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Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm

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Abstract

Endocrine-disrupting chemicals (EDCs), among which is the antiandrogen vinclozolin (VCZ), have been reported to affect the male reproductive system. In this study, VCZ was administered to pregnant mice at the time of embryo sex determination, and its possible effects on the differentially methylated domains (DMDs) of two paternally (*H19* and *Gtl2*) and three maternally (*Peg1*, *Snrpn*, and *Peg3*) imprinted genes were tested in the male offspring. The CpGs methylation status within the five gene DMDs was analyzed in the sperm, tail, liver, and skeletal muscle DNAs by pyrosequencing. In the sperm of controls, the percentages of methylated CpGs were close to the theoretical values of 100 and 0% in paternally or maternally imprinted genes respectively. VCZ decreased the percentages of methylated CpGs of *H19* and *Gtl2* (respective values 83.1 and 91.5%) and increased those of *Peg1*, *Snrpn*, and *Peg3* (respective values 11.3, 18.3, and 11.2%). The effects of VCZ were transgenerational, but they disappeared gradually from F1 to F3. The mean sperm concentration of the VCZ-administered female offspring was only 56% of that of the controls in the F1 offspring, and it was back to normal values in the F2 and F3 offspring. In the somatic cells of controls, the percentages of methylated CpGs were close to the theoretical value of 50% and, surprisingly, VCZ altered the methylation of *Peg3*. We propose that the deleterious effects of VCZ on the male reproductive system are mediated by imprinting defects in the sperm. The reported effects of EDCs on human male spermatogenesis might be mediated by analogous imprinting alterations.

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Introduction

Exposure to environmental pollutants may induce changes in gene expression not involving changes in the underlying DNA sequence, but involving the so-called epigenetic changes. Imprinting is an epigenetic form of gene regulation that mediates a parent-of-origin-dependent expression of the alleles of a number of genes. It occurs at specific sites within or surrounding the gene called differentially methylated domains (DMDs). Within a DMD, one parental allele is methylated on all or the majority of its CpG dinucleotides, and the opposite parental allele is methylated on none or a small percentage of its CpG dinucleotides. DNA methylation of imprinted gene DMDs results in the silencing of the methylated allele (Li *et al.* 1993, Kaneda *et al.* 2004). An epigenetic reprogramming initiated by the erasure of preexisting DNA methylation marks takes place during the embryonic development in primordial germ cells. Imprint reestablishment follows in both the female and the male germline during gametogenesis (Lee *et al.* 2002).

In the past century, a decline in sperm counts has been documented in young healthy men of industrialized countries, which could be responsible for the currently observed decline in fertility rates. The rapidity of the changes suggested that environmental factors may play a role (Andersson *et al.* 2008). Among them are the endocrine-disrupting chemicals (EDCs), which are substances in our environment and food that interfere with the biosynthesis, metabolism, and action of hormones. The EDCs stand for a broad class of molecules among which are the organochlorinated pesticides (Diamanti-Kandarakis *et al.* 2009). Several studies performed in humans reported that exposure to pesticides may decrease spermatogenesis and male fertility (Roeleveld & Bretveld 2008).

It has recently been shown that imprinting defects are associated with decreased spermatogenesis. Indeed, partial methylation errors affecting the CpGs of paternally or maternally methylated genes (Roeleveld & Bretveld 2008) and methylation defects in the maternally

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methylated *H19* gene (Marques *et al*. 2010) were observed in the sperm of oligospermic or azoospermic patients respectively.

Studies in rodents strongly suggest a link between EDCs and the reproductive system. Vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione, VCZ), a fungicide used to treat fruits and vegetables, was found to exert significant antiandrogenic effects. When administered to pregnant female mice during the gestational period of gonadal sex determination, VCZ was found to affect the offspring. It masculinized females (longer urethras), feminized males (hypospadias), and increased the expression of progesterone receptors in both genders (Buckley *et al*. 2006). It also induced decreases in the anogenital distance and in the prostate weight (Buckley *et al*. 2006, Elzeinova *et al*. 2008). Eventually, VCZ affected spermatogenesis inducing decreases in sperm number and motility, sperm head abnormalities, and apoptosis in the seminiferous tubules germ cells (Shimamura *et al*. 2002, Anway *et al*. 2005, 2006, 2008, Buckley *et al*. 2006, Elzeinova *et al*. 2008). An intriguing feature of VCZ is that the effects on male genital tract and spermatogenesis were found to be transgenerational, extending from F1 to F4 (Anway *et al*. 2005, 2006, 2008). An outcross of a VCZ generation female with a wild-type male had no phenotype, suggesting that the male germline is responsible for the transmission of the phenotype (Anway *et al*. 2006). Studies performed using the same protocol, i.e. VCZ administration to pregnant female rats during the gestational period of gonadal sex determination, failed to demonstrate any change in spermatogenesis. The reason for this discrepancy has not been identified (Schneider *et al*. 2008, Inawaka *et al*. 2009).

In the mouse studies in which a transgenerational effect of VCZ was observed, the possibility of an epigenetic effect of the endocrine disruptor on the male germline was investigated. A methylation-sensitive restriction enzyme study revealed altered methylation patterns on various DNA fragments, called 'imprinted-like gene/DNA sequences', in the F1–F3 offspring sperm (Anway *et al*. 2005). However, these studies were only preliminary, and it is still not known whether or not these DNA fragments are associated with genes exhibiting imprinted (i.e. monoallelic, parent-of-origin dependent) expression.

A few mouse and human DMDs have been well characterized. They are, in particular, the DMDs of the maternally expressed paternally methylated *H19* (Tremblay *et al*. 1997) and *Gtl2* (Li *et al*. 2004) genes and the paternally expressed maternally methylated small nuclear ribonucleoprotein polypeptide N (*Snrpn*) (Shemer *et al*. 1997), *Peg1* (Kaneko-Ishino *et al*. 1995), *Peg3* (Kuroiwa *et al*. 1996), and the potassium channel 1 (*Kcnq1*) (Smilinich *et al*. 1999) genes.

The aim of the present study was to evaluate, more systematically than ever before, the possible deleterious effects of VCZ administration in mice during pregnancy on imprinted genes. The study was performed by testing the possible methylation defects in the DMDs of two paternally (*H19* and *Gtl2*) and three maternally (*Peg1*, *Snrpn*, and *Peg3*) imprinted genes in the sperm as well as in the tail, liver, and skeletal muscle DNAs of the male offspring over three generations.

Results

VCZ or vehicle only was administered to five pregnant female mice during the gestational period of gonadal sex determination, and the methylation status of the paternally methylated *H19* and *Gtl2* genes and that of the maternally methylated *Peg1*, *Snrpn*, and *Peg3* genes were studied by DNA bisulfite treatment in male offspring over three generations (F1–F3). For each imprinted gene, a number of CpGs varying between 5 and 23 were analyzed. The DNA pyrosequencing technique allows to measure the amount of methylated CpGs as compared to total (methylated and nonmethylated) CpGs.

In the sperm of control male offspring, the numbers of methylated CpGs were close to the theoretical values of 100 and 0% of the total CpGs in paternally or maternally methylated genes respectively (Fig. 1). VCZ induced dramatic changes in the sperm of male offspring (F1). The number of methylated CpGs of *H19* and *Gtl2* were decreased (respective values 83.1 and 91.5%), and those of *Peg1*, *Snrpn*, and *Peg3* increased (respective values 11.3, 18.3, and 11.2%). The VCZ effects were all highly significant ($P<0.001$; Fig. 1). As demonstrated by the s.e.m., the inter-individual variations were relatively small. It is noteworthy that all the sperm methylated

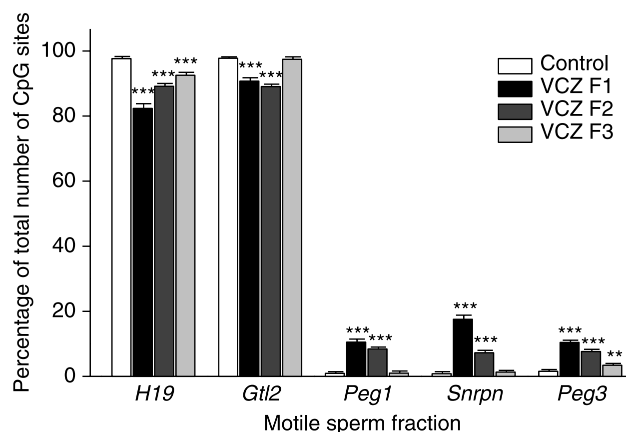

Figure 1 Methylation status of the paternally methylated *H19* and *Gtl2* genes and of the maternally methylated *Peg1*, *Snrpn*, and *Peg3* genes in the motile sperm fraction of the F1–F3 offspring of control and of vinclozolin-administered female mice. Control: vehicle only. VCZ: vinclozolin. The results are the means ± s.e.m. of 8–15 mice. They represent the number of methylated CpG sites and are expressed as the percentage of the total number of CpG sites. $^{***}P<0.001$ and $^{**}P<0.01$ versus respective controls.

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CpG values of the VCZ-administered female offspring differed from control values with no overlap in the sperm methylated CpG values between the two groups ($n=10$ and 15 respectively). The modifications of each gene methylation status were found to be evenly distributed between the CpGs, with no particular one being more affected than the others. Therefore, no specific CpG sites were found to be more or less sensitive to VCZ than the overall sites within the region studied. The effects of VCZ were still present and highly significant ($P<0.001$) in the F2 offspring sperm (Fig. 1). However, a trend toward a recovery between F1 and F2 sperm was observed, with the methylated CpG values being significantly higher in the F2 offspring than in the F1 offspring for *H19* ($P<0.001$) and lower in the F2 offspring than in the F1 offspring for *Peg1*, *Snrpn*, and *Peg3* ($P<0.05$, $P<0.001$, and $P<0.001$ respectively). Only *Gtl2* was as much affected in the F2 offspring as in the F1 offspring. In the F3 offspring, the effects of VCZ could not be detected anymore in *Gtl2*, *Peg1*, and *Snrpn*, and they were significantly less marked in *H19* and *Peg3* ($P<0.01$ and $P<0.001$ respectively). An outcross of a VCZ generation F1 female with a wild-type male resulted in a methylation pattern of *H19*, *Gtl2*, *Peg1*, *Snrpn*, and *Peg3* in the sperm of the F2 offspring that did not differ from that of the control (not shown; Anway *et al.* 2006).

In the tail, which represents a heterogenous sample of somatic cells, the amount of methylated CpGs in control offspring was close to 50% of the total methylated+nonmethylated CpGs in all the imprinted genes tested. This value is in total agreement with the theoretical value expected for imprinted genes in which DNA methylation/unmethylation is present on one of the two parental alleles only (Fig. 2). A few differences were observed between control and VCZ-administered female offspring. In the F1 offspring, the methylated CpG values were significantly higher in *H19* and lower in *Snrpn* than in the controls. In the F2 and F3 offspring, they were significantly lower in *Peg3* than in the controls (Fig. 2).

To investigate further the finding that the VCZ-induced changes in the methylation patterns of imprinted genes are not restricted to male germline cells, the methylation patterns of our five target genes were analyzed in liver and skeletal muscle. As observed in the tail, the amount of methylated CpGs in the control offspring was close to the theoretical value of 50% of the total CpGs in all the imprinted genes tested (Figs 3 and 4). Again, a few differences were observed between control and VCZ-administered female offspring. In the liver of the F1 offspring, the methylated CpG values were significantly lower in *Snrpn* than in the controls. In the F2 offspring, they were significantly lower in *Peg1* than in the controls, and in the F2 and F3 offspring, they were significantly lower in *Peg3* than in the controls (Fig. 3). In the skeletal muscle of the F1 offspring, the methylated CpG values were significantly lower in *Snrpn* than in the

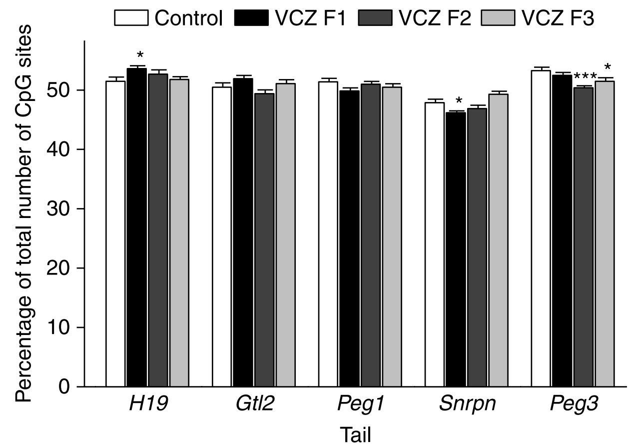

Figure 2 Methylation status of the paternally methylated *H19* and *Gtl2* genes and of the maternally methylated *Peg1*, *Snrpn*, and *Peg3* genes in the tail of the F1–F3 offspring of control and vinclozolin-administered female mice. The results are the means±S.E.M. of 8–15 mice. They represent the number of methylated CpG sites and are expressed as the percentage of the total number of CpG sites. ***$P<0.005$ and *$P<0.05$ versus respective controls.

controls. In the F2 offspring, they were significantly lower in *Snrpn* and *Peg3* than in the controls (Fig. 4). It is noteworthy that the changes induced by VCZ in the somatic cells are not as erratic as they look, with them being reproducibly observed for the F1 offspring *Snrpn* in the tail, liver, and muscle, and for the F2 offspring *Peg3* in the tail, liver, and muscle.

The possibility that the observed changes in the methylation pattern of imprinted genes in the sperm might have biological consequence was tested. A sperm count was performed in five controls and in six to eight

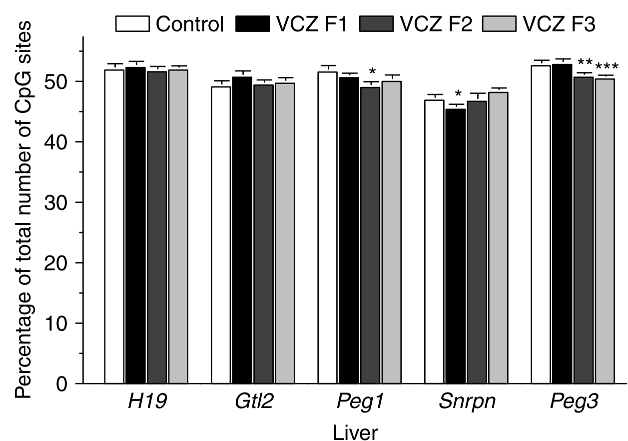

Figure 3 Methylation status of the paternally methylated *H19* and *Gtl2* genes and of the maternally methylated *Peg1*, *Snrpn*, and *Peg3* genes in the liver of the F1–F3 offspring of control and vinclozolin-administered female mice. The results are the means±S.E.M. of 8–15 mice. They represent the number of methylated CpG sites and are expressed as the percentage of the total number of CpG sites. ***$P<0.001$; **$P<0.01$, and *$P<0.05$ versus respective controls.

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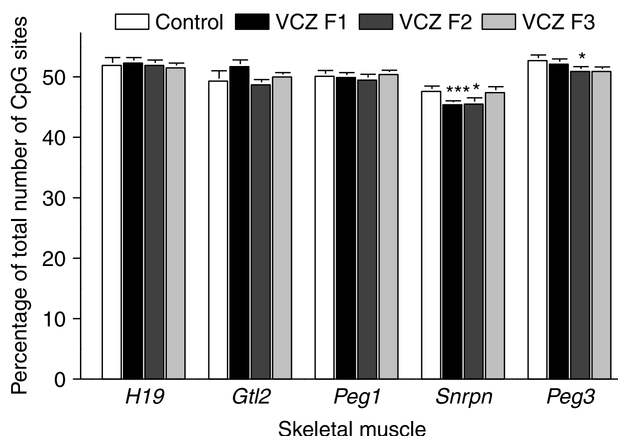

Figure 4 Methylation status of the paternally methylated *H19* and *Gtl2* genes and of the maternally methylated *Peg1*, *Snrpn*, and *Peg3* genes in the skeletal muscle of the F1–F3 offspring of control and vinclozolin-administered female mice. The results are the means ± S.E.M. of 8–15 mice. They represent the number of methylated CpG sites and are expressed as the percentage of the total number of CpG sites. ***$P<0.001$ and *$P<0.025$ versus respective controls.

F1, F2, and F3 offspring of pregnant VCZ-administered female mice. As shown in Fig. 5, the mean sperm concentrations of the VCZ female offspring were only 56% in the F1, 90% in the F2, and 100% in the F3 of that of the controls.

Discussion

The current study is the first to address the possible effects of VCZ administration to pregnant female mice on canonical imprinted genes in different tissues, including the male gametes, over three generations.

The pyrosequencing technique was chosen and used throughout this study since it was previously shown to yield results similar to those obtained by the subcloning–sequencing technique (Stouder *et al*. 2008).

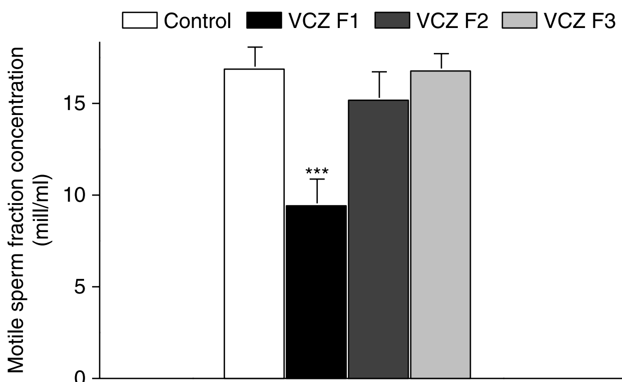

Figure 5 Motile sperm concentration, in millions of spermatozoa per milliliter of sperm, in the F1, F2, and F3 offspring of control and vinclozolin-administered female mice. The results are the means ± S.E.M. of 5–8 mice. ***$P<0.005$.

VCZ was shown to affect the imprinting status of the two paternally (*H19* and *Gtl2*) and three maternally (*Peg1*, *Snrpn*, and *Peg3*) imprinted genes in the sperm of the offspring. These data corroborate the finding by Anway *et al*. (2005) who showed that VCZ administration to pregnant female mice alters the methylation patterns on 'imprinted-like gene/DNA sequences' in the sperm of the offspring (Anway *et al*. 2005) and extend it to the well-characterized imprinted genes. Our outcross experiment, showing no phenotype in the sperm of the F2 offspring, confirmed the results of Anway *et al*. (2006), which suggested that the male germline is responsible for the transmission of the phenotype. In a previous study conducted on the sperm of superovulated female offspring, we described methylation disturbances that affected both paternally and maternally methylated genes in 75% or more of the F1 offspring (Stouder *et al*. 2008). The effects of VCZ, described in this study, are more marked and affect 100% of the F1 offspring.

It is generally admitted that due to the mechanisms of erasure and reprogramming, abnormal epigenetic states are not transmitted to the next generation. However, it has been shown that in some occasions, such as VCZ administration or superovulation in pregnant female mice, epigenetic mark defects can survive transgenerational reprogramming (Anway *et al*. 2005, Stouder *et al*. 2008). The possible mechanism of this resistance to reprogramming is not known (Youngson & Whitelaw 2008). One hypothesis is that it is due to modified expressions of genes coding for proteins involved in the epigenetic reprogramming. In this study, the imprinting defects induced by VCZ in the sperm of the offspring were transgenerational. The methylation, however, showed normal pattern in F3, for three of five imprinted genes. The latter observation suggests that the resistance to reprogramming is reversible and little by little eroded by the successive reprogramming periods occurring during each gestation.

Another finding of this study is that the imprinting errors observed in the sperm of VCZ-administered female mice might have biological consequences as suggested by a decrease in sperm count in the F1 offspring. However, in the F2 and F3 offspring, the sperm count was normalized. There is therefore a discrepancy between the effects of VCZ on the methylation status of sperm imprinted genes which persist in the F2 and F3 offspring and the effect of VCZ on the sperm count, which has already disappeared in the F2 offspring. The lack of correlation between epigenetic and biological changes does not necessarily mean that the decrease in the sperm count observed in the F1 offspring is not related to the changes in the methylation status of sperm imprinted genes. It would be interesting to study whether qualitative parameters of the sperm morphology are or not transgenerationally affected by VCZ.

The methylation errors described in this study have the same characteristics as those reported previously in the offspring of superovulated mothers (Stouder *et al*. 2008):

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they affect only a fraction of the total number of CpG sites, but they are highly significant and evenly distributed among the offspring.

Kobayashi *et al.* (2007) described aberrant methylation patterns of paternally and maternally imprinted genes, and Marques *et al.* (2010) described a hypomethylation of the paternally imprinted gene *H19* and of its CTFC-binding site 6 in the sperm of patients with abnormal spermatogenesis. In both papers, aberrant methylations as low as 8–10% of the total number of CpGs were considered in the association studies. Therefore, it is quite possible that small changes in the methylation pattern of imprinted genes, such as those described in this study, might have biological consequences.

In the somatic cells, two different types of changes are observed depending on whether F1 or F2/F3 generations are considered.

In the F1 offspring, the methylation pattern of *Snrpn* was altered in all somatic cells studied, i.e. those of the tail, liver, and muscle. The occurrence of this alteration in F1 offspring shows that it is not mediated by the germline. It might be the result of a direct toxic effect of VCZ on somatic cells. The reason why this effect targets *Snrpn* is unknown. The direct alteration of somatic cells is not transmitted to the next generations in the tail and in the liver.

In the F2 offspring, a prominent alteration in the methylation pattern of somatic cells is that occurring in *Peg3*, which is observed in the tail, liver, and skeletal muscle. In the F3 offspring, interestingly, this alteration is still observed in the tail and liver. The reason why this effect targets *Peg3* is unknown. The latter gene encodes a zinc-finger protein that is ubiquitously expressed, although at the highest mRNA levels in the placenta, uterus, ovary, testis, and brain (Hiby *et al.* 2001). The high conservation of PEG3 protein between mice and humans suggests that it is critical for cellular function. $Peg3^{+/-}$ mice demonstrated impaired maternal behavior (Li *et al.* 1999), impaired olfactory processing (Swaney *et al.* 2007), a lower growth rate, a delayed puberty, and a tendency to become obese (Curley *et al.* 2005). The deficits observed in $Peg3^{+/-}$ mice might be explained by an increased postnatal apoptosis in the neuroanatomical structures that support reproductive behavior and metabolic homoeostasis (Broad *et al.* 2009). Therefore, prenatal exposure to VCZ might affect, in addition to sperm concentration, reproductive behavior and energy balance in the offspring. The effects observed in somatic cells of the F2 and F3 offspring are probably transmitted through the germline. We therefore deal with a phenomenon that mimics a DNA mutation, with the important difference, however, being that it seems to be reversible.

Environmental factors affect the methylation pattern of not only imprinted genes but also a number of other target genes throughout the genome (Szyf *et al.* 2007). Therefore, our observations, on a selection of strategic sites, might represent only a small fraction of the genome-wide effects of VCZ.

This study shows that VCZ administration to pregnant female mice induces alterations in the imprinted status of five canonical paternally and maternally imprinted genes in the sperm of the offspring which are transgenerational but disappear gradually from F1 to F3. These effects are paralleled by a decrease in the sperm concentration observed only in F1. Therefore, it proposes a mechanism for the previously observed effects of VCZ on the rodent reproductive system (Shimamura *et al.* 2002, Anway *et al.* 2005, 2006, 2008, Buckley *et al.* 2006, Elzeinova *et al.* 2008). By analogy, we can postulate that the reported effects of EDCs on human male spermatogenesis and fertility rates (Roeleveld & Bretveld 2008) might involve imprinting alterations in the sperm.

Materials and Methods

Mice

Normal FVB/N mice were purchased from Charles River (Arbresle, France). Two-month-old female mice were naturally mated with male mice of the same age. Females with a copulation plug the next morning were separated into two groups. The dose of VCZ reported to affect the male offspring reproductive system when administered to pregnant female rats is 100 mg/kg per day (Anway *et al.* 2005, 2006, 2008). Since the mice might be more sensitive to VCZ than the rats and to minimize the possible nonspecific toxic effects of the drug, a dose of 50 mg/kg per day was chosen. VCZ (Sigma–Aldrich) was suspended in corn oil and administered i.p. to the first group of pregnant mice in doses of 50 mg/kg per day from days 10 to 18 of pregnancy. The second group consisted of control mice that were injected with only corn oil from days 10 to 18 of pregnancy. To study the possible transgenerational effects, F1 males were analyzed or subsequently crossed with FVB/N females in order to obtain F2 males. Both F1 and F2 offspring were used at the age of 2 months. An outcross of a VCZ generation F1 female with a control male was also performed. Animal protocols used in these studies were approved by the Commission d'Ethique de l'Expérimentation Animale of the University of Geneva Medical School and by the Geneva Veterinarian Office.

Sperm collection

The mouse vas deferens and epididymis were dissected out, placed into a Petri dish, scored with a razor blade in a droplet of PBS into small pieces, and left for 30 min to allow sperm to diffuse into the medium. The latter was then transferred into a microcentrifuge tube, and the fragments were allowed to sediment for 30 min at 37 °C. The supernatant was carefully transferred to another tube, and this procedure was repeated three times. The final supernatant containing the motile sperm fraction was carefully removed and centrifuged at 6000 ***g*** for 10 min to pellet the sperm.

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Sperm count

In some experiments, an aliquot of the supernatant's motile sperm fraction was used for sperm counting using a hemocytometer. The corresponding sperm concentration was calculated using the formula: cells per ml = the average count per square × dilution factor × 10^4 (counts ten squares).

Tissue collection

Fragments of 5×5 mm size of tail, liver, and tibialis anterior muscle were collected, cut into small pieces in a Petri dish, and transferred into DNA-extraction buffer.

DNA isolation

Motile sperm fraction DNA was extracted using the QIAamp DNA microkit (Qiagen). Total genomic DNA was extracted from tail, liver, and tibialis anterior muscle tissues by incubation for 3 h at 55 °C in DNA-extraction buffer (50 mM Tris–HCl, pH 8, 100 mM EDTA, and 0.5% SDS) followed by phenol–chloroform–isoamyl alcohol extractions and ethanol precipitation.

Bisulfite treatment

Using the EZ Methylation Gold Kit (Zymo Research, Orange, CA, USA), the extracted DNA was treated with sodium bisulfite in order to convert unmethylated cytosine residues to uracil. The converted DNA was eluted in 10 µl TE buffer (10 mM Tris–HCl and 0.1 mM EDTA, pH 7.5). Two microliters of the post-bisulfite-treated DNA were used for subsequent PCR amplification.

PCR amplification of bisulfite-treated DNAs for subsequent pyrosequencing

The PCR amplifications aimed at pyrosequencing were performed starting from 100 to 140 ng of bisulfite-treated sperm, tail, liver, and skeletal muscle DNAs. The PCR conditions were the same for all the genes tested, i.e. 94 °C for 15 min, followed by 40 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; and by a 72 °C 10-min final extension step.

The characteristics of the amplicons and the oligonucleotides chosen are presented in Table 1.

A diagram showing the locations of the DMDs, within the corresponding genes, and of the DNA fragments of the five genes amplified by PCR has been presented in a previous study (Stouder *et al*. 2008).

All reactions were performed with a PCR mixture (total volume 25 µl) containing oligonucleotides at 0.5 mM concentration and 12.5 µl HotStarTaq Master Mix (Qiagen).

The biotinylated PCR products were purified using streptavidin–sepharose beads (Amersham) and were sequenced using the PSQ 96 Gold reagent kit (Biotage AB, Uppsala, Sweden) with the following primers:

H19: 5′-GTGTAAAGATTAGGGTTGT-3′, *Gtl2*: 5′-GTTATGGATTGGTGTTAAG-3′, *Peg1*: 5′-TCAATATCAACTAAATAATC-3′, *Snrpn*: 5′-GAATTGGAGTTGTGTGG-3′, and *Peg3*: 5′-AATTGATAAGGTTGTAGATT-3′.

Table 1 Amplicon and oligonucleotide characteristic

H19
255 bp amplicon
6 CpGs
Oligonucleotide forward: 5′-GGGGGGTTATAAATGTTATTAGGGGGGTAGG-3′
Oligonucleotide reverse: 5′-biotin-7AACCCCTAACCTCATAAAACCCATAACTATAAAATCA-3′
Gtl2
316 bp amplicon
23 CpGs
Oligonucleotide forward: 5′-GTGGTTTGTTATGGGTAAGTTTT-3′
Oligonucleotide reverse: 5′-biotin-7CTTCCCTCACTCCAAAAATTAAA-3′
Peg1
180 bp amplicon
5 CpGs
Oligonucleotide forward: 5′-biotin-7TTTGTTGTAGTTTTGGTTTTGTAA-3′
Oligonucleotide reverse: 5′-CCCTCTCTCAAATACTAACATTTT-3′
Snrpn
202 bp amplicon
5 CpGs
Oligonucleotide forward: 5′-TTTGGGGAATTGGAGTTG-3′
Oligonucleotide reverse: 5′-biotin-7TCTCCCCCAAACATAAACTAA-3′
Peg3
103 bp amplicon
6 CpGs
Oligonucleotide forward: 5′-GGTTTTTAAGGGTAATTGATAAGG-3′
Oligonucleotide reverse: 5′-biotin-7CCCTATCACCTAAATAACATCCC-3′

The degree of methylation at each CpG site was determined using Pyro Q-CpG Software (Biotage AB). All samples were analyzed in duplicate.

In a previous study, the results obtained were double-checked by comparing the techniques used above, i.e. DNA bisulfite treatment and PCR amplification followed directly by pyrosequencing, with PCR amplification followed by amplicon subcloning and sequencing. The results were found to be similar.

Statistical analysis

Significances were evaluated using unpaired Student's *t*-test and set at $P<0.05$.

Declaration of interest

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

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