Long non-coding RNA H19-mediated mouse cleft palate induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Abstract. Long non-coding RNAs (lncRNAs) are a novel class of transcripts, which are pervasively transcribed in the genome and a have greatly unknown biological function. Previous studies have identified that lncRNAs serve an important role in embryonic development. However, the function and mechanism of lncRNAs in the development of palate remains unclear. The aim of the present study was to investigate the role of lncRNA H19 in cleft palate (CP) development in mice. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a well-known teratogen that can induce CP. After establishing a CP mouse model by oral administration of TCDD *in vivo*, no significant differences were detected in the tail length and body weight of fetuses between the TCDD-treated and control groups during the embryonic days 12 to 17. Furthermore, the expression levels of lncRNA H19 and target gene insulin-like growth factor 2 (IGF2) presented specific embryo age-associated differences during the entire development of CP in mice. An inverse correlation was identified between lncRNA H19 and IGF2 expression levels in the CP model. In conclusion, these findings revealed that lncRNA H19 mediated the CP induced by TCDD in mice.

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Introduction

Long non-coding RNAs (lncRNAs) are transcripts composed of >200 nucleotides (1), which do not contain functional open reading frames, cannot encode proteins (2), and are pervasively transcribed throughout the entire genome (3). Several lncRNAs have been found to serve a critical role in embryonic development (4), while the dysfunction of lncRNAs is associated with widespread conditions, such as birth defects (5). Cleft palate (CP) is the most common congenital malformation in the oral and craniofacial region, and may occur at any stage of the palate development, including the palatal shelf growth, elevation or fusion (6). In addition, the loss of medial edge epithelial cells or failure of mesenchymal consolidation can cause CP. However, the etiology of CP is complex and remains poorly understood.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental contaminant (7) that is a teratogen (8) and has been found to cause CP at high rates in mice and humans (8-12). Low levels of TCDD are able to inhibit medial edge epithelial cell apoptosis and suppress the mesenchymal growth of the palatal shelves, which leads to the failure of palatal fusion (13-15). The identification of lncRNAs has provided novel opportunities for the investigation of the precise etiology and pathogenesis of CP, which remain unclear. However, although a great number of studies have investigated the underlying mechanisms of TCDD-induced CP (8,14,16-18), the involvement of lncRNAs in TCDD-induced CP has been rarely reported (6).

lncRNA H19 is a 2,300 bp non-coding RNA (ncRNA), which is transcribed from the maternal allele, with a high expression observed prenatally and a low expression observed postnatally. lncRNA H19 is evolutionarily conserved at the nucleotide level of humans and rodents, and is not translated into a protein (19). The lncRNA H19 gene is pervasively expressed in various tissues in embryos, including the palate (20). However, whether the expression of lncRNA H19 in TCDD-induced mouse CP varies with different developmental stages has yet to be examined. lncRNA H19 is expressed from the imprinted gene locus that also contains the reciprocally imprinted insulin-like growth factor 2 (IGF2) gene (21). Genetic evidence indicates that the parent of origin-dependent expression patterns of the

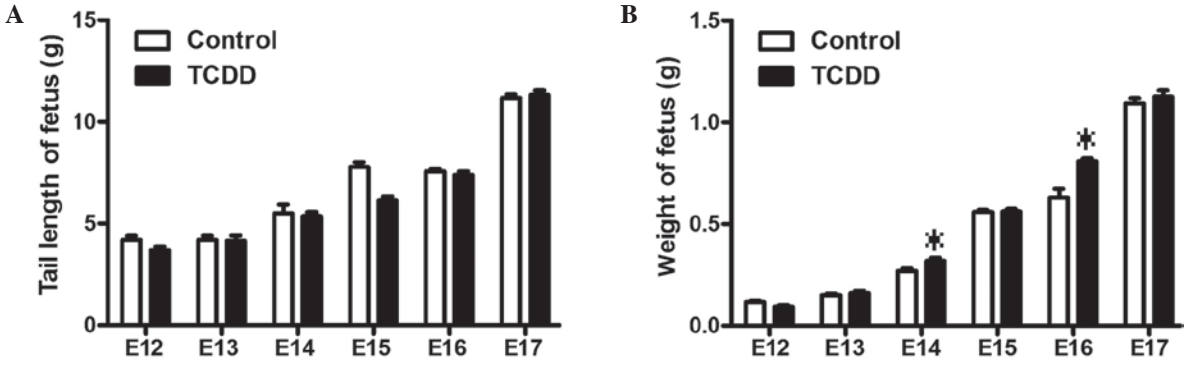

Figure 1. Fetal morphology in the control and TCDD-treated groups, shown by the (A) tail length and (B) weight of the fetus at time points E12 to E17. The TCDD-treated group was treated with a single dose of TCDD (64 μg/kg body weight) and the same amount of corn oil was administered to the control mice. No differences were observed in the tail length and body weight of the fetal mice. Data are presented as the mean ± standard deviation of two replicate experiments. *P<0.05 vs. the corresponding control values. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; E, embryonic day.

lncRNA H19 and IGF2 genes is coordinated in mice (22). The IGF2 gene codes for a growth factor that serves an important role during prenatal development (23).

In the present study, we speculated that lncRNA H19 may have an important role in the process of TCDD-induced mouse CP and thus investigated this effect in a mouse model of CP.

Materials and methods

Animals. Pregnant Kunming mice (n=48; age, 8-12 weeks; weight, ~25 g) were obtained from the Henan Laboratory Animal Center of Zhengzhou University (Zhengzhou, China). All experiments were performed in accordance with the Experimental Animal Center Guide for the Care and Use of Laboratory Animals, and the Institutional Ethical Guidelines for Experiments with Animals. The day of vaginal plug confirmation was determined as the embryonic day 0 (E0). The mice were housed at a constant temperature of 28±2˚C and relative humidity of 60±10% with a 12 h light/dark cycle.

Construction of CP model and sample collection. The pregnant mice were divided into control (untreated) and TCDD-treated groups (n=24 in each group). TCDD (dissolved in 100 μg/ml dimethyl sulfoxide suspension; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in corn oil and was prepared into a 6.0-mg/ml suspension. This suspension was administered orally to each pregnant mouse (64 μg/kg body weight) in the TCDD-treated group on embryonic day 10 (E10). The same volume of corn oil (cat no. 383756; Jinlongyu Oil And Grain Food Convenience Store, Qinghuangdao, China) was administered to the control mice. On time points E12, E13, E13.5, E14, E14.5, E15, E15.5, E16 and E17, pregnant mice from the two experimental groups were sacrificed (4 mice were sacrificed at each time point from E12-E17). The fetuses were first removed from the uterus of the sacrificed mice. Subsequently, the palates of the fetuses were harvested from the two experimental groups under a stereomicroscope (model no. SZ61TRC-SET; Olympus Corporation, Tokyo, Japan) and immediately preserved in RNAlater solution (AM7020 product no. AM7020; Ambion; Thermo Fisher Scientific, Inc., Austin, TX, USA) at 4˚C and stored at -80˚C for further RNA isolation. Certain palate samples (n=5) from fetuses at E13.5 up to E16.5 from the control and TCDD groups were fixed with 10% formalin for histological examination. The lengths of fetuses was measured by vernier caliper (cat no. 500-752-10, Mitutoyo, Tokyo, Japan), and the weights of the fetuses were measured by electronic balance (cat no. AX224ZH, Ohaus Corporation, Parsippany, NJ, USA) on E12, E13, E14, E15, E16 and E17, respectively.

Histological analysis. After palate samples were fixed with 10% formalin for 2 days, then flushed with water overnight. The samples were then dehydrated as follows: Firstly, samples were treated in 70% ethanol for ~5 min and then transferred to 80% ethanol for 5 min. Next, samples were transferred to 95% ethanol for 5 min. Subsequently, samples were transferred to 100% ethanol for 5 min and then transferred again into fresh 100% ethanol for 5 min, followed by two changes of xylene for 5 min per change. Finally, the samples were fixed in paraffin (cat no. 8002-74-2, Kunlun, Daqin, China) at 60˚C for 3 h. Then, samples were cut into 5 μm thick sections,and placed on glass slides (cat no. HDMED7101, Yancheng, Jiangsu, China). Samples were deparaffinized in three changes of xylene for a duration of 5 min per change. The samples were dehydrated as follows: Firstly, slides were transferred through two changes of 100% ethanol for 5 min per change and then transferred to 95% ethanol for 5 min. Subsequently, samples were transferred to 80 and 70% ethanol, respectively, each for 5 min. Slides were rinsed in running distilled tap water at room temperature for at least 5 min. Samples were then stained in hematoxylin (cat no. H9627) solution for 3 min, then stained the samples in working eosin solution (cat no. H004642), both Sigma-Aldrich, for 2 min. Slides were rinsed with water at room temperature for at least 5 min. Finally, a drop of neutral balsam (cat no. 33645; Ningbo, Shanghai, China) was administered to the tissue on each slide, and a coverslip was placed on top. Slides were viewed on an electron microscope (model no. CX41; Olympus Corporation; magnification, x40).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Palates (n=10) were harvested from the TCDD-treated and control mice from E13.5-E15.5 due to the formation of bud palatal shelves, growth and perfusion at the aforementioned time points, which are important to

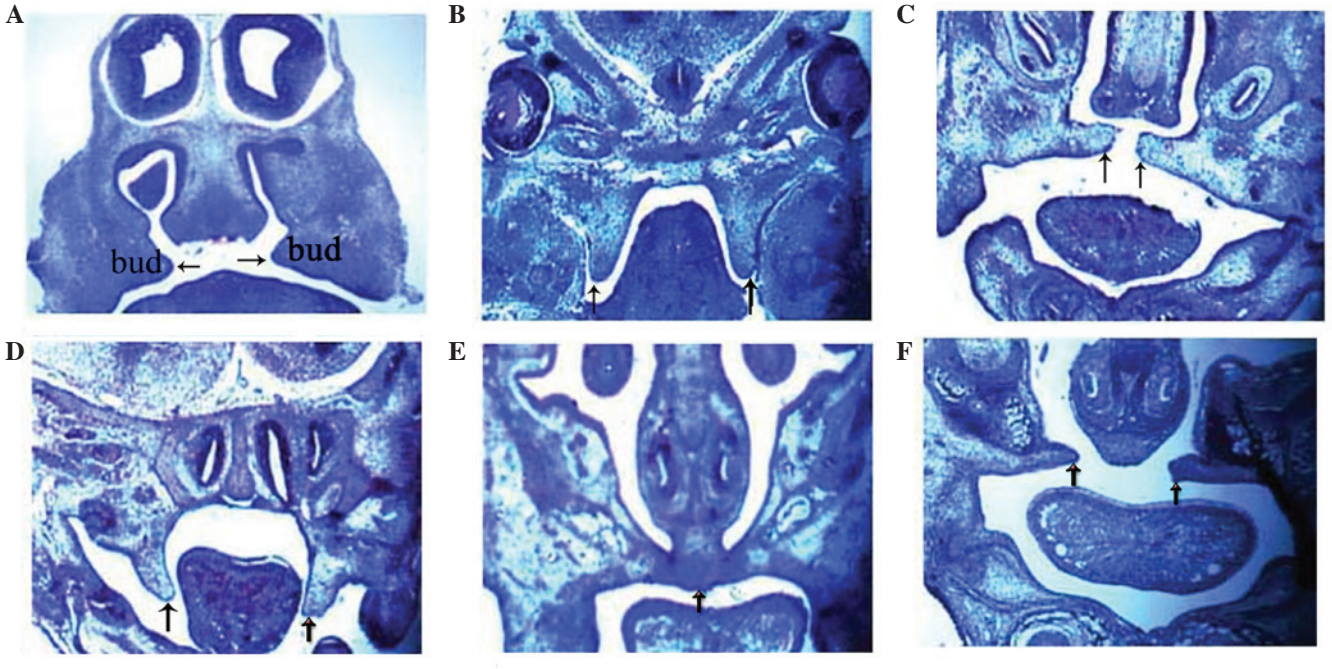

Figure 2. Histological analysis of palatal shelves in the control and TCDD-treated group between the time points E13.5 and E15.5. On E13.5, (A) the palate shelves of the fetuses elevated in the control group, whereas (B) failing to elevate in the TCDD-treated group. On E14.5, (C) the palate shelves of the fetuses were elevated and reached a horizontal position above the dorsum of the tongue in the control group, whereas (D) delayed elevation was observed in the TCDD-treated group. On E15.5, (E) the palate shelves of the fetuses were fused together in the control group, whereas (F) failure to fuse was observed in the TCDD-treated group, resulting in cleft palate. In the two groups, hematoxylin and eosin staining was used (magnification, x40). E, embryonic day; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

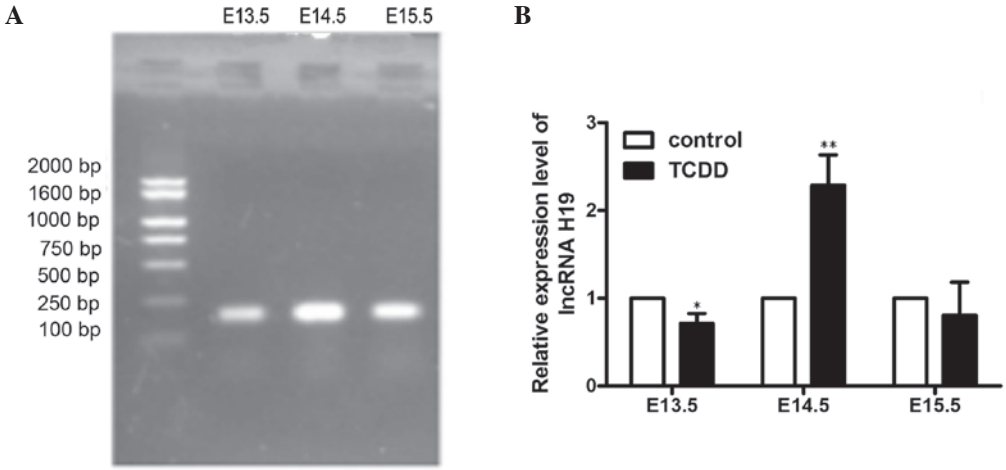

Figure 3. Expression levels of long non-coding RNA H19 in the control (untreated) and TCDD-treated groups between E13.5 and E15.5, as determined by reverse transcription-quantitative polymerase chain reaction. (A) The final polymerase chain reaction product was confirmed as lncRNA H19 and (B) the expression levels of lncRNA H19 in the control and TCDD-treated groups Data are presented as the mean ± standard deviation of three replicate experiments. *P<0.05 and **P<0.01 vs. the corresponding control values. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; E, embryonic day.

the development of palates. Palates were lysed in TRIzol lysis solution (cat no. 15596-018; Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). Total RNA of the palates was isolated according to the manufacturer's instructions, and then dissolved in nuclease-free water. In order to detect the expression of lncRNA H19, first-strand cDNA was synthesized using a PrimeScript II 1st strand cDNA Synthesis kit (cat no. 6210A; Takara Bio Inc., Otsu, Japan) and then amplified by qPCR with the SYBR Premix Ex Taq kit (cat no. DRR420A, Takara) using an ABI 7900 Prism Real-Time PCR system (model no. 7900HT; Applied Biosystems; Thermo Fisher Scientific, Inc.). 18s rRNA was used as an internal control. The conditions of qPCR for lncRNA H19 and IGF2 amplification were as follows: Polymerase activation for 15 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec, 56˚C for 20 sec and 72˚C for 30 sec. The threshold cycle (Cq) value of the PCR amplification curve of the target gene was analyzed using the $2^{-\Delta\Delta Cq}$ method (24). All primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and were as follows: Forward, 5'-CGGACATCTAAGGGCATCA-3' and reverse, 5'-AAG

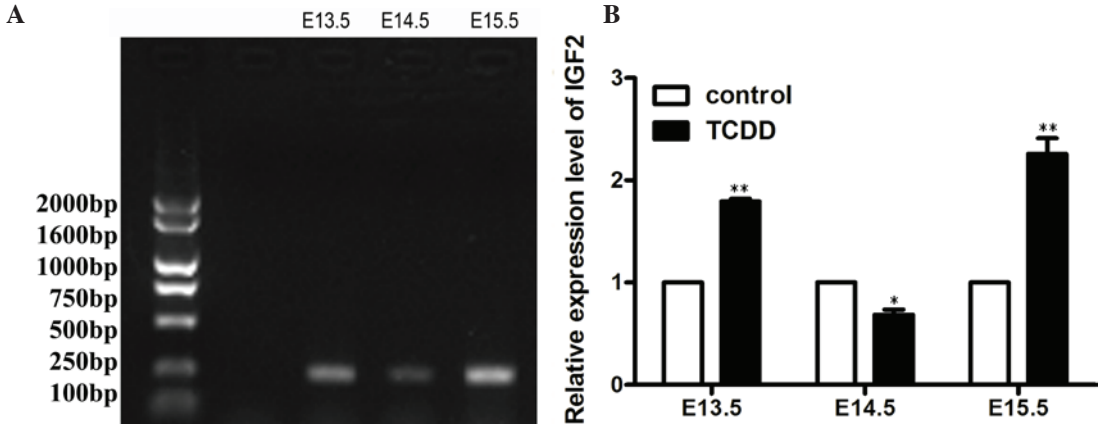

Figure 4. Relative expression of IGF2 in different development stages of palatogenesis, as determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (A) IGF2 was amplified by RT-qPCR, and the final PCR product was confirmed to be IGF2. (B) The expression levels of IGF2 were compared in the control and TCDD-treated groups between E13.5 and E15.5. Data are presented as the mean ± standard deviation of three replicate experiments. *P<0.05 and **P<0.01 vs. the corresponding control values. IGF2, insulin-like growth factor 2; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; E, embryonic day.

ACGGACCAGAGCGAAA-3' for 18s rRNA; forward, 5'-TAC CCCGGGATGACTTCATC-3' and reverse, 5'-TATCTCCGG GACTCCAAACC-3' for lncRNA H19; and forward, 5'-TAT CTCCGGGACTCCAAACC-3' and reverse, 5'-CAAATG TGGGGACACAGAGG-3' for IGF2. The final PCR product were confirmed to be lncRNA H19 and IGF2 by agarose gel electrophoresis.

Statistical analysis. All data were compared using double-sided Student's t test or one-way analysis of variance. Tukey's post hoc test was used to determine the differences between the two groups. The choice of tests was performed automatically using the SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation of two or three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of TCDD on fetal mice. The appearance, feeding, drinking and activity of 12 pregnant mice in the control and TCDD-treated groups did not present any evident differences. At time-points E12, E13, E14, E15, E16 and E17, the fetuses were removed from the uterus of control and TCDD-treated mice. There were no differences in tail length and birth rates between control and TCDD-treated mice. As shown in Fig. 1, there were also no statistically significant differences in the tail length and body weight of the fetal mice at the various time points.

Morphological and histological observations of palatal development. For the fetuses from TCDD-treated mice, the detected rate of CP was 80% (272/340). Their morphological and histological alterations were examined and the results are shown in Fig. 2. Formation of the bud of palatal shelves began on E13.5 in the fetuses from the control group (Fig. 2A). By contrast, the formation of palatal shelves in the fetuses from TCDD-treated mice was interrupted on E13.5 (Fig. 2B). The construction of palate shelves in normal fetuses began on E14.5 (Fig. 2C), whereas the palate shelves remained separated on E14.5 and E15.5 in the fetuses from TCDD-treated mice (Fig. 2D). On E15.5, the palatal shelves of the fetuses from the untreated mice were completely fused, which was not observed in the fetuses from TCDD-treated mice, indicating CP formation (Fig. 2E and F).

Expression levels of lncRNA H19 at different developmental stages of CP. In order to investigate the expression patterns of lncRNA H19 parallel to the CP formation induced by TCDD treatment, RT-qPCR was performed on palate tissues from E13.5, E14.5 and E15.5. The primers for lncRNA H19 are on an exon and cDNA sequencing of the final PCR products confirmed lncRNA H19 (Fig. 3A) The results showed that the expression levels of lncRNA H19 varied with the development of palate (Fig. 3B). The relative expression of lncRNA H19 in the TCDD-treated group was found to be reduced on E13.5 (0.29±0.16-fold vs. control; P<0.05) and on E15.5 (0.19±0.53-fold vs. control). However, the relative expression levels of IGF2 were significantly increased on E14.5 (2.29±0.49; P<0.01) compared with the corresponding control palate tissues.

Expression of insulin-like growth factor 2 (IGF2) in the development of CP. In order to investigate the biological function of lncRNA H19, one of the lncRNA H19 target genes, IGF2, was also investigated. IGF2 is a mitogen for a variety of cell types and is required for normal embryonic growth. IGF2 was amplified by RT-qPCR, and the final PCR product was confirmed to be IGF2. The results indicated that the expression of IGF2 was embryo age-specific during the development of the palate (Fig. 4A and B). More specifically, the expression levels of IGF2 gene in the TCDD-treated group were significantly increased on E13.5 (1.79±0.04-fold vs. control; P<0.01) and E15.5 (2.26±0.22-fold vs. control; P<0.01). However, the expression levels of IGF2 were significantly reduced on E14.5 (0.69±0.07-fold; P<0.05) compared with the corresponding control palate tissues, respectively.

Discussion

ncRNAs account for ~98% of the entire genome (25), and lncRNAs are evidently the most numerous and functionally

diverse amongst the multiple ncRNA classes (26). Previous studies have shown that lncRNAs serve a vital role in the development in embryonic development (27-29), such as heart and body-wall development (30) and other features of organogenetic development (31). Cleft palate (CP) is one of the most common birth defects, with a complex genetic and environmental etiology (32). However, few studies have investigated the pattern of lncRNA expression during the development of the palate. The lncRNA H19 gene was initially identified as a CP gene in transforming growth factor-β3-knockout mice by RNA sequencing analysis (6). However, whether lncRNA H19 is involved in H19 gene expression in TCDD-induced CP has yet to be investigated.

In the present study, a mouse CP model was initially established, which was induced by oral administration of TCDD, and subsequently the role of lncRNA H19 was determined. The results demonstrated that TCDD resulted in CP development and the rate was 80%, which is in accordance with previous findings stating that TCDD induced CP at a high rate in mice (33). In addition, the present study found that there were no differences in the tail length and body weight of the fetal mice between the control and TCDD-treated groups, which coincided with an absence in differences in litter size and body weight between the TCDD fetal mice and control mice (34). Furthermore, the current study showed that the expression levels of lncRNA H19 varied with the stages of TCDD-induced palatogenesis between the time points E13.5 and E15.5. It has been established that the period from E13.5 to E15.5 is important for the development of palate. For example, both sides of the palates lift above the tongue on E13.5 and grow rapidly on E14.5, and begin to touch each other on E15.5. Notably, the expression levels of lncRNA H19 in TCDD-treated mice were lower on E13.5 and E15.5 compared with those of the control, while a high expression was observed on E14.5. These results are similar to those of a previous study, which stated that the expression level of lncRNA H19 increased between E14 and E15 (6). Therefore, lncRNA H19 is suggested to be the primary contributor to the development of CP induced by TCDD, including in the stages of palatal convergence, adhesion and fusion.

As mentioned earlier, lncRNA H19 may play an important role in the pathogenesis of CP induced by TCDD; however, the mechanisms underlying lncRNA H19-regulated CP in TCDD-treated mice remain elusive. In the present study, the expression levels of IGF2 gene were found to be high on E13.5 and E15.5, whereas a low expression was detected on E14.5. Thus, the IGF2 gene showed the opposite expression pattern to that of lncRNA H19, which suggested lncRNA H19 may function through interaction with IGF2. However, the precise mechanisms through which lncRNA H19 regulates TCDD-induced CP require further investigation.

In conclusion, the present study revealed that lncRNA H19 mediates TCDD-induced CP, which provides a new insight into the role of lncRNA in CP.

Acknowledgements

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