2,3,7,8-Tetrachlorodibenzo-p-dioxin and *TGFβ3*-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 effector for cleft palate. Transforming growth factor β3 (*TGF-β3*) is an essential growth factor for palatogenesis. The objective of this study is to clarify the effects of TCDD and *TGF-β3* in mouse embryonic palatal mesenchymal (MEPM) cells. The effects of 10 nM TCDD, 10 ng/mL *TGF-β3*, or a combination of 10 nM TCDD and 10 ng/mL *TGF-β3* on MEPM cells were revealed by cell and biological methods. With the increase in TCDD (0.5-10 nM), the expression of *TGF-β3* increased, but at TCDD concentrations greater than 10 nM, the expression of *TGF-β3* reduced. The viabilities of MEPM cells decreased in the 10 nM TCDD-treated group. But the viabilities increased in the 10 ng/mL *TGF-β3*-treated group, and the viabilities were intermediate in the group treated with a combination of 10 nM TCDD and 10 ng/mL *TGF-β3*. This phenomenon was the same as that of the motilities. In addition, we found that the expression of p-Smad2, p-Smad3,and Smad7 were increased by TCDD, *TGF-β3*, combination of TCDD and *TGF-β3*, but the expression of Smad4 were decreased by TCDD, *TGF-β3*, combination of TCDD and *TGF-β3*. These data revealed that TCDD and *TGF-β3* interacted and affected MEPM cells.

Keywords

TCDD, MEPM cells, *TGF-β3*, TGF-β/Smad signaling

Introduction

Palatogenesis is a tightly regulated process, in which multipotential mesenchymal cells can differentiate into chondrocytes to form cartilage.[1,2] The secondary palates of the 2 palatal shelves grow and fuse by mesenchymal cell proliferation in mammals. Interestingly, transforming growth factor β3 (*TGF-β3*) is expressed in the palatal mesenchyme during palatal growth and elevation.[3] Transforming growth factor β3 plays a very important role in a variety of cellular processes including cell proliferation, differentiation, apoptosis, migration, invasion, matrix synthesis, and immune response.[4-6] Transforming growth factor β3 was believed to play a very important role in the development of palatogenesis.[7] *TGF-β3* was an important to palatal fusion in mice transforming growth factor β3 was an important palatal fusion in mice and it had been shown that *TGF-β3*-knockout mice exhibited cleft palate,[8] but exogenous *TGF-β3* treated *TGF-β3*-knockout mice and was sufficient to rescue palatal fusion. Moreover, *TGF-β3* was also the most effective in inducing human palatal mesenchymal cell proliferation.[9] Transforming growth factor β3 can initiate diverse cellular responses by binding and activating specific cell-surface receptors, which also can activate TGF-β receptors to stimulate the phosphorylation of receptor-regulated Smad proteins, such as phosphorylation of transcription factors Smad2 and Smad3. Phospho-Smad2/3 (p-Smad2/3) in turn formed complexes with Smad4, which accumulated in the nucleus and regulate the transcription of target genes. The actions of TGF-β were antagonized by Smad7, which can prevent phosphorylation of Smad2/3, thereby blocking TGF-β/Smad signaling.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a well-known teratogenic effector of cleft palate. Morphological studies performed in vivo revealed that TCDD caused cleft palate by not only disturbing palatal shelf growth but also inhibiting

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the fusion of palatal shelves by a variety of effects.[10] Many genes played important roles in palatogenesis, such as *TGF-β3* and *KLF4*.[7,11] But there were few reports about the relationships between TCDD and *TGF-β3* in mouse embryonic palatal mesenchymal (MEPM) cells. In the present study, we found the possible relationships between TCDD and *TGF-β3* in MEPM cells.

Materials and Methods

Cell Culture and Treatment

MEPM cells were derived from palatal tissue on 13-day-old 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 method by Feng et al.[12] The MEPM cells were cultured in flasks with DMEM/F12 medium (Hyclon, Logan, Utah) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China). The MEPM cells were placed in a humidified incubator at 37°C in 5% CO_2 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.[13,14] Others were treated with 10 nM TCDD (DD-2378-S, Sigma, Saint Louis, Missouri), 10 ng/mL *TGF-β3* (cyt-143; PROSPEC, Zion, Israel), or a combination of 10 nM TCDD and 10 ng/mL *TGF-β3* for further analysis. Control cells were treated with DMSO (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 the expression of *TGF-β3*, first strand 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). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The qRT-PCR conditions were as follows: polymerase activation at 95°C 15 minutes and 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-β3*-forward: 5′-CCTGGCCCTGCTGAA CTTG-3′, and reverse, 5′-TTGATGTGGCCGAAGTCCAAC-3′; GAPDH-forward: 5′-TGACGTGCCGCCTGG AGAAAC-3′, and reverse, 5′-CCGGCATCGAAGGTGGAAGAG-3′.

Cell Viability Assays

To evaluate the effect of TCDD, the viabilities of MEPM cells were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide (MTT), (JT343; Genview, CA,) assay. The third passage of MEPM cells (5×10^3 cells per well) was seeded in 96-well plates (Nunc, Roskilde, Denmark). The cells were treated with 10 nM TCDD, 10 ng/mL *TGF-β3*, a combination of 10 nM TCDD and 10 ng/mL *TGF-β3*, or DMSO (≤0.05%). After 72 hours of incubation, the cell viabilities were detected by MTT assay according to the manufacturer's protocols.

Scratch Wound-Healing Motility Assay

Mouse embryonic palatal mesenchymal cells were seeded on 6-well plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and washed with PBS 3 times to remove cell debris. The cells were treated with 10 nM TCDD, 10 ng/mL *TGF-β3*, or a combination of 10 nM TCDD and 10 ng/mL *TGF-β3*. After being maintained 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 (http://rsb.info.nih.gov/ij/).

Western Blot Analysis

Total cellar protein extraction from different treated MEPM cells using 5 × sodium dodecyl sulfate-lysis buffer supplemented with protease inhibitors (M250; Amresco, Ohio). Protein concentration was determined using a standard BSA protein assay (DingGuo, Beijing, China). Then 40 μg of proteins were fractionated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis 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-CZ5616 R, Santa Cruz, California), Smad4 (PB0446; Boster Biotech, Wuhan, China), and Smad7 (sc-365846; Santa Cruz, California). β-Actin was probed as a loading control. Then membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibody (sc-2004 or sc-2005; Santa Cruz, California). 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 1-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 at $P < .05$.

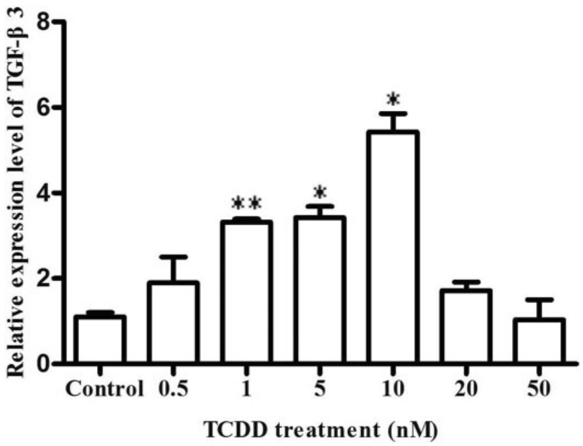

Figure 1. The effect of *TGF-β3* by TCDD induced in MEPM cells . 0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM TCDD, or DMSO (≤0.05%) treated MEPM cells as the experiment group and control group, respectively. After treatment for 72 hours, the expression of *TGF-β3* was measured by qRT-PCR. Data were mean values (standard deviation) of 3 replicate experiments. *$P < .05$ or **$P < .01$ versus the corresponding control values. TCDD indicates 2,3,7,8-tetrachlorodibenzo-p-dioxin; *TGF-β3*, transforming growth factor β3; MEPM, mouse embryonic palatal mesenchymal; 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.[15] We explored the expression of *TGF-β3* in MEPM cells using 0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM TCDD for 72 hours. As shown in Figure 1, we found that the mRNA levels of *TGF-β3* significantly increased 1.90 (0.86) fold, 3.32 (0.10) fold, 3.42 (0.37) fold, and 5.43 (0.61) fold in MEPM cells by 0.5 nM, 1 nM, 5 nM, and 10 nM TCDD induced compared with the corresponding control cells, respectively. The mRNA levels of *TGF-β3* decreased 1.72 (0.28) fold and 1.04 (0.66) fold at 20 nM and 50 nM TCDD compared with 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 a 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, TCDD group was decreased by 13.23%, compared with the corresponding control cells, but proliferation rate of *TGF-β3* group was increased by 56.34%, while the combination of TCDD and *TGF-β3* group of cells was increased by 11.56%.

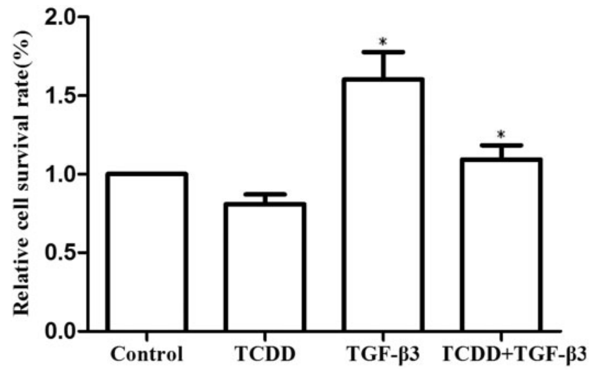

Figure 2. The effects of cell proliferation in MEPM cells. 10 nM TCDD, 10 ng/mL *TGF-β3*, or combination of 10 nM TCDD and 10 ng/mL *TGF-β3*, or DMSO (≤0.05%) treated MEPM cells as the experiment group and control group, respectively. After treatment for 72 hours, the cell proliferation was determined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

The Effects of Cell Motility in MEPM Cells

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

The Effects of TGF-β/Smad in MEPM Cells

Smads were intracellular effectors of TGF-β/Smad signaling. Smad2 and Smad3 can be activated and form heteromeric complexes with Smad4. Smad7 plays 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 genes in MEPM cells. As shown in Figure 4, we found that the expression of p-Smad2 increased 1.21 (0.02) fold, 1.38 (0.11) fold, and 1.31 (0.01) fold in 10 nM TCDD, 10 ng/mL *TGF-β3*, and combination of 10 nM TCDD and 10 ng/mL *TGF-β3* groups compared with the corresponding control cells, respectively. The expression of p-Smad3

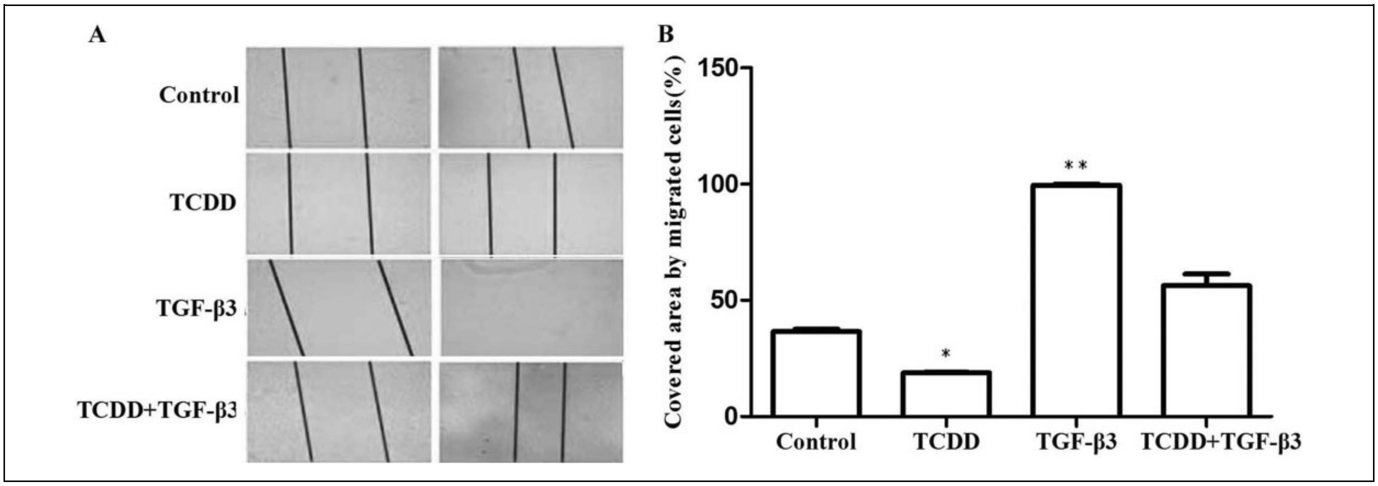

Figure 3. The effects of cell migration in MEPM cells. When MEPM cells were seeded and grew to confluence, the cells were scratched with a pipette tip, and 10 nM TCDD, 10 ng/mL *TGF-β3*, or combination of 10 nM TCDD and 10 ng/mL *TGF-β3*, or DMSO ($\leq$0.05%) treated MEPM cells as the experiment group 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 (standard deviation) of 3 replicate experiments. $^{*}P < .05$ or $^{**}P < .01$ versus the corresponding control values. TCDD indicates 2,3,7,8-tetrachlorodibenzo-p-dioxin; MEPM, mouse embryonic palatal mesenchymal.

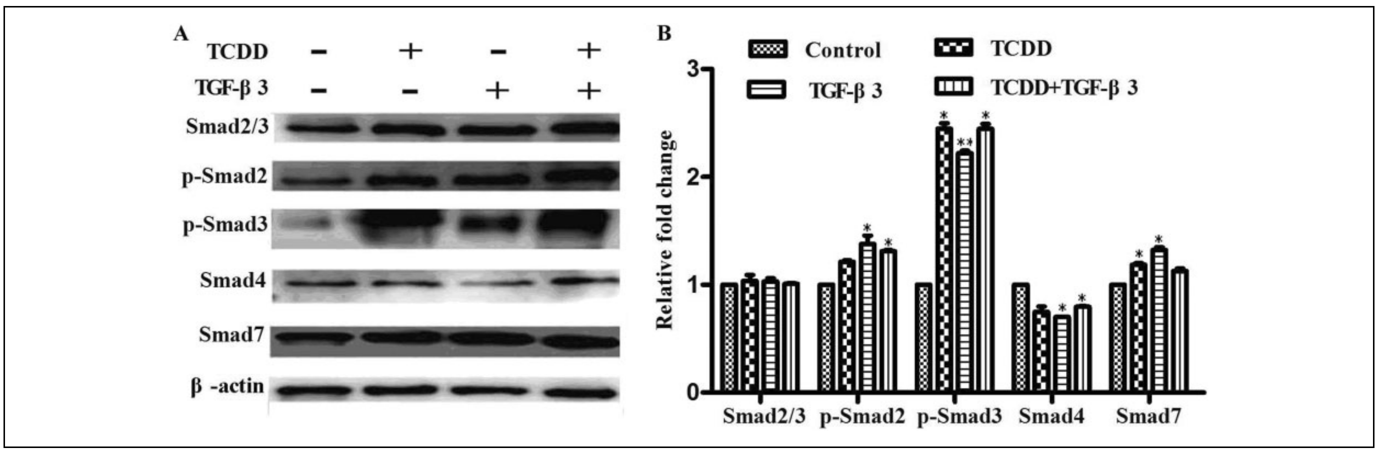

Figure 4. The effects of TGF-β/Smad Signaling molecules in MEPM cells. MEPM cells were treated with 10 nM TCDD, 10 ng/mL *TGF-β3*, and a combination of 10 nM TCDD and 10 ng/mL *TGF-β3*, or DMSO($\leq$0.05%), respectively. After 72 hours treatment, the expression of Smad2/3 and phosphorylation of Smad2 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 levels of Smad2/3, phosphorylation of Smad2, Smad3, and Smad7 proteins were calculated after normalization with the loading control β-actin. The data are the mean values (standard deviation) of 3 replicate experiments. $^{*}P < .05$ or $^{**}P < .01$ versus the corresponding control values. TCDD indicates 2,3,7,8-tetrachlorodibenzo-p-dioxin; *TGF-β3*, transforming growth factor β3; MEPM, mouse embryonic palatal mesenchymal.

increased 2.45 (0.07) fold, 2.22 (0.03) fold, and 2.45 (0.06) fold, respectively. But the expression of Smad4 decreased 0.25 (0.07) fold, 0.20 (0.001) fold, and 0.20 (0.006) fold, respectively. The expression of Smad7 increased 1.19 (0.02) fold, 1.33 (0.04) fold, and 1.13 (0.03) fold, respectively.

Discussion

The MEPM cells are very important in palatogenesis. Mouse embryonic palatal mesenchymal 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-*β*3 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,[16,17] and *TGF-β3* was also upregulated in the palatal tissues of people with cleft palate.[17] Moreover *TGF-β3* exposure completely prevented the dioxin-induced block of palatal fusion in this system.[18] Transforming growth factor β3/Smad signaling pathway may mediate cleft palate.[19-21] Until now, no reports have been published about the relationships between TCDD and *TGF-β3* in MEPM cells. We found that when MEPM cells were exposed to different concentrations of TCDD (0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, and 50 nM), 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 to the fact that *TGF-β3* expression upregulates cleft palates induced by TCDD in mice,[17] which implicated that the *TGF-β3*/Smad signaling pathway might have an important role in MEPM cells by TCDD and *TGF-β3* induced.

The relationship between TCDD and *TGF-β3* remained obscure. Very low-dose TCDD can affect palatogenesis and lead to malformations in the early stages of cartilage development.[22] Numerous studies have demonstrated that TCDD inhibited programmed cell death of MEPM cells in palatal shelves, and TCDD can alter MEPM cell differentiation.[23-25] However, few reports revealed that TCDD and *TGF-β3* explored to MEPM cells. In the current study, we focused on the interactive effects between TCDD and *TGF-β3*. We found that TCDD inhibited the MEPM cell proliferation, which was same that TCDD inhibited urogenital sinus mesenchymal cell proliferation.[26] However, 10 ng/mL *TGF-β3* promoted the MEPM cell proliferation, which is consistent with the fact that *TGF-β3* can stimulate the proliferation of mesenchymal cells in vaginal thread.[27] The combination of 10 nM TCDD and 10 ng/mL *TGF-β3* also promoted MEPM cell proliferation, but the rate of cell viability was between that of TCDD or *TGF-β3* treated alone, which might be consistent with *TGF-β3* as an effective antidote to dioxin-induced MEPM cells.[28] This phenomenon was same as the effect of motilities in MEPM cells by 10 nM TCDD, 10 ng/mL *TGF-β3*, or a combination of 10 nM TCDD and 10 ng/mL *TGF-β3*. TCDD inhibited the MEPM cell motility, which concurred with TCDD-mediated the inhibition of MCF-7 cell motility. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibited the MEPM cell motility, which concurred with TCDD-mediated the inhibition of MCF-7 cell.[29] *TGF-β3* promoted MEPM cell motility which are aligned to *TGF-β3* activating the palatal midline epithelial seam cell motility. Transforming growth factor β3 promoted MEPM cell motility which are aligned to *TGF-β3* activating the palatal midline epithelial seam cell.[30] The combination of TCDD and *TGF-β3* also promoted MEPM cell motility and the combination of TCDD and *TGF-β3* also promoted MEPM cell motility, which might be the *TGF-β3* playing mainly important role in MEPM cells to antagonize TCDD.

2,3,7,8-Tetrachlorodibenzo-p-dioxin increased the mRNA expression of *TGF-β3* in MEPM cells, and TCDD might function through the TGF-β/Smad pathway. Phospho-Smad2/3 can bind Smad4 to form a heteromeric complex in the nucleus, where they act as transcription factors.[31] Smad7 was an inhibitor of TGF-β/Smad signaling pathway.[32] The current study showed that TCDD increased the expression of p-Smad2/3 and inhibited the expression of Smad4. TGF-β/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. Which suggests TCDD and *TGF-β3* mediated TGF-β/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.[13] The mRNA levels of Smad2, Smad3, Smad4, and Smad7 were lower in MEPM cells by TCDD induced compared with the corresponding control. They believed that TCDD did not make function by TGF-β/Smad signaling.[13,33] 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 posttranscriptional level. However, TCDD receptor (Aryl hydrocarbon receptor, AhR) can inhibit the downregulation of the TGF-β/Smad pathway in human glioblastoma cells.[34] Expression of genes in the TGF-β signaling pathway is significantly deregulated in smooth muscle cells from aorta of AhR knockout mice by TCDD induced.[35] Moreover, inhibition of TGF-β signaling pathway also inhibited TCDD-induced Treg activity.[36]

In conclusion, we found that TCDD and *TGF-β3* might have a negative relationship in MEPM cells by TGF-*β*/Smad signaling, but the precise mechanisms of TCDD and *TGF-β3* mediated-cleft palate in MEPM cells require further investigation.

Authors' Note

Liyun Gao conceived the original idea and designed and performed the experiments.

Declaration of Conflicting Interests

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

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