Original Research

Correlation between TGF-β2/3 promoter DNA methylation and Smad signaling during palatal fusion induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin

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Impact statement

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a persistent organic pollutant, and mammals exposed to TCDD can cause a variety of developmental toxicity including cleft palate. Previous studies have found that TGF-β2 and TGF-β3 also play a vital role in the development of the palatal. In this study, TCDD exposure model was constructed to investigate the effects of TCDD on the epigenetics of TGF-β2 and TGF-β3. We found that TCDD exposure can methylate part of the CpG sites of TGF-β2 and TGF-β3 promoters, simultaneously reduce TGF-β2 and TGF-β3 mRNA expression, inhibit mouse palate mesenchymal cell proliferation, and the expression of Smad pathway signaling molecules. This may indicate that TCDD affects the development of the palate by altering the epigenetic mechanism of TGF-β2 and TGF-β3, which may involve the Smad signaling pathway. We feel our study is of importance for exploring the mechanism of TCDD and transforming growth factors in palatal development.

Abstract

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent organic pollutant that is strongly associated with a number of human diseases and birth defects, including cleft palate. Transforming growth factor (TGF) plays a significant role during mammalian palatogenesis. However, the epigenetic mechanism of transforming growth factors in the process of TCDD-induced cleft palate is unclear. The purpose of this research was to investigate the relationship and potential mechanism between TGF-β2/3 promoter DNA methylation and Smad signaling during TCDD-induced cleft palate. Pregnant C57BL/6N mice were exposed to 64 μg/kg TCDD on gestational day 10 (GD10) to establish the cleft palate model and palatal tissues of embryos were collected on GD13, GD14, and GD15 for subsequent experiments. TGF-β2/3 mRNA expression, TGF-β2/3 promoter methylation, and Smad signaling molecules expression were assessed in the palate of the two groups. The results showed that the incidence of cleft palate was 94.7% in the TCDD-treated group whereas no cleft palate was found in the control group. TCDD-treated group altered specific CpG sites of TGF-β2/3 promoter methylation. Compared to the control group, the proliferation of mouse embryonic palate mesenchymal stromal cells (MEPM), the expressions of TGF-β2/3, p-Smad2, and Smad4 were all reduced, while the expression of Smad7 was significantly increased in the atAR group. Smad signaling was downregulated by TCDD. Therefore, we suggest that TGF-β2/3 promoter methylation and Smad signaling may be involved in TCDD-induced cleft palate formation in fetal mice.

Keywords: Cleft palate, 2,3,7,8-trtrachlorodibenzo-p-dioxin, TGF-β2, TGF-β3, DNA methylation

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Introduction

Cleft lip with or without cleft palate is among the most prevalent congenital deformities in humans, affecting an estimated 10.63 per 10,000 live births.[1] Despite its relatively high rate of occurrence, cleft palate (CP) exhibits a complex pathogenesis that is shaped by both environmental and

genetic factors.[2] In mammals, secondary palate formation is tightly regulated and initiates with the downward vertical growth of the palate shelves, after which tissue remodeling and the horizontal growth of the shelves towards the midline is initiated, followed by midline contact and shelf fusion that coincides with epithelial seam degradation.[3] The disruption of any of these steps or their underlying regulatory processed has the potential to cause CP. Intrauterine exposure to smoking byproducts, alcohol, infections, retinoic acid, or dioxin can additionally induce CP.[4,5] In addition to these environmental factors, both genetic and epigenetic variation can shape secondary palate development.

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is an organic pollutant that is generated through industrial and combustion-related processed, whereupon it serves as a highly persistent teratogen that can drive CP development.[6] Some studies reported that females exposed to TCDD during pregnancy can induce CP.[7,8] Histone modification and DNA methylation are the primary epigenetic factors that regulated gene expression,[9] and recent work suggests that in murine model systems, TCDD can influence palatogenesis by modulating DNA methylation and thereby causing CP.[10] The genome contains large GC-rich unmethylated regions known as CpG islands, and the methylation of those CpG islands located with the gene promoter regions can suppress gene expression.[11] Patterns of the DNA methylation arise early during embryonic development and are responsive to environmental influences.[12] Prior studies have found that the establishment of inappropriate methylation patterns is associated with the incidence of CP and other craniofacial abnormalities.[13,14] However, the role of epigenetic mechanisms for transforming growth factors (TGF) in TCDD-induced palatal malformation in mammal remains unclear.

Transforming growth factor (TGF)-β serves as an essential mediator of palatogenesis-related signaling.[15] TGF-β2/3 expression can be detected in both palatal mesenchymal and medial epithelial (MEE) cells in the context of palate fusion.[16,17] CP and other craniofacial deformities have been detected in mice in which TGF-β2 or TGF-β3 have been knocked out, underscoring the functional role of this growth factor.[17,18] Gaido *et al.*[19] further found that the TCDD treatment of human keratinocytes was sufficient to suppress TGF-β2 expression. Through cDNA microarray analyses, Nogai *et al.* further detected TGF-β3 upregulation in the palatal shelves, and mice lacking TGF-β3 have also been shown to exhibit impaired cellular proliferation and CP,[18,20,21] indicating that TGF-β2/3 signaling is an essential mediator of palatal development in mice. The impact of TCDD treatment on mouse embryonic palate mesenchymal (MEPM) cell TGF-β2/3 expression, however, has yet to be established.

Lee *et al.*[22] demonstrated that TCDD can affect TGF-β2 promoter activity, and thus down-regulate TGF-β2 transcription. TCDD treatment has been linked to gradual TGF-β3 up regulation, suggesting that TCDD-mediated modulation of TGF-β3 expression dynamics contributes to the pathogenesis of CP[23]. Liu *et al.* additionally determined that TCDD was able to inhibit human fetal palate mesenchymal cell differentiation through the TGF-β/Smad signaling pathway resulting in CP development.[7] There have been few reports to date, however, regarding TGF-β2 and TGF-β3 expression and methylation patterns in response to TCDD exposure in the context of palatogenesis. Whether the TCDD-mediated inhibition of the proliferation of MEPM cells *in vivo* is linked to changes in TGF-β2/3 expression of to the suppression of Smad signaling also remains to be established. Herein we sought to explore the relationship between TGF-β2/3 promoter methylation status and the incidence of TCDD-induced CP by measuring the expression of Smad signaling cascade-related genes and the promoter methylation status of TGF-β2/3 in a mouse model system.

Materials and methods

Animal model establishment

C57BL/6N mice (six to eight weeks old) were obtained from Vital River Laboratory Animal Technology (Beijing, China) and allowed to acclimatize in a climate-controlled facility ($22 \pm 2^{\circ}$C, $50 \pm 10\%$, 12-h light/dark) for seven days prior to experimentation. Two female mice were then housed overnight with one male mouse, and were inspected the following morning for the presence of a vaginal plug. When a plug was observed, this was denoted gestational day 0 (GD0). Pregnant mice were randomized into control and TCDD treatment groups ($n = 21$ each). TCDD (Sigma-Aldrich, MO, USA) was dissolved in DMSO (Sigma-Aldrich, MO, USA) and then diluted in corn oil and administered orally (64 μg/kg) to individual mice of TCDD group on GD10, whereas an equal volume of corn oil was given to control animals. The mice were sacrificed via cervical dislocation on GD13, GD14, GD15, and palatal shelves were dissected from fetuses using microscissors and stored at -80°C for further analysis.

All animals were housed as per the US NIH Guide for the Care and Use of Laboratory Animals. The Animal Ethics Board of Zhengzhou University (No. ZZUIRB2021-04) approved this study.

Histochemical staining

After collection, embryonic heads were fixed using 4% paraformaldehyde (PFA), dehydrated via ethanol gradient, and paraffin-embedded as per standard laboratory procedures. Deparaffinization of sections (with a thickness of 5 μm) was then performed and hydrated stepwise, immersed in hematoxylin, washed in differentiation liquid, and then immersed in eosin. Following gradual dehydration, the sections were blocked with neutral gel and observed under a microscope.

BrdU incorporation assay

A BrdU uptake assay was performed to evaluate cell proliferation based on provided directions. Pregnant mice (GD13-15) were euthanized via cervical dislocation, and embryonic heads were collected, fixed with 4% PFA for 8 h, paraffin embedded, and 0.1% TritonX-100 was

permeated for 5 min and washed with PBS three times. Tissue sections were next incubated for 30 min in 1 M HCL at 37°C, washed thrice with PBS, blocked for 30 min in 3% BSA at 37°C. Coronal sections (5 μm) were incubated with mice anti-BrdU (ab8039, Abcam, CA, USA), then stained for 10 min with DAPI to detect nuclei. Finally, a fixed area was selected from palatal mesenchyme. Fluorescence microscopy was used to observe and count the BrdU positive cell rate in TCDD-treated group and control group.

DNA methylation analysis

Collected embryonic tissues were isolated from TCDD-treated or untreated pregnant female mice between GD13 and GD15, quickly rinsed with PBS, and digested for 15 min at 37°C in Dispase II (2.4 U/mL; Roche Diagnostics) to separate mesenchymal and epithelial cells. MEPM cell DNA was then isolated with a QIAamp DNA Mini Kit (QIAGEN) based on instructions. Absorbance at 260 nm and 280 nm was measured to gauge DNA concentration and purity, after which bisulfite was used to treat 1.5 μg of DNA per sample based on the directions provided with the EZ DNA Methylation-Gold Kit (Zymo Research). TGF-β2-promoter and TGF-β3-promoter (Gen-Bank Accession Number: NM_009368.3) methylation in MEPM cells was then examined in a quantitative fashion with the Sequenom MassARRAY platform (Capitalbio, Beijing, China) consisting of MALDI-TOF mass spectrometry and RNA base-specific cleavage, PCR primers preparation was conducted using EpiDesigner (http://www.epidesigner.com), with a T7 promoter tag being forward primers were amended with a 10 mer tag to melting temperatures. Primers used in this analysis are shown in Table 1. MassARRAY Compact MALDI-TOF (Sequenom, CA, USA) was used for mass spectra acquisition, and spectra methylation ratios were generated using the EpiTYPER program (v 1.0, Sequenom). For validation of these results, six samples were randomly selected, including three controls and three TCDD-treated samples.

qPCR

Total RNA was isolated from palatal samples with Trizol (Life Technologies, CA, USA) based on provided directions, after which a PrimeScript RT reagent Kit (TaKaRa, Dalian, China) was used to prepare cDNA. All qPCR analyses of TGF-β2 and TGF-β3 expression were conducted using the SYBR Premix Ex Taq kit (TaKaRa) and the Mx3000P platform (Agilent Stratagene, CA, USA). Thermocycler settings were: 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. The comparative Ct method was employed to assess relative gene expression, and β-actin served as a normalization control. Table 2 details the primers used in this study.

Western blotting

MEPM cells were lysed using a lysis buffer containing protease inhibitors (EpiZyme, Shanghai, China), and BCA assay (Solarbio, Beijing, China) was conducted to measure sample protein concentrations. Equivalent protein quantities (30 μg) were then separated via 12% SDS-PAGE (EpiZyme, Shanghai, China) and transferred onto nitrocellulose membranes that were then blocked for 2 h using 5% non-fat milk at 37°C prior to being probed overnight with primary antibodies specific for Smad2 (ab33875, Abcam, MA, USA), Smad7 (ab216428, Abcam), Smad4 (ab40759, Abcam), p-Smad2 (S465, Abcam), and GAPDH (AB-P-R-001, Good Here, Hangzhou, China) at 4°C. HRP-conjugated secondary antibody (sc-2007, Santa Cruz, CA, USA) was incubated 1 h at 37°C, and the immunoreactive bands were detected by FemtoLight Chemiluminescence

Table 1. Primer lists of TGF-β2 and TGF-β3 for methylation.

Amplicon name	Left primer	Right primer	Direction	Target length	Left primer plus tag	Right primer plus tag
TGF-β2-01	TTTTGGGTTTTAATGG AATTATGAA	AAAAAACTAAACAAAAA ACTAAAACTCC	Forward	477	aggaagagagTTTTGGGTTTT AATGGAATTATGAA	cagtaatacgactcactatagggag aaggctAAAAAACT AAACAAAAAACTAAAACTCC
TGF-β2-02	GGGAGTTTTAGTGA ATTTTTAGGGT	AAACCCCAACCTCC TACTCAAC	Reverse	330	aggaagagagGGGAGTTTTAG TGAATTTTTAGGGT	cagtaatacgactcactatag ggagaaggctAAA CCCCAACCTCCTACTCAAC
TGF-β3-01	TTTAGAAAGGGTTT AGGAGATTTGG	CATTCCAAACCCC AAAATAAAAC	Forward	391	aggaagagagTTTAGAAAGGG TTTAGGAGATTTGG	cagtaatacgactcactatagggg agaaggctCATT CCAAACCCCAAAATAAAAC
TGF-β2-02	TGGTTGTTTATTTGG AAAGTTTTTT	CCAACCTCCATACCTC TATCACTAA	Reverse	365	aggaagagagTGGTTGTTTAT TTGGAAAGTTTTTT	cagtaatacgactcactatagg gagaaggctCCAA CCTCCATACCTCTATCACTAA

Table 2. The RT-PCR primers.

Gene	Forward (5′–3′)	Reverse (5′–3′)
TGF-β2	GCTGAGCGCTTTTCTGATCCT	CGAGTGTGCTGCAGGTAGACA
TGF-β3	GGACTTCGGCCACATCAAGAA	TAGGGGACGTGGGTCATCAC
β-actin	CTGTGCCCATCTACGAGGGCTAT	TTTGATGTCACGCACGATTTCC

Kit (EpiZyme, Shanghai, China) and quantified via scanning densitometry. Protein bands were normalized to β-actin.

Statistical analysis

Student's *t*-tests and one-way ANOVAs with Fisher's least significant difference (LSD) test were used to compare data in SPSS 19.0. Values are presented as means $\pm$ SD, and $P < 0.05$ served as the significance threshold.

Results

TCDD treatment on GD10 induces cleft palate development in mice

To explore the effect of TCDD on palate fusion, we treated pregnant mice with 64 μg/kg TCDD according to previous studies[23,24] on GD10 and found that compared with the control group, TCDD group showed CP (94.7%, 71/75) with low mortality (5.3%, 4/75); embryos in the control group exhibited no evidence of CP, whereas CP rates were significantly elevated among embryos in the TCDD-treated group ($P < 0.05$, Table 3). The HE section staining results of embryonic plate on GD13, GD14, and GD15 are shown in Figure 1, and there was no significant difference in palatal morphological development between TCDD-treated group and control group on GD13 (Figure 1(a) and (b)). On GD14 and GD15, in control embryos, palatal shelves were elevated into a horizontal apposition over the tongue, thereby allowing them to contact one another and fuse (Figure 1(c) and (e)). In contrast, the palatal shelves of TCDD-exposed embryos failed to make contact and fuse despite being elevated above the tongue, resulting in CP (Figure 1(d) and (f)).

Table 3. The incidence of cleft palate in each group.

Group	Total	Cleft palate	Stillbirth or absorbed fetus	Incidence of cleft palate (%)
Control	78	0	0	0.0
TCDD (64 μg/kg)	75	71	4	94.7[a]

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin.
[a]$P < 0.05$ compared with the control group.

TCDD treatment is associated with significantly reduced embryonic MEPM cell proliferation

When analyzing the results of a BrdU uptake experiment, we calculated the relative frequency of BrdU-positive cells in a defined 100 μm × 100 μm region in the mesenchyme proximal to the MEE. This analysis revealed that TCDD treatment was associated with a significant reduction in the frequency of BrdU-positive MEPM cells on GD13-15 relative to control embryos (Figure 2(d)).

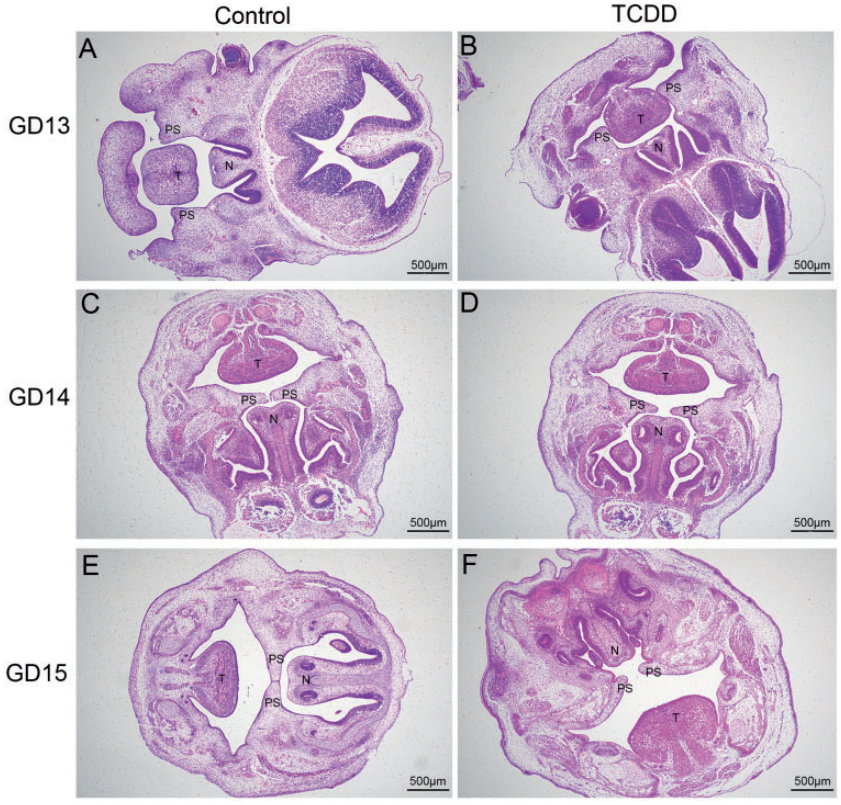

Figure 1. Assessment of palatogenesis in control and TCDD-treated embryos. Embryos were assessed via histological staining of the frontal and posterior palatal regions on GD13-15 in the control (a, c, e) and TCDD-treated groups (b, d, f). PS: palatal shelf; T: tongue; N: nose. (A color version of this figure is available in the online journal.)

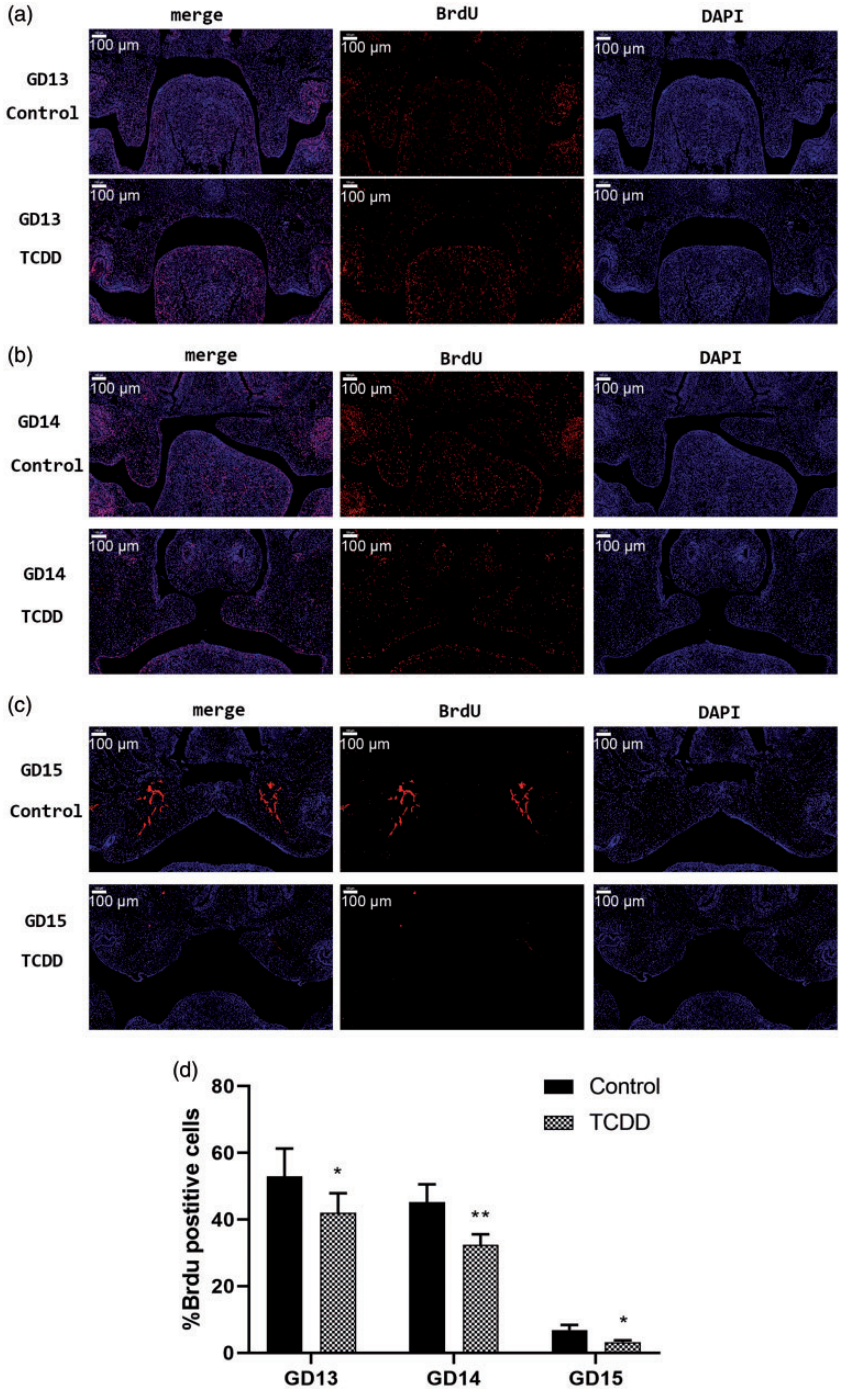

Figure 2. Assessment of the association between TCDD treatment and the proliferation of MEPM cells. (a–d) MEPM cell proliferation was assessed in control and TCDD-treated groups (treated with corn oil or TCDD, respectively, on GD10) via immunofluorescent staining on GD13 (a), GD14 (b), and GD15 (c). Cells positive for BrdU staining are shown in red, while nuclei were stained with DAPI (blue). Data are means ± SD. *$P < 0.05$, **$P < 0.01$; Student's *t*-test. (A color version of this figure is available in the online journal.)

TCDD treatment alters the methylation status of the TGF-β2/3 promoters on GD14

Next, the methylation status of the TGF-β2/3 promoters was evaluated on GD14 in control ($n = 3$) and the TCDD-treated ($n = 3$) embryo samples. Analyzed regions in the TGF-β2 and TGF-β3 genes included a 477-bp/391-bp region harboring 31 CpG/19 CpG sites in the forward chain and a total 330-bp/365-bp region containing 34 CpG/25 CpG sites in the reverse chain, respectively. Figure 3 shows that although TCDD treatment did not significantly change the overall methylation status of TGF-β2 ($\text{mean}_{\text{TCDD}} = 4.37\%$, $\text{mean}_{\text{control}} = 4.28\%$; $P = 0.141$) and TGF-β3 ($\text{mean}_{\text{TCDD}} = 7.40\%$, $\text{mean}_{\text{control}} = 8.43\%$; $P = 0.321$), TCDD exposure was linked to significant alterations in the CpG methylation of this regions, with TCDD-related decreases in methylation being detected for the 10th CpG sites ($P = 0.001$) in TGF-β2 region-01 (Figure 4(a)), and

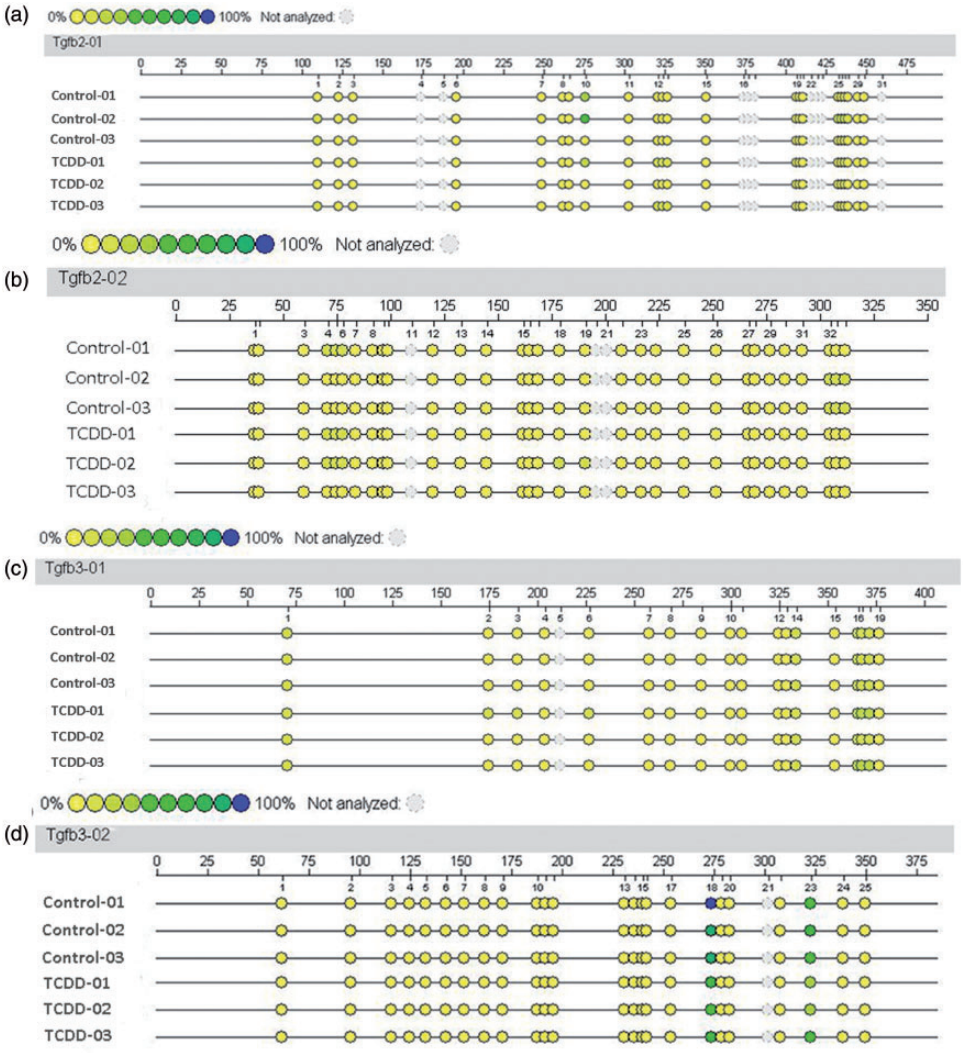

Figure 3. Assessment of TGF-β2 and TGF-β3 promoter CpG methylation. TGF-β2 and TGF-β3 promoter CpG methylation in control and TCDD-treated embryos were evaluated on GD14. Quantitative methylation analyses were performed with the SequenomMassARRAY platform. Lines indicate the TGF-β2 promoter region-01 (a), TGF-β2 promoter region-02 (b), TGF-β3 promoter region-01 (c), and TGF-β3 promoter region-02 (d) CpG methylation profiles in the control (Control-1 to Control-3) and the TCDD-treated (TCDD-1 to TCDD-3) groups. Circle colors correspond to the degree of CpG methylation, with yellow, green, and dark blue corresponding to 0%, 50%, and 100% methylation, respectively. White circles indicating missing data for a given CpG site. (A color version of this figure is available in the online journal.)

the 18th CpG site ($P = 0.013$) in TGF-β3 promoter region-02 (Figure 4(d)) relative to controls ($P < 0.05$), whereas 14th CpG ($P = 0.039$), 18th and 19th CpG ($P = 0.013$), 25th CpG ($P = 0.039$), and 29th CpG ($P = 0.039$) in TGF-β2 promoter region-02 (Figure 4(b)), and the 2nd, 3rd CpG ($P = 0.023$) for TGF-β3 promoter region-01 (Figure 4(c)), and the 16th, 17th, 18th CpG ($P = 0.013$) for TGF-β3 promoter region-02 (Figure 4(d)) in TCDD samples were increased compared with the control samples ($P < 0.05$).

TCDD treatment inhibits embryonic MEPM TGF-β2 and TGF-β3 expression

Real-Time PCR was used to analyze the mRNA expression level between GD13 and GD15 to investigate whether DNA methylation directly affected the expression of MEPM in control and TCDD-exposed group. As shown in Figure 5, compared to control MEPM samples, those from TCDD-exposed embryos exhibited significantly reduced TGF-β2 and TGF-β3 mRNA levels ($P < 0.05$).

TCDD inhibits MEPM Smad signaling on GD14

Next, we analyzed Smad-signaling related protein levels in MEPM cells via Western blotting in order to understand whether the TCDD-mediated suppression of MEPM cell proliferation and TCDD-induced increases in TGF-β2/3 promoter methylation were associated with the dysregulation of TGF-β/Smad signaling activity. As shown in Figure 6, TCDD treatment was associated with significant reductions in p-Smad2 and Smad4 protein expression, whereas Smad7 expression in TCDD-exposed embryos was significantly enhanced ($P < 0.05$).

Discussion

Mammalian palatogenesis is a heterogeneous and dynamic process involving abundant signal molecules and transcription factors. This process requires precise interaction between extracellular matrix, mesenchymal cells, and epithelial cells, and failure in any of these processes can lead to

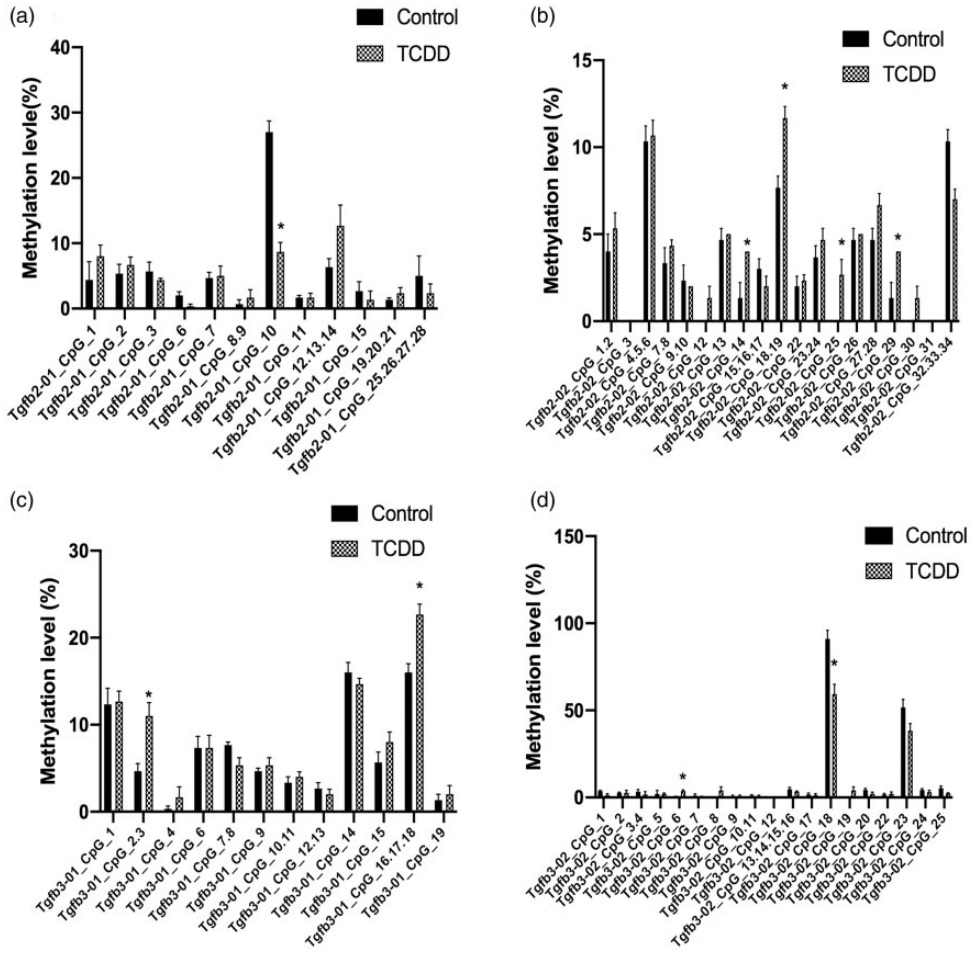

Figure 4. Analysis of TCDD-related changes in TGF-β2/3 promoter CpG methylation status. The methylation status of TGF-β2 region-01 (a), TGF-β2 promoter region-02 (b), TGF-β3 promoter region-01 (c), and TGF-β3 promoter region-02 (d) were calculated. Data are means $\pm$ SD. *$P < 0.05$ vs. control; Student's t-test. $n_{control} = 3$ (Samples: Control-1 to Control-3); $n_{TCDD} = 3$ (TCDD-1 to TCDD-3).

CP.[21,25] The normal development of the primary and secondary palatal shelves is the basis of complete palatal formation in mammals. Although a large number of studies on secondary palatal development in recent years have provided abundant clues to the occurrence of CP, there are still major gaps in the molecular mechanisms of CP palatogenesis, particularly regarding the mechanisms involving gene-environment interactions. Herein, we explored the functional importance of the epigenetic control of TGF-β2/3 expression in the context of MEPM cell proliferation and secondary palate development in C57BL/6N mice.

We used the CP model constructed by 64 μg/kg TCDD to explore the potential biological mechanism of CP. We found that the incidence of CP was 94.7% and no other malformations in the TCDD group. We selected GD14 as the primary developmental time point of interest in this study given that this is the critical period for palatal-shelf adhesion and fusion in mice. Indeed, the occurrence of murine CP has been attributed to impaired mesenchymal cell proliferation on GD14[20,26,27] To explain the TCDD-induced the mechanism of CP formation, we hypothesized that TCDD may be increasing apoptosis and/or inhibiting proliferation. Our BrdU incorporation results showed that the proliferation of MEPM cells was significantly inhibited in the TCDD-treated group compared with the control group.

Several studies to date have highlighted the ability of TCDD to impact growth factor-dependent signaling pathways.[7,28] Therefore, we focused on these genes—TGF-β2/3—and examined the potential means by which their expressions are regulated in tissue during TCDD-induced palatal malformation. In this study, we provided evidence that the TGF-β2 and TGF-β3 expression during embryonic palatogenesis treated with TCDD is partly subject to epigenetic control. The results of this study demonstrating that TCDD exposure can alter TGF-β2 and TGF-β3 promoter methylation in embryonic MEPM cells while simultaneously impairing the ability of these cells to proliferate. While no significant differences in TGF-β2 and TGF-β3 promoter overall methylation were detected when comparing embryos from the control and TCDD-treated groups, the methylation of individual CpG residues was significantly altered following TCDD treatment. Such CpG methylation is known to suppress gene expression especially when DNA methylation occurs in promoter regions known as "CpG island," where CpG occurs with high frequency,[29,30] and consistent with this, we observed significant decreases

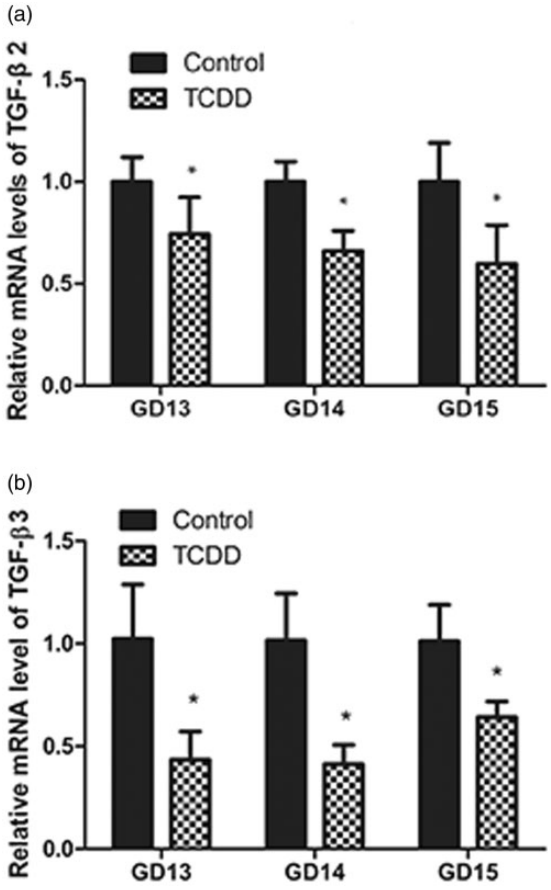

Figure 5. Assessment of TCDD-related changes in TGF-β2/3 expression in the mouse palatal mesenchyme from GD13 to GD15. A qPCR approach was used to assess TGF-β2 (a) and TGF-β3 (b) mRNA expression in the indicated samples, with GAPDH serving as a normalization control. Data are means $\pm$ SD. $^{*}P < 0.05$; Student's *t*-test.

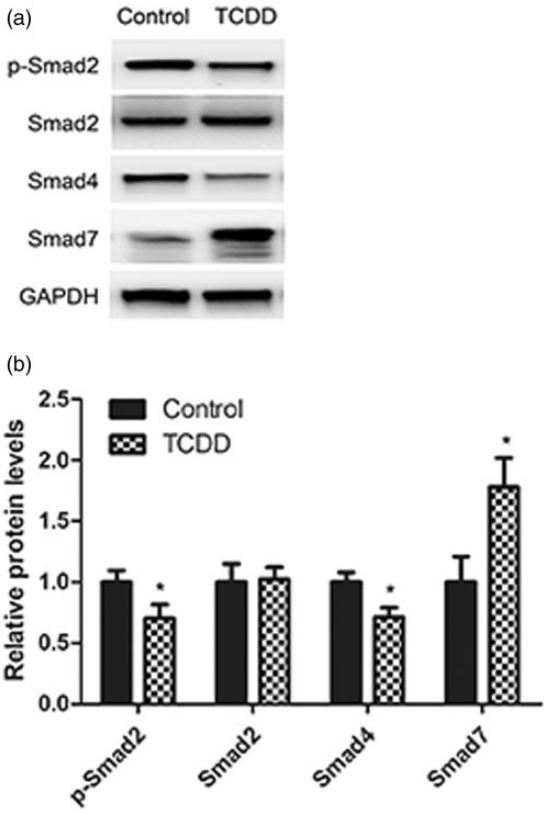

Figure 6. TCDD suppresses murine palatal Smad signaling in the mesenchyme on GD14. Western blotting was used to assess the levels of key Smad signaling proteins (p-Smad2, Smad2, Smad4, and Smad7) in the palatal mesenchyme of embryonic mice in the control and TCDD-treated groups on GD14. $^{*}P < 0.05$; Student's *t*-test.

in TGF-β2/3 mRNA expression levels in MEPM tissue samples from TCDD-treated embryos on GD14 relative to levels in control embryos. Also, *in vivo* and *in vitro* studies published by predecessors, we also reached the results that TCDD treatment decreases the TGF-β2 and/or TGF-β3 mRNA expression levels.[22,31] By studying the effect of TCDD on the deletion of a series of TGF-β2 gene promoters at some sites upstream of the transcription start point, Lee *et al.* found that TCDD caused the transcription downregulation of TGF-β2 gene by the reduction in rather than a complete elimination of TGF-β2 promoter function.[22] Therefore, we speculated that the mRNA expression levels of TGF-β2 and TGF-β3 were significantly decreased in the TCDD-treated group on GD14, which may be related to the methylation of individual CpG sites.

Knocking out TGF-β2 results in CP but with incomplete penetrance (~25%), and in the model, clefting was associated with palatal hypoplasia,[17] although the array of defects observed in the knockout model suggests that CP in this model is syndromic and secondary to other craniofacial defects. In our study, the level of reduction in TGF-β2 expression observed here (30–40% in the TCDD treatment group) does not seem adequate to cause CP, given that previous evidence in a TGF-β2 knockout mouse suggests that CP only exhibited ~25% penetrance.[17] A knockout model of Foxf2 demonstrated mesenchymal hypoplasia in the palatal shelves which the authors indirectly attributed to reduced TGF-β2 expression and Smad2 signaling.[32] From what has been discussed above, we speculated that TGF-β2 may not be directly involved in mesenchymal proliferation and palatal shelf growth.

Palatal shelf growth is controlled by positive feedback interactions between the palatal epithelium and the palatal mesenchyme, with TGF-β3 signaling in the medial edge epithelium being a key driver of palatal fusion following growth/elevation.[3,33] Moreover, TGF-β3 expression is critically important to midline epithelial seam (MES) degeneration and that TGF-β3 knockout mice exhibit failure of palatal fusion characterized by persistence of the MES.[18] Among the several mechanisms of MES degeneration, epithelial mesenchymal transdifferentiation (EMT) has been extensively studied. Nakajima *et al.* used β-galactosidase and Dil cell lineage markers to find that TGF-β3 may be strongly related to EMT in palatogenesis,[34] whereas Liu *et al.* found that the expression of TGF-β3 decreased in palatal mesenchymal cells when atRA induced CP[27]. It seems that reduced TGF-β3 expression in the present study would be more likely to interfere with palatal fusion as opposed to palatal growth. The expression of TGF-β2 is restricted to the palatal mesenchyme, whereas TGF-β3 expression is evident in the palatal epithelium and mesenchyme.[16,27] This

suggests that TCDD may influence the occurrence of CP through its combined effects on the epigenetic control of both TGF-β2 and TGF-β3.

TGF-β family cytokines control embryonic development and other cellular processes through both Smad-dependent or Smad-independent signaling mechanisms.[35] TGF-β can be presented to the transmembrane TβR-I and TβR-II signaling receptors by TβR-III, which is membrane-anchored. Such presentation triggers receptor activation and intracellular phosphorylation and activation of the closely related Smad2 and Smad3 proteins. Smad2 and/or Smad3 can then interact with the shared Smad4 mediator protein and undergo nuclear translocation whereupon they can control transcriptional activity in a cell-type-dependent manner by interacting with a range of transcription factors. In contrast, Smad7 supresses TGF-β-induced Smad signaling.[35–37] Such TGF-β/Smad signaling is known to play a central role in palatogenesis and the incidence of CP.[16,38] We have also demonstrated that this TGF-β/Smad signaling pathway can substantially impact mesenchymal cells in the context of palatal development in prior studies.[7,27,39]

We additionally evaluated MEPM Smad signaling cascade protein expression on GD14 and observed significant TCDD exposure-related reductions in p-Smad2 and Smad4 levels, while Smad7 expression was enhanced. The results are consistent with previous results obtained from human fetal palatal mesenchymal cells *in vitro*.[7] Prior studies have shown that the overexpression of Smad2 in TGF-β knockout mice can rescue CP phenotypes.[25,40] In summary, we hypothesize that TCDD can modulate TGF-β2/3 expression in MEPM cells, thereby suppressing Smad signaling pathway activation and compromising the proliferation of these cells such that normal palatogenesis is disrupted.

Conclusions

In conclusion, the results of this study demonstrated that 64 μg/kg TCDD successfully interfered with the palatogenesis of fetal mice and resulted in CP by inhibiting MEPM cell proliferation. TCDD exposure can alter the CpG methylation status of the TGF-β2 and TGF-β3 promoters, potentially thereby inhibiting the expression of these two growth factors in palatal tissues in developing murine embryos. We therefore speculate that TCDD can cause CP through the disruption of palatal mesenchyme proliferation and growth either indirectly or directly through alterations in TGF-β2 expression, and via the disruption of palatal fusion directly via disrupting normal TGF-β3 expression, which may be mediated by Smad-dependent signaling pathways. While exposure to TCDD can alter the methylation status of TGF-β2 and TGF-β3, further research will be necessary to understand the mechanistic basis for these epigenetic changes in the context of mammalian palatal development, and we will continue to pay attention to this issue in the future.

AUTHORS' CONTRIBUTIONS

YC, ZY, XL, and ZW conceived and conducted the experiment; YC, ZY, ZW, XL, LC, ZH, ZG, LL, ZL analyzed the data and wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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