

## Transforming Growth Factor- $\beta$ 3 Restores Fusion in Palatal Shelves Exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin\*

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The pollutant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (“dioxin”), has been implicated in the etiology of a wide variety of human birth defects. In an effort to identify pharmacological blockers of dioxin-induced terata, we performed a histological and microscopic analysis of the developing murine palate that had been exposed to dioxin. In both *in vivo* and *in vitro* model systems, we observed that dioxin exposure leads to a reduction in the number of filopodial extensions at the medial epithelial edge of the developing palate. Given that this filopodial aberration is similar to the phenotype observed in *Tgfb3* null mice, a mutant known to display a 100% incidence of cleft palate, we examined the interaction between TGF $\beta$ 3 and dioxin in palatal fusion. We found that the addition of TGF $\beta$ 3 to an *in vitro* palate culture model prevented the dioxin-induced reduction in filopodial density. Moreover, TGF $\beta$ 3 exposure completely prevented the dioxin-induced block of palatal fusion in this system. Although these data do not point to a direct cellular or molecular mechanism for TGF $\beta$ 3 dioxin antagonism, these results do suggest that TGF $\beta$ 3 or stimulators of this signaling pathway hold potential as antidotes for dioxin-induced terata and that this opposing pharmacology may extend to additional toxicological endpoints.

Halogenated dioxins and related halogenated dibenzofurans are persistent chemicals that are widely dispersed in the global environment (1, 2). These compounds are introduced as byproducts of certain industrial processes as the result of the municipal handling of waste materials or because of their trace contamination of certain commercial products.<sup>1</sup> In addition to chronic sources of exposure, there have been several environmental accidents in which human populations have been exposed to higher concentrations of these contaminants. These include the exposure of citizens and soldiers during the Vietnam War to dioxin<sup>2</sup> contamination of the defoliant known as “Agent Orange” (3–6), the exposure of populations in both

China and Japan when halogenated dioxins and halogenated dibenzofurans were accidentally introduced into cooking oil (7–9), and the exposure of citizens of Seveso, Italy after an explosion at a nearby chlorinated-phenol plant (10–12).

Epidemiological studies performed after many of these high exposure incidents suggest that halogenated dioxins and halogenated dibenzofurans are linked to human toxicity and increases in congenital anomalies (13, 14). Putative responses include changes in menses, low sperm count, and delayed time to pregnancy (15, 16). Evidence of human teratogenicity from halogenated dioxins and halogenated dibenzofurans include the following: 1) reports of hyperpigmentation, gingival hyperplasia, hyperkeratosis, skull calcification, and perinatal teeth (17); 2) reports of abnormalities in musculoskeletal development (18–20); 3) reports of low birth weight and length (18); 4) reports of fragile teeth and finger and toenail abnormalities (18); and 5) reports of delayed cognitive and behavioral development (21–23). Thus, existing epidemiological data suggest that prenatal and postnatal populations are sensitive to halogenated dioxin/halogenated dibenzofuran exposure and that these compounds may be significant human teratogens.

The identification of blockers of dioxin-induced toxicity/teratogenicity could have significant benefit to human and wildlife populations. If such antidotes can be developed with a high therapeutic index, they could be used to minimize consequences in situations where dioxin exposure is known and an individual or developing fetus is at high risk. To work toward this goal, we have begun using late stage developmental endpoints and *in vitro* organ culture models in a search for pharmacological blockers of dioxin teratogenicity. In this effort, we identified TGF $\beta$ 3 as an effective antidote to dioxin-induced cleft palate.

### MATERIALS AND METHODS

**Mice**—Animals were housed in a specific pathogen-free facility on corn-cob bedding with food and water *ad libitum* according to the rules and regulations set by the University of Wisconsin. All of the C57BL/6J mice were obtained originally from Jackson Laboratories (Bar Harbor, ME) and bred in-house.

**Cleft Palate Assessment in Vivo**—Female mice were weighed at E0. The E0 time point is defined as the time directly prior to females being mated to male mice for 12 h. At E10, females were weighed and those gaining at least two grams were assumed pregnant. Pregnant females were dosed intraperitoneally with 64.0  $\mu$ g of dioxin/kg body weight. At E18, the embryos were harvested. Normal palatogenesis was assessed based on gross examination of the palate surface after an incision was made through the temporal-mandibular joint. A diagnosis of “cleft palate” was made if there was no fusion between the secondary palatal shelves.

**Scanning Electron Microscopy**—Scanning electron microscopy (SE) was used to characterize the fine surface structure of the palate. Micrographs were taken at the surface of the palate at E18.5 and at the medial edge epithelia (MEE) at E13.5. The SE was performed according to previously described methods (24). Samples were fixed for 12 h in modified Karnovsky’s fixative (30% paraformaldehyde, 60% glutaraldehyde, 30% 0.2 M cacodylate buffer, pH 7.4). Samples were subsequently

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<sup>1</sup> Data base of Sources of Environmental Releases of Dioxin-like compounds in the United States/EPA/2001.

<sup>2</sup> The abbreviations used are: dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF $\beta$ 3, transforming growth factor- $\beta$ 3; MEE, medial edge epithelia; E, embryonic day; Me<sub>2</sub>SO, dimethyl sulfoxide; TEM, transmission electron microscopy.

washed three times for 20 min in 0.1 M cacodylate, pH 7.4, 7.5% sucrose. After washing, the samples were incubated overnight in 2% OsO<sub>4</sub> and then washed three times for 15 min in H<sub>2</sub>O. The embryos were dehydrated through a graded series of ethanol for 10 min each until the specimen was stored in 100% ethanol. After dehydration, the samples were dried with a Samdri-780A Supercritical Point Dryer (Tousimis, Rockville, MD). Samples were "gold sputter-coated" using an Autoconductivevac IV (See Vac, Pittsburgh, PA), and images were viewed on a Hitachi S-570 with a La<sub>B</sub> emitter (Hitachi, Schaumburg, IL).

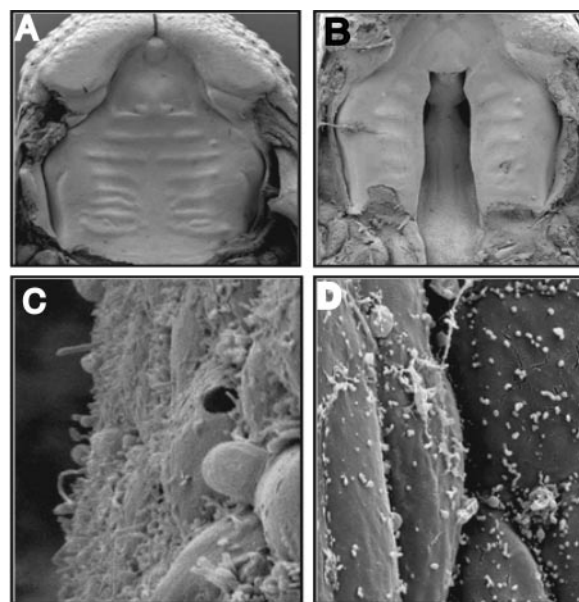
**Transmission Electron Microscopy**—Palatal shelves were fixed, processed, and viewed by transmission electron microscopy (TEM). Samples were immersed for fixation in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M NaPO<sub>4</sub> buffer (v/v), pH 7.4) for 2 h at 4 °C. Following fixation, the samples were washed in rinsing buffer (0.1 M NaPO<sub>4</sub>) and post-fixed in 2% OsO<sub>4</sub> buffered in 0.1 M NaPO<sub>4</sub> buffer for 1 h at 25 °C. Post-fixed samples were washed in rinsing buffer and dehydrated in a graded series of ethanol baths at 50, 70, 80, 90, and 95% for 7 min each step. The final dehydration was performed three times for 12 min each in 100% ethanol. All of the dehydration steps were performed at 25 °C. Final dehydration was performed twice for 7 min at 25 °C using propylene oxide as a transition solvent. Dehydrated samples were infiltrated in mixture EMbed-812 and Spurr's Low Viscosity embedding media (Electron Microscopy Sciences, Fort Washington, PA). All of the infiltration steps used a 1:1 mixture of EMbed-812 and Spurr's medium. Ultrathin sections (70 nm thickness) were collected on Pioloform (Ted Pella, Inc., Redding, CA)-coated 2 × 1-mm aperture copper electron microscope grids (Electron Microscopy Sciences). Ultrathin sectioning was performed on a Reichert-Jung Ultracut E Ultra Microtome. The sections were post-stained in uranyl acetate and lead citrate. The samples were viewed and documented on a Philips CM120 at 80 kV. Filopodial extensions and cellularity were quantified directly from the TEM micrographs and histology slides, respectively. Light microscope sections were cut at 0.5 μm and stained with 1% methylene blue and 1% AzureII.

**Palate Organ Culture**—C57BL/6J female mice were weighed at gestation day 0 and mated via cohabitation with a C57BL/6J male for 6 h. The E13.0 embryos were harvested from the uterus, and palatal shelves were dissected from the embryos. Shelves were separated from the embryo proper by an incision at the mandibular joint, and the cerebrum and the cerebellum were dissected away from the palatal shelves. The remaining opposing shelves were placed into cold phosphate-buffered saline. Suspended palate organ cultures were performed as described previously (25, 26). Shelves were cultured in 1:1 Dulbecco's modified Eagle's/F-12 medium supplemented with 1% L-glutamine, 1% ascorbate, and 1% penicillin/streptomycin. Cultures were treated with Me<sub>2</sub>SO, 3.3 nM dioxin in Me<sub>2</sub>SO, saline, and/or 10 ng/ml recombinant human TGF $\beta$ 3 in saline (R&D Systems, Minneapolis, MN). After 72 h, the organ cultures were fixed in 10% formalin and stained with hematoxylin for visualization or cultured for 48 h and processed for transmission electron microscopy. Histological examination was performed through hematoxylin and eosin-stained 5-μm serial sections of each culture condition in triplicate.

## RESULTS AND DISCUSSION

**Known Teratology of Dioxins**—Halogenated dioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, have proven to be powerful tools in understanding environmentally mediated teratogenesis. This power stems from the extensive pharmacological, genetic, and molecular evidence that the teratogenicity of dioxin is mediated through a ligand-activated transcription factor known as the aryl hydrocarbon receptor (AHR) (27–31). Two lines of experimental evidence provide compelling proof for the role of AHR in dioxin-induced terata. First, dioxin congeners that bind most avidly to the AHR are the most potent with regard to the induction of both cleft palate and hydronephrosis (32–36). Second, a naturally occurring polymorphism in the gene encoding the murine AHR yields mouse strains that bind dioxin with both high and low affinity (37–39). Mice with the high affinity receptor are ten times more sensitive to the teratogenic effects of dioxin (27). It is the working model of our laboratory that dioxin-induced up-regulation of AHR-transcriptional targets is responsible for teratological endpoints such as cleft palate (41).

An understanding of a toxicants mechanism of action has

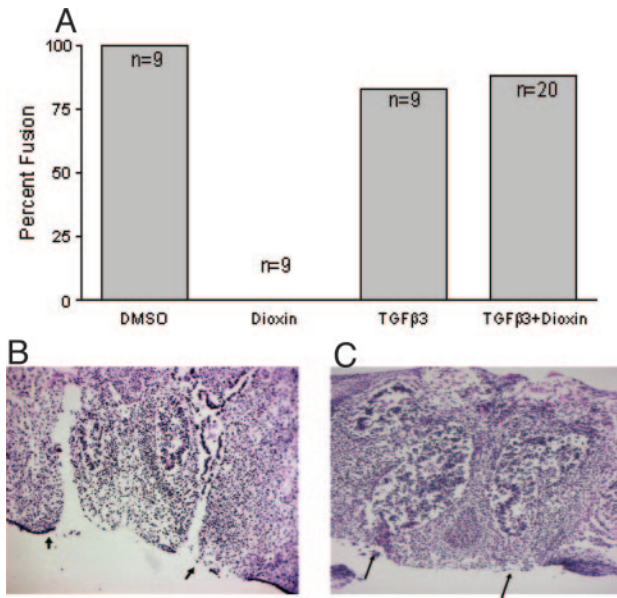


**FIG. 1. Scanning electron microscopy of palate and MEE.** Pregnant dams were dosed at E10 with Me<sub>2</sub>SO or 64 μg/kg dioxin. At E18, the embryos were harvested from Me<sub>2</sub>SO and treated dams. An incision was made through the temporal-mandibular joint, and the palates were processed for SE. **A**, Me<sub>2</sub>SO-exposed embryos exhibit normal palatogenesis. **B**, dioxin-exposed embryos show a complete cleft of the secondary palate. A second set of dams was dosed with Me<sub>2</sub>SO or 64 μg/kg dioxin, and the embryos were harvested at E13 prior to fusion. **C**, Me<sub>2</sub>SO-exposed embryos show numerous filopodia at the MEE surface (×7000). **D**, dioxin-exposed embryos show a fewer number of filopodia at the MEE surface, and the extensions that remain are shorter (×7000).

two major values. The most common application of mechanistic information is to help in estimating risk from exposure (42). For example, in the case of dioxin toxicology, mechanistic information has been used to model the dose response curve for tumor promotion based upon data from surrogate transcriptional endpoints, such as interleukin-1 $\alpha$ , plasminogen activator-1, and cytochromes P450 (43). A second use of mechanistic information in toxicology is to aid in the design of intervention strategies that can be employed in the case of accidental exposure, *i.e.* to help develop antidotes. A classic example of an antidote being developed based on mechanistic insight is the treatment of acetaminophen overdose by the permeable thiol drugs diethylthiocarbamate and dithiothreitol (44, 45). It is our proposal that similar antidotes can be developed for highly toxic receptor-mediated toxicants/teratogens such as dioxin.

**Novel Teratology of Dioxin**—Many laboratories have established that dioxin is a potent teratogen in the mouse (46–50). In an effort to better understand this model system, we initiated a thorough description of the cellular phenotypes that occur in dioxin-treated palates. The rationale was that this characterization could provide insights into the mechanism that underlies dioxin-induced teratogenesis. Earlier studies have defined a critical developmental window between E9.0 and E12.0 where the fetus is most sensitive to dioxin-induced cleft palate (50). Based on these earlier studies, we induced cleft palate *in vivo* by administration of 64 μg/kg dioxin at E10. Consistent with earlier reports, we found these conditions to yield a 100% frequency of cleft palate (data not shown). Because dioxin-induced cleft palate is known to result from failure of the palatal shelves to fuse, we found it to be present as a binary phenotype. That is, we observed either complete separation of the palatal shelves or normal fusion when assessed at E18, (Fig. 1).

In addition to the well described clefting of the secondary palate, we observed that dioxin exposure leads to a number of

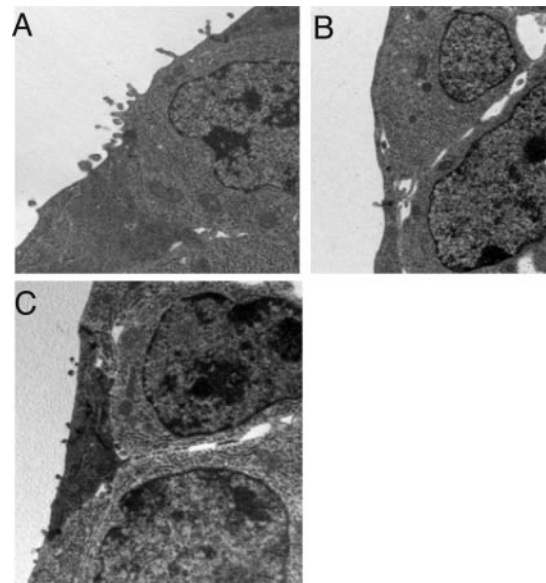


**FIG. 2. TGF $\beta$ 3 restores fusion to dioxin-treated palate culture.** Dioxin was used at a concentration of 3 nM, and TGF $\beta$ 3 was used at 10 ng/ml. After 72 h, fusion was assessed by both gross and histological examination. *A*, TGF $\beta$ 3 rescued fusion of palatal shelves 90% of the time compared with 0% when treated with dioxin. The Me<sub>2</sub>SO (*DMSO*) palates fused 100% of the time. *B*, palate fusion fails after 72 h with the addition of 3.3 nM dioxin. *Arrowheads* indicate individual palate shelves. *C*, palate fusion occurs after 72 h with the addition of 3.3 nM dioxin and 10 ng/ml TGF $\beta$ 3. *Arrows* indicate fusion between the nasal septum and the palatal shelves.

additional endpoints that had not been previously described. These endpoints include a “heart”-shaped morphology and disorganized muscle pattern of the tongue, hypermaturation of hair follicles, absence of rugae in the posterior secondary palate, and keratinization of the hair follicles and the MEE (data not shown). Most important for the experiments described here was the observation under electron microscopy of a reduction in number and length the filopodia at the MEE of the developing palate (Fig. 1, *C* and *D*).

**Dioxin and TGF $\beta$ 3 Opposition**—The reduction in filopodia at the MEE was of interest for two reasons. First, this phenotype has previously been described in the TGF $\beta$ 3 null mouse model (25, 52, 53). Second, a reduction in filopodia is consistent with a failure to fuse mechanism (52, 54, 55). It has been proposed that the filopodia at the MEE intercalate and hold the shelves in proximity, allowing fusion to proceed (53). The similarities of the palatal phenotypes between TGF $\beta$ 3 null mouse embryos and those dosed *in utero* with dioxin prompted us to examine this relationship further. In particular, this observation suggested an opposing effect of TGF $\beta$ 3 and dioxin. This led us to examine whether exogenous TGF $\beta$ 3 would restore normal fusion to dioxin-treated palates. Although some attempts were made at maternal dosing regimens for TGF $\beta$ 3, limited knowledge with respect to TGF $\beta$ 3 transport across the placenta led us to test our ideas in a more defined system. To this end, palatal shelves were excised from E13.0 embryos and placed into a suspended culture system (26, 56). Palate fusion was scored with a dissecting microscope and verified by histological examination of hematoxylin and eosin-stained serial sections through the palate organ tissue (Fig. 2, *B* and *C*). In this system, 3.3 nM dioxin completely prevents palatal fusion (Fig. 2*A*) (57, 58). More importantly, we observed that the addition of 10 ng/ml TGF $\beta$ 3 concomitant with 3.3 nM dioxin blocked the teratogenic effects of dioxin and restored palatal fusion to 90% (Fig. 2*A*).

We hypothesized that TGF $\beta$ 3 may rescue fusion in dioxin-



**FIG. 3. Transmission electron microscopy to assess filopodial extensions.** TEM was used to assess the presence or absence of filopodial extensions at the MEE of palate shelves in culture. Cultures were treated with Me<sub>2</sub>SO, dioxin, or dioxin + TGF $\beta$ 3 as previously described. *A*, representative micrograph of Me<sub>2</sub>SO-treated palate culture. *B*, representative micrograph of dioxin-treated palate culture. There is an absence of filopodial extensions. *C*, representative micrograph of dioxin + TGF $\beta$ 3-treated palate culture.

treated palate cultures through a restoration of filopodial extensions at the MEE (25). Therefore, the embryonic palatal tissues were treated with Me<sub>2</sub>SO, dioxin, and dioxin + TGF $\beta$ 3 and scored for density of filopodia at the MEE (Fig. 3, *A–C*). This quantification was performed across 242  $\mu$ m of the MEE surface extending equilaterally from the tip of the palatal shelf. The average number of filopodial extensions per shelf for a Me<sub>2</sub>SO-treated, dioxin-treated, and dioxin + TGF $\beta$ 3-treated culture was 234, 70, and 133, respectively (Fig. 4). That is, dioxin exposure led to a 3-fold reduction in the number of filopodial extensions at the MEE compared with the control. More importantly, the addition of TGF $\beta$ 3 with the dioxin treatment doubled the number of filopodial extensions compared with cultures treated with dioxin alone.

With respect to the cell biology of the MEE, the influence of dioxin and the opposing effects of TGF $\beta$ 3 extend beyond the surface of the apical membrane. We also observed that dioxin exposure increases the cellularity of the MEE and that cotreatment with TGF $\beta$ 3 normalizes this cellularity (Fig. 5). In this regard, the cell number of the MEE was counted across 81  $\mu$ m of MEE surface extending equally from the tip of the palatal shelves. These data showed a doubling in the number of cells at the medial edge of the palate in dioxin *versus* Me<sub>2</sub>SO-treated cultures ( $p < 0.003$ ). Yet, when TGF $\beta$ 3 was added along with dioxin, the cellularity was indistinguishable from control (Fig. 5).

**Potential Mechanism**—Our results suggest that dioxin causes cleft palate through its ability to block the exposure, maintenance, or formation of filopodial extensions at the MEE. This model is consistent with evidence from other laboratories, indicating that intercalation of filopodia at the MEE is essential for adherence of the opposing shelves and normal palatal fusion (25, 53, 59). In this regard, evidence has been previously published (60) to indicate that apoptosis of the outer or “peridermal” cell layer of the MEE leads to exposure of the basal epithelial cell layer, which then displays the filopodia required for palatal adherence and fusion. It has also been shown previously that dioxin exposure enhances proliferation of the MEE, prevents the degeneration of the peridermal layer, and

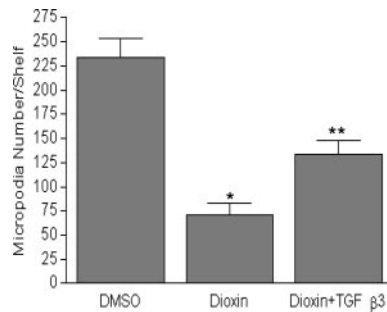


FIG. 4. **Quantification of filopodial extensions.** Filopodial extensions were quantified directly from the TEM micrographs. Filopodial extensions were quantified across 242  $\mu$ m of shelf surface from each treatment condition. The bars on the histogram represent the mean filopodia number per shelf performed in triplicate for each treatment. Me<sub>2</sub>SO (DMSO) had significantly more filopodia than dioxin- or dioxin + TGF $\beta$ 3-treated palate cultures,  $p < 0.0001$  and  $p < 0.002$ , respectively. The dioxin + TGF $\beta$ 3-treated palate shelves had significantly more filopodial extensions than the dioxin-treated cultures ( $p < 0.003$ ).

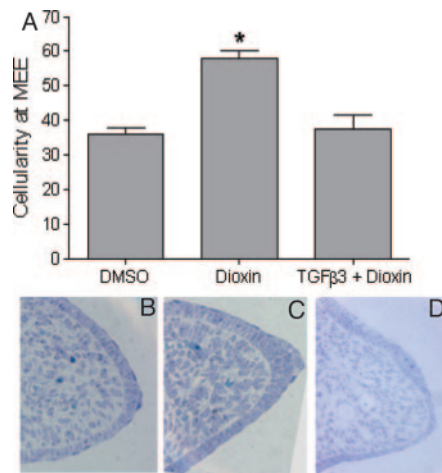


FIG. 5. **Cellularity of medial edge epithelia.** Serial sections of the palate organ cultures of each treatment were assessed to examine the morphology of the MEE. Cell counts were assessed across 81  $\mu$ m of shelf surface extending equally from the tip of the palatal shelves. The experiment was performed in triplicate, and the pictures are representative. A, histogram of the cell quantification at the medial edge of the palate shelf. The mean cellularity at the Me<sub>2</sub>SO- (DMSO), dioxin-, and TGF $\beta$ 3 + dioxin-treated shelves was 38, 58, and 38, respectively. The cellularity of shelves treated with dioxin was significantly increased (analysis of variance (ANOVA),  $p < 0.003$ ). B, the Me<sub>2</sub>SO-treated palate shelf. C, dioxin-treated palate shelf. D, dioxin + TGF $\beta$ 3-treated palate shelf.

can cause the periderm to differentiate into a stratified squamous epithelium (58). This description of the dioxin-induced response of the MEE is similar to the increase in MEE cellularity reported here (Fig. 5). We have further characterized the dioxin-induced palatal phenotype by showing that dioxin causes a reduction in filopodia at the MEE (Figs. 3 and 4). Taken together, these data are in line with a model where dioxin alters the presentation of filopodia at the MEE and prevents palatal fusion. The mechanism by which dioxin influences MEE cell biology may be through a combination of its known activities on epithelia, including stimulation of hyperplasia, metaplasia, and inhibition of apoptosis (58, 61–65).

These data are consistent with the idea that the mechanism for the opposition of TGF $\beta$ 3 and dioxin lies at the level of epithelial cell proliferation and differentiation. The observation that TGF $\beta$ 3 can block dioxin-induced cleft palate is also in line with the possibility that these two signaling molecules stimulate pathways that represent opposing forces in the presentation of filopodial extensions. Importantly, the MEE in TGF $\beta$ 3

knock-out mice shows decreased apoptosis and an attenuated epithelial-mesenchymal transition, leading to impaired intercellular adhesion and failure of palatal fusion (66).

At the present time, we do not know the molecular details of the dioxin-TGF $\beta$ 3 opposition. In preliminary experiments, we examined the hypothesis that dioxin inhibits intracellular signals initiated by TGF $\beta$ 3. To this end, we monitored levels of TGF $\beta$ 3 signaling through Western blot analysis of SMAD2 phosphorylation (67). Using the HepG2 cell culture model, we were unable to demonstrate any dioxin-induced interference on TGF $\beta$ 3 signaling. That is, in two independent experiments, TGF $\beta$ 3-induced SMAD2 phosphorylation was unaffected by dioxin treatment.<sup>3</sup> These preliminary data lead us to suggest that dioxin does not directly inhibit intracellular TGF $\beta$ 3 signaling but may influence this pathway via an indirect mechanism. It is also possible that TGF $\beta$ 3 interferes with dioxin signaling. In this regard, others (68) have reported evidence that TGF $\beta$ 3 influences the expression from the AHR promoter in a cell-specific manner.

**Conclusions**—These experiments demonstrate that dioxin and TGF $\beta$ 3 have opposing effects on the developing MEE. Specifically, filopodial density and palatal fusion are reduced, whereas MEE cellularity is increased, by dioxin exposure. These three toxic endpoints are all rescued when TGF $\beta$ 3 is coadministered with dioxin. At the present time, we do not know whether this opposition of TGF $\beta$ 3 extends to other dioxin-induced teratological or toxic endpoints that are relevant to the human population, such as hydronephrosis, porphyria, or chloracne. Given that such dioxin-induced endpoints occur at different doses and routes of administration, they were not examined in this study (69–71). Although these results are preliminary, they do demonstrate the potential for TGF $\beta$ 3 or its analogues to serve as part of interventional strategies used to treat acute dioxin exposure. Such potential may be realized if methods of safely delivering TGF $\beta$ 3 can be developed (40, 51, 72).

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