401 N. Lindbergh Blvd.
St. Louis, MO 63141
Tel.: 314.993.1700, #546
Toll Free: 800.424.2841, #546
Fax: 800.708.1364
Cell: 314.283.1983
Send via email to: jbode@aaortho.org and cyoung@aaortho.org

AAO Foundation Final Report Form
(a/o 1/3/2018)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Please prepare a report that addresses the following:

Type of Award: **Biomedical Research Award**

Name(s) of Principal Investigator(s): **Sercan Akyalcin (PI), Ariadne Letra (coI)**

Institution(s): **Tufts University (PI), UTHealth Houston (coI)**

Title of Project: **Functional impact of tooth agenesis-associated gene variants**

Period of AAOF Support: **07-01-19 to 12-31-20**

Amount of Funding: **$30,000**

Summary/Abstract

Two manuscripts have originated from this work. Both of them are attached for your review.

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript OR
   Both manuscripts are attached for a detailed review.

2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis and interpretation of results should be

included.

Response to the following questions:

1. Were the original, specific aims of the proposal realized? **Yes**
2. Were the results published? **Yes**
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers

    Manuscript #1

    Zeng Y, Baugh E, Akyalcin S, Letra A.
    Functional Effects of *WNT10A* Rare Variants Associated with Tooth Agenesis.
    Journal of Dental Research. J Dent Res. 2020 Oct 9:22034520962728.
    doi: 10.1177/0022034520962728. Online ahead of print.

    Manuscript #2

    Williams M, Zeng Y, Chiquet B, Jacob H, Kasper FK, Harrington DA, English J, Akyalcin S, Letra A. Functional characterization of *ATF1, GREM2* and *WNT10B* variants associated with tooth agenesis. Orthodontics and Craniofacial Research Journal. Online ahead of print.

    b. Was AAOF support acknowledged? **Yes, in both manuscripts.**
    c. If not, are there plans to publish? If not, why not? **Not applicable**.

3. Have the results of this proposal been presented? **Yes**
    a. If so, list titles, author or co-authors of these presentation/s, year and locations

    1) Functional Effects of *WNT10A* Rare Variants Associated with Tooth Agenesis. Letra A, Zeng Y, Baugh E, Akyalcin S, Letra A. Invited oral presentation. Society for Craniofacial Genetics and Developmental Biology. Houston TX. October 2019.

    2) Functional Genomics Approaches of Rare Pathogenic Variants in Tooth Agenesis. Letra A, Zeng Y, Williams W, Akyalcin S, Hecht JT. Oral presentation. 2020 IADR annual meeting. Washington DC (accepted for presentation; meeting cancelled due to COVID-19).

    b. Was AAOF support acknowledged? **Yes, in both instances.**
    c. If not, are there plans to do so? If not, why not? **Not applicable**.

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

The funding from the AAOF has been a significant contributor to my career development since 2011, when I was awarded my first OFDFA. In 2014, I was promoted to associate professor and in August of 2020, I finally became a "full professor" at my current institution. In these days of limited funding opportunities for orthodontic researchers, the AAOF had become a major resource for career development. My Co-I was able to purchase necessary materials and help offset the laboratory personnel costs as she supported me immensely from planning to execution of

the project. These two publications would not be possible without her involvement. I intend to further my collaboration with Dr. Letra on the genetic studies of congenitally missing teeth. We continue to learn more about the condition's etiology and impact on the orthodontic population. My thirst for knowledge has been stimulated by these studies and the support of the AAOF. It feels as if we continue collecting the pieces of a large puzzle and contributing to understanding the clinical outcomes.

Journal of Dental Research
1–8

DOI: 10.1177/0022034520962728
journals.sagepub.com/home/jdr

Functional Effects of *WNT10A* Rare Variants Associated with Tooth Agenesis

Y. Zeng[1,2], E. Baugh[3], S. Akyalcin[4], and A. Letra[1,2,5]

Abstract

Mutations in *WNT10A* have frequently been reported as etiologic for tooth agenesis (TA). However, the effects of *WNT10A* variation on gene/protein function and contribution to TA phenotypes remain poorly understood. Here, we performed bioinformatic and functional characterization analysis of *WNT10A* variants. In silico prediction of variant function was performed with VIPUR for all *WNT10A* missense variants reported in the Exome Aggregation Consortium database. Functional characterization experiments were then performed for selected *WNT10A* variants previously associated with TA. Expression vectors for wild-type and mutant *WNT10A* were made and transfected into stem cells from human exfoliated deciduous teeth (SHED) for evaluation of gene/protein function, WNT signaling activity, and effects on expression of relevant genes. While 75% of *WNT10A* variants were predicted neutral, most of the TA-associated variants received deleterious scores by potentially destabilizing or preventing the disulfide bond formation required for proper protein function. WNT signaling was significantly decreased with 8 of 13 variants tested, whereas wild-type–like activity was retained with 4 of 13 variants. *WNT10A*-mutant cells (T357I, R360C, and R379C mutants) showed reduced or impaired binding affinity to FZD5, suggesting a potential mechanism for the decreased WNT signaling. Mutant cells also had decreased WNT10A protein expression in comparison to wild-type cells. mRNA expression of *PAX9, MSX1, AXIN2*, and *RUNX2* (known tooth development genes) was perturbed in mutant cells and quite significantly for *PAX9* and *RUNX2*. Transcriptome analysis of wild-type and T357I-mutant cells identified 36 differentially expressed genes (26 downregulated, 10 upregulated) involved in skeletal system development and morphogenesis and pattern specification. *WNT10A* variants deemed pathogenic for TA likely affect protein folding and/or stabilization, leading to decreased WNT signaling and concomitant dysregulated expression of relevant genes. These findings may allow for improved interpretation of TA phenotypes upon clinical diagnosis while providing important insights toward the development of future tooth replacement therapies.

Keywords: gene function, hypodontia, oligodontia, wnt, tooth development, tooth agenesis

Introduction

Nonsyndromic tooth agenesis (TA) is a common birth defect characterized by the congenital absence of ≥1 permanent teeth (Gorlin et al. 1990). Over the years, numerous genes have been accounted for the etiology of TA (e.g., *MSX1, PAX9, AXIN2, EDA, LRP6, WNT10A, WNT10B*). More recently, however, *WNT10A* has been reported as a major etiologic gene suggested to account for as much as 50% of all TA cases worldwide (Yin and Bian 2015; Williams and Letra 2018). Rare homozygous as well as single and compound heterozygous variants in *WNT10A* have been identified in association with variable TA phenotypes (van den Boogaard et al. 2012; Arzoo et al. 2014; Song et al. 2014; Dinckan et al. 2018). Interestingly, single heterozygous variants in this gene have been reported in approximately 2.5% of individuals without TA (van den Boogaard et al. 2012).

In mice, *Wnt10a* expression in the dental epithelium was required for activation of the canonical Wnt signaling pathway activation deemed essential for tooth development (Yamashiro et al. 2007; Liu and Millar 2010). *Wnt10a* deficiency resulted in skeletal, skin, and tooth defects (e.g., supernumerary teeth and abnormal crown morphology) but no missing tooth phenotypes (Yang et al. 2015; Xu et al. 2017). In contrast, knockdown of *wnt10a* in zebrafish embryos arrested tooth development by day 5 postfertilization (Yuan et al. 2017). While these findings support a role for *WNT10A* in tooth development, a better understanding is warranted of how variants in this gene affect gene/protein function and contribute to TA.

In this study, we performed bioinformatic and functional characterization analysis of *WNT10A* variants. In silico prediction of deleteriousness of *WNT10A* variants was performed, as

[1]Center for Craniofacial Research, School of Dentistry, University of Texas Health Science Center at Houston, Houston, TX, USA
[2]Department of Diagnostic and Biomedical Sciences, School of Dentistry, University of Texas Health Science Center at Houston, Houston, TX, USA
[3]Department of Biology, New York University, New York, NY, USA
[4]Department of Orthodontics, School of Dental Medicine, Tufts University, Houston, TX, USA
[5]Pediatric Research Center, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX, USA

A supplemental appendix to this article is available online.

Corresponding Author:
A. Letra, Department of Diagnostic and Biomedical Sciences, Center for Craniofacial Research, School of Dentistry, University of Texas Health Science Center at Houston, 1941 East Road, BBSB Room 4210, Houston, TX 77054, USA.
Email: ariadne.m.letra@uth.tmc.edu

Table. Details of *WNT10A* Variants Evaluated.

Variant[a]	cDNA Change[a]	dbSNP ID[a]	Base Position[a]	Function[a]	GERP[b]	Highest MAF[c]	VIPUR Score[d]
C107*	c.321C>A	rs121908119	218,882,368	Stop gained	−0.49	<0.01	—
R113C	c.337C>T	rs141074983	218,882,384	Missense	0.47	<0.01	0.404
V145M	c.433G>A	rs543063101	218,890,040	Missense	4.12	<0.01	0.548
R171C	c.511C>T	rs116998555	218,890,118	Missense	2.96	<0.04	0.250
G213S	c.637G>A	rs147680216	218,890,244	Missense	4.13	<0.04	0.312
F228I	c.682T>A	rs121908120	218,890,289	Missense	4.13	<0.04	0.675
R232W	c.694C>T	rs193098360	218,890,301	Missense	3.06	<0.01	0.277
E233*	c.697G>T	rs121908118	218,890,304	Stop gained	4.13	<0.01	—
C276R	c.826T>A	rs1011303295	218,892,843	Missense	4.13	<0.01	0.524
W277C	c.831G>T	rs1234227647	218,892,848	Missense	4.13	<0.01	0.639
T357I	c.1070C>T	rs750190755	218,893,087	Missense	4.12	<0.01	0.708
R360C	c.1079G>T	rs893127185	218,893,095	Missense	3.00	<0.01	0.849
R379C	c.1135C>T	rs1347556761	218,893,152	Missense	4.13	<0.01	0.247

[a]Based on National Center for Biotechnology Information dbSNP database (GRCh38/hg38 assembly); variants listed represent heterozygous variants with the alternate alleles.
[b]Genomic Evolutionary Rate Profiling score ranges from −12.3 to 6.17, with 6.17 being the most conserved.
[c]Highest population minor allele frequency observed in any population, as reported in 1000 Genomes phase 3 and gnomAD databases.
[d]VIPUR scores >0.5 are predicted deleterious.

followed by functional characterization experiments of 13 variants previously associated with TA. Our findings demonstrate that *WNT10A* variants deemed pathogenic for TA present differential effects on gene/pathway activity and likely affect protein folding and/or stabilization.

Materials and Methods

In Silico Prediction of Variant Deleteriousness

We used VIPUR for in silico prediction of *WNT10A* variant function (Baugh et al. 2016). Since there are no structural models of WNT10A, modeling was performed with the best template model found, based on PDB 4F0A chain B (WNT8 from *Xenopus laevis*). This model covers most protein residues (positions 76 to 416, 341 of 417 amino acids; Appendix Fig. 1). As controls, details of all *WNT10A* variants reported in the Exome Aggregation Consortium database (http://exac.broadinstitute.org/) were retrieved for comparison. Additional details are described in the Appendix.

Expression Constructs

Thirteen TA-associated *WNT10A* variants were selected for functional characterization experiments based on their previously reported associations with TA and their predicted effects as deleterious (Williams and Letra 2018; Table). Constructs for each variant were designed with pCDNA-WNT10A-V5 vector (Addgene) as wild-type template. Mutant alleles were then generated with site-directed mutagenesis and specific primers (Appendix Table 1). Direct sequencing was performed to verify cloning efficiency and confirm the presence of *WNT10A* wild-type and mutant alleles.

Transfection and TCF Reporter Assays

SHED (stem cells from human exfoliated deciduous teeth) were cultured as described in the Appendix. At 24 h prior to transfection, cells were seeded into 96-well plates and treated with 50 ng of either pCDNA-WNT10A-V5 (wild type) or each variant construct and 0.15 µL of Lipofectamine Stem Transfection Reagent (Invitrogen) in Opti-MEM medium (Life Technologies). The TOPFlash/FOPFlash TCF Reporter Plasmid Kit (Upstate Biotechnology) was used to detect WNT signaling (Xue et al. 2015). Cotransfection of a Renilla luciferase vector (pRL-TK; Addgene) and an empty vector were used as controls. Luciferase activity was measured at 48 h after transfection with a Synergy H1 Multi-mode Microplate Reader (BioTek) and normalized against Renilla coexpression (Zhao et al. 2014; Hu et al. 2019). Results are shown as fold changes for the ratio of expression from TOPFlash to expression from FOPFlash as a precise measurement of canonical Wnt transcriptional activity.

Details on cell culture, immunocytochemistry, Western blot, coimmunoprecipitation, real-time quantitative polymerase chain reaction (qPCR), RNA sequencing, and data analysis are described in the Appendix.

Results

Most WNT10A Missense Variants Are Predicted Neutral

A total of 151 *WNT10A* missense variants were found in the Exome Aggregation Consortium database, of which 114 (75%) were predicted neutral by VIPUR (Appendix Fig. 1, Appendix Table 3). Among the variants of interest in this study (i.e., those previously associated with TA), V145M, F228I, C276R, W277C, T357I, and R360C obtained confident deleterious prediction scores (VIPUR score >0.5), although only F228I, W277C, and R360C are located in highly conserved sites. These variants appear to act by general energetic destabilization, which manifests as straining the numerous disulfide bonds and hence obtaining destabilized disulfide interpretations (since the wild-type fold cannot accommodate the

destabilizing variant and all disulfides). Therefore, a destabilized sulfide suggests that the variant acts by destabilization of a canonical disulfide bridge. The C276R variant is predicted to directly eliminate a disulfide bond, although not located at a highly conserved site. Variants R113C, G213S, R171C, and R379C had neutral predictions but could be disruptive by preventing proper disulfide bond formation, as cysteines are directly involved in disulfide bridges (Table). Only 2 cysteines in WNT10A are not involved in disulfide bridges, and both these are farther from the first disulfide bridge, suggesting that additional cysteines in the region around 96 to 416 nucleotides could lead to the formation of aberrant disulfide bonds. Out of these variants, R232W also had neutral scores despite the egregious change to tryptophan (Appendix Table 3). The frameshift mutations C107* and E233* were not assessed, as VIPUR is limited to analyzing missense mutations.

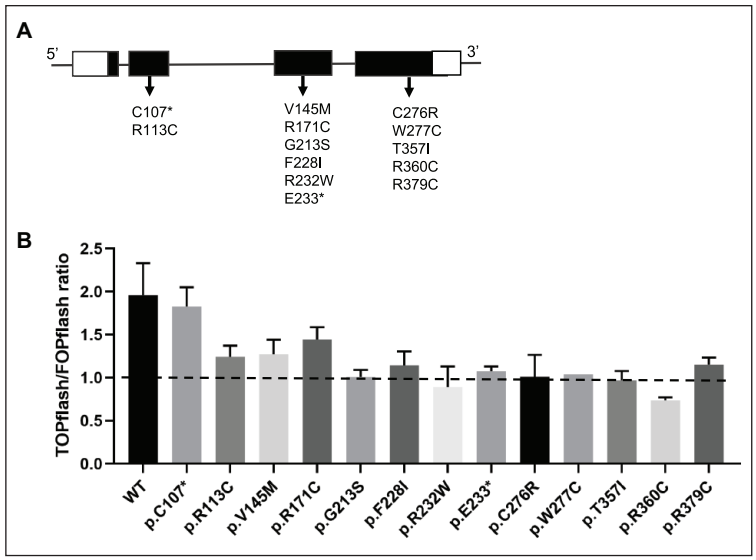


Figure 1. *WNT10A* gene variants and their effects on WNT signaling. (**A**) Schematic illustration of the human *WNT10A* gene (NT_005403). Black boxes represent exons, and black lines represent introns. Arrows denote location of variants and their corresponding amino acid substitutions in the WNT10A protein. (**B**) TOPFlash/FOPFlash expression ratios (fold change) in SHED transiently transfected with either wild-type pCDNA-WNT10A-V5 or each variant construct and cotransfected with TOPFLASH/FOPFLASH and pRL-TK control plasmids. Values are shown as mean ± SD of 3 experiments. SHED, stem cells from human exfoliated deciduous teeth.

WNT10A Variants Have Differential Effects on WNT Signaling

Constructs for wild-type and mutated *WNT10A*, as well as TOP/FOPFlash plasmids, were cotransfected into SHED to assess for variation in WNT signaling activity. TOPFlash contains a luciferase reporter gene under the control of a minimum promoter containing multiple wild-type TCF-binding sites, which become activated in response to Wnt/β-catenin pathway stimulation. As a negative control, we used FOPFlash, which contains mutant TCF-binding sites (Xue et al. 2015). Our results showed significantly increased WNT transcriptional activity in C107*-mutant cells in comparison with wild-type cells (TOP/FOP ratio ~2 ± 0.22). In R113C-, V145M-, R171C-, and R379C-mutant cells, WNT signaling was decreased, although some activity was retained (TOP/FOP ratio, ~1.1 to 1.4 ± 0.1). In contrast, minimal or no WNT transcriptional activity was detected in the G213S-, F228I-, R232W-, E233-*, C276R-, W277C-, T357I-, and R360C-mutant cells (TOP/FOP ratio, ~0.74 to 1.16 ± 0.11; Fig. 1). These findings suggest that most *WNT10A* variants lead to a truncated protein that perturbs or fails to activate WNT signaling.

WNT10A Expression and Activity Are Decreased in Mutant Cells

We sought to investigate the effects of the T357I and R360C variants due to their predicted ability to destabilize disulfide bonds (VIPUR scores, 0.71 and 0.85, respectively) and R379C, which, though predicted neutral (VIPUR score, 0.25), could disrupt proper disulfide bond formation due to the cysteine change at the terminal end of the gene (Appendix Table 3). As expected, significantly decreased protein expression was detected in T357I-, R360C-, and R379C-mutant cells by immunofluorescence ($P \leq 0.005$; Fig. 2A). Western blot analysis confirmed the decreased WNT10A expression, particularly in T357I mutants ($P = 0.006$; Fig. 2B). To clarify the potential mechanism of decreased WNT10A expression in T357I-, R360C-, and R379C-mutant cells, coimmunoprecipitation experiments were performed to evaluate WNT10A-FZD5 binding, as FZD5 is a known ligand for WNT10A (Voloshanenko et al. 2017). Our results demonstrate that FZD5 binding to mutant WNT10A was significantly decreased in T357I-, R360C-, and R379C-mutant cells ($0.00003 \leq P \leq 0.0001$), thereby providing insights into the mechanism by which *WNT10A* variation affects protein function (Fig. 2C).

Dysregulation of WNT10A Perturbs Expression of Relevant Genes

Tooth development requires coordinated expression of numerous genes; therefore, we investigated if mutant *WNT10A* could perturb expression of additional tooth development genes (*AXIN2, MSX1, PAX9, RUNX2*). Using real-time qPCR, we found significantly reduced *PAX9* mRNA expression in T357I-, R360C-, and R379C-mutant cells ($0.003 \leq P \leq 0.01$), whereas increased *RUNX2* expression was detected in R379C-mutants ($P = 0.01$). *AXIN2* was upregulated in all mutant cells, although no significant differences were found in comparison with wild type. *MSX1* expression was not significantly affected (Fig. 3).

To understand the downstream effects of mutant *WNT10A*-induced gene regulation, we used RNA sequencing for genome-wide transcriptome profiling of wild-type and T357I-mutant cells. The T357I variant was selected for these experiments, as it significantly affected protein expression and we

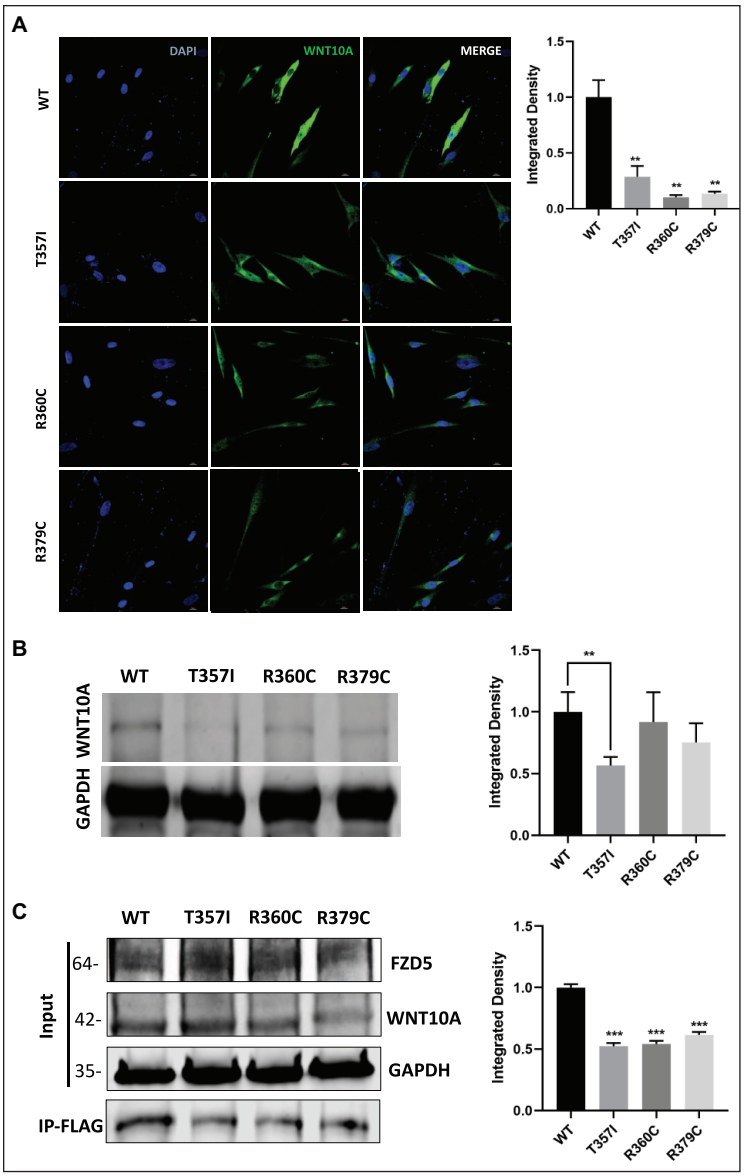


Figure 2. Expression and function of wild-type and mutant *WNT10A* in SHED. (**A**) Immunocytochemistry analysis of WNT10A expression in T357I-, R360C- and R379C-mutant cells. Fluorescence intensity (integrated density) was quantified with ImageJ software and expressed as relative values to that obtained in SHED transfected with wild-type WNT10A (WT). Scale bar = 10 µm; 60× magnification. (**B**) Western blot analysis of WNT10A and GAPDH in lysates of T357I-, R360C- and R379C-mutant cells. Relative intensities of WB bands were quantified with ImageJ. (**C**) Coimmunoprecipitation analysis of FZD5 and WNT10A in wild-type and T357I-, R360C- and R379C-mutant cells. Protein band quantification was performed with ImageJ software. Data analyses were performed with Student's *t* test. Values are shown as mean ± SD of 3 experiments. $^{**}P<0.01$. $^{***}P<0.001$. SHED, stem cells from human exfoliated deciduous teeth.

had previously identified it as the presumed driver variant in a compound heterozygous form (with G213S) leading to an oligodontia phenotype (Yuan et al. 2017).

RNA sequencing profiling identified 38 differentially expressed transcripts between wild-type and T357I-mutant cells, of which 2 were located in pseudogenes and excluded from analyses. Of the remaining 36 differentially expressed genes, 26 were downregulated and 10 upregulated in T357I mutants ($P<0.05$; Appendix Fig. 2A–C). Gene Ontology analyses of the dysregulated genes showed significant enrichment for various biological processes, cellular components, and molecular functions (e.g., skeletal system development and morphogenesis, pattern specification, response to estrogen; $P<0.05$; Fig. 4, Appendix Table 5). Reverse transcription qPCR validation experiments were also performed to confirm expression of differentially expressed genes, and they showed overall good agreement between the direction of the dysregulated expression (up- or downregulation) identified via RNA sequencing and reverse transcription qPCR (25 of 36 genes; Appendix Fig. 3).

Discussion

In this study, we demonstrate that *WNT10A* variants deemed pathogenic for TA have differential effects on gene/protein function, leading to impaired WNT signaling and protein expression and dysregulated expression of additional relevant genes. *WNT10A* has been the object of numerous genetic studies of TA; variants in this gene were associated with a range of mild to severe TA clinical phenotypes, despite also being found in unaffected individuals (van den Boogaard et al. 2012; Song et al. 2014; Yuan et al. 2017; Dinckan et al. 2018; Magruder et al. 2018). Nonetheless, the mechanisms through which these variants result in altered gene and/or protein function and likely contribute to TA are largely unknown (Williams and Letra 2018). Understanding the biological effects of *WNT10A* variants will provide important insights into the role of this gene in tooth development and the variable expressivity of TA.

Overall, the results of our in silico analysis are consistent with the expectation that the majority of genetic variants found in the general population are not deleterious and, indeed, 75% of *WNT10A* variants are predicted neutral. Despite this expected trend, there were still several deleterious variants with relatively high allele frequencies in the general population (Appendix Table 3). Out of the 151 missense variants found for *WNT10A*, 37 obtained deleterious prediction scores in VIPUR, of which 11 had been previously reported in association with TA (Williams and Letra 2018), and 6 of these (V145M, F228I, C276R, W277C, T357I, R360C) obtained confident deleterious scores due to predicted effects on destabilization and/or elimination of disulfide bonds in the protein structure. Interestingly, variants that consisted of cysteine substitutions (R113C, R171C, R379C) and were expected to have damaging effects by preventing proper disulfide bond formation received neutral scores. For WNT10A, the fold appears to

be maintained heavily by the numerous disulfide bridges, so variants that disrupt this—either by destabilization of the protein fold that prevents disulfide formation or by introducing a new cysteine that can form disulfide bonds—can be disruptive and prevent it from folding and interacting properly with its downstream binding partners in the WNT signaling pathway.

Activation of the canonical Wnt signaling pathway is critical for tooth development, and *Wnt10a* appears to be a key mediator in this process (Yamashiro et al. 2007; Liu and Millar 2010). To characterize the functional impact of *WNT10A* variants, we overexpressed wild-type and mutant *WNT10A* in SHED. SHED are highly proliferative and capable of differentiating into many cell types, including neural cells, adipocytes, and odontoblasts (Miura et al. 2003; Sakai et al. 2010). They express key components of the Wnt signaling pathway and are capable of responding to signaling initiated by Wnt, therefore constituting an excellent in vitro model system for testing the effects of TA-associated variants (Zhang et al. 2016). Our results of the luciferase reporter assays suggest that most TA-associated variants (G213S, F228I, R232W, E233*, C276R, W277C, T357I, R360C) affect gene/protein function, possibly leading to perturbed or lack of WNT signaling. The C107* variant, predicted to result in a premature stop codon, was still able to retain signaling activity, indicating that compensating mechanisms may exist to rescue the variant-induced suspected loss of function. Collectively, these findings are in general agreement with our in silico predictions and previous reports (Dinckan et al. 2018; Grejtakova et al. 2018). The variants R113C, V145M, R171C, and R379C also appear to retain some wild-type–like activity able to sustain protein function. To explore a potential mechanism for the altered WNT signaling in *WNT10A* mutant cells, we assessed the ability of T357I-, R360C-, and R379C-mutant cells to bind to FZD5. FZD5 has been reported to interact with numerous WNT proteins, including *WNT10A*, and activate signaling cascades in various cellular and developmental processes (Voloshanenko et al. 2017; Agostino and Pohl 2019). Our results showed reduced binding affinity to FZD5 in *WNT10A*-mutant cells, hence suggesting a plausible mechanism by which *WNT10A* variants may negatively affect WNT signaling. Given the complexity of WNT signaling, it is possible that binding of mutant WNT10A to other WNT receptor genes (e.g., LRP5/6 and KREMEN1/2) may also be impaired, therefore warranting additional investigation.

Tooth development is complex and requires coordinated and reciprocal expression of numerous genes in the dental epithelium and mesenchyme (Thesleff et al. 1996). In this context, we wanted to investigate if *WNT10A* variation affected the expression of additional tooth development genes. Dysregulation of *WNT10A* expression in mutant cells led to altered expression of *AXIN2, MSX1, PAX9*, and *RUNX2*, for which a role in tooth development is well established (Yin and Bian 2015). In the presence of the 3 *WNT10A* variants tested, expression of *PAX9* was significantly decreased whereas *RUNX2* was increased. *PAX9* and *RUNX2* are transcription factors required for activation of odontogenic signals in the early tooth mesenchyme to drive tooth morphogenesis. Mice lacking *Pax9* or *Runx2* exhibit tooth developmental arrest at the early and late bud stages, respectively (Peters et al. 1998; D'Souza et al. 1999). Furthermore, in *Pax9*-null mice, altered expression of several Wnt ligands and receptor genes has been described (Jia et al. 2017). Moreover, mutations in *Pax9* were shown to result in defective interactions that disrupt the expression and/or function of additional genes (e.g., Wnts) required for tooth development (Mostowska et al. 2003; Nakatomi et al. 2010). These findings support our observations that coordinated expression of *PAX9* and *WNT10A* is required for proper tooth development and, in contrast, dysregulated expression of either gene may disrupt this process and lead to defects of tooth development. Previous studies also support a relationship between *Runx2* and *Wnt10a*, with *Wnt10a* being identified as a *Runx2*-dependent gene during bone and tooth development and with additional Wnt genes found to act upstream of *Runx2* to trigger activity (Bennett et al. 2005; Gaur et al. 2005; James et al. 2006). Our findings corroborate these previous observations and suggest that *WNT10A* variation disrupts expression of *PAX9* and *RUNX2* (and possibly other tooth development genes) and/or essential genetic interactions, thereby contributing to TA.

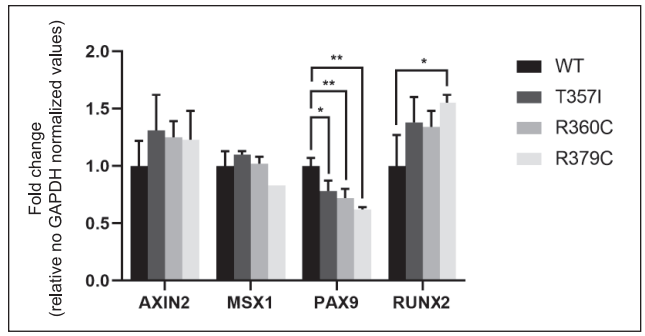


Figure 3. mRNA expression levels of *PAX9, MSX1, AXIN2*, and *RUNX2* in *WNT10A* wild-type and mutant cells obtained by quantitative real-time reverse transcriptase polymerase chain reaction. Data analysis was performed with the $\Delta\Delta C_T$ method. Results are shown as fold changes per expression of each target gene relative to endogenous GAPDH mRNA expression. Values are shown as mean ± SD of 3 experiments. $^*P<0.05$. $^{**}P<0.01$.

When comparing the transcriptomes of wild-type and T357I-mutant cells, we uncovered additional genes/pathways that may have a role in tooth development and in TA. We previously identified this variant as the likely driver variant in a compound heterozygous form (with G213S) leading to an oligodontia phenotype (Yuan et al. 2017). Further, the T357I variant is located in a very constrained region in the WNT10A protein, near 3 disulfide bonds and a N-acetylglucosamine modification site, and the resulting threonine-to-isoleucine substitution appears to destabilize the protein fold, thus inhibiting normal protein function (Yuan et al. 2017). Our findings in the present study further support a functional role for this variant in dysregulation of WNT10A protein function. The results of our RNA sequencing analysis in wild-type and T357I-mutant cells revealed 36 differentially expressed genes, with roles in biological processes or molecular pathways essential for pattern specification, regionalization, and skeletal system

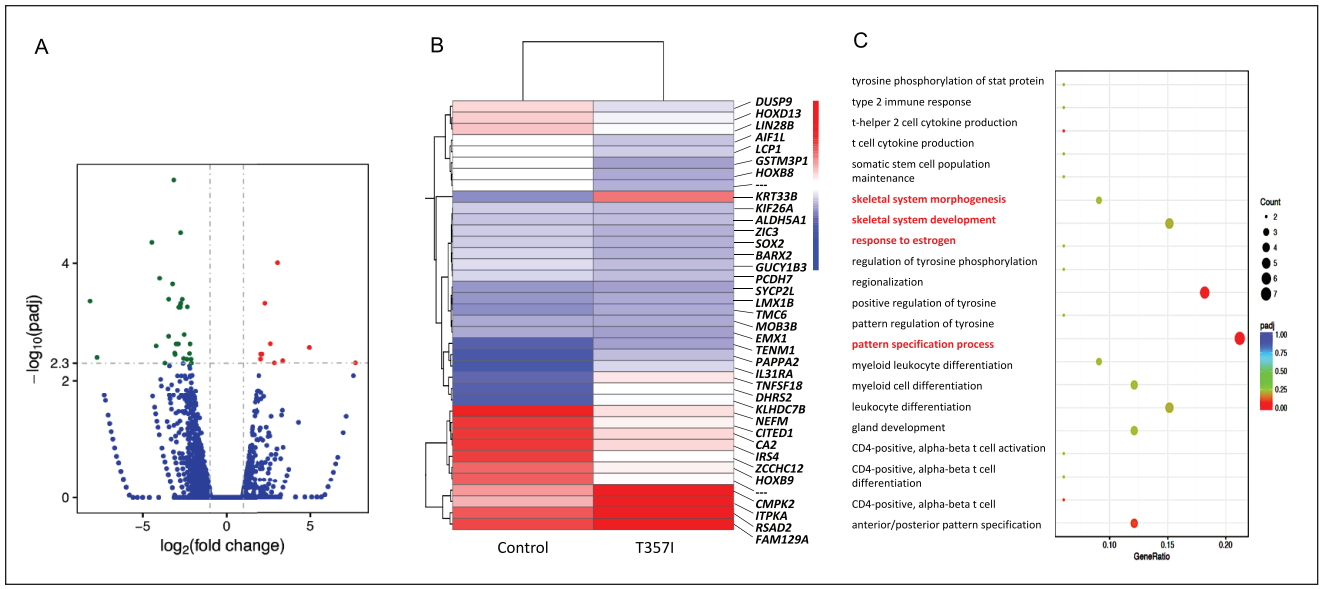


Figure 4. Transcriptome analysis of *WNT10A* wild-type and T357I-mutant cells with RNA-seq. (**A**) Volcano plot shows the number of differentially expressed genes. Vertical dashed lines correspond to 2-fold changes in expression (log2 scaled); the horizontal dashed line represents a *P* value of 0.05. Red and green dots represent significantly up- and downregulated genes, respectively. (**B**) Hierarchical clustering heat map of differentially expressed genes. Intensity of expression is shown from blue (relatively lower expression) to red (relatively higher expression). (**C**) Gene Ontology (GO) analyses of differentially expressed genes. Dot map of the top 20 significantly enriched GO terms in biological processes, cellular components, and molecular functions. "Count" is the number of differentially expressed genes concerning each GO term. "GeneRatio" is the ratio between the number of differentially expressed genes in each GO term and all differentially expressed genes that can be found in the GO database. Adjusted *P* values are represented by a color scale from purple (relatively lower significance) to red (relatively higher significance).

development and morphogenesis. Of these, previous evidence supports the role of *DUSP9* (dual specificity phosphatase 9), *SOX2* (sex determining region Y-box 2), and *PAPPA2* (pappalysin 2) in craniofacial and/or tooth development. Phenotypes associated with mutant *SOX2* and *PAPPA2* in humans and/or mice include craniofacial and dental defects, such as creased cranium length, abnormal mandible morphology, short mandible, supernumerary teeth, and retention of deciduous teeth (Numakura et al. 2010; Christians et al. 2013). *DUSP9* belongs to the DUSP gene family and acts as negative regulator of the MAPK family (mitogen-activated protein kinase). Activation of MAPK signaling by numerous stimuli (i.e., growth factors, cytokines, cellular stress) regulates essential cellular processes, such as proliferation, differentiation, survival, migration, or production of soluble factors (Bermudez et al. 2010). In a previous study, we found variations in another *DUSP* gene, *DUSP10* (dual specificity phosphatase 10), as significantly associated with TA. Furthermore, we showed for the first time that this gene was expressed during murine tooth development (Williams et al. 2018). Although the exact function of these differentially expressed genes in tooth development remains to be elucidated, these findings provide solid evidence that variation in *WNT10A* may regulate the expression and function of genes involved in tooth formation.

Taken together, our results suggest that *WNT10A* variants may affect gene/protein function in a variant-specific manner, through direct effects on WNT signaling or indirectly through effects on additional genes. It is also possible that the location of *WNT10A* variants may be correlated with the phenotypic variability and/or severity of TA. In a recent meta-analysis on the dental phenotypes of 522 patients with TA, the most frequently reported patterns of *WNT10A*-associated TA phenotypes include a variable number of missing teeth, with third molars being the most frequently affected teeth (78%), followed by mandibular premolars (71%) and maxillary lateral incisors (60%; Fournier et al. 2018). These TA patterns, however, are not unique to *WNT10A* variation and match previously reported missing tooth types in the general population (Polder et al. 2004). The C107*, R113C, and F228I mutations have been described as hotspots for TA with overlapping TA-associated patterns, whereas a preferential association between the G213S variant and maxillary canine agenesis was noted (Fournier et al. 2018). In agreement with these findings, we and others have identified the F228I mutation—as single or compound heterozygous forms or segregating with variants in other genes—in individuals with mild and severe TA and overlapping phenotypes (van den Boogaard et al. 2012; Arzoo et al. 2014; Song et al. 2014; Dinckan et al. 2018; Du et al. 2018). These studies show that the TA phenotypes associated with *WNT10A* mutations are highly variable, and while individuals presenting the same variant (e.g., F228I, V145M) may have distinct TA phenotypes, significant overlap in missing tooth patterns is also observed. Interestingly, evidence of multilocus genomic variation (i.e., the cosegregation of variants in different genes) was recently suggested to explain complex phenotypes (e.g., Bardet-Biedl syndrome), including tooth agenesis (Posey et al. 2017; Dinckan et al. 2018; Du et al. 2018). These studies highlight the heterogeneity of the condition and,

importantly, indicate that identification of *WNT10A* mutations should not be considered as the basis for exclusion in molecular diagnosis of TA patients.

The limitations of this study include the selected set of *WNT10A* variants for evaluation, leaving additional potential functional variants to be investigated. Furthermore, transiently transfected genes are expressed for a limited period, so it may be possible that transiently transfected genetic materials can be lost by exogenous factors and cell division. However, this method is safe and reliable and ensures high transfection efficiency, low toxicity, and reproducibility (Kim and Eberwine 2010).

Thus far, it seems plausible that *WNT10A* variants deemed pathogenic for TA likely affect protein folding and/or stabilization, leading to decreased WNT signaling and concomitant dysregulated expression of relevant genes: the more deleterious the effects, the stronger likelihood of contributing to more severe TA phenotypes. Understanding the effects of *WNT10A* variants in gene/protein function may allow for improved interpretation of TA phenotypes upon clinical diagnosis while providing important insights toward the development of future tooth replacement therapies.

Author Contributions

Y. Zeng, contributed to data acquisition and analysis, drafted the manuscript; E. Baugh, contributed to data acquisition, analysis, or interpretation, critically revised the manuscript; S. Akyalcin, contributed to data interpretation, critically revised the manuscript; A. Letra, contributed to conception, design, data interpretation, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

We thank Dr. Songtao Shi, University of Pennsylvania, for kindly providing SHED for this study.

Declaration of Conflicting Interests

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

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by an American Association of Orthodontists Foundation Biomedical Research Award (to S.A. and A.L.) and University of Texas School of Dentistry start-up funds (to A.L.).

ORCID iD

A. Letra https://orcid.org/0000-0002-7197-6735

References

Agostino M, Pohl SO. 2019. WNT binding affinity prediction for putative frizzled-type cysteine-rich domains. Int J Mol Sci. 20(17):4168.

Arzoo PS, Klar J, Bergendal B, Norderyd J, Dahl N. 2014. WNT10A mutations account for (1/4) of population-based isolated oligodontia and show phenotypic correlations. Am J Med Gen Part A. 164(2):353–359.

Baugh EH, Simmons-Edler R, Muller CL, Alford RF, Volfovsky N, Lash AE, Bonneau R. 2016. Robust classification of protein variation using structural modelling and large-scale data integration. Nucleic Acids Res. 44(6):2501–2513.

Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD, MacDougald OA. 2005. Regulation of osteogenesis and bone mass by WNT10B. Proc Natl Acad Sci U S A. 102(9):3324–3329.

Bermudez O, Pages G, Gimond C. 2010. The dual-specificity map kinase phosphatases: critical roles in development and cancer. Am J Physiol Cell Physiol. 299(2):C189–C202.

Christians JK, de Zwaan DR, Fung SH. 2013. Pregnancy associated plasma protein A2 (PAPP-A2) affects bone size and shape and contributes to natural variation in postnatal growth in mice. PLoS One. 8(2):e56260.

D'Souza RN, Aberg T, Gaikwad J, Cavender A, Owen M, Karsenty G, Thesleff I. 1999. Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. Development. 126(13):2911–2920.

Dinckan N, Du R, Petty LE, Coban-Akdemir Z, Jhangiani SN, Paine I, Baugh EH, Erdem AP, Kayserili H, Doddapaneni H, et al. 2018. Whole-exome sequencing identifies novel variants for tooth agenesis. J Dent Res. 97(1):49–59.

Du R, Dinckan N, Song X, Coban-Akdemir Z, Jhangiani SN, Guven Y, Aktoren O, Kayserili H, Petty LE, Muzny DM, et al. 2018. Identification of likely pathogenic and known variants in TSPEAR, LAMB3, BCOR, and WNT10A in four Turkish families with tooth agenesis. Hum Gen. 137(9):689–703.

Fournier BP, Bruneau MH, Toupenay S, Kerner S, Berdal A, Cormier-Daire V, Hadj-Rabia S, Coudert AE, de La Dure-Molla M. 2018. Patterns of dental agenesis highlight the nature of the causative mutated genes. J Dent Res. 97(12):1306–1316.

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, et al. 2005. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem. 280(39):33132–33140.

Gorlin RJ, Cohen M Jr, Levin SL. 1990. Syndromes of the head and neck. New York (NY): Oxford University Press.

Grejtakova D, Gabrikova-Dojcakova D, Boronova I, Kyjovska L, Hubcejova J, Fecenkova M, Zigova M, Priganc M, Bernasovska J. 2018. WNT10A variants in relation to nonsyndromic hypodontia in eastern Slovak population. J Genet. 97(5):1169–1177.

Hu X, Zhong Y, Kong Y, Chen Y, Feng J, Zheng J. 2019. Lineage-specific exosomes promote the odontogenic differentiation of human dental pulp stem cells (DPSCs) through TGFβ1/smads signaling pathway via transfer of microRNAs. Stem Cell Res Ther. 10(1):170.

James MJ, Jarvinen E, Wang XP, Thesleff I. 2006. Different roles of Runx2 during early neural crest–derived bone and tooth development. J Bone Miner Res. 21(7):1034–1044.

Jia S, Zhou J, Fanelli C, Wee Y, Bonds J, Schneider P, Mues G, D'Souza RN. 2017. Small-molecule Wnt agonists correct cleft palates in Pax9 mutant mice in utero. Development. 144(20):3819–3828.

Kim TK, Eberwine JH. 2010. Mammalian cell transfection: the present and the future. Anal Bioanal Chem. 397(8):3173–3178.

Letra A, Zhao M, Silva RM, Vieira AR, Hecht JT. 2014. Functional significance of MMP3 and TIMP2 polymorphisms in cleft lip/palate. J Dent Res. 93(7):651–656.

Liu F, Millar SE. 2010. WNT/β-catenin signaling in oral tissue development and disease. J Dent Res. 89(4):318–330.

Magruder S, Carter E, Williams MA, English J, Akyalcin S, Letra A. 2018. Further evidence for the role of WNT10A, WNT10B and GREM2 as candidate genes for isolated tooth agenesis. Orthod Craniofac Res. 21(4):258–263.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. 2003. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 100(10):5807–5812.

Mostowska A, Kobielak A, Trzeciak WH. 2003. Molecular basis of non-syndromic tooth agenesis: mutations of MSX1 and PAX9 reflect their role in patterning human dentition. Euro J Oral Sci. 111(5):365–370.

Nakatomi M, Wang XP, Key D, Lund JJ, Turbe-Doan A, Kist R, Aw A, Chen Y, Maas RL, Peters H. 2010. Genetic interactions between PAX9 and MSX1 regulate lip development and several stages of tooth morphogenesis. Dev Biol. 340(2):438–449.

Numakura C, Kitanaka S, Kato M, Ishikawa S, Hamamoto Y, Katsushima Y, Kimura T, Hayasaka K. 2010. Supernumerary impacted teeth in a patient with SOX2 anophthalmia syndrome. Am J Med Gen Part A. 152(9):2355–2359.

Peters H, Neubuser A, Kratochwil K, Balling R. 1998. PAX9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev. 12(17):2735–2747.

Polder BJ, Van't Hof MA, Linden FPGM, Kujipers-Jagtman AM. 2004. A meta-analysis of the prevalence of dental agenesis of permanent teeth. Community Dent Oral Epidemiol. 32(3):217–226.

Posey JE, Harel T, Liu P, Rosenfeld JA, James RA, Coban Akdemir ZH, Walkiewicz M, Bi W, Xiao R, Ding Y, et al. 2017. Resolution of disease phenotypes resulting from multilocus genomic variation. N Engl J Med. 376(1):21–31.

Sakai VT, Zhang Z, Dong Z, Neiva KG, Machado MA, Shi S, Santos CF, Nor JE. 2010. SHED differentiate into functional odontoblasts and endothelium. J Dent Res. 89(8):791–796.

Song S, Zhao R, He H, Zhang J, Feng H, Lin L. 2014. WNT10A variants are associated with non-syndromic tooth agenesis in the general population. Hum Gen. 133(1):117–124.

Thesleff I, Vaahtokari A, Vainio S, Jowett A. 1996. Molecular mechanisms of cell and tissue interactions during early tooth development. Anat Rec. 245(2):151–161.

van den Boogaard MJ, Creton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK. 2012. Mutations in WNT10A are present in more than half of isolated hypodontia cases. J Med Gen. 49(5):327–331.

Voloshanenko O, Gmach P, Winter J, Kranz D, Boutros M. 2017. Mapping of Wnt-Frizzled interactions by multiplex CRISPR targeting of receptor gene families. FASEB J. 31(11):4832–4844.

Williams MA, Biguetti C, Romero-Bustillos M, Maheshwari K, Dinckan N, Cavalla F, Liu X, Silva R, Akyalcin S, Uyguner ZO, et al. 2018. Colorectal cancer–associated genes are associated with tooth agenesis and may have a role in tooth development. Sci Rep. 8(1):2979.

Williams MA, Letra A. 2018. The changing landscape in the genetic etiology of human tooth agenesis. Genes (Basel). 9(5):255.

Xu M, Horrell J, Snitow M, Cui J, Gochnauer H, Syrett CM, Kallish S, Seykora JT, Liu F, Gaillard D, et al. 2017. WNT10A mutation causes ectodermal dysplasia by impairing progenitor cell proliferation and KLF4-mediated differentiation. Nat Commun. 8:15397.

Xue J, Chen Y, Wu Y, Wang Z, Zhou A, Zhang S, Lin K, Aldape K, Majumder S, Lu Z, et al. 2015. Tumour suppressor TRIM33 targets nuclear beta-catenin degradation. Nat Commun. 6:6156.

Yamashiro T, Zheng L, Shitaku Y, Saito M, Tsubakimoto T, Takada K, Takano-Yamamoto T, Thesleff I. 2007. WNT10A regulates dentin sialophosphoprotein mrna expression and possibly links odontoblast differentiation and tooth morphogenesis. Differentiation. 75(5):452–462.

Yang J, Wang SK, Choi M, Reid BM, Hu Y, Lee YL, Herzog CR, Kim-Berman H, Lee M, Benke PJ, et al. 2015. Taurodontism, variations in tooth number, and misshapened crowns in WNT10A null mice and human kindreds. Mol Genet Genomic Med. 3(1):40–58.

Yin W, Bian Z. 2015. The gene network underlying hypodontia. J Dent Res. 94(7):878–885.

Yuan Q, Zhao M, Tandon B, Maili L, Liu X, Zhang A, Baugh EH, Tran T, Silva RM, Hecht JT, et al. 2017. Role of WNT10A in failure of tooth development in humans and zebrafish. Mol Genet Genomic Med. 5(6):730–741.

Zhang Z, Nor F, Oh M, Cucco C, Shi S, Nor JE. 2016. WNT/β-catenin signaling determines the vasculogenic fate of postnatal mesenchymal stem cells. Stem Cells. 34(6):1576–1587.

FUNCTIONAL CHARACTERIZATION OF *ATF1, GREM2* AND *WNT10B* VARIANTS ASSOCIATED WITH TOOTH AGENESIS

Meredith Williams[1], Yu Zeng[2,3], Brett Chiquet[3,4,5], Helder Jacob[1], F. Kurtis Kasper[1,3], Daniel A. Harrington[2,3], Jeryl English[1], Sercan Akyalcin[6], Ariadne Letra[2,3,5]

1 Department of Orthodontics, University of Texas Health Science Center School of Dentistry, Houston, 77054, USA

2 Department of Diagnostic and Biomedical Sciences, University of Texas Health Science Center School of Dentistry, Houston, 77054, USA

3 Center for Craniofacial Research, University of Texas Health Science Center School of Dentistry, Houston, 77054, USA

4 Department of Pediatric Dentistry, University of Texas Health Science Center School of Dentistry, Houston, 77054, USA

5 Pediatric Research Center, University of Texas Health Science Center McGovern Medical School, Houston, 77054, USA

6 Department of Orthodontics, Tufts University School of Dental Medicine, Boston, 02155, USA

Corresponding author:

Ariadne Letra, DDS, MS, PhD

Interim Associate Dean for Research

Professor, Department of Diagnostic and Biomedical Sciences

University of Texas Health Science Center School of Dentistry

1941 East Road, BBSB Room 4210

Houston, 77054, USA

Phone: 713-486-4228 (office); 713-486-2577 (lab)

Ariadne.M.Letra@uth.tmc.edu

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/OCR.12462

Running title: Functional effects of tooth agenesis variants

Acknowledgements: We thank Dr. Songtao Shi, University of Pennsylvania, for kindly donating SHED cells for this study. This study was supported by an American Association of Orthodontists Foundation Biomedical Research Award (to SA and AL), and startup funds to AL.

DR HELDER BALDI JACOB (Orcid ID : 0000-0003-2642-0540)
DR ARIADNE LETRA (Orcid ID : 0000-0002-7197-6735)

Article type : Original Article

FUNCTIONAL CHARACTERIZATION OF *ATF1, GREM2* AND *WNT10B* VARIANTS ASSOCIATED WITH TOOTH AGENESIS

ABSTRACT

OBJECTIVE: To determine the functional effects of *ATF1, WNT10B* and *GREM2* gene variants identified in individuals with tooth agenesis (TA).

SETTINGS and SAMPLE POPULATION: Stem cells from human exfoliated deciduous teeth (SHED) were used as an in vitro model system to test the effect of TA-associated variants.

MATERIALS AND METHODS: Plasmid constructs containing reference and mutant alleles for *ATF1* rs11169552, *WNT10B* rs833843 and *GREM2* rs1414655 variants were transfected into SHED for functional characterization of variants. Allele-specific changes in gene transcription activity, protein expression, cell migration and proliferation, and expression of additional tooth development genes (*MSX1, PAX9,* and *AXIN2)* were evaluated. Data analyses was performed using one-way ANOVA, Student's *t*-test, and Student-Newman-Keuls post-hoc test. P-values ≤ 0.05 were considered statistically significant.

RESULTS: Mutant variants resulted in significantly decreased transcriptional activity of respective genes ($p<0.05$), although no changes in protein localization were found. Expression of *MSX1* was significantly decreased in *ATF1*- and *GREM2*-mutant cells, whereas *PAX9* or *AXIN2* mRNA expression was not significantly altered. Mutant *WNT10B* had no significant effect on the expression of additional TA genes. *ATF1*- and *GREM2*-mutant cells presented increased cell migration. Cell proliferation was also affected with all three mutant alleles.

CONCLUSIONS: Our results demonstrate that *ATF1, WNT10B,* and *GREM2* mutant alleles have modulatory effects on gene/protein function that may contribute to TA.
KEY WORDS: genetic variation, hypodontia, oligodontia, functional assays

INTRODUCTION

Tooth agenesis (TA) is the most common anomaly of the human dentition, and is characterized by the absence of one or more permanent teeth.[1] The prevalence of TA ranges from 1-10% in the general population, and up to 20% when missing third molars are included, with 70-80% of the cases missing one to two teeth.[2] TA can present as familial or sporadic forms; most familial cases are characterized by autosomal dominant inheritance, however X-linked and autosomal recessive inheritance has also been reported.[1] While TA may at first appear to be a harmless condition, individuals often experience psychosocial, esthetic, and functional consequences, making it a public health concern. TA has variable expressivity resulting in either hypodontia (missing 1-5 teeth), oligodontia (missing 6 or more teeth), or anodontia (the complete absence of any permanent teeth).[2]

The etiology of TA is mostly genetic and damaging mutations in numerous genes (e.g. *MSX1, PAX9, AXIN2, EDA, EDARADD, LRP6, GREM2, TSPEAR, WNT10A, WNT10B)* have been identified as causal for the condition.[3] In previous studies, we replicated the association of *WNT10A, WNT10B* and *GREM2* genes with TA phenotypes.[4] We also identified *ATF1* as a novel candidate gene for TA and showed its temporal-spatial expression during murine tooth development[5]. While the function of some TA-associated variants and their respective genes has been previously established, the role of the more recently identified variants on gene/protein function remains to be elucidated. Understanding the impact of genetic variants on gene/protein function might allow for improved interpretation of genotype-phenotype relationships.[5]

In this study, we performed functional characterization experiments of variants located in putative regulatory regions of *ATF1, WNT10B,* and *GREM2* genes and previously associated

with TA. Our findings show that all three variants may contribute to dysregulation of gene/protein function with further implications in TA.

MATERIALS AND METHODS

Details of the selected variants in *ATF1*, *GREM2* and *WNT10B* are presented on Table 1.

Cell Culture

Stem cells from human exfoliated deciduous teeth (SHED) were kindly provided by Dr. Songtao Shi, University of Pennsylvania, under IRB-approved protocols (HSC-DB-18-0765). SHED were characterized and cultured as previously described[6]. Briefly, single-cell suspensions were cultured in α-minimum essential medium (α-MEM, Gibco #12571048)), supplemented with 15% fetal bovine serum (FBS, Equitech Bio Inc.), penicillin/streptomycin 10,000U/mL each (Invitrogen), L-ascorbic acid phosphate 0.1mM (Wako Chemicals), and glutamine (2mM, Invitrogen).

Generation of Plasmid Constructs

For each variant, constructs were made by amplifying the target gene region containing the reference alleles from human genomic DNA samples as described elsewhere.[7] Mutant alleles were then obtained using site-directed mutagenesis.[8] Primers for PCR amplification of target regions were designed using Primer3 software[9] (Supplementary Tables 1 and 2). The resulting *ATF1* and *WNT10B* constructs, containing either rs11169552-C (reference) or rs11169552-T (mutant) alleles, and either rs833843-C (reference) or rs833843-T (mutant), respectively, were then inserted between NheI and HindIII restriction sites of pGL4.10[luc2] vector (Promega). The resulting *GREM2* 3'-UTR constructs containing rs1414655-T (reference) or rs1414655-C (mutant) were inserted between the XbaI and NheI restrictive sites of pLight-switch_3'UTR vector (Switchgear Genomics). DNA sequencing was used to confirm cloning efficiency.[7]

Luciferase Reporter Gene Assays

SHED were seeded at a density of 15,000 cells/well into 96-well plates and cultured for 24 hours to reach 80% confluence. Cells were then transfected with 300 ng of either wild type or each variant construct, and co-transfected with a pGL4.74[hRluc/TK] control plasmid, in a 20:1 ratio

of firefly luciferase reporter to renilla luciferase reporter in Opti-MEM medium (Life Technologies, Grand Island, NY, USA) and 0.3 μL of Lipofectamine Stem Transfection Reagent (Invitrogen, Carlsbad, CA, USA) for 48 hours at 37° C. Cells transfected with empty pGL4.10[luc2] or pLight-switch_3'UTR vectors were used as controls. Cells were incubated with 100 μL Dual-Glo Reagent for 10 min. Luciferase activity was measured using a microplate reader (Synergy H1, BioTek Instruments, Winooski, VT, USA) and then 100 μL of Dual-Glo Stop & Glo reagent was added and incubated for 10 min. Renilla luciferase activity was measured with Synergy H1 Multi-Mode Microplate reader (BioTek Instruments). Measurements obtained for firefly luciferase activity were divided by renilla luciferase activity to control for transfection efficiency, and normalized by the expression from empty vectors.[7]

mRNA Expression Analysis

Reverse transcription qPCR (RT-qPCR) was used to investigate whether variant alleles might perturb expression of the known tooth development genes *AXIN2, PAX9,* and *MSX1*. Cells were transfected with reference and mutant variant alleles as described, followed by total RNA extraction using Quick-RNA Mini Prep Kit (Zymo Research, Tustin, CA, USA). cDNA synthesis was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were performed using SYBR green chemistry and specific oligonucleotides (Supplementary Table 3) in a ViiA7 Sequence Detection Instrument (Life Technologies). Reaction conditions were: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 sec, and 55°C for 1 min. Experiments were performed in triplicates and repeated twice. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method[10] considering target gene expression levels normalized to that of *GAPDH* as endogenous control gene.

Cell Migration

SHED were seeded at a density of 3×10^3 per well into 6-well plates and incubated for 24 hours at 5% CO_2 and 37°C. Cells were then transfected with either reference or mutant alleles for each variant and incubated for 24 hours. Empty pGL4.10 vector and pLight-switch 3'UTR vector were used as controls. After 24 hours, fresh serum-free Opti-MEM was added and cells were incubated to reach 90-100% confluence. Next, a sterile micropipette tip was used to produce a vertical scratch on each well as previously described.[11] To remove free-floating cells, wells were washed with Opti-MEM and incubated in fresh Opti-MEM medium at 37°C. Cell migration was

documented at 24 and 48 hours under phase contrast microscopy (Nikon Instruments Inc, Melville, NY, USA). ImageJ software was used to measure wound closure.[12]

Cell Proliferation

Cells were transfected with either reference or mutant alleles for each variant and incubated for 24 hours. Cells were then plated at a density of 3 x 10^3 per well into a 96-well plate and incubated at 37°C for 0, 24, 48 and 72 hours, for use in cell proliferation and viability assays (CCK-8) following manufacturer's instructions. In each well, 10 µl of CCK-8 reagent (Dojindo Molecular Technologies, Rockville, MD) was added and cells were incubated for 2 hours at 37°C. The number of viable cells in each well was assessed by measurement of absorbance at 450 nm using a microplate reader (BioTek Instruments).

Immunocytochemistry

Transfected cells were assessed for expression of ATF1, WNT10B and GREM2 proteins using immunocytochemistry.[13] The following antibodies were used: for ATF1 [rabbit monoclonal anti-ATF1 (Abcam, ab#76085, 1:100 dilution) and goat anti-Rabbit IgG (H+L) Cy5 secondary antibody (Invitrogen, A#10523, 1:200 dilution)], for WNT10B [rabbit polyclonal anti-WNT10B (Abcam, #ab70816, 1:100 dilution) and goat anti-Rabbit IgG (H+L) Alexa Fluor 488 secondary antibody (Invitrogen, A#11008; dilution 1:200)], and for GREM2 [rabbit polyclonal anti-GREM2 (Invitrogen, #PA5-78559; dilution 1:100) and goat anti-Rabbit IgG (H+L) Alexa Fluor 488 (Invitrogen, A#11008, dilution 1:200)]. Signals were detected at 48 hours post-transfection and assessed using a confocal laser scanning microscope (Nikon C2+, Nikon Instruments Inc., Melville, NY, USA) with 60x magnification. Images were analyzed using ImageJ software.[12]

Data Analyses

Data analyses were performed using GraphPad Prism v.5 (GraphPad Inc). All values are represented by mean ± standard deviation (SD) and all *P* values reported are calculating from a two-sided Student's t test.

RESULTS

Mutant alleles decrease gene transcription activity

Figure 1 presents the results of dual luciferase assays for evaluation of *ATF1*, *WNT10B* and *GREM2* gene transcriptional activities in the presence of respective reference and mutant alleles. *ATF1* rs11169552-T resulted in a 1.35-fold decrease in promoter activity when compared with the reference-C allele ($p < .001$). *WNT10B* rs833843-T resulted in a 1.5-fold decrease ($p < .05$) in promoter activity when compared with the reference-C allele. *GREM2* rs1414655-C resulted in a 1.3-fold decrease ($p < .001$) in gene expression when compared with the reference T-allele. Our findings suggest that all three mutant alleles impacted gene transcription.

Protein expression

We assessed for allele-specific changes in protein expression in ATF1-, WNT10B-, and GREM2- reference and alternate allele transfected cells using immunocytochemistry. Our results showed positive expression of all proteins with both reference and alternate alleles, although decreased expression was observed with ATF1-T, WNT10B-C and GREM2-T alleles (p=.002, p=.02, and p=.02, respectively) (Figure 2).

Allele-specific cell migration and proliferation

At 24h post-transfection, *GREM2* T allele-transfected cells showed slightly lower cell migration in comparison to C allele (p=.03), meanwhile no significant allele-specific differences were found for *ATF1* and *WNT10B* variants. At 48h post-transfection, *ATF1* T allele-transfected cells showed increased cell migration when compared to the C allele (p=0.01) whereas no significant differences were detected for *WNT10B* and *GREM2* variants (Figure 3).

With regards to cell proliferation, *ATF1*-mutant cells showed increased proliferation rates at all time points in comparison with empty vector-transfected cells and cells transfected with the reference allele (p<.05). For *WNT10B*, differences in proliferation rates between reference and mutant-allele transfected cells were seen at 48h and 72h (p<.05). *GREM2* mutant allele T resulted in decreased cell proliferation at 24h and 72h (p<.05, respectively) in comparison to reference allele (Figure 4).

Expression of additional tooth development genes

Figure 5 presents the results of *MSX1, AXIN2* and *PAX9* mRNA expression analysis in SHEDs transfected with each variant reference and mutant allele constructs. Expression of *MSX1* was

significantly decreased in *ATF1*- and *GREM2*-mutant cells (p=.001 and p=.04, respectively), whereas *PAX9* or *AXIN2* mRNA expression was not significantly altered (p > .05). *WNT10B* had no significant effect on the expression of *MSX1, AXIN2* or *PAX9* (p > .05) (Figure 5).

DISCUSSION

Numerous genes have been implicated in the etiology of TA,[6,14,15] although the biological effects of putatively pathogenic TA variants on gene/protein function are not fully understood. Understanding the impact of genetic variants on gene/protein function might allow for improved clinical diagnosis and interpretation of genotype-phenotype relationships.[6]

ATF1, *WNT10B* and *GREM2* were recently suggested as candidate genes for TA,[5,8,16] and replicated in independent studies.[4,17,18] In this study, we assessed the potential allele-specific effects of the TA-associated variants *ATF1* rs11169552, *WNT10B* rs833843 and *GREM2* rs1414655, located in the regulatory regions of their respective genes and with potential functional effects on gene activity. We used SHEDs as a TA-relevant *in vitro* model system due to their source location (obtained from human exfoliated deciduous teeth), and because they are easily accessible, multipotent, and highly proliferative.[6] Tooth development is a complex process and requires the coordinated expression of numerous genes and transcription factors in the dental epithelia and subjacent mesenchyme as well as their interactions for proper tooth formation. Therefore, any disturbances in gene/protein expression and/or function may perturb this tightly controlled mechanism leading to arrest of tooth development.[15]

ATF1 is a transcription factor that regulates a variety of target genes involved in different cellular processes and which bind the consensus ATF/CRE site 'TGACGTCA'.[19] Importantly, *ATF1* has been strongly implicated in increased predisposition to colorectal cancer,[20] and independently associated with isolated TA.[5] Further, we showed for the first time, that *Atf1* was expressed in the developing mouse teeth, reinforcing the role of this gene during tooth development. In the initiation phase of tooth development, *Atf1* expression was detected in the oral epithelium and adjacent ectomesenchymal cells when the epithelium begins to thicken. During the cap and bell stages of tooth development, *Atf1* was expressed within the inner dental epithelium, stratum intermedium and is scattered throughout the dental papilla. At late bell stage, *Atf1* expression is seen in the Tome's process and in the cytoplasm of polarized ameloblasts.[5]

WNT10B belongs to the WNT/ß-catenin signaling gene family and has been implicated in regulation of cell fate and patterning during embryogenesis and oncogenesis.[21,22] Mutations in *WNT10B* have been reported in unrelated Chinese and Brazilian individuals with nonsyndromic TA, and suggested to play a role in agenesis of the permanent upper lateral incisors due to decreased activation of Wnt signaling which in turn did not effectively induce endothelial cell differentiation.[4,23] Notably in mice, Wnt10b expression was restricted to the epithelium around the area of the presumptive developing tooth and then at the cap stage of tooth development, especially in the incisor region.[8,24]

Variation in *GREM2* was first identified as a putative cause of isolated TA in a Thai population,[16] and successfully replicated in Caucasian populations.[4,25] *GREM2* is a negative regulator of BMP signaling[26,27] and may interfere with the function of *BMP4* which is known to play a role in tooth formation.[28,29] During murine tooth development, Grem2 expression was seen in the mesenchymal cells beneath the thickening of the epithelium in the oral cavity prior to the bud stage, in the dental papilla during the cap stage, and weak expression in the cervical loop and the mesenchymal cells adjacent to the cervical loop during the bell stage of murine tooth development.[16] *Grem2* knockout mice show malformation of the upper and lower incisors resulting in smaller and deformed teeth when compared to the wild type.[30]

Overall, our findings support a functional role for *ATF1*, *GREM2* and *WNT10B* variants, located in putative regulatory regions of their respective genes with allele-specific effects on gene transcription activity, cell migration and proliferation, as well as expression of additional tooth development genes. The results of our luciferase assays showed that *ATF1*, *GREM2* and *WNT10B* mutant alleles resulted in decreased gene transcription. ATF1 protein expression was also significantly decreased with the mutant allele T, meanwhile WNT10B and GREM2 protein expression were increased with the mutant alleles; this suggests that compensating mechanisms may exist to fulfill the activity of these molecules.

Cell migration and proliferation are essential processes during tooth development, and interruptions in these events have been shown to affect normal tooth formation. It has been shown that specific inhibiting of FGF and SHH signaling causes TA by interrupting cellular migration.[31] Our results suggested allele-specific effects on cellular migration and proliferation with each of the investigated variants at different time points. For all variants, cell migration and proliferation increased with time; cell proliferation peaked at 48h post transfection for both

reference and mutant alleles, decreasing thereafter. Our most significant findings at 24h include that *GREM2* mutant cells (T allele) migrated less/slower and proliferated less than those with the reference allele; whereas *ATF1* T allele-transfected cells presented increased cell migration and proliferation when compared to the C allele. In this context, numerous studies showed that *ATF1* overexpression resulted in increased cell proliferation in various cancers.[20,32,33] However, those effects seem to be site-specific, as other studies demonstrate that *ATF1* might also act as a tumor suppressor such as in breast cancer.[34] Moreover, increased *ATF1* expression in colorectal cancer tumor samples was associated with better prognosis for overall survival.[20] *GREM2* also has a documented role in altering gastric cancer stem cell proliferation and migration[35] and in inhibiting the canonical BMP pathway resulting in the proliferation of cardiac progenitor cells.[36] Taken together, these observations suggest that *ATF1* and *GREM2* variation exert tissue-specific effects on cell migration and proliferation, and could potentially impact normal tooth development.

To further explore the role of *ATF1, WNT10B,* and *GREM2* variants in tooth development, we assessed the expression of *MSX1, PAX9,* and *AXIN2,* for which a role in tooth development and TA has been established,[14,37–39] in reference and mutant allele-transfected cells. Overall, our results suggest that only *MSX1* expression is altered, with significantly decreased expression in the presence of *ATF1* and *GREM2* mutant alleles. Additional studies are warranted to clarify if direct interactions exist between *MSX1, ATF1* and *GREM2.*

CONCLUSION

Our results demonstrate that *ATF1, WNT10B,* and *GREM2* variants have functional effects on gene regulation and reveal possible mechanisms by which these variants may affect tooth development thereby leading to TA. Further, our findings expand current knowledge on the genetic etiology of TA which may provide insights into genotype-phenotype correlations in TA.

ACKNOWLEDGMENTS

Thanks to Dr. Songtao Shi (Univeristy of Pennsylvania for kindly donating SHED cells). This study was supported by an American Association of Orthodontists Biomedical Research Award (to AL and SA), and start-up funds to AL.

AUTHOR CONTRIBUTIONS

MW and YZ contributed to data acquisition and interpretation, performed statistical analyses and drafted the manuscript. BC, HJ, FKK, DAH, JE, and SA contributed to data interpretation, critically revised the manuscript. AL contributed to study conception and design, data interpretation, drafted and critically revised the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose with respect to this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Shapiro SD, Farrington FH. A potpourri of syndromes with anomalies of dentition. *Birth Defects Orig Artic Ser* 1983;19:129-140.

2. Polder BJ, Hof MAV, Linden FPGMV der, Kuijpers- Jagtman AM. A meta-analysis of the prevalence of dental agenesis of permanent teeth. *Community Dent Oral Epidemiol* 2004;32:217-226.

3. Williams MA, Letra A. The Changing Landscape in the Genetic Etiology of Human Tooth Agenesis. *Genes* 2018;9(5).

4. Magruder S, Carter E, Williams MA, English J, Akyalcin S, Letra A. Further evidence for the role of WNT10A, WNT10B and GREM2 as candidate genes for isolated tooth agenesis. *Orthod Craniofac Res* 2018;21:258-263.

5. Williams MA, Biguetti C, Romero-Bustillos M, et al. Colorectal Cancer-Associated Genes Are Associated with Tooth Agenesis and May Have a Role in Tooth Development. *Sci Rep* 2018;8:2979.

6. Miura M, Gronthos S, Zhao M, et al. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 2003;100:5807-5812.

7. Letra A, Zhao M, Silva RM, Vieira AR, Hecht JT. Functional Significance of MMP3 and TIMP2 Polymorphisms in Cleft Lip/Palate. *J Dent Res* 2014;93:651-656.

8. Yu P, Yang W, Han D, et al. Mutations in WNT10B Are Identified in Individuals with Oligodontia. *Am J Hum Genet* 2016;99:195-201.
9. Untergasser A, Cutcutache I, Koressaar T, et al. Primer3--new capabilities and interfaces. *Nucleic Acids Res* 2012;40:e115.
10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif* 2001;25:402-408.
11. Rebollo J, Geliebter J, Reyes N. ESM-1 siRNA Knockdown Decreased Migration and Expression of CXCL3 in Prostate Cancer Cells. *Int J Biomed Sci IJBS* 2017;13:35-42.
12. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671-675.
13. Donaldson JG. Immunofluorescence Staining. *Curr Protoc Cell Biol* 2015;69:4.3.1-4.3.7.
14. Vastardis H. The genetics of human tooth agenesis: New discoveries for understanding dental anomalies. *Am J Orthod Dentofacial Orthop* 2000;117:650-656.
15. Cobourne MT, Sharpe PT. Diseases of the tooth: the genetic and molecular basis of inherited anomalies affecting the dentition. *Wiley Interdiscip Rev Dev Biol* 2013;2:183-212.
16. Kantaputra PN, Kaewgahya M, Hatsadaloi A, et al. GREMLIN 2 Mutations and Dental Anomalies. *J Dent Res* 2015;94:1646-1652.
17. Kantaputra PN, Hutsadaloi A, Kaewgahya M, et al. WNT10B mutations associated with isolated dental anomalies. *Clin Genet* 2018;93:992-999.
18. Mostowska A, Biedziak B, Zadurska M, et al. GREM2 nucleotide variants and the risk of tooth agenesis. *Oral Dis* 2018;24:591-599.
19. Hai T, Hartman MG. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 2001;273:1-11.
20. Huang G-L, Guo H-Q, Yang F, et al. Activating transcription factor 1 is a prognostic marker of colorectal cancer. *Asian Pac J Cancer Prev* 2012;13:1053-1057.
21. Wu X-D, Bie Q-L, Zhang B, Yan Z-H, Han Z-J. Wnt10B is critical for the progression of gastric cancer. *Oncol Lett* 2017;13:4231-4237.
22. Madueke I, Hu W-Y, Hu D, et al. The role of WNT10B in normal prostate gland development and prostate cancer. *The Prostate* 2019;79:1692-1704.

23. Yue H, Liang J, Yang K, Hua B, Bian Z. Functional analysis of a novel missense mutation in AXIN2 associated with non-syndromic tooth agenesis. *Eur J Oral Sci* 2016;124:228-233.
24. Dassule HR, McMahon AP. Analysis of epithelial-mesenchymal interactions in the initial morphogenesis of the mammalian tooth. *Dev Biol* 1998;202:215-227.
25. Mostowska A, Biedziak B, Zadurska M, Dunin-Wilczynska I, Lianeri M, Jagodzinski P. Nucleotide variants of genes encoding components of the Wnt signalling pathway and the risk of non-syndromic tooth agenesis. *Clin Genet* 2013;84:429-440.
26. Avsian-Kretchmer O, Hsueh AJW. Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists. *Mol Endocrinol Baltim Md* 2004;18:1-12.
27. Sun J, Zhuang F-F, Mullersman JE, et al. BMP4 activation and secretion are negatively regulated by an intracellular gremlin-BMP4 interaction. *J Biol Chem* 2006;281:29349-29356.
28. Tucker AS, Matthews KL, Sharpe PT. Transformation of tooth type induced by inhibition of BMP signaling. *Science* 1998;282:1136-1138.
29. Tucker A, Sharpe P. The cutting-edge of mammalian development; how the embryo makes teeth. *Nat Rev Genet* 2004;5:499-508.
30. Vogel P, Liu J, Platt KA, et al. Malformation of incisor teeth in Grem2$^{-/-}$ mice. *Vet Pathol* 2015;52:224-229.
31. Prochazka J, Prochazkova M, Du W, et al. Migration of founder epithelial cells drives proper molar tooth positioning and morphogenesis. *Dev Cell* 2015;35:713-724.
32. Jean D, Harbison M, McConkey DJ, Ronai Z, Bar-Eli M. CREB and its associated proteins act as survival factors for human melanoma cells. *J Biol Chem* 1998;273:24884-24890.
33. Cui J, Yin Z, Liu G, et al. Activating transcription factor 1 promoted migration and invasion in lung cancer cells through regulating EGFR and MMP-2. *Mol Carcinog* 2019;58:1919-1924.
34. Haakenson JK, Kester M, Liu DX. The ATF/CREB family of transcription factors in breast cancer. In: Rebecca L. Aft, ed. Targeting New Path Cell Death Breast Cancer. London, United Kingdom: IntechOpen, 2012.
35. Ran A, Guan L, Wang J, Wang Y. GREM2 maintains stem cell-like phenotypes in gastric cancer cells by regulating the JNK signaling pathway. *Cell Cycle* 2019;18:2414-2431.
36. Bylund JB, Trinh LT, Awgulewitsch CP, et al. Coordinated Proliferation and Differentiation of Human-Induced Pluripotent Stem Cell-Derived Cardiac Progenitor Cells Depend on Bone

Morphogenetic Protein Signaling Regulation by GREMLIN 2. *Stem Cells Dev* 2017;26:678-693.

37. Peters H, Neubüser A, Kratochwil K, Balling R. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* 1998;12:2735-2747.

38. Callahan N, Modesto A, Meira R, Seymen F, Patir A, Vieira AR. Axis inhibition protein 2 (AXIN2) polymorphisms and tooth agenesis. *Arch Oral Biol* 2009;54:45-49.

39. Bergendal B, Norderyd J, Zhou X, Klar J, Dahl N. Abnormal primary and permanent dentitions with ectodermal symptoms predict WNT10A deficiency. *BMC Med Genet* 2016;17:88.

Gene	SNV Id.†	Base position†	Location in gene†	Base change ‡	Reference
ATF1	rs11169552	Chr12:50761880	5' near promoter	C/**T**	Williams et al. 2018[5]
GREM2	rs1414655	Chr1:240465161	Downstream 3'	C/**T**	Magruder et al. 2018[4]
WNT10B	rs833843	Chr12:48970608	5' near promoter	C/**T**	Magruder et al. 2018[4]

Table 1. TA variants studied.

† Based on NCBI dbSNP GRCh38 assembly; alternate alleles shown in bold

‡ Minor allele frequency based on European Caucasian (CEU) population

Figure Legends

Figure 1. Results of dual luciferase reporter assays in *ATF1*-, *WNT10B*-, and *GREM2*-mutant cells compared with reference alleles. Plasmid constructs for *ATF1* (rs11169552_C or _T), *WNT10B* (rs833843_C or _T), and *GREM2* (rs1414655_C or _T) were respectively co-transfected with a pGL4.74 renilla luciferase vector (control) into SHED. Firefly luciferase activity was normalized to that of renilla luciferase activity. Results are presented as the average of three independent experiments ± SD. *p < .05, ***p < .001.

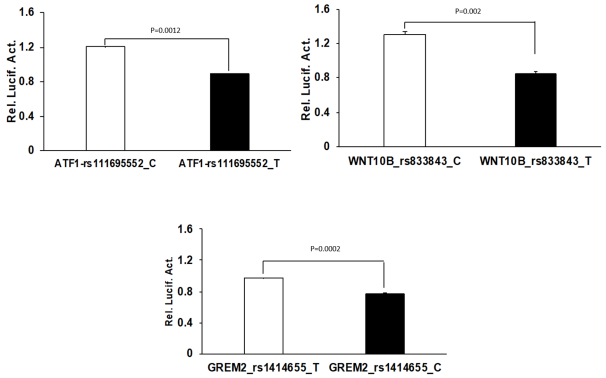
Figure 2. Immunocytochemistry analysis of ATF1, WNT10B, and GREM2 expression. Plasmid constructs for *ATF1* (rs11169552_C or _T), *WNT10B* (rs833843_C or _T), and *GREM2* (rs1414655_C or _T) were respectively co-transfected with a pGL4.74 renilla luciferase vector (control) into SHED. Fluorescence intensity (integrated density) was quantified with ImageJ software. Results are presented as the average of three independent experiments ± SD. Scale bar

= 10 um; 60X magnification.

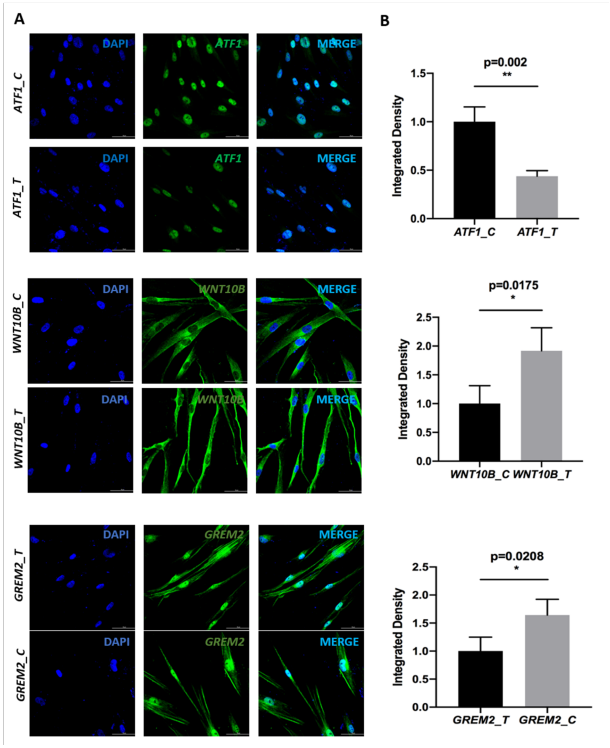
Figure 3. Representative images and migration index of wound closure in SHEDs transfected with *ATF1, WNT10B* and *GREM2* wild-type and mutant constructs. Cells transfected with empty vectors (pGL4.10 for A and B, pLightswitch-3'UTR for C) were used as controls. Cell migration was monitored every 12 hours for 48 hours. Results are presented as the average of three independent experiments ± SD. * p<.05.

Figure 4. Cell proliferation assays in *ATF1-*, *WNT10B-* and *GREM2*-wild-type and mutant cells. Cells transfected with empty vectors (pGL4.10 for A and B, pLightswitch-3'UTR for C) were used as controls. Cells were counted at 0, 24, 48, and 72 hours. Results are presented as the average of three independent experiments ± SD. * p<.05.

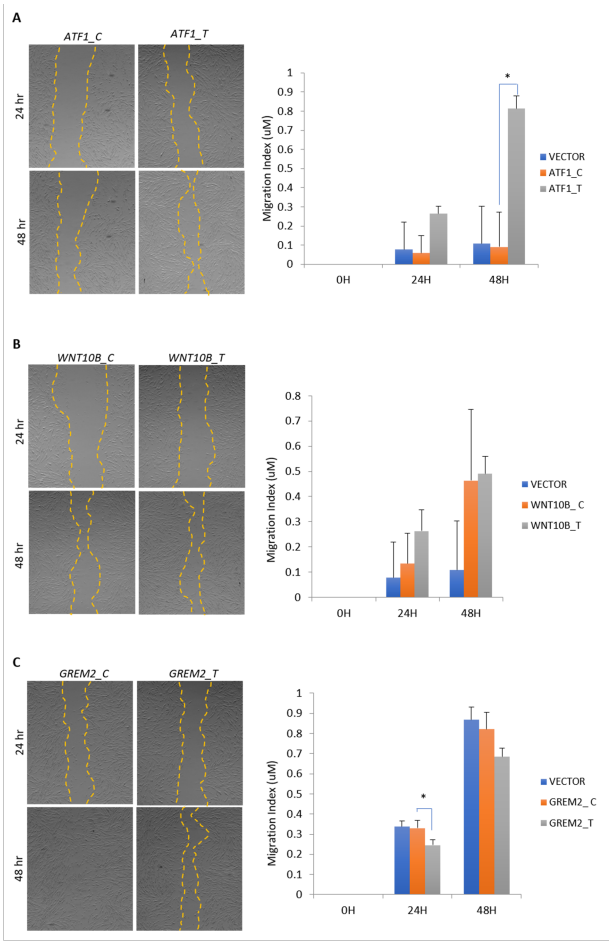
Figure 5. Reverse transcription qPCR analysis of *AXIN2, PAX9,* and *MSX1* in wild-type and mutant SHED. Expression levels of target genes were normalized to *GAPDH* expression. Results are presented as the average of three independent experiments ± SD. *p < .05, ***p < .001.



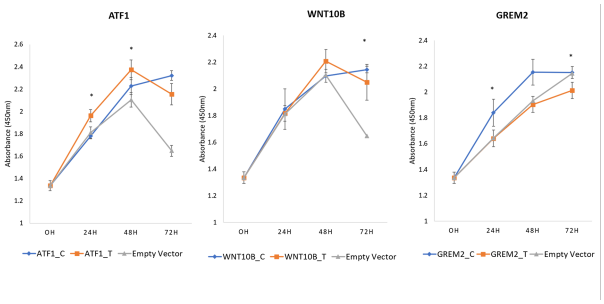
ocr_12462_f1.tif



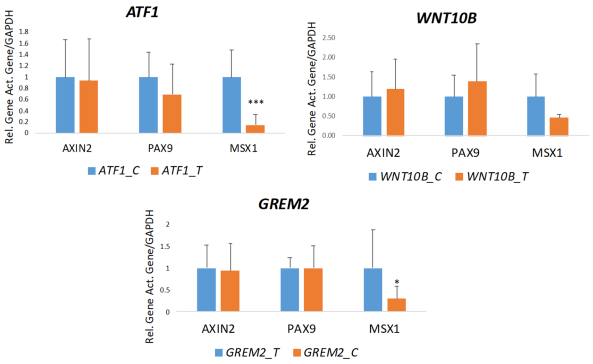
ocr_12462_f2.tif



ocr_12462_f3.tif



ocr_12462_f4.tif



ocr_12462_f5.tif