Ancestral TCDD exposure promotes epigenetic transgenerational inheritance of imprinted gene *Igf2*: Methylation status and DNMTs

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ABSTRACT

Ancestral TCDD exposure could induce epigenetic transgenerational phenotypes, which may be mediated in part by imprinted gene inheritance. The aim of our study was to evaluate the transgenerational effects of ancestral TCDD exposure on the imprinted gene insulin-like growth factor-2 (*Igf2*) in rat somatic tissue. TCDD was administered daily by oral gavage to groups of F0 pregnant SD rats at dose levels of 0 (control), 200 or 800 ng/kg bw during gestation day 8–14. Animal transgenerational model of ancestral exposure to TCDD was carefully built, avoiding sibling inbreeding. Hepatic *Igf2* expression of the TCDD male progeny was decreased concomitantly with hepatic damage and increased activities of serum hepatic enzymes both in the F1 and F3 generation. Imprinted Control Region (ICR) of *Igf2* manifested a hypermethylated pattern, whereas methylation status in the Differentially Methylated Region 2 (DMR2) showed a hypomethylated manner in the F1 generation. These epigenetic alterations in these two regions maintained similar trends in the F3 generation. Meanwhile, the expressions of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) changed in a non-monotonic manner both in the F1 and F3 generation. This study provides evidence that ancestral TCDD exposure may promote epigenetic transgenerational alterations of imprinted gene *Igf2* in adult somatic tissue.

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1. Introduction

Dioxins are a family of persistent organic pollutants (POPs) listed under the Stockholm Convention, which are also typical endocrine disrupting chemicals (EDCs). They are ubiquitously existed in the environment and released by production of chloro-organic chemicals and various industrial processes, including combustion and incineration. Dioxins can accumulate in the food chain due to their high lipophilicity and stability, which have raised public concerns. According to the risk

assessment of dioxins, the primary source of human exposure to dioxins at present is food (Parzefall, 2002).

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent dioxin. Although the cancer hazard posed by TCDD has received much attention over the past 20 years, its non-cancer hazard may be more important to the general public than the cancer hazard at current background doses (Greene et al., 2003). Exposure to high levels of dioxin has also been associated with adverse pregnancy outcomes (Tsukimori et al., 2008), including higher risk of birth defects and lower birth weight. Studies have shown that dioxins can not only affect hormone homeostasis (Gupta et al., 2006) and reproductive parameters (Mocarelli et al., 2008) in directly exposed individuals, but also have implications in hormone dysfunction (Baccarelli et al., 2008) and behavioral sexual dimorphism (Winneke et al., 2013) in the offspring.

Human and rodent research of dioxins has proven that exposures that exhibit any or no severe impact during development can cause serious influences at a later stage of life in the offspring (Ahmed, 2011; Foster et al., 2010; Schantz et al., 1996; Winneke et al., 2013). These findings are quite consistent with the Barker hypothesis (Barker, 1990). The essential aspect of the Barker hypothesis is that an abnormal physiology at a later stage of development may be due to an adverse intrauterine environment, in which the delicate embryo is subjected to an environmental stimulus or insult during a critical, sensitive period (Gillman, 2005). The mechanism which links the adult onset disease

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; ALT, alanine aminotransferase; ANOVA, analysis of variance; Arnt, aryl hydrocarbon receptor nuclear translocator; AST, aspartate aminotransferase; BSP, bisulfite genomic sequencing; CTCF, CCCTC-binding factor; DMR2, differential methylation region 2; DMSO, dimethylsulfoxide; DNMT, DNA methyltransferase; EDCs, endocrine disrupting chemicals; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GD, gestation day; ICR, imprinted control region; *Igf2*, insulin-like growth factor-2; PCR, polymerase chain reaction; ADIs, actual daily intakes; TEQ, toxic equivalents.

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and the intrauterine exposure remains unknown. Compared to the genetic mechanism (relatively low frequency and low reproducibility), the epigenetic changes (no DNA mutation, control genes to be expressed or repressed through non-genomic modifications) provide a more promising way to explain the adult onset diseases. The reversible and inherited characteristics of epigenetics allow the embryo to make adaptive responses to environmental perturbations and ultimately survive during fetal growth. The adaptive aberrant epigenetic modifications can create a memory of the received signal, allowing it to persist into later life and promote disease susceptibility in adulthood. Thus, epigenetics appears to provide plausible feasibility for the mediation of these long-term effects.

When a gestating female (F0 generation) was exposed to the environmental compound, the F1 generation embryo and F2 generation germline were directly exposed, and the F3 generation is the first generation not directly exposed. The F3 phenotype can be unequivocally defined as a transgenerational phenomenon for an embryonic exposure (Skinner, 2008). There are various studies revealed that the epigenetic changes in the germline induced by EDCs could result in transgenerational inheritance of adverse effects, especially the male reproductive system (Walker and Gore, 2011). These detrimental effects on subsequent generations can be possibly “programmed” through molecular epigenetic changes induced by ancestral exposure to EDCs, the expression of which may not manifest until adulthood (Anway et al., 2005; Stouder and Paoloni-Giacobino, 2010). Although negative results of the transgenerational adverse effect have also been reported (Inawaka et al., 2009; Schneider et al., 2008), the controversy is possibly due to detailed experimental differences (Gore, 2010).

It has been proposed that imprinted loci may be particularly vulnerable to environmental disruptions due to its monoallelic expression and epigenetic-based regulation (Young, 2001). Imprinting is generally considered “primary” imprints that are established in the germline (Kono, 1998). In the early embryo, imprinted genes are the only loci that avoiding demethylation, which could be disrupted by environmental influences. The disruptions can be maintained, and somatically inherited through many cell cycles into adult tissues. Besides, many imprinted genes are crucial for fetal growth. Thus, such environmental disruption can affect not only specific organs during fetal development but the adult phenotypes in a direct or indirect manner (Young, 2001). During sex determination, the germ cells undergo intense epigenetic programming in a sex specific manner. If the germ cells are exposed to environmental toxicants during this critical period, the imprinted genes would be affected leading to alterations in the epigenome of the germline and induction of an epigenetic transgenerational inheritance (Jirtle and Skinner, 2007). Imprinted gene insulin-like growth factor-2 (Igf2) is an important growth factor, the expression of which is epigenetically controlled by several Differentially Methylated Regions (DMRs), such as the imprinted control region (ICR) and the DMR2 in the Igf2/H19 locus (Murrell et al., 2001; Lopes et al., 2003; Han et al., 2008). The methylation patterns of DMRs are assumed to be set up during early development and faithfully maintained in somatic cells during individual development (Jirtle and Skinner, 2007), which is quite essential for normal embryo development and determines developmental potential. Thus, Igf2 is a plausible candidate for studying on the epigenetic long-term effects of intrauterine chemical exposure (Young, 2001; Somm et al., 2013; Susiarjo et al., 2013).

In summary, the disruption of establishment or maintenance of a mature imprinted gene during critical phases of development could induce abnormal epigenetic marks, which could result in somatic inheritance of the defect, causing alterations of methylation status in adult somatic tissue, along with silence or over-expression of the imprinted gene in later development. So far, the effect on imprinted gene upon ancestral TCDD exposure has been mainly focusing on the fetus (Wu et al., 2004), the reproductive organs or the germ cells (Manikkam et al., 2012). Although, one study has explored the effects of maternal exposure to TCDD on imprinted genes *Snrpn*, *Peg3* and *Igf2r* in the liver of the F1 male mouse (Somm et al., 2013), whether epigenetic

transgenerational effects existing in the somatic tissue are not yet known. Thus, the aim of our study was to evaluate the transgenerational effects of ancestral TCDD exposure on the imprinted gene *Igf2* in male rat liver, and further to explore the role of DNMTs in the methylation alterations of DMRs (ICR and DMR2) in the *Igf2/H19* locus.

2. Materials and methods

2.1. Animal transgenerational model of ancestral exposure to TCDD

The study was approved by the Ethics Committee of Tianjin Medical University. The animals were treated humanely and all efforts were made to minimize suffering during the process of experiments. Sprague–Dawley rats were purchased from the Laboratory Animal Center of the Military Academy of Medical Sciences and reared in the laboratory of the SPF level. Sexually mature rats were mated overnight at a ratio of 1:1. The sperm-positive vaginal plug was designated as gestation day 0 (GD 0). At the beginning of the animal transgenerational model, sibling sisters were randomly allocated to the control and TCDD-treated groups on GD 0 to ensure that the genetic background was similar. Four different lineages (individual F0 gestating females) were generated for both control and TCDD-treated groups.

TCDD was purchased from Cambridge Isotope Laboratory (Tewksbury, MA, USA), and its purity was 99.2%. Dimethylsulfoxide (DMSO) and corn oil, which were used for dissolving TCDD, were both obtained from Sigma (St. Louis, MO, USA). TCDD was dissolved in DMSO to make a stock solution and then further diluted with corn oil to make the TCDD treatment solutions. During GD 8–14, time-pregnant rats were given a consecutive daily dose of TCDD (200 or 800 ng/kg body weight/day, 5 mL/kg dosing preparation) or an equivalent volume of vehicle (i.e., DMSO and corn oil) in gavage.

Two of the F1 generation male offspring of each litter, designated as “TCDD-exposed” in reference to their prenatal exposure, were mated to the F1 generation control females. The F1 generation control male offspring were mated to the F1 generation control females after vehicle treatment of the F0 gestation mothers. Subsequent breeding continued for three generations with sufficient numbers of animals to avoid sibling inbreeding. The litter was randomly adjusted to eight after birth to assure uniformity and weaned on day 21. Both TCDD and the control dams or offspring were reared under the same conditions, and given a regular laboratory rodent diet and distilled water *ad libitum*.

2.2. TCDD hepatotoxicity

Male offspring from the control and TCDD groups of the F1 and F3 generation were sacrificed at 90 days of age. The rats were fasted overnight, weighed, and anesthetized with chloral hydrate (0.3 mL/100 g bw). Blood samples were obtained from the male offspring, and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined in our laboratory using a Roche Cobas Bio Analyzer (Cobas c701). The liver was excised, weighed, and dissected. A section from the liver was additionally preserved in 10% neutral buffered formalin, embedded in paraffin wax, and then cut to the thickness of 4 μ m. The liver sections were stained with hematoxylin and eosin (HE) and examined to assess the overall liver damage. The rest sections of the liver were snap-frozen in liquid nitrogen immediately after dissection and kept at -80°C until following analysis.

2.3. Bisulfite genomic sequencing (BSP) for imprinted control region (ICR) and differentially methylated region 2 (DMR2) in the *Igf2/H19* locus

Bisulfite sequencing is a powerful technique to determine DNA methylation pattern with a single CpG resolution (Grunau et al., 2001). DNA was extracted from the liver tissue using the Zymo Genomic DNA™-Tissue Miniprep kit (Zymo Research) according to the

manufacturer's protocol. The quantity and quality of the extracted DNA were assessed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Genomic DNA (20 µl) was modified by treatment with sodium bisulfite using the Zymo EZ DNA methylation kit (Zymo Research). The bisulfite treatment of denatured DNA converts all unmethylated cytosines to uracils, whereas methylated cytosine residues remain unchanged. This results in specific sequence changes, allowing the quantitative measurement of the cytosine methylation status. The MethPrimer software was used for the identification of CpG islands in the target region and designed BSP primers for the ICR and DMR2 in the Igf2/H19 locus (Li and Dahiya, 2002). CpG island was predicted using the following criteria: island size >100, GC percentage >50.0, and Obs/Exp >0.6. DNA amplification was performed through two rounds of polymerase chain reaction (PCR). The PCR products were then recovered and gel-purified. The purified fragments were ligated into the pBLUE-T vector (Beijing Zoman Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions. The plasmids were transformed into *Escherichia coli*. After incubation, 10–30 positive clones were picked from each sample and sequenced on Applied Biosystems sequencer (3730XL). Ten effective DNA methylation sequencing data per sample were randomly selected and analyzed by BiQ analyzer (Bock et al., 2005). The primer sequences specific for the bisulfite-converted DNA of the ICR and DMR2 in the Igf2/H19 locus are shown in Table 1. In the F1 generation, one male rat from each lineage (each was randomly selected from each litter) in the three groups was analyzed ($n = 4$ per group); while in the F3 generation, two male rats from each lineage (each was randomly selected from different litters of the same lineage) in the three groups were analyzed ($n = 8$ per group), which were treated as parallel samples to provide possible variations in the F3 generation.

2.4. Real-time PCR analysis of Igf2 and DNA methyltransferases

The total RNA was extracted from liver tissues using the TRIzol® Reagent (Invitrogen). cDNA was synthesized by reverse transcription of the total RNA using an oligo deoxythymidine primer and RevertAid Reverse Transcriptase (Fermentas). Real-time PCR was performed in a 20 µl reaction system containing 10 µl of 2 × All-in-One™ qPCR Mix (containing ROX, GeneCopia™), 0.4 µl of the PCR forward primer (10 pmol/µl), 0.4 µl of the PCR reverse primer (10 pmol/µl), and 5 µl of the cDNA sample. The amplification was performed on an ABI StepOne plus instrument. The PCR program consisted of an initial denaturing step at 95 °C for 10 min and 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The specificity of each PCR product was verified by melting curve analysis. The expression levels were calculated using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. The fold-change was calculated using the comparative cycle threshold (Ct) method. The same liver sample used for BSP analysis was used to measure gene expressions of Igf2 and DNMTs in the F1 and F3 generation. Each gene of interest was analyzed in triplicate for each sample. The primer sequences used for the real-time PCR of Igf2 and DNMTs are shown in Table 2.

Table 1
Primer sequences used for bisulfite sequencing PCR of ICR and DMR2.

Region	Primer sequence	Product size (bp)	CpG number
ICR	F: 5'-GTTGAATTGTTGTGTGGTTTATA-3' R: 5'-CAATCTCAATTACAATCTATTTCAACAA-3'	341	23
DMR2	F: 5'-TATTGATATTGGATGGGAGTTAG-3' R: 5'-ATACTACATTACAAAATTACCACA-3'	350	20

Note: F: forward; R: reverse.

Table 2
Primer sequences used for the real-time PCR of Igf2 and DNMTs.

Gene	Accession number	Primer sequence	Product size (bp)
Igf2	NM_001190163.1	F: 5'-CGCTTCAGTCTTGTCTGTTCCG-3' R: 5'-AACTTGCCACGGGGTATCT-3'	220
DNMT1	NM_053354.3	F: 5'-ACGCCCTGAACCTATCG-3' R: 5'-GCTTCTCATCGCTCCAGTA-3'	190
DNMT3A	NM_001003958.1	F: 5'-GGAAAGATCATGTACGTCCGG-3' R: 5'-GGAGGCGGTAGAACTCAAAGA-3'	178
DNMT3B	NM_001003959.1	F: 5'-CCGAAACAGCAACAGTATCTCC-3' R: 5'-CCTGGCTCAAGTCAACCGA-3'	239
GAPDH	NM_017008.3	F: 5'-CGCTAACATCAATGGGGTG-3' R: 5'-TTGCTGACAATCTTGAGGAG-3'	201

Note: F: forward; R: reverse.

2.5. Western blot analysis of Igf2 protein

The protein extracts from 50 mg liver tissue were diluted with 5 × loading buffer and then denatured at 100 °C for 3 min. Approximately 30 µg of total protein from each group was loaded into the lanes of a 10% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were first blocked with 5% (w/v) skim milk in phosphate buffer solution containing 0.05% Tween-20 (PBST) for 1 h at 37 °C and then incubated with specific primary antibodies (Rabbit Igf2 antibody, Santa Cruz, 1:500; Mouse β-actin antibody, Abgent, 1:10,000) overnight at 4 °C. After three 10-min washes in PBST, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody (Abgent, San Diego, CA, USA, 1:10,000). The membranes were then developed with enhanced chemiluminescence (ECL, Pierce Biotechnology Inc., Rockford, IL, USA) reagents, and the chemiluminescence signal was imaged using a laser densitometer (GeneSnap version 7.05.02; SynGene, Cambridge, UK), with β-actin considered to be the internal control. The immunoblots were quantified using Quantity One 4.6.2 software (Bio-Rad).

2.6. Statistical Analysis

The SPSS software (version 11.5, Chicago, IL, USA) was used for the statistical analysis. When indicated, the values were expressed as the means ± SD or percentages (%). The quantitative data was analyzed by one way ANOVA followed by Dunnett's test or Student-Newman-Keuls test, while the qualitative data was analyzed by chi-square test or Fisher's Exact test. A statistically significant difference was defined at $p < 0.05$.

3. Results

3.1. Effects of ancestral exposure to TCDD on liver weight and liver/body weight ratio of male rat offspring in the F1 and F3 generation

Ancestral exposure to TCDD had no significant effect on litter size after parturition ($P > 0.05$). The litter size of the control group and the 200, 800 ng/kg bw TCDD group in the F1 and F3 generation were 10.5 ± 1.8 , 11.5 ± 2.6 , 10.0 ± 1.8 and 10.1 ± 2.2 , 10.4 ± 2.1 , 11.3 ± 1.9 , respectively. The livers were weighed after excision, and the liver/body weight ratio was expressed as the percentage of the body weight of each animal. For male progenies only, as shown in Fig. 1A, the liver weight of the 800 ng/kg bw TCDD in the F1 generation was significantly lower than the control ($P < 0.05$); while in the F3 generation, the liver weight of the two TCDD group were still lower than that of the control group ($P < 0.05$). The liver/body weight ratio of the 200 ng/kg bw TCDD group was significantly lower than that of the control group in both the F1 and F3 generation ($P < 0.05$) (Fig. 1B).

Fig. 1. Effects of ancestral exposure to TCDD on liver weight and liver/body weight ratio of male rat offspring in the F1 and F3 generation. (A) The livers were weighed after excision. (B) The liver/body weight ratio was expressed as the percentage of the body weight of each animal. * $P < 0.05$ compared with the control group.

3.2. Effects of ancestral exposure to TCDD on liver histopathology and serum ALT/AST activities of male rat offspring in the F1 and F3 generation

After carefully examination of the HE-stained slides of the male liver, we discovered that compared to the control group, there were significant hepatocellular damages in the TCDD groups. In the F1 generation, the liver in the TCDD groups showed discrete necrotic foci with inflammatory cell infiltration, larger liver sinusoidal gap, congestion in the hepatic sinusoid, hydropic change, hepatocyte vacuolation and swelling (Fig. 2A). In the F3 generation, the liver pathology of the 800 ng/kg bw group still showed similar pathological changes, while that of the 200 ng/kg bw group returned to normal (Fig. 2A). Serum ALT and AST activities were measured to assess the liver function. Results showed that TCDD caused hepatocellular damage in the 800 ng/kg bw group in the F1 generation, as indicated by the elevation in both serum ALT and AST levels (Fig. 2B). In the F3 generation, the serum AST of the 800 ng/kg bw group still showed slightly increase compared to the control (Fig. 2B). In contrast, the elevation of serum ALT/AST in the 200 ng/kg bw group was not statistically significant in the F1 and F3 generation.

3.3. Ancestral exposure to TCDD alters gene and protein expressions of Igf2 in the male liver in the F1 and F3 generation

Real-time PCR was used to measure the mRNA expression level of the imprinted gene Igf2 in the liver tissues of male rat offspring. The housekeeping gene GAPDH served as the internal control. Fig. 3 shows that the Igf2 mRNA expression was decreased in both TCDD-treated groups in the F1 and F3 generation. Compared with the control group, the Igf2 mRNA levels of the 200 and 800 ng/kg bw TCDD group in the F1 generation were decreased to 0.81- and 0.39-fold of control, respectively; while in the F3 generation, those were 0.25- and 0.33-fold, respectively.

Western blot was used to measure the protein expression of Igf2. The housekeeping gene β -actin served as the internal control. Fig. 4 shows the distorted Igf2 protein expression in the F1 generation and the decreased trend in the F3 generation. Compared with the control

group, the Igf2 protein levels of the 200 and 800 ng/kg bw TCDD group in the F1 generation were 0.92- and 1.40-fold, respectively; while in the F3 generation, those were declined to 0.87- and 0.60-fold, respectively.

3.4. Ancestral exposure to TCDD changes the methylation status of ICR in the Igf2/H19 locus in the F1 and F3 generation

Igf2 is subjected to the methylation-dependent control of certain differential methylated regions within the Igf2/H19 cluster. The ICR is a methylation control region within the Igf2/H19 locus which located on chromosome 1 of the rat genome and contains 23 CpGs. For the ICR region in the F1 generation, the average methylation level (methylated CpG sites per total CpG sites) of the control group was 53.19%, while that of the 200 ng/kg bw TCDD group was 56.67%. Compared to the control group, the 800 ng/kg bw TCDD group showed marked hypermethylation, with the average methylation level of 78.41%. In the 800 ng/kg bw TCDD group, about 87% clone manifest hypermethylation ($>50\%$), with a range of 60.87–95.65%. In the F3 generation, the average methylation levels of the control, 200, 800 ng/kg bw TCDD group were 32.98, 51.09 and 82.47%, respectively. In the 200 ng/kg bw TCDD group, about 58% clone showed hypermethylation ($>50\%$), with a range of 52.17–95.65%; while that of the 800 ng/kg bw TCDD group was 97% (range 65.22–100.00%).

After individual CpG analysis of ICR (Fig. 5), most CpG sites in the ICR region showed a similar pattern of the overall methylation. Data are represented as the percentage of methylated CpG sites to the total number of CpG sites assessed for that site. Among the detected 23 CpGs, site 2, 3, 4 and 5 belong to the first CCCTC-(CTCF) binding site, while site 16, 17, 18, 19, 20 and 21 belong to the second CTCF binding site (Szabo et al., 2000). It seems that ancestral exposure to 800 ng/kg bw TCDD induced hypermethylation on the second CTCF binding site of the ICR in the Igf2/H19 locus, and this effect could still be manifested in the F3 generation.

3.5. Ancestral exposure to TCDD changes the methylation status of DMR2 in the Igf2/H19 locus in the F1 and F3 generation

DMR2 is another differential methylation region in the Igf2/H19 locus, which located within the gene body of Igf2, and contains 20 CpGs. In the F1 generation, the average methylation level of DMR2 in the male offspring manifested a decreased trend. The average methylation levels of the control, 200 and 800 ng/kg bw TCDD group were 58.00%, 52.00% and 37.75%, respectively. In the 800 ng/kg bw TCDD group, about 55% clone showed hypomethylation, with a range of 0–40%. In the F3 generation, the hypomethylation trend still existed. The average methylation levels of the control, 200 and 800 ng/kg bw TCDD group were 49.17%, 41.02% and 42.08%, respectively. About 54% clone manifested hypomethylation in the 200 ng/kg bw TCDD group, with a range of 0–45%, while in the 800 ng/kg bw TCDD group was 60% (0–45%).

After individual CpG analysis of DMR2 (Fig. 6), most CpG sites in the DMR2 region showed an overall hypomethylation in the F1 generation. Among the detected 20 CpGs, site 5–12 belong to the “core DMR2”. Several sites outside the “core DMR2” also showed certain degree of hypomethylation. This phenomenon seemed not present in the F3 generation, since only four sites showed hypomethylation in the F3 generation.

3.6. Ancestral exposure to TCDD deregulates the expression of DNMT genes in the adult rat liver in a non-monotonic manner in the F1 and F3 generation

DNMT catalyzes the process of DNA methylation, which is a key epigenetic modification of DNA. We further evaluated the corresponding DNMT1, DNMT3A, and DNMT3B gene expression levels to explore their possible role in the induction of methylation changes in the Igf2/H19 locus. As shown in Fig. 7, the expression levels of DNMTs changed

Fig. 2. Effect of ancestral exposure to TCDD on liver pathology and serum ALT and AST activities in male offspring in the F1 and F3 generation. (A) Section of the liver was fixed in 10% neutral buffered formalin, and section was prepared HE staining to assess the overall liver damage. Control liver demonstrates normal histology with uniform hepatocytes. The most conspicuous finding in the TCDD group shows discrete necrotic foci (blue arrow), congestion in the hepatic sinusoid (red arrow), hydropic change, hepatocyte vacuolation and swelling (green arrow), with inflammatory cell infiltration and larger liver sinusoidal gap (not indicated). Original magnification 400 $\times$. (B) Serum ALT and AST activities ($n = 4$ and 8 per group in the F1 and F3 generation). Data are presented as the means $\pm$ SD. * $P < 0.05$ compared with the control group.

in a non-monotonic manner in the F1 generation ($P < 0.05$). The DNMT1 expression in the 200 ng/kg bw TCDD group was 0.66-fold of that found for the control ($P < 0.05$), whereas the DNMT3A expression in the 200 and 800 ng/kg bw TCDD group was 0.62- and 0.85-fold of that found for the control ($P < 0.05$) and the DNMT3B expression level in the 800

ng/kg bw TCDD group was 1.92-fold higher than that obtained for the 200 ng/kg bw TCDD group ($P < 0.05$). In the F3 generation, only DNMT3A and DNMT3B still manifested significant changes. The DNMT3A expression in the 200 and 800 ng/kg bw TCDD group was 0.65- and 0.71-fold of that found for the control ($P < 0.05$), whereas the DNMT3B expression level in the 800 ng/kg bw TCDD group was 1.36- and 1.42-fold higher than that obtained for the control and 200 ng/kg bw TCDD group ($P < 0.05$). Overall, these results suggest that TCDD exposure may affect both maintenance and *de novo* methylation.

4. Discussion

Recent studies have emphasized the role of dioxins played by the proximity to highly contaminated sites. High blood levels of dioxins have been reported in pregnant women, due to potential background dioxins pollution (Chen et al., 2006; Wittsiepe et al., 2007; Grumetto et al., 2015). The estimated Actual Daily Intakes (ADIs) reported for local residents (pregnant women included) near a disassembly site in China was 612 pg TEQ/kg bw/day (Zhao et al., 2006), which greatly exceed the WHO reference doses (1–4 pg TEQ/kg bw/day) (WHO, 1998). Applying the formula adopted at the WHO (1998) and JMHLW/JMOE (Japan Ministry of Health, Labour and Welfare/Japan Ministry of the Environment) (1999), we could estimate the body burden of the local residents to be 1209 ng/kg. In our study, the body burden of the F0

Fig. 3. Effect of ancestral exposure to TCDD on the mRNA expression levels of Igf2 in the livers of male offspring in the F1 and F3 generation. After the liver was excised, the total RNA was extracted and real time PCR analysis was used to determine the mRNA expression of Igf2. Igf2 gene was analyzed in triplicate for each sample ($n = 4$ and 8 per group in the F1 and F3 generation). Data are presented as the means $\pm$ SD. * $P < 0.05$ compared with the control group.

Fig. 4. Effect of ancestral exposure to TCDD on the protein expression levels of Igf2 in the livers of male offspring in the F1 and F3 generation. After the liver was excised, the protein was extracted and Western blot was used to detect the protein expression of Igf2. Data are presented as the means $\pm$ SD ($n = 4$ and 8 per group in the F1 and F3 generation). * $P < 0.05$ compared with the control group.

pregnant rats in the low dose group could be estimated to be 1204 ng/kg, which was comparable to the local residents (200 ng/kg bw/day, 7 days, gastrointestinal absorption of dioxins as 86% from oral gavage with corn

oil). Besides the pregnant women, our group has recently reported high blood levels of PCDD/Fs + PCBs in the healthy non-occupational childbearing-aged women living near a pentachlorophenol plant in Tianjin, China (Chen et al., 2015), which were much higher than the TEQ reference background level for the USA (Lorber et al., 2009) and Taiwan (Hsu et al., 2009). Therefore, the highly contaminated environment may pose great health risk to the pregnant and childbearing-aged women, and may have health implications for the next generation. In our study, animal model was explored to ascertain further the adverse effects of exposure to these dioxins for offspring, especially with such high exposure levels. In addition, considering that rodents require appreciably higher doses to reach the same body burdens at steady state as in humans to suffer any harmful effects (half-life: humans $>>$ rodents) (Hurst et al., 2000), the F0 pregnant rats in our model were exposed to a relative high range of doses. Therefore, it should be noted that there are uncertainties when extrapolating the results of animal studies to the human, the environmental relevance comparison should be made in caution.

So far, the reproductive organs and germ cells are intensively studied in the field of transgenerational effects of environmental toxicants. Our study reported the epigenetic transgenerational effects on imprinted gene Igf2 in adult male liver, which could provide possible epigenetic indicators for studying the mechanism for the adult onset diseases induced by ancestral TCDD exposure. During early development of the embryo, the epigenome is most reactive and plastic while the whole genome undergoes intense reprogramming. GD 8–14 is a critical window for gonadal sex determination and establishment of imprinted gene, which early developmental stage was chosen in this study.

We found severe pathological changes concomitantly with increased activities of hepatic enzymes in F1 male offspring upon high level

Fig. 5. Individual CpG site analysis of ICR in the liver of male offspring in the F1 and F3 generation. 10 positive clones per sample were randomly selected ($n = 4$ and 8 per group in the F1 and F3 generation). Data are represented as the percentage of methylated CpG sites to the total number of CpG sites assessed for that site. * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with the 200 ng/kg bw TCDD group.

Fig. 6. Individual CpG site analysis of DMR2 in the liver of male offspring in the F1 and F3 generation. 10 positive clones per sample were randomly selected ($n = 4$ and 8 per group in the F1 and F3 generation). Data are represented as the percentage of methylated CpG sites to the total number of CpG sites assessed for that site. * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with the 200 ng/kg bw TCDD group.

intrauterine exposure. Some of the liver pathology was still manifested in the F3 generation, indicating slight but persistent hepatic effect of ancestral TCDD exposure. Igf2 is an important growth factor. Disruptions at imprinted gene loci Igf2 have been demonstrated to significantly disrupt allometric growth and development of specific relevant organs such as liver, heart and kidney (Sinclair et al., 2000; Young and Fairburn, 2000). This could directly result in adult onset diseases or manifest in abnormal phenotype due to pleiotropic effects of the gene imprinted in the fetus (Young, 2001). In this study, we found that hepatic Igf2 was down-regulated after ancestral exposure to TCDD, and this effect was still manifested in the F3 generation after two generations' transmission. The decreasing tendency observed in the liver/body weight ratio and the hepatic damage may be partly explained by the decrease of Igf2. Accompanied with the gene expression changes of Igf2, the methylation status of ICR and DMR2 also manifested similar alterations in the F1 and F3 generation. Theoretically, DNA methylation of imprinted gene should be 50% in somatic cells for its monoallelic expression, although the average methylation levels of DMR in healthy somatic tissues could be varied along with different genes or tissue types (Schneider et al., 2010). The majority of clones manifested hyper/hypomethylated trend in the ICR/DMR2 in the F1 and F3 TCDD lineages, indicating that both the maternal and paternal alleles might be affected during embryonic development. Furthermore, there could be a range of "threshold value" for the ICR or DMR2, with higher or lower than that could affect the normal expression of Igf2 (Benchai et al., 2005). Although the changes of DNA methylation were relatively mild, it could have produced significant biological effects (Kobayashi et al., 2007).

Igf2 and H19 are reciprocally expressed imprinted genes which are located in the Igf2/H19 imprinted control cluster and share a downstream enhancer. ICR is located 2–4 kb upstream of the H19 transcription start

site between Igf2 and H19. It functions as a methylation-regulated enhancer blocker to mediate the monoallelic expression of Igf2 and H19 through four CTCF binding sites within the ICR (Han et al., 2008). CTCF can remotely control Igf2 through long-range chromatin interaction (Han et al., 2008) and maintain the hypomethylation of the ICR in somatic tissues (Engel et al., 2006). In short, the DNA methylation of the ICR may up-regulate transcription by facilitating a gene enhancer to interact with the gene promoter (Schoenherr et al., 2003). Decreased mRNA and protein levels of Igf2, in combination with the hypomethylation of ICR, have been observed in the resorbing embryo and spermatozoa of tamoxifen-treated rats (Pathak et al., 2010). In our study, ancestral exposure to TCDD during gonadal sex determination could significantly induce hypermethylation in the second CTCF binding site in the ICR of the F1 and F3 adult livers. Opposite to the expectation, the mRNA expression of Igf2 was decreased with the hypermethylation of ICR. Wu et al. previously reported that preimplantation mouse embryos exposed to TCDD also exhibit an increased methylation level of ICR accompanied with decreased Igf2 and lower fetus body weight (Wu et al., 2004). Despite the TCDD dose used in Wu's study was much higher than the actual TCDD concentration in the environment, similar Igf2 expression and epigenetic results were found in the fetus (Wu et al., 2004) and the adulthood of the offspring (Figs. 3 and 5). With the exception of this result, consistent conclusions have been found in other studies on E14 fetuses of maternal diabetes (Shao et al., 2008) and maternal insulin exposure (Shao et al., 2007). Thus, the trend of Igf2 expression could be opposite to the methylation status of the ICR.

DMR2 is an intragenic methylated region in the body of the Igf2 gene. A methylated core region of DMR2 (referring to sites 5–12 in our study) may act as a positive regulator for the up-regulation of Igf2 and is accompanied with a methylated ICR in mouse (Murrell et al.,

Fig. 7. Effect of ancestral exposure to TCDD on the mRNA expression levels of DNMTs (DNMT1, DNMT3A and DNMT3B) in the livers of male offspring in the F1 and F3 generation. After the liver was excised, the total RNA was extracted and real time PCR analysis was used to determine the mRNA expression of DNMTs. Each gene of interest was analyzed in triplicate for each sample ($n = 4$ and 8 per group in the F1 and F3 generation). Data are presented as the means $\pm$ SD. * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with the 200 ng/kg bw TCDD group.

2001; Lopes et al., 2003). Studies have proven that the progressive methylation of CpGs in the DMR2 is required for high level of Igf2 gene transcription (Dejeux et al., 2009). It is possible that hypermethylated DMR2 can also interact with the Igf2 promoter and the downstream enhancer to further influence the chromatin structure, which in turn would affect Igf2 expression. There was also an inconsistent conclusion that in utero TCDD exposure can cause hypomethylated DMR2 in the fetal liver accompanied with the up-regulation of Igf2 (Wang et al., 2007). We speculate the inconsistency may be due to different TCDD dosage (10 μ g/kg vs. 200/800 ng/kg), expose period (GD 10 vs. GD 8–14), endpoint (GD 20 fetus vs. adult) and developmental stage-specific expression of imprinted genes (embryonic vs. adult).

Our result shows that the decreased Igf2 gene expression in the adult male liver was consistent with the methylation status of DMR2 with a distorted methylation pattern of the ICR. One possible reason is that the imprinted Igf2 and H19 and their control elements (silencers, activators, enhancers, other DMRs) occur in clusters in the mammalian genome, all of which are under the control of epigenetic regulation (Murrell et al., 2001). Other DMRs, such as DMR0 and DMR1, located in the upstream or promoter region of the Igf2 gene (Murrell et al., 2001) cooperate with each other exclusively after fertilization during somatic development in a hierarchical fashion (Lopes et al., 2003). Higher TCDD exposure in utero may affect other control regions, which may partly explain the distorted methylation of the ICR, hence more comprehensive studies are required to illustrate the mechanisms. Another reasonable explanation would be the involvement of other epigenetic modifications. CTCF within the ICR can recruit active histone tail modification marks to the ICR locus, suppressing transcription through

repression by H3K27me3 independently of repression by DNA hypermethylation (Han et al., 2008). Imprinted ncRNA may also interact with cellular RNA targets and contribute to genomic imprinting control (Royo and Cavaille, 2008). A recent study has demonstrated that miR-101a and miR-122 are differentially down-regulated in TCDD-induced liver damage (Yoshioka et al., 2011). Thus, it is possible that ncRNA could target Igf2 or its imprinting control region, and further affect the expression of Igf2. Furthermore, TCDD is also an environmental endocrine disruptor which can potentially affect the functions of transcription factors. Through the transactivation of C/EBP beta, prenatal exposure to TCDD may induce Igf2 gene expression in the fetal liver (Wang et al., 2011); thus, transcription factors may play a role in the long-lasting developmental effects of TCDD.

In our result, there is inconsistency in the Igf2 gene and protein expression trend in the higher group of the F1 generation. We speculate the pathway TCDD exerts its toxicity and other epigenetic effect could be account for the special effect in the higher group. TCDD exerts its toxic effects through AhR, which is translocated to nucleus in complex with the molecular chaperone Hsp90 (Tsuji et al., 2014). Hsp90 could be temporally induced as a protective mechanism against TCDD induced hepatotoxicity, and then be depressed with prolonged exposure (Kim et al., 2012). Study has shown that inactivation of Hsp90 could inhibit transcription of chromatin (Ruden and Lu, 2008). These effects involve in the post-translational modifications of histone (Ruden and Lu, 2008), which has been shown to be related to the imprinted marks. In our study, TCDD in the higher group might further inhibit the transcription of imprinted Igf2 gene through the action of Hsp90. As for the different effects between the lower and higher dose group, we assume it could be the endocrine disrupting effect of TCDD during the critical developmental window in utero, manifesting unique non-monotonic dose-response relationship.

DNA methyltransferases play an important role in the establishment and maintenance of imprinted gene (Kaneda et al., 2004). Studies have shown that the activities of DNMTs were inhibited along with the decreased mRNA expression of DNMTs (Chen et al., 2013; Nakano et al., 2013). The present study showed that DNMTs were changed in a non-monotonic manner in the F1 generation, and significant changes of DNMT3A and DNMT3B still manifested in the F3 generation. DNMT1 is quite important for the maintenance of the methylation pattern of the Igf2 gene because knockout mice exhibit abnormal expression of Igf2 (Li et al., 1993). Study has been reported that after lactational exposure to PCBs, PCDDs and PCDFs, the DNMT1 mRNA in the liver and hypothalamic of weaning rats were 28% and 32%, compared to the control group (Desaulniers et al., 2005), which is quite consistent with our result. To the best of our knowledge, DNMT1 in somatic cells could be regulated by transcription factor Sp1 (Kishikawa et al., 2003), whereas an in vitro transcription assay proved that Sp1 and AhR/Arnt have a synergistic effect (Kobayashi et al., 1996); thus, DNMT1 mRNA may be a target for the adverse effects of exposure to AhR agonists (Wu et al., 2004; Desaulniers et al., 2005). Therefore, early exposure to environmental pollutant could alter the expression of DNMT1, and further affect DNMT1 involved methylation process. DNMT3A and DNMT3B function as *de novo* methyltransferase during development and are responsible for the establishment of methylation imprints. There was a significant correlation between the expression of DNMT3A and DMR2 methylation ($R^2 = 0.66$, $P = 0.015$) in the F1 male offspring. Hence the hypomethylation of DMR2 could be possibly associated with the alteration of DNMT3A. As for the DNMT3B, the significant higher expression trend was consistent with the hypermethylation of ICR. Several potential Sp1 sites are also reported in the promoter region of DNMT3B and it is possible that the transcription factor Sp1 can regulate DNMT3B activity (Yanagisawa et al., 2002) and further affect the methylation process in somatic cells. It is worth mentioning that DNMT3B is highly expressed in early embryo and embryonic stem cell, and maintain lower expression level in most somatic tissues in adults (Ishida et al., 2003). Thus, the elevated DNMT3B

expression observed in higher TCDD group in our study should be paid more attention.

5. Conclusions

In summary, our study provides evidence that ancestral exposure to TCDD may promote epigenetic transgenerational inheritance of imprinted gene *Igf2*, accompanied with abnormal *Igf2* expression and liver damage. DNMTs may get involved in this epigenetic regulation. Additional research is necessary to elucidate the interplay between epigenetics and apical endpoints, while these results in present study may provide new and valuable clues which could be used in future studies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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