

EFFECTS OF AROCLOR 1254 ON THE DNA METHYLATION OF IMPRINTED GENES IN THE ADULT MOUSE SPERM

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Abstract. As estrogen-like and anti-androgenic endocrine-disrupting chemicals, polychlorinated biphenyls have been reported to affect the male reproductive axis resulting in infertility. In the present study, Aroclor 1254 was administered to adult male mice, and its possible effects on the differentially methylated regions of two paternally (H19 and Gtl2) and three maternally (Mest, Snrpn and Igf2r) imprinted genes were tested in the sperm DNA. In the control, the percentages of methylated CpGs were close to 100% and 0% in paternally or maternally imprinted genes respectively. Aroclor 1254 significantly decreased the percentages of methylated CpGs of H19 and Gtl2 and markedly increased those of Mest, Snrpn, and Igf2r. In addition, dramatically increased mRNA expression of estrogen receptor α and β were obtained in the treatment. We postulate that the effects of PCBs on male spermatogenesis and fertility rates might involve imprinting alterations in the sperm.

Keywords: *polychlorinated biphenyls; endocrine disrupting chemicals; epigenetics; CpG island; sperm cell*

Introduction

Genomic imprinting is an epigenetic mechanism through which a subset of mammalian genes is expressed in a parental allele-specific manner. The parental-specific expression of genes is regulated by parent-specific epigenetic markers such as DNA methylation in the differentially methylated region (DMR) or imprinting control region (ICR) (Paoloni-Giacobino and Chaillet, 2006). These markers are independently established during gametogenesis (Morison et al., 2001). After fertilization in mice, the paternal genome undergoes active demethylation and the maternal genome undergoes passive demethylation, without affecting imprinting marks (Hammoud et al., 2010). Thus, the DNA methylation differences between maternal and paternal alleles of many imprinted genes are inherited from the male and female gametes and subsequently maintained during development (Surani et al., 1986; Lucifero et al., 2002). It is also reversible in that the methylation imprints are switched in the germ line to reflect the sex of the parent (Li et al., 1993). Evidence of sex-specific

differences in imprint acquisition suggests that male and female germ cells may be susceptible to perturbations in imprinted genes at specific prenatal and postnatal stages (Lucifero et al., 2004). Germ cells are a unique cell type that can transmit genetic information to the next generation. Germ cell development and early embryogenesis are crucial windows in the erasure, acquisition and maintenance of genomic imprints (Lucifero et al., 2002). Moreover, imprinted genes have been shown to play a key role in the regulation of embryonic growth and placental function during development (Strogantsev and Ferguson-Smith, 2012). The paternal-derived imprints are biased toward development of extra embryonic lineages, thereby promoting embryo growth (McGrath and Solter, 1984). The disruption of imprinting of genes in spermatogenesis commonly result in infertility and developmental problems (Hammoud et al., 2010; Hitchins and Moore, 2004).

Polychlorinated biphenyls (PCBs) are a group of halogenated aromatic hydrocarbons, synthetic chemicals which do not occur naturally in the environment. They belong to a group of widespread environmental pollutants, and their ecotoxicological impact on living organisms was of great interest (Safe, 1994). The manufacture of PCBs was stopped and restricted in many countries since the 1970s. However, they continue to be detected as major global pollutants in the environment and biological samples and cause harmful health effects (Rudel and Perovich, 2009). Although PCBs are not necessarily fatal to wildlife and human, they are biomagnified along food chains, increasing a risk of human exposure due to their ubiquitous, persistent and lipophilic characters (Zhou and Zhang, 2005). PCBs are considered potential endocrine disruptors. They are estrogen-like and anti-androgenic chemicals in the environment contain potentially hazardous effects on male reproductive axis resulting in infertility and other hormonal dependent reproductive functions (Pflieger-Bruss and Schill, 2000; Fielden et al., 2001).

Evidence in animal models suggests that endocrine disruptors may affect not only the exposed individual but also the offspring and subsequent generations (Schug et al., 2011). The mechanism of transmission involves non-genomic modifications of the germ line such as changes in DNA methylation and histone acetylation. Several studies have shown an alteration in DNA methylation in the male germ cells on exposure to endocrine disruptors. Anway et al have revealed an association between altered methylation patterns in testicular germ cells and reduced male fertility in rats on exposure to endocrine disruptors, methoxychlor, and vinclozolin (Anway et al., 2005). Recent study has shown that in utero exposure of male rats with an antiandrogenic compound, vinclozolin, results in epigenetic transgenerational disease phenotype involving alterations in DNA methylation patterns at various loci in the spermatozoa (Chang et al., 2006). Reduced DNA methylation at imprinted loci in rat spermatozoa upon tamoxifen treatment indicated a role of estrogen-associated signaling in the acquisition of paternal-specific imprints during spermatogenesis (Pathak et al., 2009). In addition, association between DNA methylation and postimplantation loss suggests that errors in paternal imprints at imprinted loci could affect embryo development (Pathak et al., 2009). These experiments indicate that normal methylation patterns of imprinted

genes in the male germ line are crucial for normal spermatogenesis and embryogenesis. The establishment of DNA methylation imprints in the male germ cells in adulthood and the possible influence of endocrine disruptors on male germ cell methylation suggest that exogenous epigenetic insults could be transmitted to the progeny, thereby affecting its developmental potential.

Aroclor 1254 is a pool of more than 60 congeners used for in vitro studies because its composition is representative of PCBs environmental pollution. Previous studies revealed the adverse in vivo effects of Aroclor 1254 on testicular germ cell development and spermatogenesis (Mi and Zhang, 2005), as well as the in vitro toxic and estrogenic actions of Aroclor 1254 on ovarian germ cell (Xie and Zhang, 2004). The above results indicated that Aroclor 1254 exposure imposed both toxic and hormonal effects on embryonic testicular germ cell proliferation, which may cause reproductive disorder and even infertility at adulthood. However, the effects of PCBs on DNA methylation patterns of imprinted genes in the male germ cells remain unknown. In the present study, five imprinted genes, H19, Gtl2, Mest, Snrpn and Igf2r were chosen, and the effects of PCBs on DNA methylation patterns of sperm cells were evaluated at their DMR or ICR loci using bisulphate modification and cloning analysis.

Methods

Animals and treatment

Animals were cared for humanely according to the guidelines for animal experiments of the Academy of Military Medical Sciences. Male Kunming strain mice (age, 8 wk) were obtained from Biological Product Institute (Changchun, China). The mice were housed in standard polypropylene cages in a temperature-controlled room (22°C) with a 12:12-h light-dark cycle. Commercial mouse pellets and water were provided *ad libitum*. The study was approved by the Institutional Animal Ethics Committee.

Adult male mice were administered 50 µg/kg body weight/day of Aroclor 1254 (Sigma Chemicals, Shanghai, China), by intraperitoneal injection, 5 days a week for 60 days (Mi and Zhang, 2005; Zhang et al., 2012). The chemical was suspended uniformly in arachis oil by sonication. Controls received arachis oil alone.

Male mouse sperm cells collection

The treated and control males were killed, and the vas deferens and cauda epididymis were dissected out, placed into a Petri dish, scored with a razor blade in a droplet of Phosphate Buffer Saline (PBS) and left at 37°C for 30 min to allow motile sperm to move into the medium. The latter was then transferred into a microcentrifuge tube and the tissue fragments were allowed to sediment during 30 min at 37°C. The motile sperm remained in the supernatant, which was carefully transferred to another tube, and this procedure was repeated 3 times. The final supernatant was carefully removed and centrifuged at 6000×g for 10 min to pellet the sperm.

DNA isolation and bisulfite sequencing

Genomic DNA was extracted from male mouse sperm cells using EasyPure Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The isolated DNA was treated with sodium bisulfite from an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Subsequently, the modified DNA sample immediately used in BS-PCR amplification or stored at -20°C. Three separate bisulfite modification treatments were performed for each DNA sample.

The bisulfite converted DNA was amplified by nested PCR for Igf2-H19 ICR, Dlk1-Gtl2 IG-DMR III, Mest promoter and exon1, Snrpn DMR1 and Igf2r DMR2 with primers. Specific primers for BS-PCR amplification were designed using Methyl Primer Express® Software v1.0 (Applied Biosystems, Foster City, CA, USA) and listed in table (Table 1). To generate the product for each imprinted gene, two rounds of PCR were done with fully nested primer pairs. The outside primer pairs were used for the first-round PCR, whereas the inside primer pairs were used for the second-round PCR.

Table 1. Primers for bisulfite PCR

Gene	Primer set (5' to 3')	Product size(bp)	T _{a1} /T _{a2} (°C)	GenBank accession no.
Igf2-H19 ICR	Outside forward: TTTTGGTTATTGAATTTTAAAAATTAG	608	53/55	U19619
	Inside forward: GTGGTTTATTATAGGAAGGTATAGAAGT			
	Inside reverse: ACCTAAAATACTCAAACCTTATCACAAC			
	Outside reverse: ACCATTCCCTAAAATATCACAAATACC			
Dlk1-Gtl2 IG-DMR III	Outside forward: GTATTGTAATATAGGTTAGGTG	499	53/52	AB291769
	Inside forward: GTATTGTAATATAGGTTAGGTG			
	Inside reverse: CACAACCTACACAAAATACTAC			
	Outside reverse: CTACATAATACCATATAAACATATCTC			
Mest promoter and exon1	Outside forward: GATTTGGGATATAAAAGGTTAATGAG	563	53/53	AF017994
	Inside forward: TTTTAGATTTTGAGGGTTTATAGGTTG			
	Inside reverse: AATCCCTTAAAAATCATCTTTCACAC			
	Outside reverse: TCATTAAAAACACAAACCTCCTTTAC			
Snrpn DMR1	Outside forward: TGTAATATGATATAGTTTAGAAATTAG	420	52/54	AF063659
	Inside forward: AATTTGTGTGATGTTTGTAATTATTTGG			
	Inside reverse: ATAAAATACACTTTCACCTACTAAAATCC			
	Outside reverse: ATAAACCCAAATCTAAAATATTTTAATC			
Igf2r DMR2	Outside forward: TTAGTGGGGTATTTTATTTGTATGG	206	52/53	L06446
	Inside forward: GTGTGGTATTTTATGTATAGTTAGG			
	Inside reverse: AAATATCCTAAAAATACAAACTACAC			
	Outside reverse: AAATATCCTAAAAATACAAACTACAC			

Ta1/Ta2 represents the annealing temperatures for the 1st and 2nd PCR, respectively.

Each 50 μ L PCR reaction contained 2 μ L bisulfite-treated DNA, 5 μ L 10 $\times$ PCR buffer, 4 μ L (2.5 mM each) dNTPs (Takara, Dalian, China), 2 μ L of both forward and reverse primers, 34.5 μ L dH₂O, and 0.5 μ L Hot start DNA Taq Polymerase (Tiangen, Beijing, China).

Thermal cycling was carried out with a 5 min denaturation step at 94°C, followed by 30 three-step cycles (30 s at 94°C, 30 s at different annealing temperatures listed in *Table 1*, and 30 s at 72°C), and final extension at 72°C for 10 min. For the second round of PCR, 1 μ L of the first-round sample was used and the conditions for the PCR were the same as above.

The PCR products were resolved on 1.2% agarose gels to confirm the specific amplification of the products by size, and then gel purified using the TIANGel Midi Purification Kit (Tiangen, Beijing, China). Purified products were cloned into pMD18-T vectors (Takara, Dalian, China) and plated onto AMP-X-GAL plates for blue-white screening. Positive colonies were reinoculated and then sequenced (Shenggong, Shanghai, China). Ten clones were analyzed for each sample for each imprinted gene.

RNA extraction and quantitative PCR

Total RNA was extracted by an EasyPureTM RNA Kit (TransGen, Beijing, China), and cDNA was synthesized by a TransScriptTM First-Strand cDNA Synthesis SuperMix Kit (TransGen, Beijing, China).

The expression level of estrogen receptors (ER α and ER β) were quantified on a StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TransStartTM Green qPCR SuperMix (TransGen, Beijing, China). The primers for quantitative PCR were designed using Primer Express[®] Software v3.0 for Real-Time PCR (Applied Biosystems, Foster City, CA, USA) and listed in table (*Table 2*).

Table 2. Primers for quantitative real-time PCR

Gene	Primer set (5' to 3')	Product size(bp)	T _a (°C)	GenBank accession no.
ER α	Forward: AGGCATGGTGGAGATCTTTGA Reverse: AATGATGGATTTGAGGCACACA	100	60	BC167246
ER β	Forward: CAAGTCCGCCTCTTGGAAG Reverse: GGTCTGGAGCAAAGATGAGCTT	100	60	BC141075
β -actin	Forward: TCTGGCACCACCTTCTACAA Reverse: TTTTCACGGTTGGCCTTAGG	100	60	BC138611

Ta represents the annealing temperature for the quantitative real-time PCR.

Reaction mixture (25 μ L) contained 2 μ L cDNA template, 12.5 μ L TransStartTM Green qPCR SuperMix (2 $\times$), 0.5 μ L of both PCR forward and reverse primers (10 μ M), 0.5 μ L Passive Reference Dye (50 $\times$), and 9 μ L dH₂O. Thermal cycling conditions were 95°C for 1 min, followed by 40 PCR cycles of 5 s at 95°C for DNA denaturation, and 30 s at 60°C for primer annealing and extension. The melting protocol was 65°C to 95°C (in increments of 0.5°C/5 s). Transcript of ERs were quantified in three replicates and

calculated relative to the transcription in sample of the housekeeping gene, β -actin (endogenous control).

Statistical analysis

Every independent experiment was duplicated at least three times, and the data were presented as mean $\pm$ SD. Significances were evaluated by a Student's t-test and set at $P < 0.05$.

Results

Methylation status and levels in the differentially methylated regions of the imprinted genes

The DNA methylation status and levels of H19 ICR, Dlk1-Gtl2 IG-DMRIII, Mest promoter and exon1, Snrpn DMR1 and Igf2r DMR2 in the mouse sperm cells were analysed by bisulfite PCR (BSP). Methylation data from BSP sequencing were analysed by computing the percentage of methylated CpGs of the total number of CpGs.

The modifications of each imprinted gene methylation status were found to be evenly distributed between the CpGs (Figs.1, 2), with no particular one being more affected than the others. Therefore, no specific CpG sites were found to be more or less sensitive to Aroclor 1254 than the overall sites within the region studied.

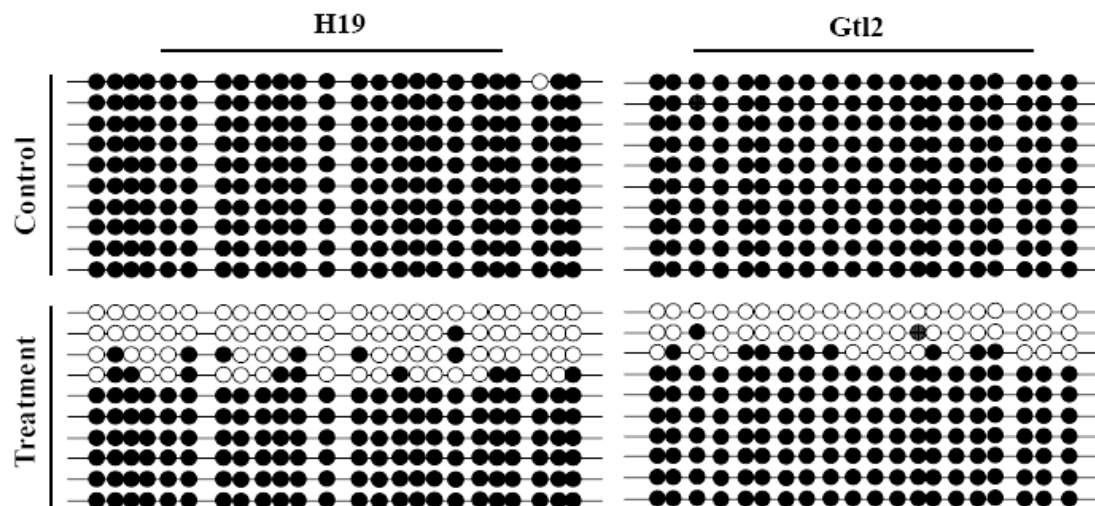


Figure 1. DNA methylation status of *Igf2*-H19 ICR and *Dlk1*-Gtl2 IG-DMRIII in the sperm cells of the control and Aroclor 1254-treated mice analyzed by BSP. Each line represents a single sequenced clone. Filled circles correspond to methylated cytosines and open circles represent unmethylated cytosines

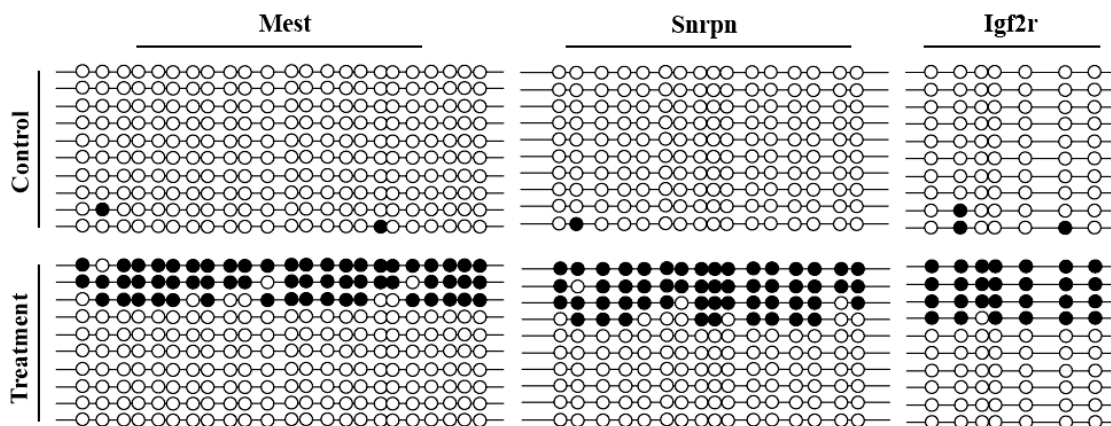


Figure 2. DNA methylation status of *Mest* promoter and exon1, *Snrpn* DMR1 and *Igf2r* DMR2 in the sperm cells of the control and Aroclor 1254-treated mice analyzed by BSP. Each line represents a single sequenced clone. Filled circles correspond to methylated cytosines and open circles represent unmethylated cytosines

In the sperm of controls, the percentages of methylated CpG sites were close to the values of 100% and 0% of the total CpGs in paternally or maternally imprinted genes respectively (Figs. 3, 4). Aroclor 1254 induced dramatic changes of DNA methylation of imprinted genes in the sperm of treated male mice. The number of methylated CpGs of *H19* and *Gtl2* were decreased (respective mean values 67.33% and 76%), and those of *Mest*, *Snrpn*, and *Igf2r* increased (respective mean values 26.09%, 33.96%, and 38.57%). The Aroclor 1254 effects were all highly significant ($P < 0.01$; Figs. 3, 4).

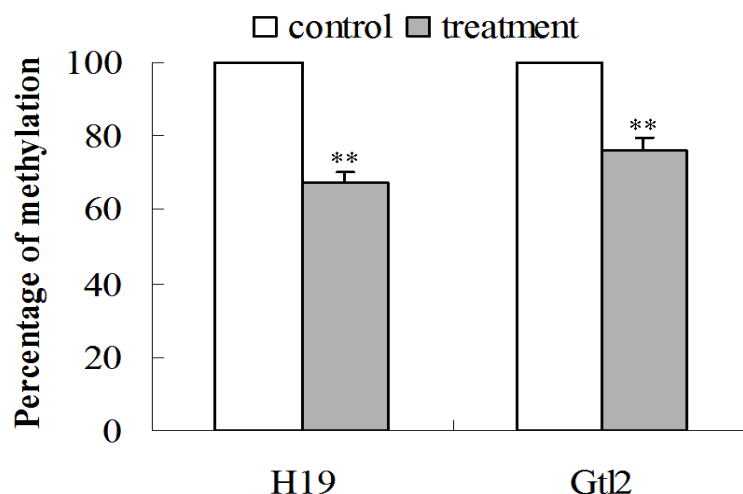


Figure 3. DNA methylation levels of *Igf2-H19* ICR and *Dlk1-Gtl2* IG-DMRIII in the sperm cells of the control and Aroclor 1254-treated mice analyzed by BSP. Statistically significant differences to the control are presented (“**” for $P < 0.01$)

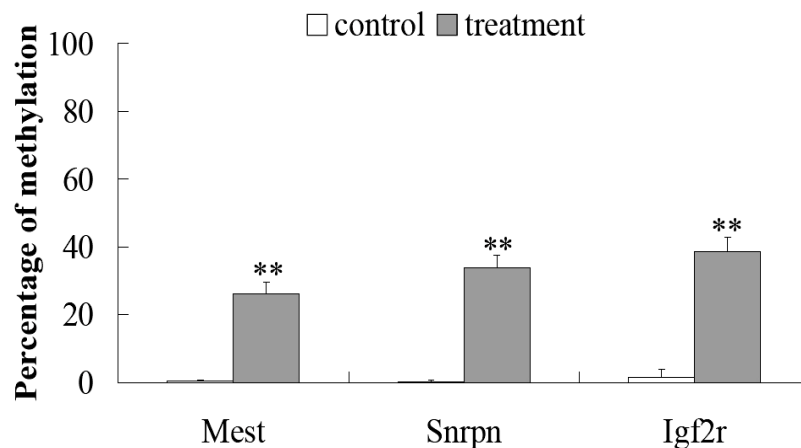


Figure 4. DNA methylation levels of *Mest* promoter and exon1, *Snrpn* DMR1 and *Igf2r* DMR2 in the sperm cells of the control and Aroclor 1254-treated mice analyzed by BSP. Statistically significant differences to the control are presented (“**” for $P < 0.01$).

Relative mRNA expression levels of estrogen receptor (ER) genes

ER is regarded as a ligand-dependent transcription factor, since it recruits coactivator complexes with histone acetyltransferase or methyltransferase activities to valid downstream target genes (Hall and McDonnell, 2005). Here, we tried to determine whether Aroclor 1254, an environmental xenoestrogen, exerted its biological function via ER signaling pathways. The results demonstrated that Aroclor 1254 administration significantly up-regulated ER α and ER β at the mRNA levels, in the adult male germ cells, 2-fold and 2.98-fold over control levels, respectively. ($P < 0.01$; Fig. 5).

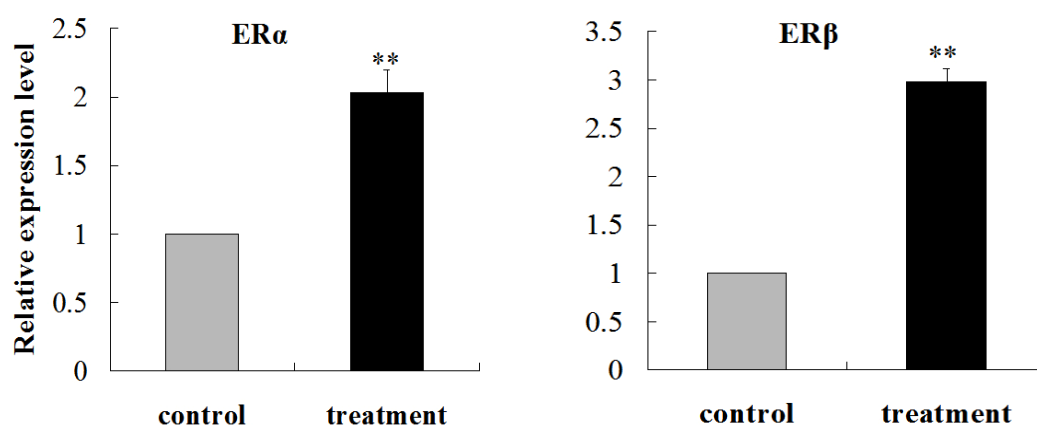


Figure 5. The relative mRNA expression levels of estrogen receptors (ER α and ER β) in mouse sperm cells. Aroclor 1254 administration significantly up-regulated ER α and ER β at the mRNA levels. The results were presented as mean $\pm$ SD. “**” indicates significance of $P < 0.0$

Discussion

A decline in sperm counts 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, which are substances in our environment and food that interfere with the biosynthesis, metabolism, and action of hormones. As estrogen-like and anti-androgenic endocrine-disrupting chemicals, PCBs continue to be of concern to biologists due to the reports that exposure to relatively low levels may be associated with reproductive failure in wild animals, especially in fish-eating bird (Clark et al., 1998). Preliminary results suggested that Aroclor 1254 could remarkably affect the fertilization ability of sperm, and then cause male infertility (Sikka and Wang, 2008).

DNA methylation plays a key role in regulating eukaryotic gene expression and acts as an important molecular marker underlying the parental-specific expression of genes in growth, differentiation, development and other processes (Reik and Walter, 2001). Genomic imprinting is erased in primordial germ cells in mice, and then re-established during gametogenesis (Hajkova et al., 2002). In mammals, imprinted genes show a unique expression pattern depending upon the parental origin of the derived allele (Delaval and Feil, 2004). This unique pattern of gene expression is regulated by a CpG-rich domain neighbouring the imprinted gene or gene clusters that are differentially modified by germline-specific de novo methylation mechanisms during male and female gametogenesis. The specific DNA methylation patterns in the DMRs or ICRs of imprinted genes provide a convenient way to distinguish the paternal allele from the maternal one (Bird, 1986). Patterns of DNA methylation frequently change in response to cell differentiation, disease and environmental influences. In the present study, significant decrease in methylation of two paternally (H19 and Gtl2) imprinted genes and dramatic increase in methylation of three maternally (Mest, Snrpn, and Igf2r) imprinted genes in spermatozoa were observed following Aroclor 1254 treatment. A normal level of DNA methylation is required for controlling differential expression of the paternal and maternal alleles of imprinted genes (Li et al., 1993). Imprinting defects in cases of disrupted spermatogenesis raised the possibility that they could be associated with infertility itself (Marques et al., 2004). The importance of genomic imprinting during spermatogenesis for male fertility has been postulated as decreased methylation of the paternal Igf2 / H19 imprinting control region 1 (ICR1) and Gtl2 imprints have been found in spermatozoa of men with disturbed spermatogenesis (Kobayashi et al., 2007). Poplinski et al determined that low sperm counts were clearly associated with Igf2 / H19 ICR1 hypomethylation and, even stronger, with Mest hypermethylation and idiopathic male infertility is strongly associated with imprinting defects at Igf2 / H19 ICR1 and Mest, with aberrant Mest methylation being a strong indicator for sperm quality (Poplinski et al., 2010). Stouder and Paoloni-Giacobino's studies using the xenoestrogen vinclozolin (Stouder and Paoloni-Giacobino, 2010) and methoxychlor (Stouder and Paoloni-Giacobino, 2011) have also shown changes in sperm imprinted genes that can be maintained up to the F2 generation. Indeed, methylation defects in the maternally

methyated H19 gene were observed in the sperm of oligospermic or azoospermic patients respectively (Marques et al., 2010). And these effects on the methylation pattern of the five imprinted genes tested were paralleled by a decrease in the sperm concentration. These findings indicate that the sperm DMRs provide potential epigenetic biomarkers for transgenerational disease and/or ancestral environmental exposures (Manikkam et al., 2013) and have implications for the human population that is exposed to these compounds and is experiencing significant decline in fertility and incidence of adult onset disease. Hypomethylation at the paternally imprinted loci and hypermethylation at the maternally imprinted loci indicates that Aroclor 1254 exposure dramatically interfered with the DNA methylation at the imprinted loci during spermatogenesis and may decrease the fertilizing ability or ability to produce viable embryos.

The maintenance and establishment of genomic methylation pattern in animals are catalyzed by DNA methyl transferases (Bestor, 2000). Dnmt1 is primarily responsible for maintenance methylation, and Dnmt3 family methyl transferases (Dnmt 3a and 3b) are considered responsible for de novo methylation (Bird, 2002). Dnmt 3L, a regulator of methylation contributes to acquisition of DNA methylation at paternally imprinted loci during spermatogenesis (Kylie et al., 2005). Altered methylation at the imprinted loci in spermatozoa as observed with Aroclor 1254 treatment suggests an effect of Aroclor 1254 on DNA methylation machinery during spermatogenesis. These effects may originate in methylation errors induced in the sperm by the hormonal stimulation of Aroclor 1254. The effects of Aroclor 1254 on differential DNA methylation patterns between paternally and maternally imprinted genes may be mediated by changes of expression and activity of DNA methyl transferases. However, in the present study, we cannot clarify which methyltransferase gene was responsible for dramatically altered methylation of the imprinted loci by Aroclor 1254 exposure. The mechanism of control for methylation needs further research.

As an estrogenic environmental toxin, Aroclor 1254 may exert its biological activities via estrogen receptors. There are two classical nuclear estrogen receptors, ER α and ER β . Since Aroclor 1254 is an estrogen-like compound, the relationship between ER and Aroclor 1254 was analyzed in our study. It revealed that Aroclor 1254 significantly increased ER α and ER β expression ($P < 0.01$). ER over-expression and DNA methylation modification of imprinted genes regulate the relative gene expression (Zhang et al., 2012). Reduced DNA methylation at imprinted loci in rat spermatozoa upon tamoxifen treatment indicated a role of estrogen-associated signaling in the acquisition of paternal-specific imprints during spermatogenesis (Pathak et al., 2009). The Aroclor 1254 exposure led to a significant reduction in the methylation patterns at the differentially methylated region of paternally imprinted genes H19 and Gtl2 and a significant increase in the methylation patterns of maternally inherited genes Mest, Snrpn, and Igf2r. This may be due to influences of Aroclor 1254 through the classical nuclear estrogen receptors, ER α and ER β . However, it is unknown whether the expression of ER is regulated by the DNA methylation. But preliminary study suggested that ER was the mediator in the process of an environmental xenoestrogen induced

hypomethylation of imprinted genes (Chao et al., 2012). Therefore, we proposed that Aroclor 1254 influences DNA methylation of the imprinting genes via regulating ER expression.

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 PCBs Aroclor 1254.

In summary, this is the first study, to our knowledge, to provide evidence that Aroclor 1254 administration can alter the genomic DNA methylation status of imprinted genes in the sperm of adult male mouse. Our results suggest that Aroclor 1254 alters epigenetic information by inducing the paternally imprinted locus-specific DNA hypermethylation and the maternally imprinted locus-specific DNA hypomethylation in the spermatozoa. These could strengthen the premise that abnormal epigenetic reprogramming of the male germ line as a possible mechanism of compromised male fertility. In addition, because Aroclor 1254 is a SERM, changes in DNA methylation status suggest involvement of estrogen associated signaling in establishment/maintenance of the imprint. This also raises a possibility of a link between environmental estrogen and increased infertility, which could be mediated by errors in imprinting in the male germ line.

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