

# 2,3,7,8-Tetrachlorodibenzo-p-Dioxin and TGF- $\beta$ 3 Mediated-Mouse Embryonic Palatal Mesenchymal Cells

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## Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a well-known environmental teratogenic agent for cleft palate. But transforming growth factor  $\beta$ 3 (TGF- $\beta$ 3) is an essential growth factor for palatogenesis. This study is to clarify effects of TCDD and TGF- $\beta$ 3 in mouse embryonic palatal mesenchymal (MEPM) cells. The result showed that with increase of TCDD (0.5 nM-10 nM), the expression of TGF- $\beta$ 3 increased, but after 10 nM TCDD, the expression of TGF- $\beta$ 3 reduced. The viabilities of MEPM cells decreased in 10 nM TCDD-treated group. But the viabilities increased in 10 ng/mL TGF- $\beta$ 3-treated group, or the viabilities were between that of them in combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3-treated group. This phenomenon was the same as the motilities. In addition, we found that the expression of phosphorylated Smad2/3 and Smad7 was increased by 10 nM TCDD, 10 ng/mL TGF- $\beta$ 3, or combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3 induced, but the expression of Smad4 was decreased. These data revealed that the TGF- $\beta$ /Smad signaling pathway affected TCDD and TGF- $\beta$ 3 in MEPM cells.

## Keywords

TCDD, MEPM cells, TGF- $\beta$ 3, TGF- $\beta$ /Smad signaling

## Introduction

Palatogenesis is a tightly regulated process, in which multi-potential mesenchymal cells can differentiate into chondrocytes to form cartilage.<sup>1,2</sup> The secondary palates of the palatal shelves grow and fuse in mesenchymal cell proliferation. Interestingly, transforming growth factor  $\beta$ 3 (TGF- $\beta$ 3) is expressed in the palatal mesenchyme during palatal growth and elevation.<sup>3</sup> Transforming growth factor  $\beta$ 3 plays a very important role in a variety of cellular processes including cell proliferation, differentiation, apoptosis, migration, invasion, matrix synthesis, and the immune response.<sup>4-6</sup> It was also thought that TGF- $\beta$ 3 played a very important role during palatogenesis.<sup>7</sup> Transforming growth factor  $\beta$ 3 was an important regulator of palatal fusion, and TGF- $\beta$ 3-knockout mice exhibited cleft palate, but exogenous TGF- $\beta$ 3 treated TGF- $\beta$ 3-knockout mice can rescue palatal fusion.<sup>8</sup> Moreover, TGF- $\beta$ 3 was also the most effective for human palatal mesenchymal cell proliferation.<sup>9</sup> Transforming growth factor  $\beta$ 3 can initiate diverse cellular responses by binding and activating specific cell surface receptors, which also can activate TGF- $\beta$  receptors to stimulate the phosphorylation of receptor-regulated Smad proteins, such as Smad2 and Smad3. Phospho-Smad2/3 (p-Smad2/3) formed complexes

with Smad4, which accumulated in the nucleus and regulated the transcription of target genes. The actions of TGF- $\beta$  were antagonized by Smad7, which can prevent phosphorylation of Smad2/3, thereby blocking TGF- $\beta$ /Smad signaling.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a well-known teratogenic agent of cleft palate. Morphological studies revealed that TCDD caused cleft palate by disturbing palatal

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shelf growth and inhibiting the fusion of palatal shelves.<sup>10</sup> Many genes played important roles in palatogenesis such as TGF- $\beta$ 3, KLF4.<sup>7,11</sup> But there were few reports about the relationships of TCDD and TGF- $\beta$ 3 in mouse embryonic palatal mesenchymal (MEPM) cells. In this study, we found the possible effects of TCDD and TGF- $\beta$ 3 in MEPM cells.

## Materials and Methods

### Cell Culture and Treatment

Mouse embryonic palatal mesenchymal cells were derived from palatal tissue on 13-day C57BL/6 mice embryos (Henan Laboratory Animal Center of Zhengzhou University, 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 method of MEPM cell culture was according to the detail by Feng et al.<sup>12</sup> The MEPM cells were cultured in flasks with DMEM (Dulbecco's Modified Eagle's medium)/F12 medium (Hyclon, Logan, Utah) supplemented with 10% fetal calf serum (Sijiqing, Hangzhou, China). The MEPM cells were placed in a humidified incubator at 37°C with 5% CO<sub>2</sub> atmosphere with media replaced every other day. The third passage cells were seeded. Some cells were treated with 0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM TCDD, and TCDD concentration was selected according to some reports,<sup>13,14</sup> others were treated with 10 nM TCDD (DD-2378-S, Sigma, Saint Louis, Missouri), or 10 ng/mL TGF- $\beta$ 3 (cyt-143, PROSPEC, Zion, Israel), or combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3 for further analysis. Control cells were treated with DMSO (Dimethyl sulfoxide) (D2650; Sigma).

### Quantitative Real-Time Polymerase Chain Reaction

Total RNAs were isolated from MEPM cells using Trizol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. To detect expression of TGF- $\beta$ 3, first strand complementary DNA (cDNA) was synthesized using a PrimeScript II 1st strand cDNA Synthesis Kit (6210A, TaKaRa Biotechnology, Kyoto, Japan), and then amplified by quantitative real-time polymerase chain reaction (qRT-PCR) with the SYBR Premix Ex Taq Kit (DRR420A; TaKaRa) through ABI 7900 PRISM system (7900HT; Applied Biosystems, Carlsbad, California). GAPDH was used as an internal control. The qRT-PCR conditions were as follows: polymerase activation for 15 minutes at 95°C, 40 cycles at 95°C for 15 seconds, 56°C for 20 seconds, and 72°C for 30 seconds. Polymerase chain reaction products were identified by melting curve analysis. All primers were synthesized by Invitrogen. The primer sets were as follows: TGF- $\beta$ 3-forward: 5'-CCTGGCCCTGCTGAACCTG-3', and reverse, 5'-TTGATGTGGCCGAAGTCCAAC-3'. GAPDH-forward: 5'-TGACGTGCCGCCTGGAGAAAC-3', and reverse, 5'-CCGGCATCGAAGGTGGAAGAG-3'.

### Cell Viability Assays

To evaluate the effect of TCDD, the viabilities of MEPM cells were determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (JT343, Genview, California) assay. The third passage of MEPM cells ( $5 \times 10^3$  cells per well) were seeded in 96-well plates (Nunc, Denmark). The cells were treated with 10 nM TCDD, 10 ng/mL TGF- $\beta$ 3, or combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3, or DMSO ( $\leq 0.05\%$ ). After 72 hour-incubation, the cell viabilities were detected by MTT assay according to the manufacturer's protocols.

### Scratch Wound-Healing Motility Assay

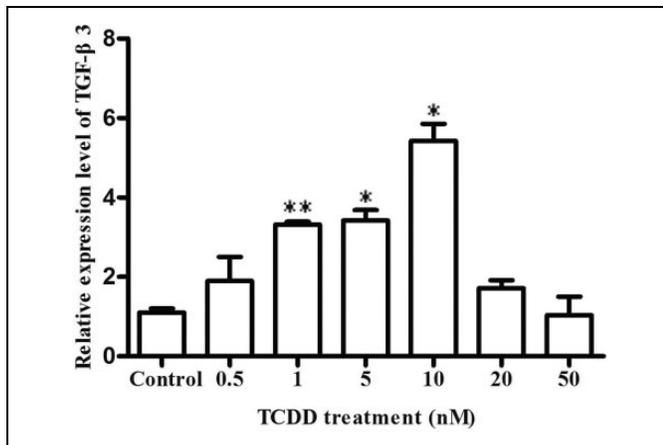
The MEPM cells were seeded on 6-well plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and the cells were washed with PBS 3 times to remove cells debris. The cells were treated with 10 nM TCDD, or 10 ng/mL TGF- $\beta$ 3 or combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3, respectively. After maintaining under standard conditions for 24 hours, plates were washed once with fresh medium to remove nonadherent cells and then photographed. The percentages of open spaces covered by migrated cells were determined using Image J software (1.37 National Institutes of Health, Bethesda, MD, USA) (<http://rsb.info.nih.gov/ij/>).

### Western Blot Analysis

Total lysates from different treated MEPM cells using  $5 \times$  SDS-lysis buffer supplemented with proteases inhibitors (M250, Amresco, Ohio) were determined. Protein concentration was determined using a standard BSA protein assay (Dingguo, Beijing, China). A total of 40  $\mu$ g proteins were fractionated on 12% SDS-PAGE, and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were immunoblotted with the primary antibodies: Smad2 (ab71109, Abcam, Massachusetts), p-Smad2 (sc101801, Santa Cruz, California), Smad3 (BM3559, Boster Biotech, Wuhan, China), p-Smad3 (GD-CZ5616R, Santa Cruz), Smad4 (PB0446, Boster Biotech), and Smad7 (sc-365846, Santa Cruz).  $\beta$ -actin was probed as a loading control. Then membranes were washed and incubated with HRP-conjugated secondary antibody (sc-2004 or sc-2005, Santa Cruz), Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor Lincoln, Nebraska).

### Statistical Analysis

All data were compared using either double-sided Student *t* test or one-way analysis of variance. The choice of tests was performed automatically using SPSS software, version 13.0 (SPSS, Chicago, Illinois). All data were presented as mean (standard deviation) of 3 independent experiments. Differences were considered to be statistically significant when  $*P < .05$ .



**Figure 1.** The effect of TGF-β3 by TCDD induced in MEPM cells. 0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM and 50 nM TCDD, or DMSO ( $\leq 0.05\%$ )-treated MEPM cells as the experiment group and control group, respectively. After treated for 72 hours, the expression of TGF-β3 was measured by qRT-PCR. Data were mean values  $\pm$  standard deviation of 3 replicate experiments. \* $P < .05$  or \*\* $P < .01$  versus the corresponding control values. MEPM indicates mouse embryonic palatal mesenchymal; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TGF-β3, transforming growth factor β3; qRT-PCR, quantitative real-time polymerase chain reaction.

## Results

### The Effect of TGF-β3 by TCDD in MEPM Cells

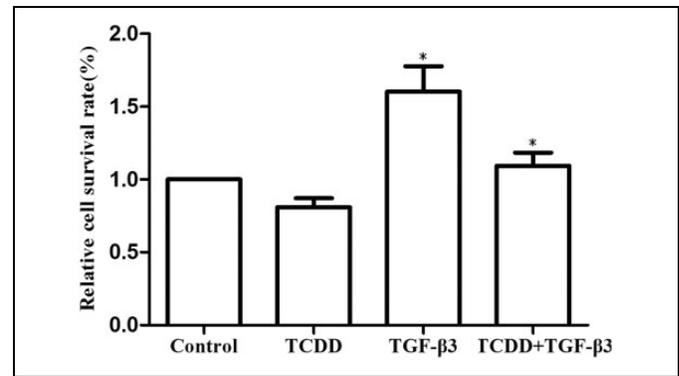
Transforming growth factor β3 was the essential growth factor for palatogenesis.<sup>15</sup> We explored the expression of TGF-β3 in MEPM cells by 0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM TCDD induced for 72 hours. As shown in Figure 1, we found that the mRNA levels of TGF-β3 significantly increased ( $1.90 \pm 0.86$  fold), ( $3.32 \pm 0.10$  fold), ( $3.42 \pm 0.37$  fold), and ( $5.43 \pm 0.61$  fold) in MEPM cells for 0.5 nM, 1 nM, 5 nM, and 10 nM TCDD induced compared to the corresponding control cells, respectively. The mRNA levels of TGF-β3 decreased ( $1.72 \pm 0.28$  fold) and ( $1.04 \pm 0.66$  fold) at 20 and 50 nM TCDD compared to the corresponding control cells, respectively.

### The Effects of Cell Proliferation in MEPM Cells

In the experiments, we evaluated the effect of cell proliferation at 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3 in MEPM cells. After treatment for 72 hours, the cell proliferation was measured by MTT assay. As shown in Figure 2, we found that the relative cell survival rate of TCDD group was decreased ( $13.23 \pm 11.40\%$ ) compared to the corresponding control cells, but proliferation rate of TGF-β3 group was increased ( $56.34 \pm 1.56\%$ ), while that of the combination of TCDD and TGF-β3 group of cells was increased ( $11.56 \pm 42.41\%$ ).

### The Effects of Cell Motility in MEPM Cells

To determine whether 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3 affected

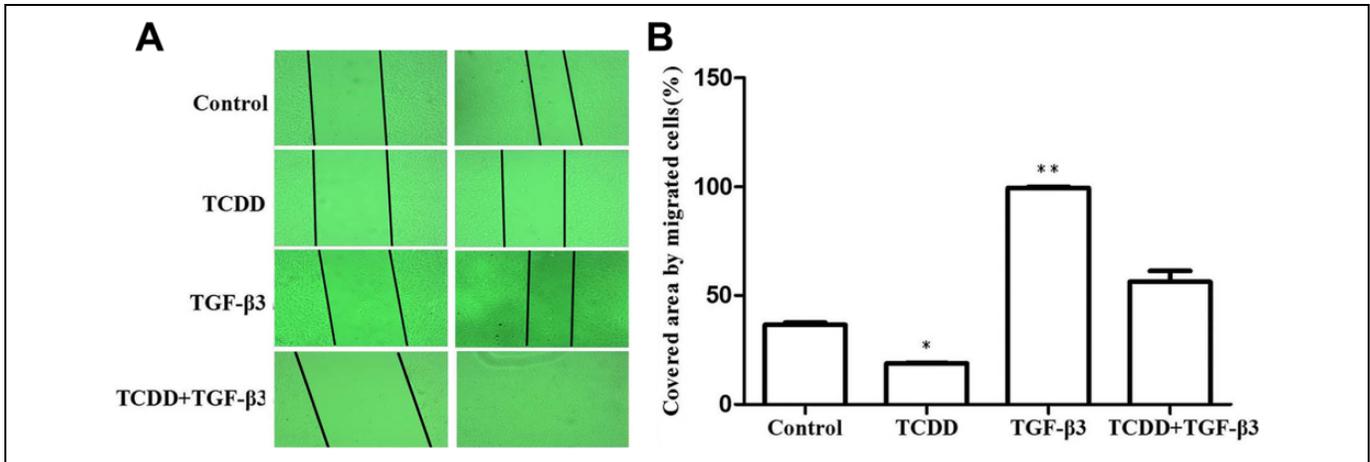


**Figure 2.** The effects of cell proliferation in MEPM cells. 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3, or DMSO ( $\leq 0.05\%$ ) treated MEPM cells as the experiment group and control group, respectively. After treated for 72 hours, the cell proliferation was determined by MTT assay according to the manufacturer's protocols. Data were mean values  $\pm$  standard deviation of three replicate experiments. \* $P < .05$  versus the corresponding control values. MEPM indicates mouse embryonic palatal mesenchymal; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

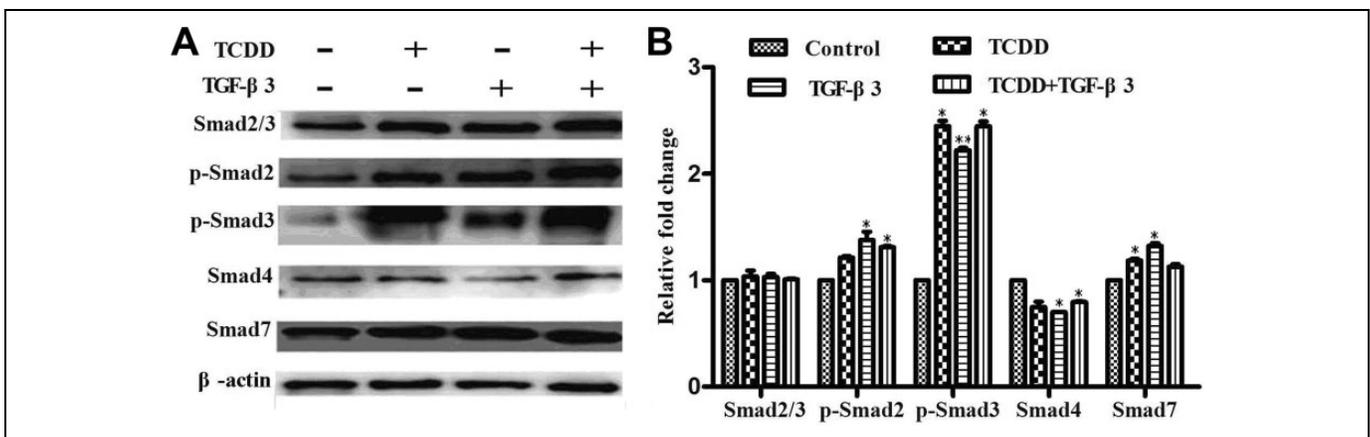
the motilities of MEPM cells. We used wound-scratch healing assay to detect the cell motility. After 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3 exposed to MEPM cells for 24 hours, the scratch test was performed. As shown in figure 3, we found that the covered area by migrated cells in the control group was ( $36.53 \pm 1.41\%$ ) compared to corresponding control cells. We found that the covered area in the 10 nM TCDD group was ( $18.80 \pm 0.29\%$ ) compared to the corresponding control cells. We found that the covered area in the combination of 10 nM TCDD and 10 ng/mL TGF-β3 group was ( $56.29 \pm 7.07\%$ ) compared to the corresponding control cells. We found that the covered area in the combination of 10 ng/mL TGF-β3 group was ( $99.50 \pm 0.71\%$ ) compared to the corresponding control cells.

### The Effects of TGF-β/Smad in MEPM Cells

TGF-β/Smad pathway may exist in MEPM cells by TCDD and TGF-β3 induced. Smads were intracellular effectors of TGF-β/Smad signaling. Smad2 and Smad3 can be activated and form heteromeric complexes with Smad4. Smad7 played a key role in the regulation of TGF-β/Smad signaling, but it was involved in negative feedback. Therefore, we assessed the protein expression of these TGF-β/Smad pathway gene in MEPM cells. As shown in Figure 4, we found that the expression of p-Smad2 increased ( $1.21 \pm 0.02$  fold), ( $1.38 \pm 0.11$  fold), and ( $1.31 \pm 0.01$  fold) in 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3 group compared to the corresponding control cells, respectively. The expression of p-Smad3 increased ( $2.45 \pm 0.07$  fold), ( $2.22 \pm 0.03$  fold) and ( $2.45 \pm 0.06$  fold), respectively. But the expression of Smad4 decreased ( $0.25 \pm 0.07$  fold), ( $0.20 \pm 0.001$  fold), and ( $0.20 \pm 0.006$  fold), respectively. The expression of



**Figure 3.** The effects of cell migration in MEPM cells. When MEPM cells were seeded and grew to confluence, the cells were scratched with a pipette tip, and 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3, or DMSO ( $\leq 0.05\%$ ) treated MEPM cells as the experiment group and the control group, respectively. After 24 hours treatment, the cells were photographed. The wound closure was determined using ImageJ software. Data were mean values  $\pm$  standard deviation of 3 replicate experiments. \* $P < .05$  or \*\* $P < .01$  versus the corresponding control values. MEPM indicates mouse embryonic palatal mesenchymal; TGF-β3, transforming growth factor β3; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.



**Figure 4.** The effects of TGF-β/Smad signaling molecules in MEPM cells. MEPM cells were treated 10 nM TCDD, 10 ng/mL TGF-β3, or combination of 10 nM TCDD and 10 ng/mL TGF-β3, or DMSO ( $\leq 0.05\%$ ), respectively. After 72 hours treatment, the expression of Smad2/3, phosphorylation of Smad2 and Smad3, Smad4 and Smad7 proteins were measured by Western blot, with β-actin as loading controls, and band fluorescence intensity was analyzed and quantified using Odyssey Infrared Imaging System. The relative expression level of Smad2/3, phosphorylation of Smad2 and Smad3, and Smad7 proteins were calculated after normalization with the loading control β-actin. The data are the mean values  $\pm$  standard deviation of three replicate experiments. \* $P < .05$  or \*\*\* $P < .01$  versus the corresponding control values. MEPM indicates mouse embryonic palatal mesenchymal; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TGF-β3, transforming growth factor β3.

Smad7 increased ( $1.19 \pm 0.02$  fold), ( $1.33 \pm 0.04$  fold), and ( $1.13 \pm 0.03$  fold) respectively.

## Discussion

The MEPM cells are very important in palateogenesis. The MEPM cells can undergo programming cell death, migration, epithelial-mesenchymal transition and differentiation, which are coincident with the process of palatal fusion and disappearance of MEPM cells. Transforming growth factor β3 has been indicated to play an essential role in the development of palatal shelves. For example, the expression of TGF-β3 had been

identified to upregulate in fetal mouse palatal shelves,<sup>16,17</sup> and TGF-β3 was also upregulated in the palatal tissues of people with cleft palate.<sup>17</sup> Moreover, TGF-β3 exposure completely prevented the dioxin-induced block of palatal fusion in this system.<sup>18</sup> Transforming growth factor β3/Smad signaling pathway may mediate cleft palate.<sup>19-21</sup> Up to present, no reports are about the relationships of TCDD and TGF-β3 in MEPM cells. We found that when different concentrations of TCDD (0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM) are exposed to MEPM cells, the expression of TGF-β3 gene was always higher than the control, and the expression of TGF-β3 was highest in MEPM cells by 10 nM TCDD induced. The results

were also identical with TGF- $\beta$ 3 expression upregulates on cleft palates by low-dose TCDD induced in mice.<sup>17</sup> The cleft palates by TCDD-induced are thought to result from a failure of the fusion of the palatal shelves, and the TGF- $\beta$ 3 is essential during palatal fusion stage. Therefore, high concentration of TCDD causes a decrease in TGF- $\beta$ 3.

The relationship between TCDD and TGF- $\beta$ 3 remained obscure. Low-dose TCDD can affect palatogenesis and lead to malformations in the early stages of cartilage development.<sup>22</sup> Numerous studies demonstrated that TCDD inhibited programmed cell death of MEPM cells in palatal shelves, and TCDD can alter MEPM cell differentiation.<sup>23-25</sup> However, few reports revealed that TCDD explored to MEPM cells. In this study, we focused on the interactive effects between TCDD and TGF- $\beta$ 3. We found that 10 nM TCDD inhibited the MEPM cell proliferation, which was same that TCDD inhibited urogenital sinus mesenchymal cell proliferation.<sup>26</sup> Instead, 10 ng/mL TGF- $\beta$ 3 promoted the MEPM cell proliferation, which was consistent that TGF- $\beta$ 3 can stimulate the proliferation of mesenchymal cells in vaginal thread.<sup>27</sup> The combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3 also promoted MEPM cell proliferation, but the rate of cell viability was between TCDD and TGF- $\beta$ 3 treated alone, which might be consistent with TGF- $\beta$ 3 as an effective antidote to dioxin-induced MEPM cells.<sup>28</sup> This phenomenon was same as the effect of motilities in MEPM cells by 10 nM TCDD, 10 ng/mL TGF- $\beta$ 3, or combination of 10 nM TCDD and 10 ng/mL TGF- $\beta$ 3. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibited the MEPM cell motility, which concurred with the fact that TCDD-mediated the inhibition of MCF-7 cell migration.<sup>29</sup> Transforming growth factor  $\beta$ 3 promoted MEPM cell motility, which is aligned to TGF- $\beta$ 3 activating the palatal midline epithelial seam (MES) cell migration.<sup>30</sup> And the combination of TCDD and TGF- $\beta$ 3 also promoted MEPM cell motility, which might be because the TGF- $\beta$ 3 plays an important role in MEPM cells to antagonize TCDD.

2,3,7,8-Tetrachlorodibenzo-p-dioxin increased the mRNA expression of TGF- $\beta$ 3 in MEPM cells, and TCDD might make function by TGF- $\beta$ /Smad pathway. Phospho-Smad2/3 can bind Smad4 to form a heteromeric complex in the nucleus, where they act as transcription factors.<sup>31</sup> Smad7 was an inhibitor of TGF- $\beta$ /Smad signaling pathway.<sup>32</sup> The current study showed that TCDD increased the expression of p-Smad2/3 and inhibited the expression of Smad4. Activation of the TGF- $\beta$ /Smad signaling pathways was transfection with a dominant negative Smad4.

Taken together, we demonstrated that cellular levels of p-Smad2/3 were activated by 10 nM TCDD, and Smad4 was inhibited by 10 nM TCDD. Transforming growth factor  $\beta$ 3 was revealed to play an important role in MEPM cells to antagonize TCDD. These suggested that TCDD and TGF- $\beta$ 3 mediated TGF- $\beta$ /Smad signaling pathways. But some researchers detected the mRNA expression levels of Smad2, Smad3, Smad4, and Smad7 in the palates of fetuses by TCDD induced at E13.5, E14.5, and E15.5.<sup>13</sup> The mRNA levels of Smad2, Smad3, Smad4, and Smad7 were lower in MEPM cells by

TCDD induced compared to the corresponding control. They believed that TCDD did not make function by TGF- $\beta$ /Smad signaling.<sup>13,33</sup> However, there are 2 kinds of cells in the mouse palatal shelves, such as medial edge epithelial cells and MEPM cells. But the authors did not tell about which kinds of cells making mainly function in tissues of mouse palatal shelves. In addition, the expression of gene in transcription level was different from that of the post transcriptional level. However, TCDD receptor (aryl hydrocarbon receptor [AhR]) can down-regulate the TGF- $\beta$ /Smad pathway in human glioblastoma cells.<sup>34</sup> Expression of genes in the TGF- $\beta$  signaling pathway was significantly deregulated in smooth muscle cells from aorta of AhR knockout mice by TCDD induced.<sup>35</sup> Moreover, inhibition of TGF- $\beta$  signaling pathway also inhibited TCDD-induced Treg activity.<sup>36</sup>

In conclusion, we found that TCDD and TGF- $\beta$ 3 might had negative relationship in MEPM cells by TGF- $\beta$ /Smad signaling, but the precise mechanisms of TCDD and TGF- $\beta$ 3 mediated-cleft palate in MEPM cells require further investigation.

### Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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