Teratogenic Drug Topiramate Upregulates TGFβ1 and SOX9 Expression in Primary Palatal Mesenchyme Cells

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Abstract

Topiramate is a commonly prescribed anti-epileptic drug with over 8 million prescriptions dispensed annually. Topiramate use during pregnancy has been linked to significantly increased risk of babies born with orofacial clefts (OFCs). However, the exact molecular mechanism of topiramate teratogenicity is unknown. We used an unbiased antibody array analysis to test the effect of topiramate on human embryonic palatal mesenchyme (HEPM) cells. This analysis identified 40 differentially expressed proteins, showing strong connectivity to known OFC genes. However, among known OFC genes, only TGFβ1 was significantly upregulated in the antibody array analysis. Next, we validated that topiramate could increase expression of TGFβ1 and of downstream target phospho-SMAD2 in primary mouse embryonic palatal mesenchyme (MEPM) cells. Furthermore, we showed that topiramate treatment of primary MEPM cells increased expression of SOX9. SOX9 overexpression in chondrocytes is known to cause cleft palate in mouse. We propose that topiramate mediates upregulation of TGFβ1 signaling through activation of γ-aminobutyric acid (GABA) receptors in the palate. TGFβ1 and SOX9 play critical roles in orofacial morphogenesis, and their abnormal overexpression may underlie the teratogenic effects of topiramate.

Keywords

Topiramate, birth defects, teratogenicity, TGFβ signaling, orofacial clefts, cleft lip and palate

Introduction

Topiramate is an anti-epileptic drug that was approved by the U.S. Food and Drug Administration (FDA) for the treatment of partial onset or primary generalized tonic-clonic seizures in 1996 and for migraine prophylaxis in 2004. Topiramate is currently used either as a monotherapy or an adjunctive therapy to treat partial-onset seizures, primary generalized tonic-clonic seizures, and other seizure disorders. In addition, topiramate has been used off-label for binge-eating disorder [1], bulimia nervosa [2], alcohol use disorder [3], anti-psychotic induced weight gain [4], and essential tremor [5]. Verispan's Vector One®: National (VONA) and IQVIA's Total Patient Tracker® (TPT) reported that, between January 2007 and December 2010, approximately 4.3 million patients filled 32.3 million topiramate prescriptions (FDA Drug Safety Communication, 03-04-2011). This number has risen to approximately 4.1 million patients receiving a prescription for topiramate between March 2014 and February 2016 (FDA Pediatric Postmarketing Pharmacovigilance and Drug Utilization Review for topiramate; June 20, 2016). According to estimates in the United States alone, approximately 1.3 million epileptic women are of childbearing age [6], and approximately 24,000 children are born annually to epileptic mothers (North American Antiepileptic Drug Pregnancy Registry) [7]. Thus, control of seizures during pregnancy is an important public health challenge, as women with epilepsy have a higher risk of peripartum complications including stillbirth, preeclampsia, preterm labor, and a 10-fold increase in mortality compared to women without epilepsy [8].

Topiramate use during pregnancy has been linked to a significantly increased risk of birth defects affecting orofacial, cardiac and urogenital development [9-11]. Multiple studies have reported that the incidence of oral clefts in particular is increased in topiramate-exposed pregnancies [9, 10, 12,

13]. In the North American Antiepileptic Drug Pregnancy Registry [14], the relative risk of oral clefts in topiramate-exposed pregnancies was ~13-fold higher than the general risk in births [15]. The FDA has now changed the classification of topiramate from a pregnancy-C category to a pregnancy-D category drug, warning that topiramate can cause fetal harm when administered to a pregnant woman (FDA Drug Safety Communication, 03-04-2011). Despite broad use and teratogenic potential of topiramate, the molecular mechanism(s) underlying the increased occurrence of major congenital malformations is not understood [6, 7, 10, 12, 16].

Normal palate development during embryogenesis is a multistep process that begins with bilateral vertical growth of the palatal shelves adjacent to the tongue till embryonic day 13.5 (E13.5). These shelves then elevate above the tongue, move horizontally and fuse in the midline to form the palate by E15.5. The palatal shelf is mainly composed of the oral ectoderm derived epithelial cells and the neural crest derived mesenchymal cells. Defects in neural crest function are sufficient to cause cleft palate [17, 18]. To investigate the potential effect of topiramate exposure on palate development, we first treated human embryonic palate mesenchyme (HEPM) cells with a high dose of topiramate, then performed an unbiased exploratory antibody-array approach to identify misregulated proteins. Our analysis showed upregulation of transforming growth factor beta one (TGFβ1) expression and altered activation of cell survival networks. We then validated our findings in primary mouse embryonic palate mesenchyme (MEPM) cells following exposure to a range of topiramate concentrations. We showed that TGFB1 levels are indeed upregulated even at physiological 50μM topiramate treatment for six hours. Topiramate treatment of primary MEPM cells also increased expression of SOX9, a known clefting gene and TGFB1 target. We propose that perturbation of TGFβ pathway and SOX9 expression through γ-aminobutyric acid

(GABA) receptors in the palate represents a plausible etiologic mechanism underlying topiramate-induced oral clefts.

Materials and Methods

Culture of HEPM cell line

Human embryonic palatal mesenchyme (HEPM) cells (ATCC, CRL-1486) were cultured in DMEM media, with high concentration of glucose and pyruvate, supplemented with 10% fetal bovine serum and penicillin/streptomycin. Topiramate (Sigma-Aldrich, St. Louis, MO) was resuspended in ethanol. HEPM cells were treated with 1000μM topiramate and cultured at 37°C for 6 hours. Cells were briefly washed with PBS, scraped and flash-frozen for subsequent RNA and protein extraction.

Isolation and culture of Primary MEPM Cells

Palatal shelves were excised from wildtype E13.5 mouse embryos. The mesenchyme was dissociated into a single-cell suspension by incubating the palatal shelves in 0.25% Trypsin for 10 minutes and then vigorously pipetting up and down to mechanically separate the cells. The resulting Mouse Embryonic Palatal Mesenchyme (MEPM) cells were then cultured in DMEM media with high glucose and supplemented with 10% Fetal Bovine Serum and Penicillin/Streptomycin. MEPMs were used fresh, never-passaged, before drug treatment. MEPM cells were treated with 25, 50 or 100 μM topiramate (ApexBio, Houston, TX) and incubated for 6 hours at 37°C. Ethanol was used for vehicle treatment.

Antibody array analysis

Protein extracts from HEPM cells with vehicle (Control) or with topiramate treatment (1000 μM for 6 hours) were analyzed by Full Moon BioSystems (Sunnyvale, CA) Cell Signaling Explorer antibody arrays (SET100), according to manufacturer's protocol. Briefly, control and topiramate-treated HEPM cell lysates were labeled with Cy3 fluorophore using the antibody array assay kit from Full Moon BioSystems (KAS02). Antibody array slides were independently incubated with labeled lysates, washed, and scanned using microarray scanner from Agilent (Santa Clara, CA).

Statistical analysis of antibody array data

Each antibody array comprised of two technical replicates. The experiment was performed in biological duplicates for downstream statistical analysis. Each probe from each of the biological and technical replicates was first determined to be significantly expressed relative to its background intensity. This significance calculation was based on a ≤ 0.05 cutoff of the Benjamini and Hochberg [19] adjusted p-value of the t-statistic of the difference between the mean expression (F532 mean) and mean background (B532 mean) intensities. A gene was regarded as not significantly expressed under a particular treatment if any one of its biological replicates was not significantly expressed. The first filtering step removed all genes that were not significantly expressed in either one of the treatment groups. The remaining probes were background corrected using a modified Robust Multi-array Average (RMA) [20] algorithm for protein arrays. The background adjusted probes were log transformed (base 2) and quantile normalized. Technical replicates were then averaged (geometric average) to give the background adjusted normalized expression for each biological replicate. A two-way ANOVA model (factor 1: treatment with levels Control and topiramate treated; factor 2: antibody array with levels array 1 and array 2) was fitted to the data to determine the gene level significance of the difference in expression between

Control and topiramate treated samples. Protein expression with a p-value ≤ 0.05 and absolute fold change ≥ 1.15 were deemed sufficiently differentially expressed for protein arrays, yielding 40 proteins for further analysis.

Gene interaction analysis

The Ingenuity Pathway Analysis software (IPA, Qiagen, www.qiagenbioinformatics.com) was used to build a gene interaction network of the 40 differentially expressed proteins and 107 known OFC genes identified by IPA. The aim was to establish a single network of interactions between as many of the 40 differentially expressed proteins from the antibody array and the 107 OFC genes. IPA performs this task with the aid of its knowledge database consisting of literature-based curated information on genes and gene product interactions.

Western Blotting

For protein extraction, MEPM cells were briefly washed with PBS, scraped and either flash-frozen or lysed immediately. Cells were lysed by suspension in radioimmunoprecipitation assay (RIPA) buffer with HALT protease inhibitor Cocktail (Thermo Scientific, Waltham, MA) and by agitation for 30 minutes at 4°C. Cell lysates were centrifuged for 10 minutes at 13,000 rcf and the protein extracts (supernatant) collected. Lysates were then electrophoresed in 4-15% gradient Mini-Protean TGX Stain-Free precast gels (Bio-RAD, Hercules, CA). After electrophoresis, the gels were exposed to UV light for 45 seconds to develop the total protein signal and imaged on a ChemiDoc System (Bio-RAD, Hercules, CA) before being transferred onto Immobilon PVDF membranes (EMD Millipore, Billerica, MA). PVDF membranes were then blocked in Odyssey Blocking Buffer (Li-Cor, Lincoln, NE) either overnight at 4°C or at room

temperature for 1 hour. Primary antibodies used were anti-TGFβ1 (1:1000; Abcam, Cambridge, MA), anti-phospho-SMAD2 (1:5000; Cell Signaling Technologies, Danvers, MA), and anti-SOX9 (1:5000; Abgent, San Diego, CA), and anti-SOX10 (1:5000; Aviva Systems, San Diego, CA). Secondary antibodies used were HRP-linked goat anti-rabbit IgG (1:10,000; Cell Signalling Technologies, Danvers, MA) and HRP-linked goat anti-mouse IgG (1:10,000; Santa Cruz Biotechnologies, Dallas, TX). Femto SuperSignal West ECL reagent (Thermo Scientific, Waltham, MA) was used to develop the signal. Image Lab software (Bio Rad, Hercules, CA) was used to quantitate total protein and western blot intensity. Each blot was normalized to the total protein loaded, and then fold change calculated by dividing total drug-treated samples by vehicle-treated sample.

Immunofluorescence and Imaging Analysis

MEPM cells cultured as described above, and were fixed in 4% paraformaldehyde (PFA) for 10 min, blocked in phosphate buffered saline (PBS) with 1% goat serum and 0.1% Tween, and stained using Anti-TGFβ1 (1:1000; Abcam, Cambridge, MA). After staining, coverslips were mounted in containing DAPI (Vector Labs, Burlingame, CA). Individual cells were imaged, and the levels of TGFB1 fluorescence were quantitated using NIH ImageJ software.

Results

Antibody-array-based analysis of HEPM cells following topiramate treatment

Protein extracts from HEPM cells with supraphysiological topiramate treatment (1000 μM for 6 hours) or without the treatment (Control) were assayed by Full Moon BioSystems (Sunnyvale, CA) Cell Signaling Explorer antibody-array. The Cell Signaling Explorer array includes

antibodies for 1358 individual proteins, in two technical replicates, encompassing 20 cellular pathways. The antibody array experiment was performed with two biological replicates. The results were analyzed for statistical significance as described in the Materials and Methods section. Protein levels of 57 gene products were significantly altered ($p<0.05$, Supplementary Table S1). We used a 1.15-fold cutoff ($|FC|\geq1.15$) [21, 22], resulting in 40 differentially expressed proteins with 19 proteins downregulated and 21 upregulated (Table1). To determine the importance of these 40 altered gene products to orofacial morphogenesis and OFCs, we used Ingenuity Systems pathway analysis (IPA) as well as manual literature curation.

IPA analysis of proteins with altered expression following topiramate treatment shows connectivity with OFC genes

The antibody array analysis identified statistically significant changes in expression of 40 proteins following topiramate treatment (Table 1; $p<0.05$, $|FC|\geq1.15$). Separately, IPA software reported 107 known OFC genes (Supplementary Table S2). Only 18 of the 107 OFC genes were represented on the Full Moon BioSystems Cell Signaling Explorer antibody array. Among these 18 proteins encoded by OFC genes, only TGFβ1 was significantly altered (increased) upon topiramate treatment of HEPM cells.

We also analyzed the 40 gene products with IPA for changes in diseases and bio-functions (Supplementary Table S3). The top predicted categories were related to "cell death and survival", and "organismal growth and development". Overall, IPA predicted that topiramate treatment decreased cell viability (Supplementary Table S3; IPA Ranks 13, 29) and increased apoptosis (Supplementary Table S3; IPA Rank 37). However, IPA also correctly predicted increased cell viability of neurons (Supplementary Table S3; IPA Rank 7) [23], consistent with a cell-type

specific effect of topiramate. The top IPA predicted networks also indicated an effect on cell growth and survival pathways (Supplementary Table S4). Next, we looked at connectivity of the genes encoding the 40 altered proteins to the IPA-reported 107 OFC genes (Figure 1). TGFβ1 was the only OFC gene among the 40 topiramate-altered proteins in our analysis. A single network was able to connect 22 (9 downregulated, 13 upregulated) of these 40 (55%) genes encoding differentially expressed proteins to 87 OFC genes (81%), either directly or indirectly. TGFβ1 showed the highest number of connections to known OFC genes (Figure 1). Therefore, we focused our validation studies on TGFβ1.

Validation of TGFβ1 upregulation in primary mouse embryonic palate mesenchyme (MEPM) cells following treatment with physiological levels of topiramate

Our analysis indicated TGFβ1 as the central molecule affected by topiramate treatment of HEPM cells. To confirm that our finding was not an artifact of using an immortalized cell line and supraphysiological levels of topiramate, we used primary MEPM cells and physiological topiramate doses. The peak serum level for even topiramate monotherapy is approximately 25-50 μM [24-26]. Therefore, we initially included both the physiological 50 μM concentration and two supraphysiological concentrations to treat fresh primary MEPM cells from E13.5 embryonic palates for 6 hours (Figure 2A-E). Following treatment, cells were fixed and immunostained using an anti-TGFB1 antibody (Figure 2A-D). Individual cells were imaged, and the levels of TGFB1 were quantitated using NIH ImageJ software. The experiment was repeated three times with different primary MEPM isolations. We saw a significant increase in intracellular TGFβ1 level at the physiological 50μM topiramate treatment (Figure 2E).

We also performed Western blot analysis following 25μM and 50μM topiramate treatments (Figure 2F) and showed a significant increase with 50μM topiramate (Figure 2G). To test for an upregulation of the TGFβ1 signaling pathway, we also assessed levels of phospho-SMAD2 (P-SMAD2), a downstream effector molecule (transcription factor) of the ligand-bound TGFβ receptor that requires phosphorylation to translocate to the nucleus. Indeed, P-SMAD2 levels were increased following both 25μM and 50μM topiramate treatment (Figure 2F,G), indicating a strong upregulation of TGFβ signaling via SMAD-dependent pathway.

Topiramate treatment of primary MEPM cells results in increased SOX9 expression

After validating the involvement of TGFβ signaling, we next wanted to look at a TGFβ target gene with a role in orofacial clefting. We decided to look at expression of SOX9, a transcription factor belonging to the SOXE group with a key role in regulating chondrocyte function [27, 28]. *SOX9* mutations have been identified in patients with Pierre Robin sequence [29] and Campomelic (or acampomelic) dysplasia with or without sex reversal [30, 31]. Pierre Robin sequence is a series of defects including small jaw, a posteriorly placed tongue and cleft palate [29]. Campomelic dysplasia is an autosomal dominant skeletal disorder that is characterized by bowed limbs, hypoplastic or hypomineralized bones, and small chest size. Cleft palate, micrognathia (including Pierre Robin sequence), flat face and hypertelorism are also frequent features of Campomelic dysplasia. Interestingly, *SOX9* mutations in patients with Pierre Robin sequence were located in the regulatory region affecting *SOX9* expression. Furthermore, both knockdown and overexpression of *Sox9* in mouse has been shown to result in cleft palate phenotypes [27, 28, 32]. Therefore, an effect on SOX9 expression downstream of TGFβ1 in our system would be consistent with a plausible pathogenetic mechanism of topiramate-based facial

clefts. Indeed, we found that SOX9 expression is increased following topiramate treatment (Figure 3A,B), which is consistent with a role for TGFβ1 in stabilizing SOX9 protein [33]. Expression of another SOXE transcription factor group member, SOX10, is not altered upon topiramate treatment (Figure 3A,C), showing specificity for SOX9 upregulation. Our results with topiramate suggest that TGFβ1-mediated altered expression of SOX9 may underlie the teratogenicity of the antiepileptic drug.

Discussion

We utilized an unbiased cell signaling antibody array to identify 40 proteins with altered expression following topiramate treatment of HEPM cells. Although a smaller number of proteins were analyzed with the antibody array compared to an RNA-based analysis, our data suggest that the protein-based analysis provides a clearer view of signaling changes. Our antibody array data analysis showed statistically significant upregulation of TGFβ1 ligand expression.

To validate that TGFβ1 increase may play a physiological role in the etiology of OFC, we utilized primary MEPM cells. We used physiologically relevant doses of topiramate (25 and 50μM) and showed that TGFβ1 level was increased. During palatogenesis, all three TGFβ ligands - TGFβ1, TGFβ2 and TGFβ3 - are expressed in the palatal shelves. While *Tgfb3* and *Tgfb2* mouse mutants show cleft palate, the mutants for *Tgfb1* do not [34, 35]. TGFβ1 has been shown to be a potent inducer of growth inhibition in various cell types [36-38]. However, in palate development, some evidence suggests TGFβ ligands can promote cell proliferation [39]. TGFβ1 is strongly expressed in the distal pre-fusion palatal shelves [40]. In contrast, TGFβ3 expression follows that of TGFβ1 in the medial edge epithelium [35, 39]. Regardless of the complex function of individual TGFβ

pathway molecules in the palatal shelves, perturbation of TGFβ signaling affects many OFC genes [39, 41] that can negatively impact palatogenesis.

To show that TGFβ1 increase can affect downstream genes, we looked at expression of SOX9 transcription factor. Deficiency of SOX9 leads to Campomelic dysplasia, characterized by facial and skeletal anomalies, including cleft palate, midface hypoplasia, short stature and short and bowed limbs [30, 31]. However, we actually observed an increase in SOX9 levels following topiramate treatment. TGFβ-mediated increase in SOX9 expression has also been recently shown in mesenchymal fibroblasts to promote renal fibrosis [42]. Interestingly, when SOX9 is overexpressed in all chondrocytes, by insertion of *Sox9* cDNA into the *Col2a1* locus, it also results in cleft palate phenotype [27]. Thus, perturbation of SOX9 expression in either direction affects palatogenesis. A sudden increase in TGFβ1 level may therefore disrupt this delicate regulation of SOX9 expression in the palate mesenchyme, and result in OFC.

We also looked at phospho-SMAD2, a downstream effector of the TGFβ pathway. Phosphorylation of SMAD2 by TGFβ is necessary for the stabilization of *Sox9* in palatogenesis [33]. Phospho-SMAD2 was upregulated in topiramate-treated MEPM cells, validating that topiramate increases TGFβ signaling cascade. In a recent study examining the effects of topical topiramate on wound healing using mice found significantly increased levels of TGFβ and SOX2 in epidermal cells treated with topiramate [43], consistent with a topiramate-TGFβ-SOX cascade observed in our analysis.

Antiepileptic drugs ameliorate CNS excitatory seizures by dampening overall neuronal activity. This includes downregulation of excitatory signals from glutamate receptors and upregulation of inhibitory signals from GABA receptors [44]. Topiramate has been reported to upregulate GABA levels and $GABA_A$ receptor-based signaling [45]. Importantly, increased GABA signaling

upregulates TGFβ levels [46, 47]. While mouse mutants for some components of the GABA signaling pathway result in cleft palate phenotype [48-50], the effect of GABA upregulation on palatogenesis has not been explored. Recently, upregulation of GABA receptor activity was shown to decrease cell proliferation of embryonic and neural crest cells, as well as of blastocysts [51]. These results are consistent with a potential inhibitory effect of GABA upregulation on overall embryonic development and, in particular, on neural crest-influenced orofacial morphogenesis [17]. Also in support, several studies indicate tissue-specific activation of cell death following upregulation of TGFβ signaling, as reviewed in Schuster and Krieglstein (38). Thus, our data suggest that topiramate-based upregulation of GABA signaling can increase TGFβ signaling, which in turn results in tissue-specific changes in expression of genes, such as SOX9, involved in palatogenesis.

To assess the impact of the genes encoding the 40 differentially expressed proteins on orofacial clefting, we assayed their connectivity to the 107 known OFC-related genes reported by IPA. A single IPA connectivity network included over 55% of both sets of genes (Figure 1). This high level of connectivity argues that exposure to topiramate has the potential to perturb many OFC-related genes and pathways in the developing palate. The highest connectivity in this network was centered on TGFβ1 ligand. We also considered the possibility that the upregulation extended beyond TGFβ1 ligand to the entire pathway. We reasoned that if TGFβ signaling was indeed upregulated in topiramate-treated HEPM cells, we would find evidence for upregulation of downstream factors from our antibody array data. Indeed, a manually curated network showed evidence for upregulation of TGFβ signaling both upstream and downstream of TGFβ1 ligand (Figure 4) [45, 46, 52-59]. We were able to directly confirm nine molecules that were consistent

with upregulation of TGFβ signaling. We also found literature support for the topiramate-based increase in GABA signaling [45, 52] to in turn upregulate levels of TGFβ1 [46]. Together, this network not only shows a broad upregulation of the TGFβ signaling pathway following topiramate treatment of HEPM cells, but also suggests perturbation of other signaling pathways important in embryogenesis, including Phospholipase-C-based PKC/PKA and Sonic hedgehog signaling.

The genetic network affected by topiramate treatment of palate mesenchyme cells provides an important framework to study the OFC-related teratogenic effects. Future *in vitro* and *in vivo* studies are required to elucidate the role of increased TGFβ signaling in the teratogenicity of topiramate. Topiramate is one of many anti-epileptic and mood-stabilizing drugs that have recently been associated with structural birth defects. All of these drugs share certain molecular characteristics and targets. Therefore, understanding the molecular genetic mechanism behind topiramate teratogenicity may also provide clues to the general link reported among a broad class of antiepileptic drugs and birth defects.

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Author contributions:

S.K.R. conceived the project. I.S. and S.K.R. conceived and designed the experiments. S.K.R., J.P.G., A.J.O., L.A.H., N.J.E., N.R.W. and E.G.H. performed the experiments. S.G. performed the statistical analysis on the antibody array data. S.K.R, J.P.G., A.J.O., L.A.H., N.J.E., N.R.W. and I.S. analyzed the data. S.K.R., J.P.G., L.A.H., S.G. and I.S. wrote the paper. N.J.E., A.J.O., N.R.W. and E.G.H. reviewed and edited the manuscript.

Conflict of Interest

The authors do not have any competing financial interests pertaining to the studies presented here.

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Table 1: List of 40 proteins with statistically significant change in expression and a 1.15-fold-change cut-off in HEPM cells following topiramate treatment.

Protein Name	*Symbol*	*UniProtKB ID*	*Antibody Array ID*	*Fold Change*
Checkpoint kinase 2	CHEK2	O96017	659	1.96
Low-density lipoprotein receptor class A domain-containing protein 1	LDLRAD1 / LRP1	Q5T700	109	1.64
Aldehyde dehydrogenase 7	ALDH3B1	P43353	92	1.46
NADPH oxidase 5	NOX5	Q96PH1	282	1.46
Proto-oncogene tyrosine-protein kinase receptor Ret	RET	P07949	490	1.42
Cardiac troponin I	TNNI3(cTnl)	P19429	146	1.39
Transforming Growth Factor beta 1	TGFB1	P01137	377	1.39
Unconventional myosin Id	MYO1D	O94832	276	1.37
Tyrosine-protein kinase receptor 3	TYRO3	Q06418	478	1.33
Cyclosome 1	APC1	Q9H1A4	1093	1.32
Superoxide dismutase 1	SOD1	P00441	996	1.31
Oral cancer-overexpressed protein 1	ORAV1 / ORAOV1	Q8WV07	1078	1.30
Growth arrest and DNA damage-inducible proteins-interacting protein 1	GADD45GIP1	Q8TAE8	1285	1.27
Phosphatidylinositol-glycan biosynthesis class H protein	PIGH	Q14442	453	1.25

CD30 Ligand	CD153	P32971	220	1.24
Patched	PTCH1	Q13635	35	1.23
Cadherin-10, T2-cadherin	CDH10	Q9Y6N8	415	1.21
ATP synthase subunit delta, mitochondrial	ATP5D	P30049	261	1.19
TNF Receptor1-associated DEATH domain protein	TRADD /TNFR1	Q15628	716	1.19
Alcohol dehydrogenase class 4 mu/sigma chain	ADH7	P40394	91	1.19
Interferon Regulatory Factor 4	IRF4	Q15306	730	1.18
Bcl10-interacting CARD protein	C9ORF89	Q96LW7	422	-1.15
S-phase kinase-associated protein 1	SKP1A/p19	P63208	363	-1.16
B melanoma antigen 3/ Cancer/testis antigen 2.3	BAGE3	Q86Y29	420	-1.20
Cancer-associated Gene Protein	ATBP3 / MTUS1	Q7Z7A3	907	-1.20
SUMO1-activating enzyme 2	UBA2	Q9UBT2	874	-1.22
Tissue inhibitor of metalloproteinases 2	TIMP2	P16035	537	-1.22
RING finger and WD repeat domain protein 2	RFWD2	Q8NHY2	1107	-1.23
Coagulation factor VII (light chain, Cleaved-Arg212)	FA7	P08709	1156	-1.29
Rho guanine nucleotide exchange factor 3	ARHGEF3	Q9NR81	1123	-1.30

Cytochrome c oxidase assembly protein COX11	COX11	Q9Y6N1	911	-1.31
Diacylglycerol Kinase eta	DGKH	Q86XP1	908	-1.36
ATP-binding cassette sub-family A member 8	ABCA8	O94911	848	-1.37
Shugoshin-like 1	SGOL1	Q5FBB7	895	-1.37
Guanylate Cyclase Beta 1	GUCY1B3	Q02153	853	-1.38
Cell division cycle 7 related kinase	CDC7	O00311	1044	-1.38
Aldo-keto reductase family 1 member C-like protein 1	AKR1CL1	Q5T2L2	246	-1.41
Interleukin 2	IL2	P60568	832	-1.55
Cytochrome P450 7B1	CYP7B1	O75881	802	-1.82
Ubiquitin-protein ligase E3B	UBE3B	Q7Z3V4	291	-2.19

Figure Legends

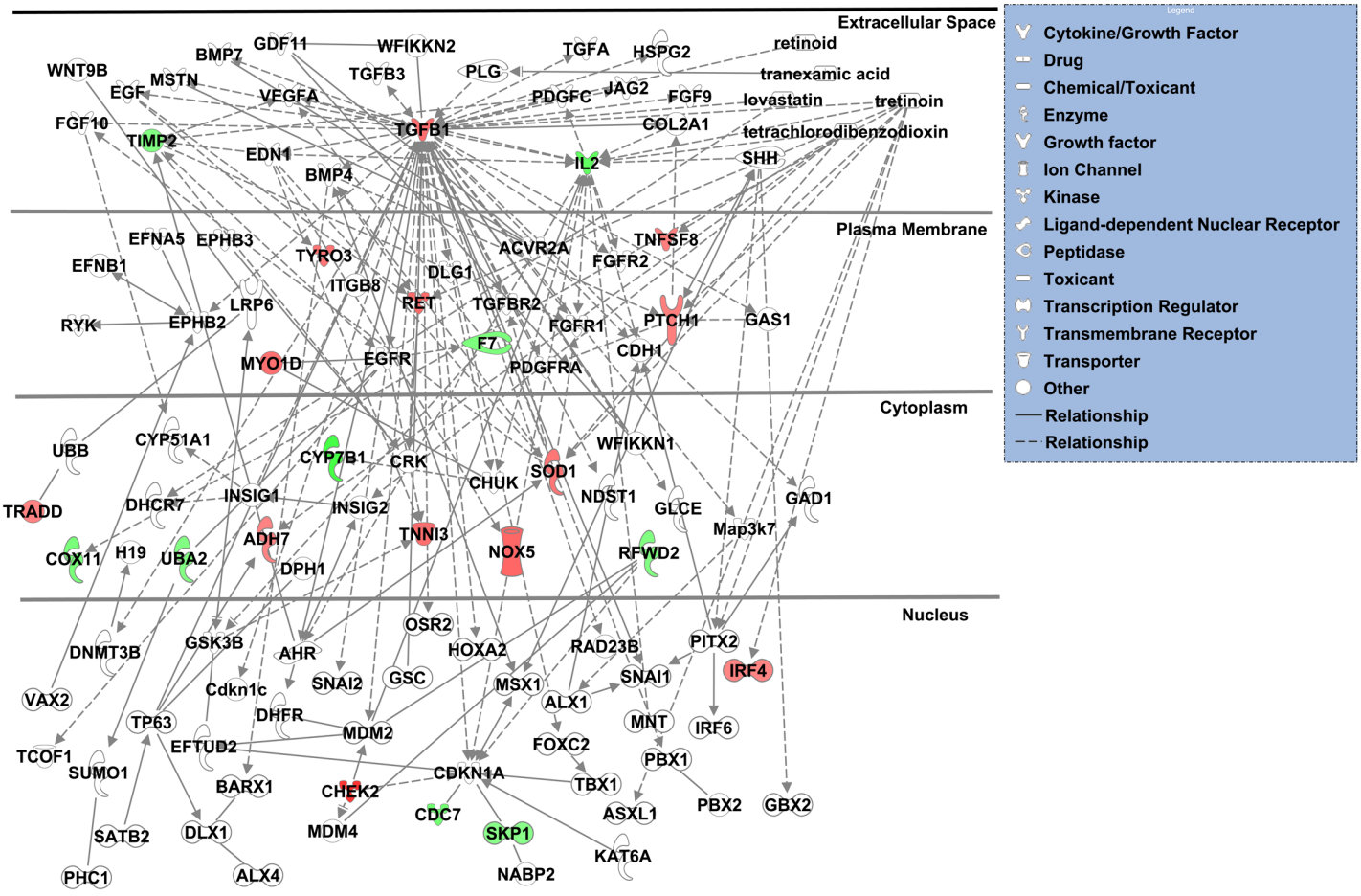
Figure 1: TGFβ1 showed highest connectivity in IPA-generated network of differentially expressed gene products from topiramate-treated HEPM cells and known orofacial clefting-associated genes. The 40 gene products with differential expression following 6-hour, 1000μM topiramate treatment of HEPM cells were analyzed with Ingenuity Systems pathway analysis (IPA) software for possible interaction with 107 IPA-identified OFC genes. A single resulting network accounted for 22 (55%) of the topiramate-treated HEPM genes in association with 87 (81%) known OFC-related genes. The upregulated or downregulated genes from the HEPM data were colored red or green, respectively. The only gene common between the two datasets was *TGFB1,* which also displays the highest connectivity in the network.

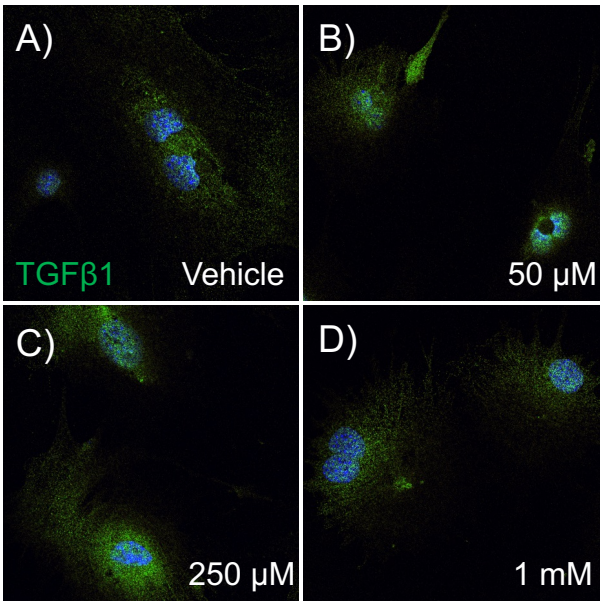
Figure 2: Topiramate treatment of primary MEPM cells upregulated TGFβ1 expression. To validate upregulation of TGFβ1, we isolated primary mouse embryonic palate mesenchyme (MEPM) cells from E13.5 embryos and treated them with 25μM, 50μM, 250μM or 1mM topiramate for 6 hours, as indicated. These cells were analyzed for TGFβ1 expression by immunostaining (A-E) and Western blotting (F-G). There was a significant increase in intracellular TGFβ1 expression upon as little as 50μM topiramate treatment (A-E), which was quantitated in at least 30 cells per treatment from 3 independent experiments (**, $p<0.003$; ***, $p<0.0003$). Western blot analysis also showed a significant increase upon treatment with 50μM topiramate (F, G). We also showed increased phospho-SMAD2 (P-SMAD2) levels with both 25μM and 50 μM topiramate treatments (F, G), indicating an upregulation of TGFβ1 signaling cascade (*, $p<0.019$).

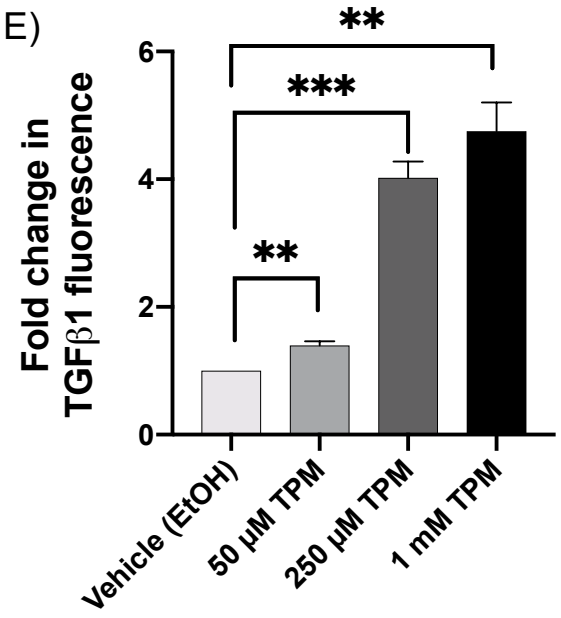
Figure 3: Topiramate treatment of primary MEPM cells resulted in increased SOX9 expression. We looked at expression of SOX9, a TGFβ1 target gene involved in orofacial clefting. Western blot analysis of topiramate-treated primary MEPM cells resulted in statistically significant increase in SOX9 expression (A,B; **, $p<0.006$). In contrast, expression of SOX10 is not altered upon topiramate treatment (A,C; ns, not significant).

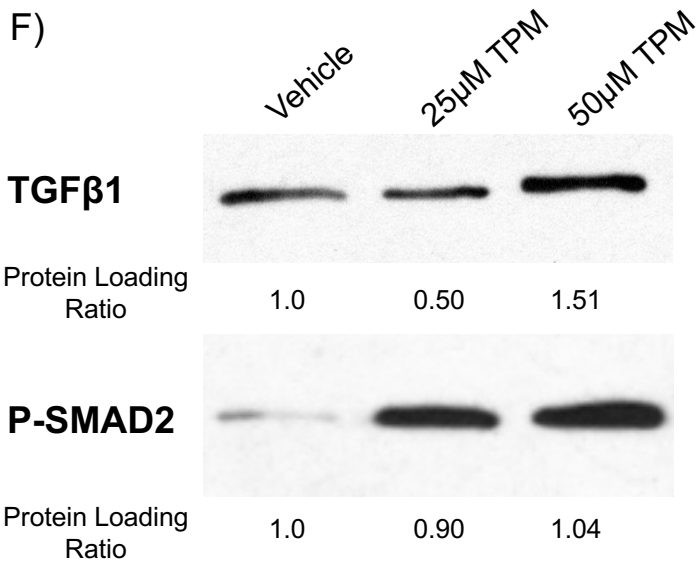
Figure 4: Model of topiramate action on MEPM cells. Our model predicts that topiramate is capable of stimulating GABA receptors in the palate to upregulate TGFβ1 expression. TGFβ1 expression is tightly regulated during craniofacial morphogenesis. Misregulation of TGFβ1 signaling can lead to altered expression of orofacial clefting genes. For example, we show that topiramate leads to upregulation of SOX9 expression, which is sufficient to result in cleft palate in mice. In order to gather corroborating evidence for the upregulation of TGFβ signaling from the 39 (excluding TGFβ) differentially expressed gene products, we performed a manual curation of the literature for connectivity to TGFβ1. Our analysis revealed that changes in an additional seven (18%) of the proteins are consistent with upregulation of TGFβ1 signaling. Moreover, these molecules affect TGFβ1 signaling both upstream and downstream of the TGFβ1 ligand, suggesting a concerted global upregulation of the pathway. The solid-colored molecules are from the antibody-array or western blot results, while the spotted molecules are changes in upstream effectors predicted from literature. Citation number (#) corresponds to listed references.

Rafi_Figure 1




A)
TGFβ1
Vehicle
B)
50 μM
C)
250 μM
D)
1 mM


E)
Fold change in TGFβ1 fluorescence
**
***
**
0
2
4
6
Vehicle (EtOH)
50 μM TPM
250 μM TPM
1 mM TPM


F)
Vehicle
25μM TPM
50μM TPM
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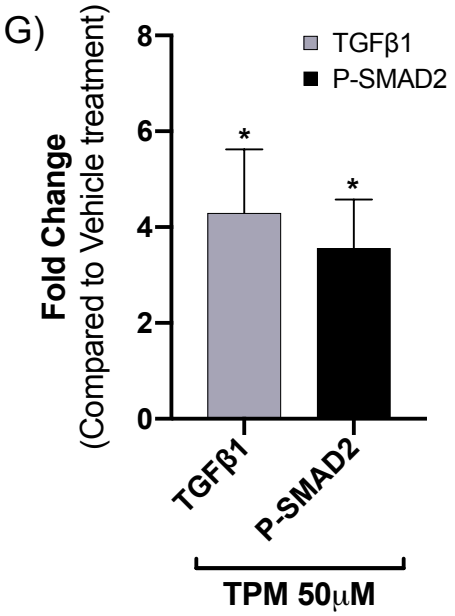
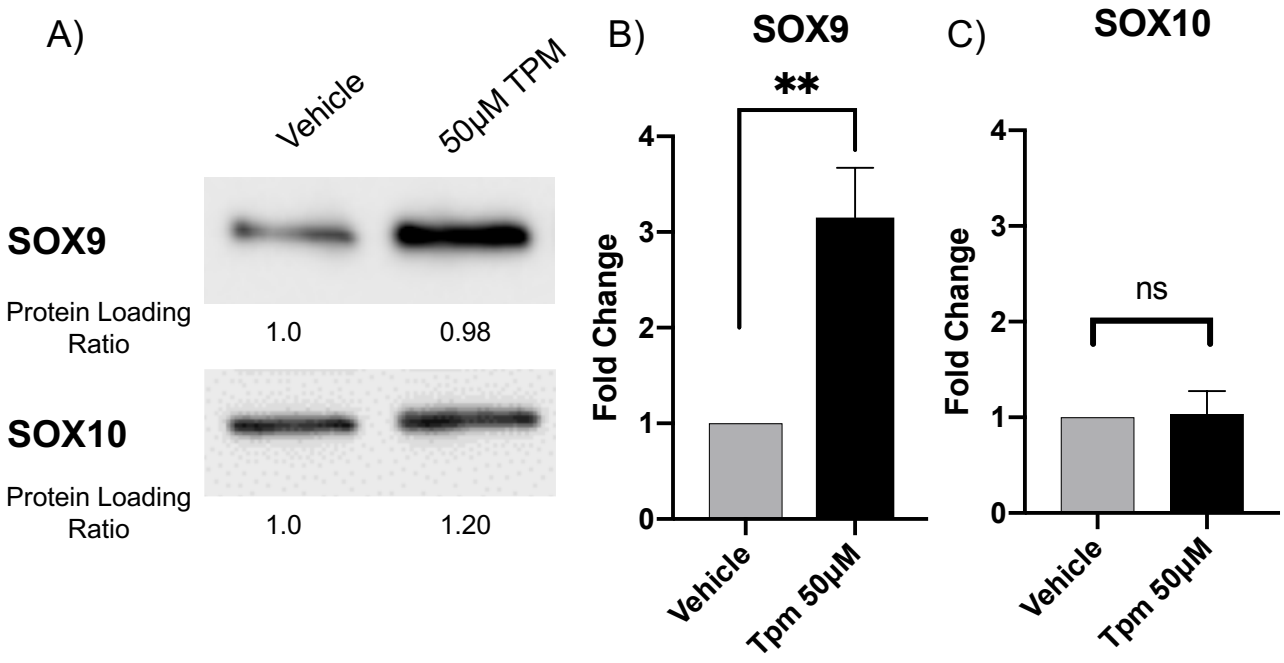
Protein Loading Ratio
1.0
0.50
1.51
P-SMAD2
Protein Loading Ratio
1.0
0.90
1.04


G)
TGFβ1
P-SMAD2
Fold Change
(Compared to Vehicle treatment)
0
2
4
6
8
*
*
TGFβ1
P-SMAD2
TPM 50μM

Rafi_Figure 3



Rafi_Figure 4