray custom CDF environment for directly mapping Affymetrix gene to Entrez gene identifiers (hta20\_Hs\_ENTREZG version 22.0.0).<sup>41</sup> Briefly, genes showing a signal  $\geq$  background cutoff (median of the normalized dataset, cutoff 5.04) and a fold-change  $\geq 3$  in at least one pairwise comparison were selected. To define the set of 4,264 differentially expressed genes displaying significant statistical changes across

early embryo development stages, the LIMMA package was used (FDR-adjusted  $F$ -value  $\leq 0.01$ )<sup>42</sup> implemented in AMEN.<sup>39</sup>

## 2.6.2 | Genes associated with embryo development

A list of 969 human genes known to be associated with the embryo development process were selected based on their association with the “embryo development” Gene Ontology term (GO:0009790) according to the ‘gene2go’ file downloaded from the NCBI website.

## 2.6.3 | Imprinted genes

A list of 91 human maternally and paternally imprinted genes has been gathered from the Geneimprint web-based portal (<http://www.geneimprint.com/site/genes-by-species.Homo+sapiens>).<sup>43</sup>

# 3 | RESULTS

## 3.1 | Patient and sperm collection

Sperm characteristics of the chemotherapy-exposed patient with TGCT including time of semen collection, volume, and sperm quality are presented in Table 1. After cancer diagnosis and one month prior to chemotherapy the sperm concentration ranged from 110 to 140 million spz/mL, while it dropped to 10 million spz/mL 6 months after the treatment and progressively increased up to 70 million spz/mL at month 24. Therefore, there was a general reduction in sperm count after the chemotherapy treatment followed by a steady increase in the 2 years following the treatment, as previously described in other patients.<sup>10</sup>

## 3.2 | Low but genome wide demethylation of the sperm chromatin after chemotherapeutic treatment

We used RRBS to profile the methylation status of the CT patient's sperm DNA at the single cytosine level and investigate the potential epigenomic impact of the chemotherapeutic treatment. Following sequencing and quality controls, a total of 67–106 million reads per sample (Figure 2A; Table 2) were next mapped on the converted human genome (mapping efficiency ranging from 60.8%–64.9%), enabling the analysis of 483–779 million of cytosines, among which 83–125 million (16.0%–17.7%) corresponded to CpGs (Figure 2B). We limited the coverage of CpGs to the 90% quantile for further analysis to reduce biases due to unusually high coverages. The median coverage of those CpGs ranged from 12–17 reads across the six samples (Figure 2C). Finally, we defined a set of 69,675 CpG islands corresponding to 1,058,776 spatially correlated CpGs for which smoothed methylation status could be compared between all samples.

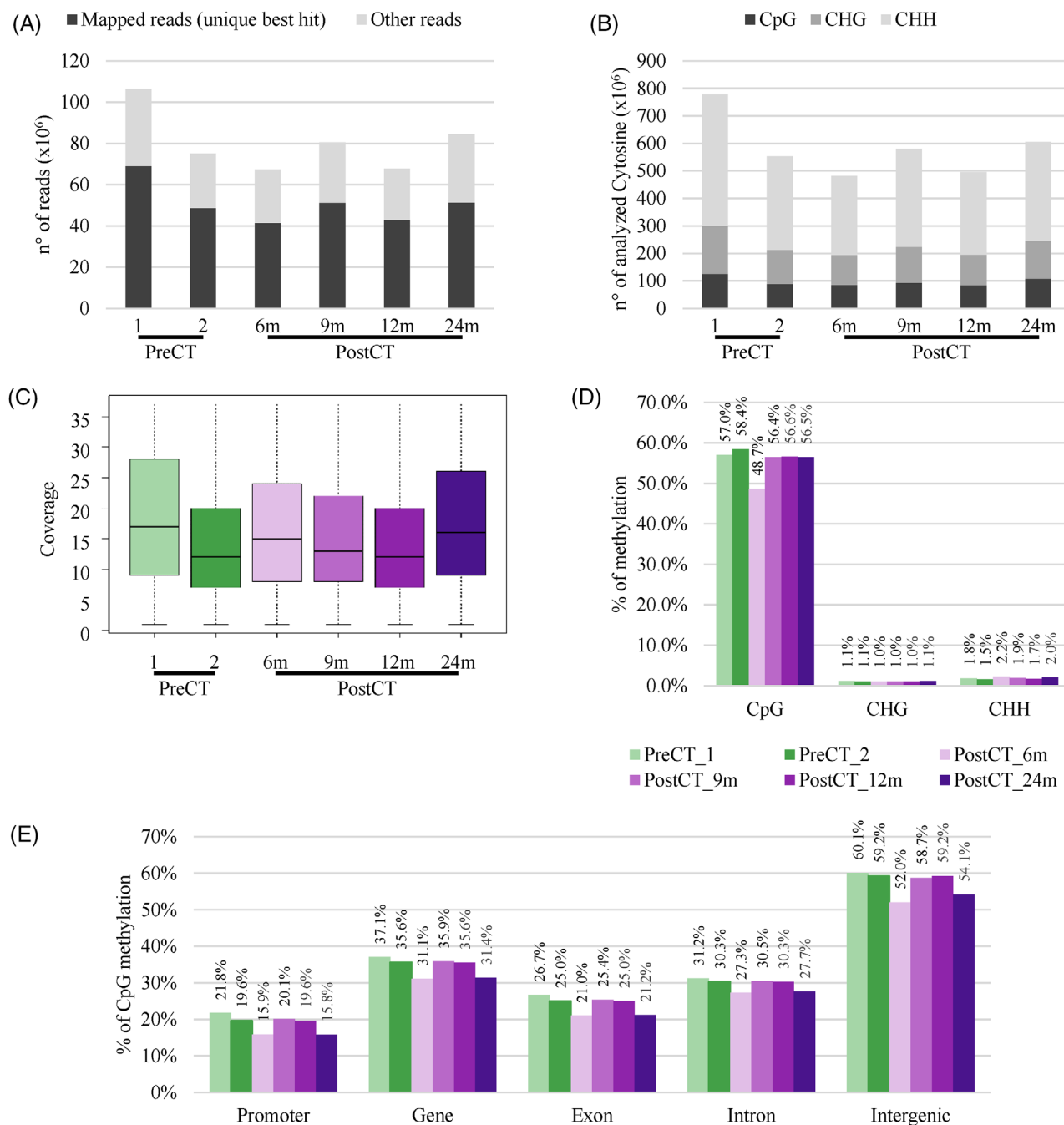
**TABLE 1** Semen characteristics before and after chemotherapy according to the time of follow-up.

Sperm samples	Date	Volumes (mL)	Sperm[C] ( $10^6$ /mL)	Mobility a+b or P (%)	Mobility cor NP (%)	Mobility d or l (%)	Typical forms	MAI	n° total ( $10^6$ /sperm straw)
PreCT_1	12-02-2015	4.6	140	49	1	50	53	1.36	8.4
PreCT_2	17-02-2015	4.4	110	58	2	40			6.6
PostCT_6 m	24-09-2015	3.52	10	50	4	50	60	1.4	0.6
PostCT_9 m	21-12-2015	6.32	30	37	3	60	44	1.29	1.8
PostCT_12 m	01-04-2016	4.1	55	74	1	25	55	1.67	3.3
PostCT_24 m	28-03-2017	3.69	70	60	5	35			4.2

Note: P, progressive motility; NP, non-progressive motility; l, immotile; MAI, multiple anomalies index. PreCT, pre-chemiotherapy semen samples; PostCT, post-chemiotherapy semen samples. \_6 m, \_9 m, \_12 m, and \_24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

Abbreviation: NA, Not available.





**FIGURE 2** Read mapping and methylation statistics. (A) Read mapping statistics for each individual sample. (B) Sample-wise numbers of CpG (or CG), CHG and CHH (where H corresponds to A, T or C). (C) Sample-wise coverage distribution of CpG sites. Distribution of coverage across samples after coverage limitation (90% quantile). (D) Proportion of methylated CpG, CHG, and CHH for each sample. (E) Distribution of methylated CpGs among genomic features. A minimum depth of four reads is required for each CpG. CpG methylation levels are averaged by feature to produce feature-associated methylation levels. PreCT, pre-chemotherapy semen samples (colored in green in panels C–E); PostCT, post-chemotherapy semen samples (colored in purple in panels C–E). \_6 m, \_9 m, \_12 m, and \_24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

It is important to note that only 1.1% of CHG and 1.5%–1.8% of CHH (where H corresponds to A, T, or C) were methylated, whereas 57.0% and 58.4% of all CpGs were methylated before a chemotherapeutic treatment (PreCT\_1 and PreCT\_2), respectively (Figure 2D). The global methylation level of CpGs decreased 6 months after the chemotherapy (48.7%) but returned close to the PreCT levels from 9

months onward (56.4%–56.6%) (Figure 2D). As the CpG methylation status of the human genome has become increasingly investigated, it has emerged that differential methylation is not only restricted to the CpG islands, but also extends to CpG regions, for example, at enhancers or across gene bodies.<sup>44,45</sup> We therefore executed the MethGO tool<sup>38</sup> to perform methylation calls for each type of genomic features, that is,

**TABLE 2** Overview of read numbers and mapping statistics of the sperm samples.

Sample	Total read numbers	Alignment w/ unique best hit	Mapping efficiency	C analyzed	C (CpG) meth	C (CHG) meth	C (CHH) meth	C (CpG) unmeth	C (CHG) unmeth	C (CHH) unmeth
PreCT_1	106436954	69110632	64.90%	778705427	71413272	1903803	8479740	53875724	172542424	470490464
PreCT_2	75158930	48618089	64.70%	552997295	51832601	1308423	5258670	36857678	122796167	334943756
PostCT_6 m	67406100	41488376	61.50%	482585530	41350774	1144074	6476325	43595438	108378866	281640053
PostCT_9 m	80582196	51202989	63.50%	580087194	52524632	1358030	6677337	40526864	129292525	349707806
PostCT_12 m	67730924	43078958	63.60%	495981405	47051533	1179854	5009548	36070643	111305601	295364226
PostCT_24 m	84423184	51330408	60.80%	605277808	60471251	1500762	7229269	46628671	136892428	352555427

Note: PreCT, pre-chemotherapy semen samples; PostCT, post-chemotherapy semen samples. \_6 m, \_9 m, \_12 m, and \_24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

for promoters, genes, exons, introns, and intergenic regions. A low but genome-wide hypomethylation of CpGs was observed at 6 months for all genomic features (Figure 2E).

### 3.3 | One hundred and seventy-nine genomic regions are associated with altered sperm DNA methylation profiles following chemotherapy

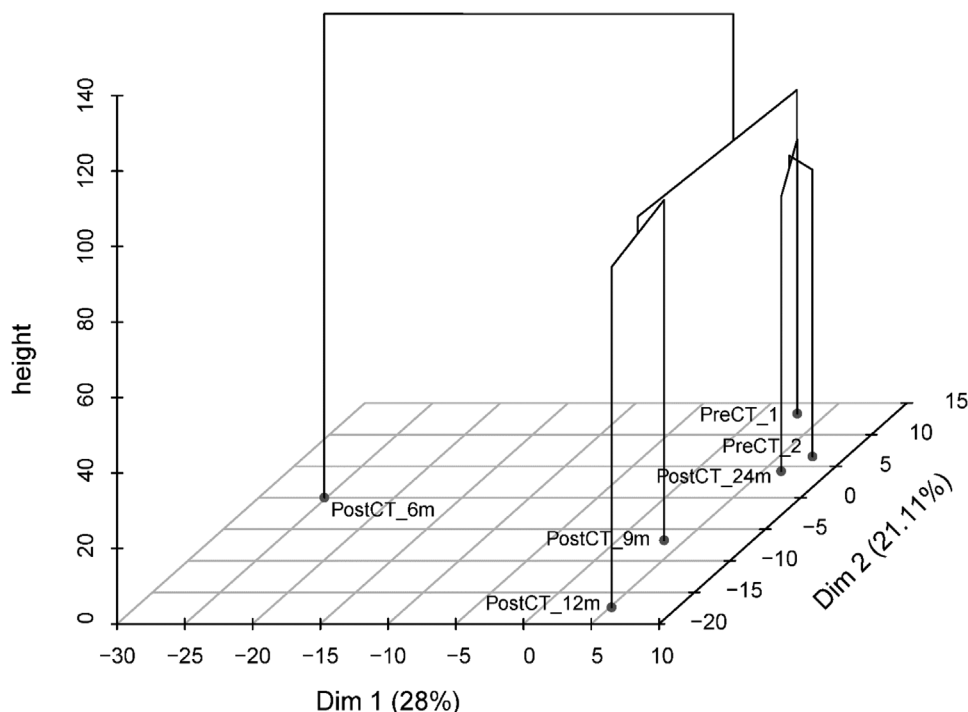
By performing all pairwise comparisons in BiSeq, we first identified 554 DMRs consisting of at least three CpG sites and covering at least 50 bp. Hierarchical clustering and principal component analysis based on these 554 DMRs revealed the proximity between the two PreCT samples and the PostCT\_24 m and their clear difference with the PostCT\_6 m, while PostCT\_9 m and PostCT\_12 m appeared in between (Figure 3). This would be suggestive of important methylation changes that occur early after the treatment followed by a progressive recovery toward a near normal methylation profile.

To further characterize chemotherapy-associated differential methylation of sperm DNA, we focused our analysis on the comparisons of PreCT samples with PostCT samples at each time point, that is, at month 6, 9, 12, or 24 after treatment (Figure S1A–D; Table 3). Altogether, these comparisons allowed us to identify 179 DMRs (FDR-adjusted  $p$ -value  $\leq 0.05$ ) associated with 102 differentially methylated genes (DMGs) following TGCT chemotherapy (Figure S1E; Table 3). Consistent with the above hypothesis, the number of DMRs was highest at 6 months. Importantly, while the number of DMRs also decreased from 9 months onward, 43 DMRs could be identified 24 months post-chemotherapy treatment. The functional analysis of the 102 DMGs did not reveal any enriched GO terms.

### 3.4 | Differential DNA methylation of thirteen genes in sperm persists up 24 months after chemotherapy

To capture the most direct and longitudinal effects of the chemotherapy on DNA methylation, we further focused on the DNA methylation changes occurring 6 months after chemotherapy (PreCT vs. PostCT\_6 m samples). Among the 179 DMRs, 74 showed a differential methylation status between the PreCT and PostCT\_6 m samples (16 hyper- and 58 hypo-methylated) associated with 49 DMGs (15 hyper- and 34 hypo-methylated) (Figure S1A; Table 3). We further sub-clustered these 74 DMRs according to their methylation profiles during the entire longitudinal study: six profiles could be identified, including three that were associated with hyper-methylation (P1-3) and three with hypo-methylation (P4-6) (Figure 4). Briefly, patterns P1 (5 DMRs; 3 DMGs) and P4 (40 DMRs; 21 DMGs) included DMRs that quickly went back to their pre-treatment methylation status just after 9th months onward. Patterns P2 (4 DMRs; 5 DMGs) and P5 (8 DMRs; 7 DMGs) corresponded to DMRs that slowly returned to their initial methylation status after 12/24 months. Finally, patterns P3 (7 DMRs; 7 DMGs including LRCOL1, PTPRS, MAP1S, PTPN23/SCAP,





**FIGURE 3** Hierarchical clustering analysis on principal components of the samples. This graph shows a combined plot of hierarchical clustering and principal component analysis (PCA) in a three-dimensional view. The two first axes of the PCA are based on the 554 DMRs. PreCT = pre-chemotherapy semen samples; PostCT = post-chemotherapy semen samples. \_6 m, \_9 m, \_12 m, and \_24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

AFAP1-AS1/AFAP1) and P6 (10 DMRs; 6 DMGs including ALG10B, BCR/FBXW4P1, PDZD2, ELFN1/ELFN1-AS1) composed of hypermethylated (P3) and hypomethylated (P6) DMRs persisting up to 24 months post-chemotherapy.

### 3.5 | The altered sperm DNA methylation of two genes important for embryogenesis persists up to 24 months post-chemotherapy

To investigate whether DMRs and their related DMGs were associated with specific biological processes, we first performed a Gene Ontology term analysis. However, no significant enrichment was found. In a second step, we wondered whether some of the 15 hyper- and 34 hypo-methylated DMGs showing altered methylation status 6 months after chemotherapy could play roles during embryogenesis. To address this issue, we first filtered a list of 4,264 genes differentially expressed during the early development.<sup>40</sup> We also assembled a set of 969 genes known to be associated with embryo development based on their associated GO terms. By intersecting those two gene lists with the set of 49 selected DMGs, we were able to identify two genes differentially expressed during early embryogenesis (KMT2C and SCAP) (Figure S2) and five genes known to be associated with embryo development (BCR, GBX2, PTK2, SATB2, and SLC39A3) (Figure S3; Table 3). Among these genes, one DMR that mapped to one of the SCAP gene exons and one DMR that mapped to one of the BCR gene introns (into the core of the

FBXW4P1 pseudogene) remained hypomethylated 24 months after chemotherapy (Figure 5A,B).

## 4 DISCUSSION

Enhancement of early detection and improved cancer treatment protocols over the last 20 years has increased the number of men surviving TC. As progress in adjuvant TGCT treatments in combination with surgery have made TGCT one of the most treatable of all cancers, the 5-year survival rate is above 90% today. However, projection into the future and the anticipation of adverse effects after treatment has become a major issue for these patients. Indeed, post-treatment quality of life is one of the most important criteria in the management of TC in young men as fertility is often impaired after chemotherapy and radiation therapy. Cryopreservation of sperm before cancer treatment is currently the only method to preserve male fertility. Discussion about fertility preservation before starting treatment is therefore needed. Male cancer survivors are less likely to father a child compared to the background population. This is influenced by cancer site, age of onset, and parity status at diagnosis.<sup>46</sup> Still, sperm parameters typically recover after treatment, even if this recovery is low. The evaluation of post-treatment paternity in testicular tumor survivors is indeed regularly described in the literature. For instance, a retrospective and multicenter study between 1980 and 1994 in Norway involved 1814 men, regardless of treatment.<sup>47</sup> In this survey, 71% of patients

**TABLE 3** Overview of differentially methylated regions (DMRs) and associated differentially methylated genes (DMGs).

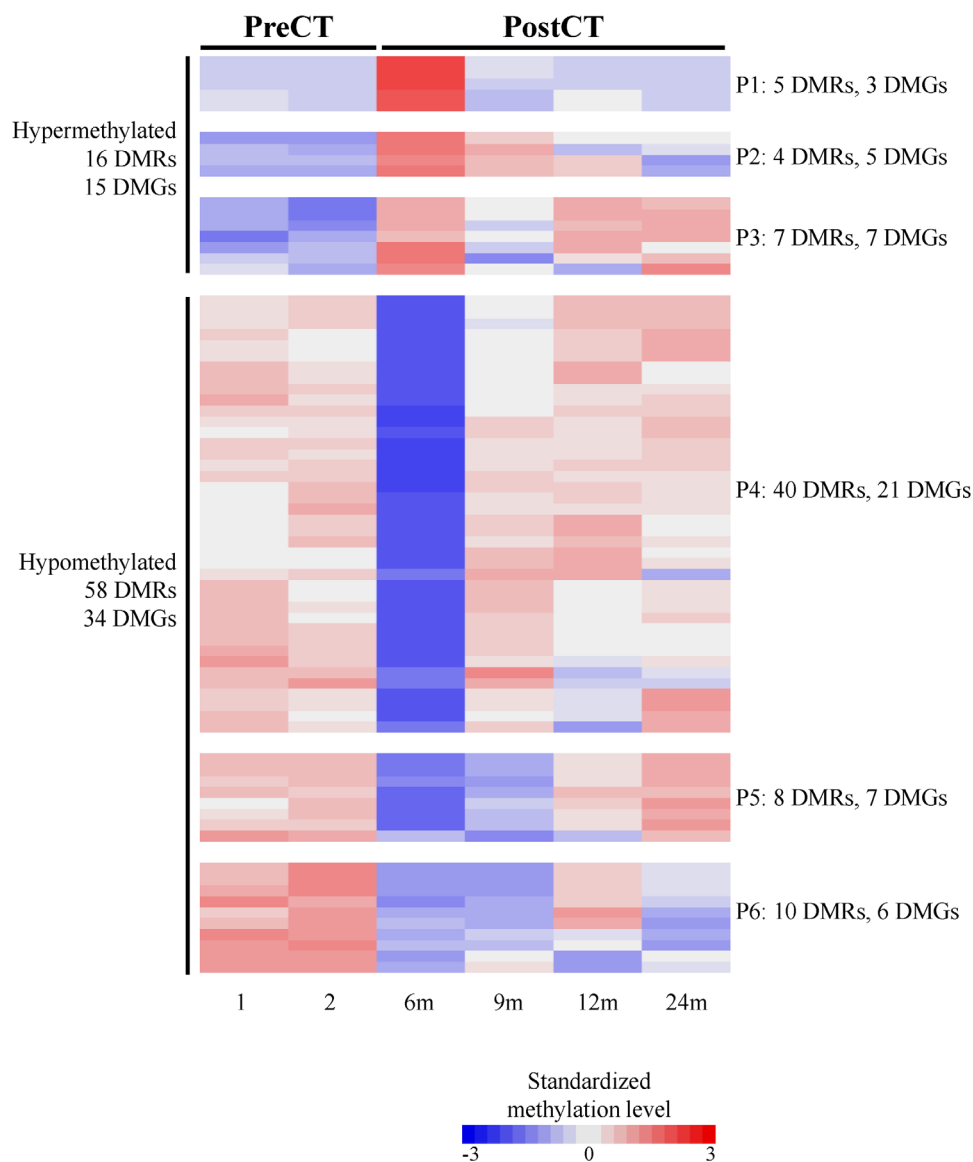
Contrasts	Methylation status	PreCT vs PostCT (all)		PreCT vs PostCT_6 m		PreCT vs PostCT_9 m		PreCT vs PostCT_12 m		PreCT vs PostCT_24 m	
		All		Hyper	Hypo	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo
n° DMRs (n° non-overlapping DMRs)		179 (104)		16 (13)	58 (34)	18 (14)	37 (20)	31 (16)	20 (16)	31 (15)	12 (9)
n° DMGs		102		15	34	13	21	13	26	15	10
Expr. in early embryo dev. (4264 genes)		3		SCAP	KMT2C	None	SH2D4B	None	SH2D4B	SCAP	None
Associated with embryo dev. (968 genes)		6		GBX2, PTK2	BCR, SATB2, SLC39A3	GBX2	SATB2	None	HPN, BCR	None	None
Imprinted genes (91)		None		None	None	None	None	None	None	None	None

Note: PreCT = pre-chemiotherapy semen samples; PostCT = post-chemiotherapy semen samples. \_6 m, \_9 m, \_12 m, and \_24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

procreated without the use of sperm straws, while only 6.3% of patients used sperm straws. Furthermore, among men who procreated without the use of sperm straws, 22% used assisted reproductive technology (ART). In another study by Huyghe et al.,<sup>48</sup> 67% of patients with TC became fathers after treatment without the use of ART.

Here, we investigated the impact of a BEP therapy on the sperm epigenome of a TGCT patient. Our longitudinal study included the use of pre-treatment samples and their comparisons to those recovered from the same patient up to 24 months after the treatment. As compared with most retrospective studies using sperm from fertile men as a control group, our experimental design should lower the possibility to identify methylation changes related to the cancer status rather than from the treatment itself. Still, it is important to remember that the DNA methylation pattern of samples before chemotherapy cannot be considered per se as the 'normal' physiological DNA methylation status since the testicular tumor itself can affect sperm DNA methylation. Furthermore, it cannot be completely ruled out that some of the changes we observed, especially those showing a persistent methylation alteration 2 years after chemotherapy, could be the result of an evolution of the cancer, or of a subsequent disruption of the gonadal environment of the patient. It was indeed demonstrated that TC patients treated with 2–4 cycles of BEP displayed increased follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels for at least 24 months.<sup>16</sup> Epigenetic changes related to the environment are well known in animal models. Environmental factors can indeed induce transgenerational inheritable DNA methylation changes in mammalian germline.<sup>49,50</sup> As the patient made no major changes in his personal way of life, we assumed that most sperm parameter variations, including methylation changes, could be attributable to chemotherapy. Following four cycles of BEP, the patient's sperm count drastically decreased at month 6 and progressively increased from 9 months onward, albeit at lower levels than before treatment. This is consistent with a previous study on 129 volunteer TGCT patients and a control group of 257 fertile men that showed a decrease of sperm characteristics after BEP treatment, with lowest values at 3 and 6 months.<sup>10</sup> In the latter study, sperm count indeed recovered to pre-treatment values at 12 months after treatment after two or fewer BEP cycles, but not after radiotherapy or more than two BEP cycles.

Sperm cells are particular cells able to transmit information from one generation to the next. Any alteration of the epigenome will be transmitted to the zygote and then to the embryonic cells. Hence, gamete exposure has consequences on epigenetic markers and may interfere with the inheritance of specific features to the offspring. Several players implicated in this non-genetic inheritance have been described so far: DNA and RNA methylation, histone modifications, non-coding RNAs and extracellular vesicles.<sup>51</sup> DNA methylation regulates gene expression and genome activity without modification of the coding sequence. DNA methylation is technically easy to investigate and therefore the most documented epigenetic mark. DNA methylation involves methyl groups that are attached to a DNA molecule. While BEP has been reported to affect sperm chromosomes and to alter DNA status,<sup>10,15,21,54</sup> to the best of our knowledge no study specifically investigated its impact on DNA methylation. In the

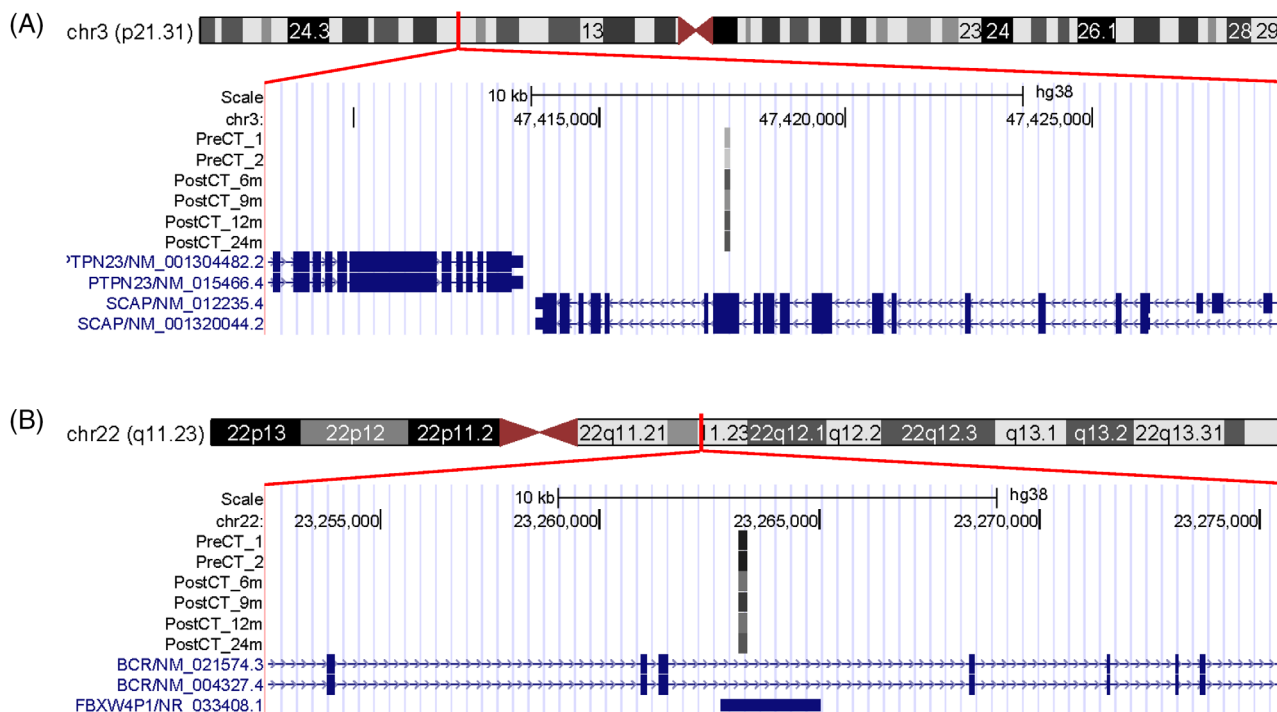


**FIGURE 4** Methylation profiles of DMRs showing altered methylation 6 months after chemotherapy. A false-color heatmap shows the standardized methylation level of 74 DMRs organized in six methylation patterns (P1–P6). Each line corresponds to a DMRs and each column to a sample. The number of DMRs and related DMGs are given for each of the six resulting methylation patterns on the right. Standardized methylation levels are displayed according to a color code ranging from blue (hypomethylation) to red (hypermethylation). PreCT, pre-chemotherapy semen samples; PostCT, post-chemotherapy semen samples. 6 m, 9 m, 12 m, and 24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

current study, the most important methylation changes were observed 6 months after chemotherapy: these were mainly associated with a demethylation of CpG islands which returned to normal after 24 months for most of them. Among the 49 DMGs associated with the 74 DMRs at 6 months, we identified two genes expressed during early embryonic stages (KMT2C and SCAP) and five genes known to be involved in the embryo development (BCR, GBX2, PTK2, SATB2, SLC39A3). Altering the methylation status of such genes might modify their expression pattern and thus affect key stages of embryogenesis and lead to potential developmental disorders or miscarriages.<sup>52–54</sup> We also highlighted 13 DMGs associated with 17 DMRs that show a persistent methylation alteration even 24 months after chemotherapy.

Although these genes are not necessarily involved in early development their altered methylation patterns could be passed on to the offspring and eventually have an impact at a later stage.

One of the two DMGs expressed during early embryonic stages is KMT2C (Lysine Methyltransferase 2C), a member of the mixed-lineage leukemia (MLL) family encoding a histone methyltransferase that regulates gene transcription by modifying chromatin structure. It is interesting to note that its loss decouples enhancer H3K4 monomethylation, H3K27 acetylation, and gene activation during embryonic stem cell differentiation.<sup>55</sup> KMT2C mediates mono and tri-methylation of histone H3 at lysine 4. It has been shown that loss of KMT2C causes defects in lung maturation in KO mice, leading to immediate death



**FIGURE 5** Sustained methylation alterations of the DMRs associated with SCAP and BCR. A screen capture of the UCSC genome viewer showing the methylation level of DMRs associated with SCAP (panel A, hypermethylated) and BCR (panel B, hypomethylated) in each sample, based on a color code ranging from white (0% of methylation) to black (100% of methylation). Corresponding transcript structures, including exons and introns, are shown at the bottom in blue. PreCT, pre-chemotherapy semen samples; PostCT, post-chemotherapy semen samples. \_6 m, \_9 m, \_12 m, and \_24 m correspond to semen samples recovered 6, 9, 12, and 24 months after the chemotherapy, respectively.

after birth.<sup>56</sup> The encoded methyltransferase is also implicated in early embryogenesis and spermatogenesis by setting methylation marks in nucleosomes.<sup>57</sup> Male mice lacking a fully functional enzyme showed reduced fertility.<sup>58</sup> Genome-wide association study (GWAS) linked non-synonymous single-nucleotide polymorphisms (SNPs) in KMT2C gene to fertility variation between fertile and infertile men.<sup>59</sup> The second DMG expressed during early embryonic stages is SCAP (SREBF chaperone) which encodes a Sterol regulatory element-binding protein (SREBP) cleavage-activating protein. In cholesterol-depleted cells, SCAP binds to SREBPs and mediates their transport from the endoplasmic reticulum to the Golgi apparatus. SREBPs are then proteolytically cleaved and stimulate sterol biosynthesis. It is well documented that impaired sterol synthesis results in developmental defects in humans and animals. Lipid homeostasis should be critical for normal development, because over regulation of cholesterol and phospholipid synthesis is required to satisfy the demand for membrane biogenesis during periods of rapid cell proliferation.<sup>60</sup> Interestingly, long-term dysregulation of SCAP/SREBP signaling was reported as a common feature in mice born from assisted reproductive technologies.<sup>61</sup>

Among DMGs involved in the embryo development, the Breakpoint Cluster Region protein (BCR) is one of the two genes in the BCR-ABL complex, which is associated with the Philadelphia chromosome. While the BCR gene product harbors a serine/threonine kinase activity, its function is not clear. The literature is rich on the association between BCR-ABL and oncohematological pathologies, but little is known about its role during embryonic development. BCR

contributes to cell division and may play a role during early embryonic development or spermatogenesis as it is expressed by preimplantary embryos in bovine<sup>62</sup> and is differently methylated in sperm of subfertile bulls leading to altered gene expression.<sup>63</sup> BCR-ABL gene product appears to have a lethal effect during this period of development by introducing the BCR gene into mouse embryos.<sup>64</sup> Gastrulation Brain Homeobox 2 (GBX2) is a homeobox gene involved in the normal development of rhombomeres 1–3 which is the mid/hindbrain region. This gene is a dosage-dependent transcription factor involved in the regulation of proper expression of other genes. GBX2 expression occurs during gastrulation and continues to be expressed in the late stages of embryogenesis.<sup>65</sup> GBX2 inhibits the expression of LHX9, which is a LIM/homeobox protein and plays a role in the gonadal development. Moreover, GBX2 is involved in pluripotency and may be an actor of iPSC.<sup>66</sup> Methylation of GBX2 mediates the effect of the lifestyle intervention in pregnant women with obesity on lean mass in the offspring and early growth.<sup>67</sup> The PTK2 gene encodes a cytosolic protein tyrosine kinase, that is, found concentrated in the focal adhesions that form among cells attaching to extracellular matrix constituents. The encoded protein is a member of the FAK family which plays a regulatory role in modulation of cellular proliferation, protection from apoptosis, adhesion, spreading and migration and is important for the development of the placenta, as well as of several organ systems, like the musculoskeletal, nervous, cardiovascular, genitourinary, and respiratory organ systems.<sup>68</sup> Additionally, FAK has been shown to be implicated in the pathophysiology of pregnancy-related

disorders and congenital neonatal diseases and defects.<sup>69</sup> The special AT-rich sequence-binding protein 2 (SATB2) encodes a DNA-binding protein that specifically binds nuclear matrix attachment regions and is involved in transcriptional regulation and chromatin remodeling. Defects in human SATB2 cause cognitive deficits, craniofacial dysmorphism, behavioral changes, and osteoporosis.<sup>70</sup> Finally, the SLC39A3 gene encodes a Zinc transporter ZIP3 protein for which physiological functions remain unknown. A study has reported that embryos developed abnormally in ZIP1, ZIP3 double-knockout mice if dietary zinc was limited during pregnancy.<sup>71</sup> SLC39A3 may be involved in psychiatric phenotypes and has been associated with bipolar disorders in humans.<sup>72</sup>

It is important to notice that our analysis did not find any methylation changes on the genes subjected to parental imprint (Table 3). Any failure to establish correct germline-specific DNA methylation patterns will have serious consequences for post-fertilization development, primarily due to the necessity for epigenetic marking of genomic imprints. Genomic imprinting refers to the epigenetic mechanism that results in the parent-of-origin monoallelic expression of autosomal genes.<sup>73</sup> It is a particularly unique epigenetic mechanism, since an individual somatic cell has both active and repressed alleles of the same gene. Imprinted DMRs are established in the male and female gametes. It was reported that the promoters of many key genes involved in the early embryonic development are hypomethylated in mature sperm.<sup>74</sup> These observations support the hypothesis that paternal genome methylation pattern could influence embryonic development through regulation of developmental gene expression.<sup>75</sup> Together with the fact that most of the methylation alterations regained the initial state of pre-chemotherapy, our results are rather reassuring for any project of children for the current patient. It would be necessary however to confirm our observations with other patients. Notably, our data are insufficient to conclude on whether the sperm quality of patients treated for TC is similar to that of untreated men and further studies are also needed to investigate the impact of such epigenetic modifications on embryogenesis. A statistically significant but modest increase in the risk of major congenital abnormalities among offspring of males with a history of cancer, independent of the mode of conception has been observed.<sup>76</sup> The increase in prevalence of birth anomalies among children of fathers with malignancy might be due to cancer per se or a common underlying paternal factor, for example, genomic instability.<sup>77</sup> On the other hand, no additional increased risk of congenital malformations was observed in children of men with testicular germ-cell cancer treated with radio- or chemotherapy, reassuring concerned patients.<sup>78</sup> However, a close follow-up of children could be important in the long term since too few studies have sought to identify the genetic and/or malformation risks of children born using the spermatozoa of cancer survivors. In this context, patients should receive information on the potentially increased risk of sperm DNA damage before and after gonadotoxic therapy while the development of less gonadotoxic protocols in the management of cancers should be studied to improve the post-cancer quality of life of patients.

## AUTHOR CONTRIBUTIONS

Frédéric Chalmel and Célia Ravel supervised the research and designed the study. Anne-Sophie Neyroud, Célia Ravel and Frédéric Chalmel wrote the manuscript. Bertrand Evrard prepared the samples. Frédéric Chalmel, Antoine D. Rolland, and Nathan Alary prepared, analyzed, and interpreted data. Anne-Sophie Neyroud, Louis Bujan and Célia Ravel interpreted data. Nathalie Dejucq-Rainsford, Louis Bujan, Nathan Alary, and Antoine D. Rolland contributed to the manuscript. All authors approved the final version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ReproGenomics Viewer at <https://rgv.genouest.org/>.

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## REFERENCES

1. Sant M, Allemani C, Santaquilani M, et al. EUROCare-4. Survival of cancer patients diagnosed in 1995–1999. Results and commentary. *Eur J Cancer*. 2009;45(6):931–991.
2. Bosetti C, Bertuccio P, Chatenoud L, Negri E, La Vecchia C, Levi F. Trends in mortality from urologic cancers in Europe, 1970–2008. *Eur Urol*. 2011;60(1):1–15.
3. Parkin DM, Ferlay J, Curado MP, et al. Fifty years of cancer incidence: c15 I–IX. *Int J Cancer*. 2010;127(12):2918–2927.
4. Bray F, Richiardi L, Ekblom A, Pukkala E, Cuninkova M, Møller H. Trends in testicular cancer incidence and mortality in 22 European countries: continuing increases in incidence and declines in mortality. *Int J Cancer*. 2006;118(12):3099–3111.
5. Znaor A, Lortet-Tieulent J, Jemal A, Bray F. International variations and trends in testicular cancer incidence and mortality. *Eur Urol*. 2014;65(6):1095–1106.



6. Nigam M, Aschebrook-Kilfoy B, Shikanov S, Eggener S. Increasing incidence of testicular cancer in the United States and Europe between 1992 and 2009. *World J Urol*. 2015;33(5):623-631.
7. Verhoeven R, Houterman S, Kiemeny B, Koldewijn E, Coebergh JW. Testicular cancer: marked birth cohort effects on incidence and a decline in mortality in southern Netherlands since 1970. *Int J Cancer*. 2008;122(3):639-642.
8. Huyghe E, Plante P, Thonneau PF. Testicular cancer variations in time and space in Europe. *Eur Urol*. 2007;51(3):621-628.
9. Auger J, Sermondade N, Eustache F. Semen quality of 4480 young cancer and systemic disease patients: baseline data and clinical considerations. *Basic Clin Androl*. 2016;26:3.
10. Bujan L, Walschaerts M, Moinard N, et al. Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. *Fertil Steril*. 2013;100(3):673-680.
11. Jeruss J, Woodruff T. Preservation of fertility in patients with cancer. *N Engl J Med*. 2009;360(9):902-911.
12. Loren AW, Mangu PB, Beck LN, et al. Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol*. 2013;31(19):2500-2510.
13. Martinez G, Walschaerts M, Le Mitouard M, et al. Impact of Hodgkin or non-Hodgkin lymphoma and their treatments on sperm aneuploidy: a prospective study by the French CECOS network. *Fertil Steril*. 2017;107:341-350.
14. Lee SJ, Schover LR, Partridge AH, et al. American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *J Clin Oncol Off J Am Soc Clin Oncol*. 2006;24(18):2917-2931.
15. Arnon J, Meirow D, Lewis-Roness H, Ornoy A. Genetic and teratogenic effects of cancer treatments on gametes and embryos. *Hum Reprod Update*. 2001;7(4):394-403.
16. O'Flaherty C, Hales BF, Chan P, Robaire B. Impact of chemotherapeutics and advanced testicular cancer or Hodgkin lymphoma on sperm deoxyribonucleic acid integrity. *Fertil Steril*. 2010;94(4):1374-1379.
17. Smit M, van Casteren NJ, Wildhagen MF, Romijn JC, Dohle GR. Sperm DNA integrity in cancer patients before and after cytotoxic treatment. *Hum Reprod*. 2010;25(8):1877-1883.
18. Ståhl O, Eberhard J, Cavallin-Ståhl E, et al. Sperm DNA integrity in cancer patients: the effect of disease and treatment. *Int J Androl*. 2009;32(6):695-703.
19. Shnorhavorian M, Schwartz SM, Stansfeld B, Sadler-Riggleman I, Beck D, Skinner MK. Differential DNA methylation regions in adult human sperm following adolescent chemotherapy: potential for epigenetic inheritance. *PLoS One*. 2017;12(2):e0170085.
20. Berthaut I, Montjean D, Dessolle L, et al. Effect of temozolomide on male gametes: an epigenetic risk to the offspring? *J Assist Reprod Genet*. 2013;30(6):827-833.
21. Rives N, Walschaerts M, Setif V, et al. Sperm aneuploidy after testicular cancer treatment: data from a prospective multicenter study performed within the French Centre d'Étude et de Conservation des Oeufs et du Sperme network. *Fertil Steril*. 2017;107(3):580-588.
22. Martos SN, Tang WY, Wang Z. Elusive inheritance: transgenerational effects and epigenetic inheritance in human environmental disease. *Prog Biophys Mol Biol*. 2015;118(1-2):44-54.
23. Hanson MA, Skinner MK. Developmental origins of epigenetic transgenerational inheritance. *Environ Epigenet*. 2016;2(1):dvw002.
24. Nilsson EE, Sadler-Riggleman I, Skinner MK. Environmentally induced epigenetic transgenerational inheritance of disease. *Environ Epigenet*. 2018;4(2):dvy016.
25. Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R. Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res*. 2005;33(18):5868-5877.
26. Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*. 2011;27(11):1571-1572.
27. Hebestreit K, Dugas M, Klein HU. Detection of significantly differentially methylated regions in targeted bisulfite sequencing data. *Bioinformatics*. 2013;29(13):1647-1653.
28. Eckhardt F, Lewin J, Cortese R, et al. DNA methylation profiling of human chromosomes 6, 20 and 22. *Nat Genet*. 2006;38(12):1378-1385.
29. Hansen KD, Langmead B, Irizarry RA. BSmooth: from whole genome bisulfite sequencing reads to differentially methylated regions. *Genome Biol*. 2012;13(10):R83.
30. Jaffe AE, Murakami P, Lee H, et al. Bump hunting to identify differentially methylated regions in epigenetic epidemiology studies. *Int J Epidemiol*. 2012;41(1):200-209.
31. Watt F, Molloy PL. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes Dev*. 1988;2(9):1136-1143.
32. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3(1):1-25.
33. Yates A, Akanni W, Amode MR, et al. Ensembl 2016. *Nucleic Acids Res*. 2016;44(D1):D710-D716.
34. Brown GR, Hem V, Katz KS, et al. Gene: a gene-centered information resource at NCBI. *Nucleic Acids Res*. 2015;43(D1):D36-D42.
35. Pruitt KD, Brown GR, Hiatt SM, et al. RefSeq: an update on mammalian reference sequences. *Nucleic Acids Res*. 2014;42(D1):D756-D763.
36. Rosenbloom KR, Armstrong J, Barber GP, et al. The UCSC genome browser database: 2015 update. *Nucleic Acids Res*. 2014;43:D670-D681.
37. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc*. 2012;7(3):562-578.
38. Liao WW, Yen MR, Ju E, Hsu FM, Lam L, Chen PY. MethGo: a comprehensive tool for analyzing whole-genome bisulfite sequencing data. *Bmc Genomics*. 2015;16(12):S11.
39. Chalmel F, Primig M. The annotation, mapping, expression and network (AMEN) suite of tools for molecular systems biology. *BMC Bioinf*. 2008;9:1-11.
40. Xie D, Chen CC, Ptazek LM, et al. Rewirable gene regulatory networks in the preimplantation embryonic development of three mammalian species. *Genome Res*. 2010;20(6):804-815.
41. Dai M, Wang P, Boyd AD, et al. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res*. 2005;33(20):1-9.
42. Ritchie ME, Phipson B, Wu D, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
43. Bajrami E, Spiroski M. Genomic imprinting. *Open Access Maced J Med Sci*. 2016;4(1):181-184.
44. Johnson KC, Houseman EA, King JE, Von Herrmann KM, Fadul CE, Christensen BC. 5-Hydroxymethylcytosine localizes to enhancer elements and is associated with survival in glioblastoma patients. *Nat Commun*. 2016;7:1-11.
45. Pennacchio LA, Bickmore W, Dean A, Nobrega MA, Bejerano G. Enhancers: five essential questions. *Nat Rev Genet*. 2013;14(4):288-295.
46. Tang SW, Liu J, Juay L, et al. Birth rates among male cancer survivors and mortality rates among their offspring: a population-based study from Sweden. *BMC Cancer*. 2016;16:196.
47. Brydøy M, Fosså SD, Klepp O, et al. Paternity following treatment for testicular cancer. *J Natl Cancer Inst*. 2005;97(21):1580-1588.
48. Huyghe E, Matsuda T, Daudin M, et al. Fertility after testicular cancer treatments: results of a large multicenter study. *Cancer*. 2004;100(4):732-737.



49. Gillette R, Son MJ, Ton L, Gore AC, Crews D. Passing experiences on to future generations: endocrine disruptors and transgenerational inheritance of epimutations in brain and sperm. *Epigenetics*. 2018;13(10-11):1106-1126.
50. Nilsson E, King SE, McBirney M, et al. Vinclozolin induced epigenetic transgenerational inheritance of pathologies and sperm epimutation biomarkers for specific diseases. *PLoS One*. 2018;13(8):e0202662.
51. Montjean D, Neyroud A, Yefimova M, Benkhalifa M, Cabry R, Ravel C. Impact of endocrine disruptors upon non-genetic inheritance. *Int J Mol Sci*. 2022;23:3350.
52. Khambata K, Raut S, Deshpande S, et al. DNA methylation defects in spermatozoa of male partners from couples experiencing recurrent pregnancy loss. *Hum Reprod Oxf Engl*. 2021;36(1):48-60.
53. Zhou Q, Xiong Y, Qu B, Bao A, Zhang Y. DNA methylation and recurrent pregnancy loss: a mysterious compass? *Front Immunol*. 2021;12:738962.
54. Carrell DT, Salas-Huetos A, Hotaling J. Increasing evidence of the role of the sperm epigenome in embryogenesis: oligoasthenoteratozoospermia, altered embryo DNA methylation, and miscarriage. *Fertil Steril*. 2018;110(3):401-402.
55. Boileau RM, Chen KX, Blieloch R. Loss of MLL3/4 decouples enhancer H3K4 monomethylation, H3K27 acetylation, and gene activation during embryonic stem cell differentiation. *Genome Biol*. 2023;24:41.
56. Ashokkumar D, Zhang Q, Much C, et al. MLL4 is required after implantation, whereas MLL3 becomes essential during late gestation. *Dev Camb Engl*. 2020;147(12):dev186999.
57. Bae S, Lesch BJ. H3K4me1 distribution predicts transcription state and poising at promoters. *Front Cell Dev Biol*. 2020;8:289.
58. Lee S, Lee DK, Dou Y, et al. Coactivator as a target gene specificity determinant for histone H3 lysine 4 methyltransferases. *Proc Natl Acad Sci U S A*. 2006;103(42):15392-15397.
59. Greither T, Behre HM, Herlyn H. Genome-wide association screening determines peripheral players in male fertility maintenance. *Int J Mol Sci*. 2022;24(1):524.
60. Kanungo S, Soares N, He M, Steiner RD. Sterol metabolism disorders and neurodevelopment—an update. *Dev Disabil Res Rev*. 2013;17(3):197-210.
61. Le F, Wang N, Wang Q, et al. Long-term disturbed expression and DNA methylation of SCAP/SREBP signaling in the mouse lung from assisted reproductive technologies. *Front Genet*. 2021;12:566168.
62. Graf A, Krebs S, Zakhartchenko V, Schwab B, Blum H, Wolf E. Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proc Natl Acad Sci U S A*. 2014;111(11):4139-4144.
63. Štiavnická M, Chaulot-Talmon A, Perrier JP, et al. Sperm DNA methylation patterns at discrete CpGs and genes involved in embryonic development are related to bull fertility. *Bmc Genomics*. 2022;23:379.
64. Heisterkamp N, Jenster G, Kioussis D, Pattengale PK, Groffen J. Human bcr-abl gene has a lethal effect on embryogenesis. *Transgenic Res*. 1991;1(1):45-53.
65. Millet S, Campbell K, Epstein DJ, Losos K, Harris E, Joyner AL. A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. *Nature*. 1999;401(6749):161-164.
66. Swaidan NT, Salloum-Asfar S, Palangi F, et al. Identification of potential transcription factors that enhance human iPSC generation. *Sci Rep*. 2020;10:21950.
67. Jönsson J, Renault KM, García-Calzón S, et al. Lifestyle intervention in pregnant women with obesity impacts cord blood DNA methylation, which associates with body composition in the offspring. *Diabetes*. 2021;70(4):854-866.
68. Peng X, Kraus MS, Wei H, et al. Inactivation of focal adhesion kinase in cardiomyocytes promotes eccentric cardiac hypertrophy and fibrosis in mice. *J Clin Invest*. 2006;116(1):217-227.
69. Chatzizacharias NA, Kouraklis GP, Theocharis SE. The role of focal adhesion kinase in early development. *Histol Histopathol*. 2010;25(8):1039-1055.
70. Leoyklang P, Suphaphetiporn K, Srichomthong C, et al. Disorders with similar clinical phenotypes reveal underlying genetic interaction: SATB2 acts as an activator of the UPF3B gene. *Hum Genet*. 2013;132(12):1383-1393.
71. Dufner-Beattie J, Huang ZL, Geiser J, Xu W, Andrews GK. Mouse ZIP1 and ZIP3 genes together are essential for adaptation to dietary zinc deficiency during pregnancy. *Genes N Y N*. 2006;44(5):239-251.
72. Baum AE, Hamshire M, Green E, et al. Meta-analysis of two genome-wide association studies of bipolar disorder reveals important points of agreement. *Mol Psychiatry*. 2008;13(5):466-467.
73. Monk D. Germline-derived DNA methylation and early embryo epigenetic reprogramming: the selected survival of imprints. *Int J Biochem Cell Biol*. 2015;67:128-138.
74. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature*. 2009;460(7254):473-478.
75. Jenkins TG, Carrell DT. The sperm epigenome and potential implications for the developing embryo. *Reprod Camb Engl*. 2012;143(6):727-734.
76. Ståhl O, Boyd HA, Giwercman A, et al. Risk of birth abnormalities in the offspring of men with a history of cancer: a cohort study using Danish and Swedish national registries. *J Natl Cancer Inst*. 2011;103(5):398-406.
77. Al-Jebari Y, Rylander L, Ståhl O, Giwercman A. Risk of congenital malformations in children born before paternal cancer. *JNCI Cancer Spectr*. 2018;2(2):pk027.
78. Al-Jebari Y, Glimelius I, Berglund Nord C, et al. Cancer therapy and risk of congenital malformations in children fathered by men treated for testicular germ-cell cancer: a nationwide register study. *PLoS Med*. 2019;16(6):e1002816.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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