Transcriptome Profiling of Cleft Palate in TGF-beta3 Knockout Mice Alleles: RNA-SEQ Analysis of TGF-beta3 Mice

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TRANSCRIPTOME PROFILING OF CLEFT PALATE IN TGF-β3 KNOCKOUT MICE
ALLELES: RNA-SEQ ANALYSIS OF TGF-β3 MICE

By

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A THESIS

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Background: Orofacial clefts are the most common craniofacial birth defect with a complex, combinatorial etiology. Tgf-β3 regulates palatal fusion in mice; Tgf-β3 knockout mice have cleft palate (CP) lacking other major deformities. The genes downstream of Tgf-β3 during palatogenesis remain largely unexplored. Our objective was to analyze the global transcriptome changes and their contribution to CP and identify novel Tgf-β3 associated genes involved in formation of CP.

We used RNA-sequencing to analyze and compare the whole transcriptome of Tgf-β3 alleles during palatal growth and fusion in mice.

Results: The whole transcriptome analysis of Tgf-β3 mice (C57BL/6) alleles revealed over 6000 significantly differentially expressed genes from 14.5 and 16.5 days post coitum (dpc), in wild type (WT) and homozygous (HM) genotypes. A majority of differentially expressed genes were upregulated (WT=2421; HM=3153) compared to downregulated (WT=1694; HM= 2151) over time. With a 2.0 fold-change cut-off, downregulated genes decreased dramatically (WT=134, HM=191) compared to upregulated genes (WT=1675; HM=1936). Comparatively, gene expression differences between WT and HM were minimal. Using Ingenuity® Pathway Analysis (IPA) we identified genes which may function as primary contributors to the development of CP.

Conclusions: Using RNA-seq analysis, we provided a global analysis of transcriptome changes between and within WT and HM in the Tgf-β3 mouse model system at critical stages of palate development. We identified genes that likely play key regulatory roles during palatogenesis downstream of Tgf-β3 bolstering our knowledge of CP.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................ iii

TABLE OF CONTENTS .................................................................................. iv

LIST OF FIGURES ........................................................................................ vii

LIST OF TABLES ........................................................................................... viii

1 CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction ............................................................................................... 1

1.2 Oral Clefts ................................................................................................. 2

1.3 TGF-β3 ..................................................................................................... 6

1.4 TGF-β3 Signaling and Downstream Molecules ......................................... 6

1.5 Craniofacial Development ...................................................................... 9

1.6 Mouse Model System ............................................................................ 12

1.7 Conclusion ............................................................................................... 14

2 CHAPTER 2: OBJECTIVES AND AIMS OF THE STUDY

2.1 Objectives and Specific Aims ................................................................. 15

2.2 Hypothesis ............................................................................................. 15

3 CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction ........................................................................................... 16

3.2 Animal Selection and Breeding ............................................................. 16
3.3 Dissection and Collection of Palatal Shelves.......................... 17
3.4 Genotype Confirmation.................................................. 19
3.5 RNA Extraction and Purification...................................... 21
3.6 Preparation of cDNA Library........................................... 21
3.7 RNA-Sequencing Analysis............................................... 21
3.8 Comparison of Differential Gene Expression...................... 22
3.9 Biological Evaluation and Pathway Analysis......................... 23
3.10 Confirmation of Dysregulated Genes with Real-Time PCR...... 24
3.11 Statistical Analysis of Real-Time PCR.............................. 24

4 CHAPTER 4: RESULTS
4.1 RNA-Sequencing Quality Analysis................................. 26
4.2 RNA-Sequencing Transcript Quantification......................... 28
4.3 Differential Expression Analysis...................................... 30
4.4 Real-Time PCR Confirmation of Dysregulated Genes............. 33

5 CHAPTER 5: DISCUSSION
5.1 Known Cleft Palate Genes.............................................. 38
5.2 Genes Upregulated at 16.5dpc vs. 14.5dpc in HM Palates........... 41
5.3 Genes Downregulated at 16.5dpc vs. 14.5dpc in HM Palates........... 44
5.4 Genes Upregulated at 16.5dpc vs. 14.5dpc in WT Palates............. 47
5.5 Genes Downregulated at 16.5dpc vs. 14.5dpc in WT Palates........... 49
5.6 Dysregulated Genes independent of TGF-β……………………………52
5.7 Direct Comparison of HM and WT Palates at 14.5 and 16.5dpc………………53
5.8 Significantly Dysregulated GO Categories & KEGG Pathways……………57
5.9 Functional Analysis of SDEGs in WT and HM at 16.5 vs. 14.5dpc………57
5.10 Real-time PCR confirmation of Gene Dysregulation…………………..65

6  CHAPTER 6: CONCLUSIONS

6.1 Conclusions…………………………………………………………………67
6.2 Future Research and Study Limitations…………………………………67

7  BIBLIOGRAPHY………………………………………………………………68

8  APPENDIX ……………………………………………………………………84
LIST OF FIGURES

CHAPTER 1
Figure 1…………………………………………………………………………… 5
Figure 2…………………………………………………………………………… 8
Figure 3…………………………………………………………………………… 11
Figure 4…………………………………………………………………………… 13

CHAPTER 3
Figure 5…………………………………………………………………………… 18
Figure 6…………………………………………………………………………… 20

CHAPTER 4
Figure 7…………………………………………………………………………… 27
Figure 8…………………………………………………………………………… 29
Figure 9…………………………………………………………………………… 32
Figure 10………………………………………………………………………… 36
Figure 11………………………………………………………………………… 37

CHAPTER 5
Figure 12………………………………………………………………………… 59
Figure 13………………………………………………………………………… 60
Figure 14………………………………………………………………………… 61
Figure 15………………………………………………………………………… 62
Figure 16………………………………………………………………………… 63
Figure 17………………………………………………………………………… 64
LIST OF TABLES

CHAPTER 4

Table 1................................................................................................................. 31
Table 2................................................................................................................. 34
Table 3................................................................................................................. 35

CHAPTER 5

Table 4................................................................................................................. 56
CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

One in every thirty-three babies born in the United States has a birth defect; birth defects are a leading cause of infant mortality and lifelong disability. Orofacial clefts are the most commonly treated craniofacial defect in the pediatric hospital setting with a lifetime cost estimated at approximately $100,000 (Jugessur et al., 2009). According to national prevalence estimates, cleft lip with or without cleft palate is the second most common defect with a prevalence ranging from 1/500 to 1/2500 based on geographic origin, racial and ethnic background, and socioeconomic status (Schutte & Murray, 1999, Parker et al., 2010). Both the financial burden and prevalence of orofacial clefts warrant investigation into the underlying cause of the deformity with the goal of improving prevention, treatment, and prognosis for affected individuals.

Orofacial clefts are a congenital disruption to facial structure. In addition to the notable facial deformity, orofacial clefts cause difficulty feeding, speaking, hearing, and socially interacting requiring highly-coordinated, interdisciplinary care throughout life. Treatment including surgery, dental treatment, speech therapy and psychosocial intervention start as early as ten weeks of age and continue through adulthood (Dixon et al., 2011). The causes of orofacial clefts are a complex combination of genetic and environmental factors lending to difficulty in fully understanding the role of genes and the environment in this embryological disturbance (Schutte & Murray, 1999). Previous research has identified several genes that play significant roles in palatogenesis, such as TGF-β3, and are likely implicated in cleft palate formation (Dixon et al., 2011).

Members of the TGFβ family play crucial roles in facial development as demonstrated in several studies. The Tgf-β3 knockout mouse model results in the phenotype of cleft palate. In chickens where the palate is normally cleft, exogenous Tgf-β3 has been shown to induce palate fusion (Sun et al., 1998). Further, various authors have confirmed that mutations involving TGF-
β3 are associated with cleft palate in humans (Zhu et al., 2012b). Research continues to show that TGF-β3 is fundamentally important in successful palate development; however we have yet to completely explore the biological mechanisms downstream of TGF-β3. A complete gene analysis of Tgf-β3 mouse alleles will allow us to elucidate genes under control of TGF-β3 in proper palate formation and those in the knockout model implicated in cleft formation.

1.2 Orofacial Clefts

Reported prevalence of orofacial clefts varies worldwide; orofacial clefts occur in approximately one in 700 live births. Prevalence in gender and sidedness also differ with 2:1 male to female ratio in cleft lip, 1:2 male to female ratio in cleft palate, and 2:1 ratio of left to right sided clefts (Dixon et al., 2011). Incidence is greatest in American Indians followed by Asians, Europeans and least in African Americans (Panamonta et al., 2015).

Orofacial clefts have varying degrees of phenotypic presentation as shown in Figure 1. Cleft lip can present with or without cleft palate (CL/P) or the cleft can be confined to the palate only (CPO) (Dixon et al., 2011). Orofacial clefts can occur as part of a syndrome—approximately 30% of CL/P and 50% of CPO cases are syndromic and a result of chromosomal anomalies, Mendelian syndromes, or teratogenic exposure. A majority of orofacial clefts are non-syndromic, having no other associated anomalies. These categorizations lend to four specific groups of clefts: syndromic CL/P, syndromic CPO, non-syndromic CL/P, and non-syndromic CPO (Schutte & Murray, 1999). Accurate identification of phenotype is crucial in understanding underlying genetic etiology in non-syndromic CL/P and CPO. Various presentations clinically reflect genetic heterogeneity. New studies suggest that phenotypic presentation is a more complex spectrum including minor structural variants such as lip pits, dental anomalies, defects of the orbicularis oris muscle, and speech or cognitive differences. Palatal variants can include a bifid uvula or submucous cleft. Accurate
phenotyping could lend to more evidence in twin studies and familial clustering studies (Dixon et al., 2011).

The complex, heterogeneous etiology of orofacial clefts involves several environmental teratogens and a multitude of genes. Noted teratogens, such as smoking, increase risk of orofacial clefts by as much as 6% (Beaty et al., 2006). Additionally, certain drugs, maternal alcohol use, and pesticides have the ability to disrupt normal facial development during embryogenesis resulting in a cleft. Epidemiological studies of indigent populations suggest that both socioeconomic status (SES) and maternal nutrition are risk factors of orofacial clefts; the prevalence of orofacial clefts is higher in populations of lower SES. In these populations, it is difficult to separate the risk contribution of SES and maternal nutrition; however, it is suggested that environmental factors are responsible for as much as one-third of orofacial cleft cases in indigent populations (Schutte & Murray, 1999). While the environment has notable effects on palate development, the genetic component of cleft palate (CP) is being aggressively studied.

Genetic perturbations have been associated with orofacial clefting through family, twin, and epidemiologic studies (Spritz, 2001). According to a population study in Norway, first-degree relatives (parents, full siblings, and offspring) have a 32 time higher risk with CL/P and 56 time higher risk with CPO. Relatives of patients with non-syndromic CL/P have an increased risk of recurrence; with the decreasing degree of relationship, the risk also decreases (Spritz, 2001). However, a family with numerous individuals affected is rare, suggesting that the defect is likely a result of a combination of multiple genes with additive risk, as well as its previously suggested environmental contribution. Similarly, studies involving monozygotic twins confirm that the etiology of orofacial clefts is a result of both genetic and environmental components. Concordance of monozygotic twins, 47%, was significantly greater than that of dizygotic, 8%--a purely genetic epidemiology would result in 100% concordance in monozygotic twins (Beaty et al., 2006). Research continues to uncover genetic factors contributing to cleft formation.
One major modality of research focuses on the genetic components of syndromic forms of CP. Even though CP lacks a true Mendelian inheritance pattern, studies rely on exploration of syndromes including cleft palate since underlying mechanisms of cleft formation may be similar (Iwata et al., 2011). X-linked cleft palate and Van der Woude syndrome are two examples of syndromes with similar phenotypic presentation to orofacial clefts (Schutte & Murray, 1999). Further assessment of the etiology of orofacial clefts will improve our understanding of the facial deformity and include genetic and environmental risk assessment applicable for genetic counseling and prevention (Dixon et al., 2011). Genetic linkage and association studies are conducted to identify loci and genes responsible for orofacial clefts. Due to low numbers of families and high heterogeneity, loci have not been consistently identified among studies. Human genes with identified association include TGF-β3 (Murray, 2002). The TGFβ isoforms have been shown to be intricately involved in palate fusion warranting a thorough investigation of their role and downstream effects.
Figure 1. A) Illustrations of different types of orofacial clefts; a and e show unilateral and bilateral clefts of the soft palate; b, c and d show degrees of unilateral cleft lip and palate; f, g, and h show degrees of bilateral cleft lip and palate (Dixon et al., 2011).
1.3 TGF-β3

Transforming growth factor-β3 (TGF-β3) belongs to the β growth factor superfamily consisting of more than 30 ligand proteins, signaling through Smads or MAPKs (Zhu et al., 2012b, Nawshad et al., 2004). This gene family is involved in biological activities that control cell proliferation, migration and differentiation; regulate extracellular matrix deposition; and promote epithelial-mesenchymal transformation (EMT). The three isoforms—β1, β2, β3—are highly conserved between species with 71-76% sequence identity and have distinct spatial and temporal expression (Zhu et al., 2012b). Further, phenotypes resulting from knockouts of each TGFβ isoform are distinct and cannot be compensated by other family members. Mutations of Tgf-β1 in mice do not survive beyond 11 dpc and cannot be evaluated for palatal involvement; Tgf-β2 knockout mice have defects of the maxilla and mandible with only 23% having cleft palate; and Tgf-β3 knockout mice show cleft palate only with 100% penetrance (Nawshad et al., 2004).

The TGFβ isoforms have shown to be spatially and temporally distinct. As shown in mouse palate development, Tgf-β1 is involved in both epithelial and mesenchymal tissues, Tgf-β2 is mainly in mesenchymal tissues, and Tgf-β3 is expressed in medial edge epithelium (Ichikawa et al., 2006). Further, Tgf-β3 appears to be solely responsible for fusion of palatal shelves regulating periderm sloughing and EMT of the medial edge epithelium (Nawshad et al., 2004, Hu et al., 2015a).

1.4 TGF-β3 Signaling and Downstream Molecules

Well-coordinated processes including cell migration, apoptosis, cell morphology, cell cycle progression, growth, and proliferation are essential for proper palate formation. Palatogenesis occurs in four main steps: vertical shelf growth, elevation, adhesion, and fusion (Zhu et al., 2012b). As shown in mouse models, Tgf-β3 is essential for palate fusion. Additionally, in vitro inhibition
of \( Tgf-\beta3 \) activity resulted in failure of palatal fusion of organ cultures. \( Tgf-\beta3 \) has also been shown to rescue palatal fusion in \( Tgf-\beta3 \) knockout mice (Meng et al., 2009).

The TGF\( \beta \) isoforms are uniquely able to signal via multiple pathways including SMAD, G protein/MAPK, and PI3 kinase with divergent signaling outcomes (Nawshad et al., 2005). In the SMAD-dependent pathway, illustrated in Figure 2, the TGF\( \beta \) ligands signal through serine/threonine kinase receptor complexes TGF\( \beta \) type I receptor (T\( \beta \)rI) and TGF\( \beta \) type II receptor (T\( \beta \)rII). TGF\( \beta \) ligand first binds to T\( \beta \)rI; T\( \beta \)rI becomes phosphorylated and active with the binding of the ligand (Jugessur et al., 2009). The T\( \beta \)rII is recruited to the complex forming a heterotetrameric signaling complex. This complex is internalized into an early endosome and receptors are recycled back to the membrane. In the early endosome, protein SMAD anchor for receptor activation (SARA) modulates the interaction of SMAD2/3 to TGF\( \beta \) receptors forming TGF\( \beta \)Ir/SARA/SMAD2 or 3 complex (Nawshad et al., 2005). Phosphorylation of SMAD2 and/or SMAD3 allows association with SMAD4; SMAD4 ushers the complex into the nucleus for transcriptional activation or repression of TGF\( \beta \) target genes (Cui et al., 2003). For example, target gene lymphoid-enhancing factor 1 (LEF1), has been shown to upregulate genes involved in EMT, an essential component of palatogenesis (Nawshad & Hay, 2003). Both \textit{in vivo} and \textit{in vitro} experiments confirm TGF\( \beta \)-mediated SMAD signaling is the major pathway for EMT required for palate fusion (Nawshad et al., 2004).
Figure 2. Illustration of Smad-dependent TGFβ signaling pathway during palatal EMT. TGF-β ligand bind TGF-β receptors I and II at the cell surface. After phosphorylation of TGFβIR, receptors internalize with the ligand into a plasma membrane pit forming vesicles and early endosomes in the cytosol with phosphorylated receptors and ligand attached. Protein SARA modulates cellular trafficking and transports cytoplasmic Smad2/3 to the TGF-β receptors to form TGFβIR/SARA/Smad 2 or 3 complexes. Smad2 or 3 is phosphorylated and with help of Smad4 able to enter nucleus ultimately activating EMT genes (Nawshad et al., 2005).
1.5 Craniofacial Development

Craniofacial development is a complex process that begins at the fourth week in utero. Craniofacial development relies on migration of cranial neural crest (CNC) cells to guide the patterning of the face by populating the branchial arches (Chai & Maxson, 2006). The timing and extent of migration is tightly controlled by highly conserved homeobox genes (Marazita & Mooney, 2004). Neural crest cells migrate into the developing branchial arches and are the source of skeletal and connective tissue structures (Alappat et al., 2003).

Development of the face, illustrated in Figure 3, results from harmonized growth and differentiation of five facial primordia—the medial frontonasal prominence, paired maxillary prominences, and paired mandibular prominences—surrounding the primitive oral cavity (Alappat et al., 2003). Both the maxillary and mandibular prominences grow toward each other in the midline where they will fuse forming the upper jaw and lip and lower jaw and lip, respectively. The frontonasal process forms the forehead, nose, lip, philtrum and primary palate (Cordero et al., 2011). Within the frontonasal prominence, nasal placodes form from localized thickenings of the surface ectoderm eventually forming the nasal pits, lateral and medial nasal prominences (Alappat et al., 2003). The maxillary prominences fuse with the lateral and medial nasal prominences bilaterally followed by the intermaxillary segment of the frontonasal process (Alappat et al., 2003). Failure of the maxillary prominences to fuse with the medial nasal prominence on one or both sides can result in a unilateral cleft lip or bilateral cleft lip, respectively (Alappat et al., 2003).

Palatogenesis begins during week five and continues through week twelve forming the primary and secondary palates (Chai & Maxson, 2006, Lan et al., 2015). The primary palate is anterior to the incisive foramen and consists of the philtrum, incisors, and a small portion of the hard palate. Located posterior to the incisive foramen, the secondary palate begins formation at week six of embryogenesis creating the majority of the hard palate and entire soft palate. Paired palatal shelves
start as vertical extensions of the maxillary processes on either side of the tongue. The developing lower jaw allows the tongue position to lower during the eighth week and the palatal shelves to elevate into a horizontal position. The palatal shelves grow towards each other fusing with the primary palate anteriorly, nasal septum dorsally, and each other at the midline. When the palatal shelves adhere in the midline, the periderm covering the medial edge epithelium (MEE) must be eliminated first to allow the epithelial cells to fuse forming the midline epithelial seam (MES) (Chai & Maxson, 2006, Lan et al., 2015). Although the mechanism is yet to be fully elucidated, MES cells have three possible fates to produce a confluent palate: EMT, apoptotic cell death, or migration of MES cells to oral or nasal sides of the palate (Nawshad, 2008, Lan et al., 2015). Any disruption of palatal shelf growth, elevation, or fusion can result in a CP (Lan et al., 2015). Palatal clefts can include one or more of the following: hard palate, soft palate, and uvula (Cordero et al., 2011).
Figure 3. Schematic diagram of development of the face and palate in humans; a) Developing frontonasal prominence, paired maxillary and mandibular processes with primitive oral cavity by the fourth week of embryogenesis; b) Formation of nasal pits leading to paired medial and lateral nasal processes by week five; c) Medial nasal processes and maxillary processes merged forming the upper lip and primary palate by the sixth week of embryogenesis; d) Palate formation beginning at week six as bilateral outgrowths from the maxillary processes on either side of the tongue; e) Palatal shelf horizontal elevation and contact; f) Palate fusion (Dixon et al., 2011).
1.6 Mouse Model System

Animal models have significantly improved our understanding of the genetic activity in palatogenesis. Over 200 genetically mutated mice exhibit CP phenotype (Iwata et al., 2011). Palatogenesis is remarkably similar in humans and mice providing an ideal model system to assess the role of particular genes in orofacial deformity (Nawshad et al., 2004, Chai & Maxson, 2006). Several mouse strains with CP are available; Msx1, Tgf-β3, and Ap-2 knockouts are three examples that result in cleft phenotype (Dixon et al., 2011, Schutte & Murray, 1999). Several studies have confirmed the essential role of Tgf-β3 in mouse palatogenesis; Tgf-β3 mice have CP due to failed palatal shelf fusion with the absence of any other craniofacial deformity (Nawshad et al., 2004).

The mouse is an ideal animal for the study of palatogenesis being both time- and cost-effective with several offspring—a litter produces approximately 8 pups. Mice have a short gestation period of 21-22 days with palatogenesis occurring from day 11-16dpc coordinating with human development weeks 6-12 in utero (Rabadan-Diehl & Nathanielsz, 2013). Palate formation in mice, illustrated in Figure 4, begins at 11.0-11.5dpc. Tgf-β3 is detected throughout palate development in MEE. After vertical growth is complete, palatal shelves elevate at 13.0-13.5dpc. Palatal shelves will grow towards each other and begin to contact at 14.0-14.5dpc. Tgf-β3 is found in the greatest quantity at the MEE during EMT and will persist until cells complete the transformation. By 16.0-16.5dpc, palatal fusion has occurred (Nawshad et al., 2004). The use of animal models, particularly mice, with CP has allowed the discovery of both cellular and molecular mechanisms of palate formation.
**Figure 4.** A-C: Camera lucida drawings of paraffin sections of the developing rodent palate; a) Between 13 and 14dpc palatal shelves move horizontally towards each other above the tongue during which the periderm layer sloughs off the epithelium along the medial palatal edge (p); b) Epithelial seams transform to mesenchyme to become confluent between 15 and 17dpc; c) Palate shelves fuse together with nasal septum (ns); D-G: Hematoxylin and eosin-stained sections of rodent palates fixed at different stages in palatogenesis in vitro; d) palatal shelves in contact with palatal seam (white arrow); e) seam disintegration; f) continued seam disintegration; g) palate fusion with complete confluence (blue arrow) (Nawshad, 2008).
1.7 Conclusion

Non-syndromic CL/P and CP have been extensively studied. The causes of orofacial clefts are incredibly sensitive to environmental disturbances, making it difficult to identify and understand gene-gene and gene-environment interactions (Schutte & Murray, 1999). The high prevalence of orofacial clefts and their devastating malformations continues to drive research to find precise genetic causes and targets for prevention.

Several studies have confirmed that TGF-β3 is paramount to palatal fusion in both primary cells and organ culture. While we know TGF-β3 is a candidate gene for non-syndromic CP in humans, we still have yet to fully explore genes that are directly regulated by TGF-β3 in both normal palate formation and CP (Ozturk et al., 2013). In conclusion, the ability to provide an analysis of Tgf-β3 and its downstream signaling effects during palatogenesis in a mouse model can provide a deeper knowledge of the development of CP and the genes involved.
CHAPTER 2: OBJECTIVE AND AIMS OF THE STUDY

2.1 Objective and specific aims

The objective of this study was to analyze transcriptome changes and their contribution to the development of cleft palate at gestational ages 14.5dpc and 16.5dpc in Tgf-β3 knockout mice alleles. The specific aims of this project were to:

- Using RNA-sequencing we will create a genome scale analysis of the palatal transcriptome of Tgf-β3 WT and HM mice at critical stages of palatogenesis (14.5 and 16.5dpc).
- Our RNA sequencing data will show a comprehensive analysis of Tgf-β3 signaling effects during palatogenesis and genes subsequently altered in mutants causing palatal cleft.
- Our genome scale analysis of the palatal transcriptome will identify exact functional cellular changes that are active during palatogenesis and compromised in Tgf-β3 HM palates.

2.2 Hypothesis

We hypothesize that there will be a differential expression of novel genes between Tgf-β3 WT and HM mice at different time points critical to palate development (14.5 and 16.5dpc) that are specific for causing cleft phenotype.
CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

This chapter describes the materials and methods used to analyze the transcriptome changes of Tgf-β3 mice using RNA-sequencing at critical stages in palatal development. While substantial evidence shows the importance and role of TGF-β3 in palatal fusion and its implication in CP, we do not know what cellular pathways are directly or indirectly regulated by TGF-β3 during palatogenesis.

The purpose of this study is to enhance our knowledge of the role of TGF-β3 in normal palatogenesis and in formation of a cleft by exploring the differentially expressed genes at 14.5 and 16.5dpc in a mouse model. Our particular interest is any gene up-regulated or down-regulated in the Tgf-β3 WT versus HM alleles. We then hope to define the involvement of dysregulated genes in the cellular mechanisms underlying palatogenesis improving our knowledge of downstream effects of TGFβ signaling. Results from such analysis will identify new candidate genes that may aid in our attempt to fully elucidate the mechanism underlying this orofacial deformity.

3.2 Animal Selection and Breeding

Tgf-β3 heterozygous (+/-) C57BL/6J male and female mice were provided by Tom Doetschman (BIO5 Institute, University of Arizona, AZ). This specific mouse strain, C57BL/6J, has an increased susceptibility to teratogen-induced CP, making this strain ideal. Mice were kept in accredited animal facilities at the University of Nebraska Medical Center (UNMC) and all procedures were approved and in accordance with the Institutional Animal Care and Use Committee (#06-064) of UNMC.

To produce heterozygous offspring for mating, a replacement type targeting vector was made with exon 6 of the Tgf-β3 gene replaced by neomycin-resistance gene derived from the
pMC1neo vector. E14-1ES cells were transfected with the targeting vector with correct targeting found in 5/250 neomycin-resistant clones analyzed by Southern blot analysis. Genomic DNA was prepared and injected into C57BL/6 blastocysts and mated. Heterozygous offspring were confirmed with Southern blot analysis. To produce HM (−/−) Tgf−β3 mice, heterozygous mice were bred. Litters contained approximately 25% homozygous pups as expected from Mendelian inheritance ratio (Proetzel et al., 1995).

3.3 Dissection and Collection of Palatal Shelves

In pregnant female mice, the morning the vaginal plug was pulled was considered 0.5dpc. Pregnant female mice were euthanized by CO2 asphyxiation at 14.5 and 16.5dpc. Upon dissection, embryos were placed in Hank’s Balanced Salt Solution (SIGMA-Aldrich, St. Louis, MO) to stabilize the embryos. The embryos were dissected in sterile conditions under the Nikon SMZ1000 stereomicroscope system (Nikon, Tokyo, Japan); palatal shelves and tails were collected from 5-10 embryos per pregnancy (Figure 5). Palatal tissues were stored in Invitrogen™ RNAlater™ Stabilization Reagent (Qiagen, Hilgen, Germany) to preserve the gene expression profile. Samples were labeled to correspond to tail tissue used for genotyping.
Figure 5: Process of palatal shelf extraction. a) Sterile laboratory dissection conditions; b) Embryonic pups from E16.5 pregnant female mouse; c) Palatal cleft in E16.5 pup.
3.4 Genotype Confirmation

Embryonic genotyping was confirmed using PCR followed by gel electrophoresis. Tissue samples, embryonic tails, were isolated from embryos at 14.5 and 16.5dpc. Genomic DNA was prepared using the Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was confirmed using the NanoDrop 2000c spectrophotometer (NanoDrop, Wilmington, DE). *Tgf-β3* genotyping primers were attached to individually labeled tissue samples as follows:

TGF-β3 Forward 5’ TGG GAG TCA TGG CTG TAA CT 3’
TGF-β3 Reverse 5’ CAC TCA CAC TGG CAA GTA GT 3’

These primers amplified 1200bp and 400bp for HM and WT alleles, respectively shown in Figure 6. PCR conditions were one cycle of 95°C for 1.5 minutes followed by 35 cycles of 95°C for 30 seconds, 57°C for 50 seconds, 72°C for 1.5 minutes, and ended with one cycle of 72°C for 5 minutes. Fifteen mL of PCR product from each reaction was loaded onto 1% agarose gel. Electrophoresis was run at a constant 100V, 400mA and 400W for 1 hour and evaluated with Kodak Gel Logic 100 Imaging System (Kodak, Rochester, NY).
**Figure 6:** Genotype confirmation using RT-PCR with gel electrophoresis.
3.5 RNA Extraction and Purification

Total proteins were collected from previously stored palatal shelves of 14.5 and 16.5dpc pups. RNA was extracted using PicoPure™ RNA Isolation Kit (ThermoFisher Scientific, San Francisco, CA). Further purification was done using DNase Treatment (Qiagen, Hilden, Germany) as described previously (LaGamba et al., 2005, Zhu et al., 2012a). Purity and concentration were measured by ultraviolet spectroscopy using the Nano Drop 2000c spectrophotometer (NanoDrop, Wilmington, DE).

3.6 Preparation of cDNA Library

RNA was sent to University of Nebraska Medical Center Bioinformatics and Systems Biology Core Facility. Libraries were prepared using Illumina mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA) according to manufacturer instructions. Poly(A)+ RNA was recovered from 1 µg of total RNA using two rounds of hybridization with oligo-dT-cated Sera-Mag magnetic beads. The recovered poly(A)+ RNA was then chemically fragmented and next converted to cDNA using SuperScript II and random primers. The second strand was synthesized using RNaseH and DNA Pol I. The ends of cDNA were repaired using T4 DNA polymerase, T4 polynucleotide kinase, and Klenow DNA polymerase. A single adenosine was added to the 3’ end using Klenow fragment (3’ to 5’ exo minus). Adapters were attached to both ends of cDNA using T4 DNA ligase. RNA fragments were extracted from a 2% low range ultra agarose sizing gel and amplified by 15 cycles of PCR using Phusion® DNA Polymerase (Thermo Fisher Scientific, San Francisco, CA). Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to validate libraries. Libraries were diluted and applied to Illumina flow cell using the IlluminaCluster Station (Illumina, San Diego, CA) as previously described (Ozturk et al., 2013).

3.7 RNA-Sequencing Analysis & Comparison of Differentially Expressed Genes
RNA-sequencing (RNA-seq) was conducted using Illumina HiSeq2000 (Illumina, San Diego, CA) for Tgf-β3 (-/-) HM and Tgf-β3 (+/+) WT samples profiled at 14.5 and 16.5dpc. RNA-seq uses high-throughput sequencing technology providing the ability to investigate RNA content from sequencing of cDNA and the ability to measure transcript quantity in a single assay (Roberts et al., 2011, Trapnell et al., 2012). RNA-seq allows the rapid, comprehensive discovery of novel genes for a reasonable cost; simply, RNA-seq quantifies how often each gene is represented in a sample (Marguerat & Bahler, 2010). Two independent biological and technical replicates were assayed at each time point for each both HM and WT to evaluate reproducibility of samples and procedures resulting in eight (n = 2 x 2 x 2) samples for sequencing performed in 2x101bp paired-end mode. Raw reads were analyzed with FASTQC (v. 0.11.5) for quality control (Andrews, 2010). Overrepresented sequences, for example those containing adapter or primer sequences, remaining in the raw reads were assessed and subsequently removed using Timmomatic (v 0.36) in the palindrome mode based on default alignment detection and scoring parameters (Bolger et al., 2014). Trimmomatic was also used for low-quality base filtering. Maximum information quality filtering was employed with a minimum average read quality threshold of 25. Following technical sequence and low-quality base removal, reads that were shorter than 36bp were removed. Transcript quantification was done based on the GRCm38.p5 reference genome using Salmon (v. 0.8.2) with default parameters (Patro et al., 2017). Salmon uses sample-specific models such as correction for GC-content bias that improves the accuracy of transcription abundance estimates. We use Transcripts Per Million (TPM) in Salmon’s output as the relative abundance measure employed in our downstream analysis.

3.8 Comparison of Differential Gene Expression

Differential gene expression is evaluated using software tool DESeq2. This program allows the analysis of read counts per gene based on set parameters, such as required fold-change (Love et al., 2014). DESeq2 uses a negative binomial model to assess differential expression and
employs the Benjamini Hochberg procedure for multiple hypotheses testing correction (Benjamini & Hochberg, 1995). When comparing the transcription abundance between two groups of samples, we used the adjusted p-value cut-off of 0.05 to define statistically significant differential expression. RVennDiagram was used to generate highly-customizable, high-resolution Venn diagrams for each experimental group with a fold change (FC) of 2.0 or greater (Ozturk et al., 2013). This allows a visual grouping of dysregulated genes by distinct region to compare HM and WT at two different time points.

Clustering of samples was done using the Unweighted Pair Group Method with Arithmetic-mean method using Pearson’s correlation as the distance measure (Sneath, 1973). The expression data matrix was row-normalized prior to the application of average linkage clustering. The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang da et al., 2009) was used for functional analysis of the gene lists interrogating Biological Process (BP), Molecular function (MF), and Cellular Component (CC) Gene Ontology (GO) categories (Ashburner et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa & Goto, 2000a). Biologically relevant categories that are over-represented in the gene set and therefore may be of further interest were assessed using Expression Analysis Systematic Explorer (EASE) score in the DAVID tool. The EASE score is the upper bound of the distribution of Jackknife iterative resampling of Fisher exact probabilities with Bonferroni multiple testing correction. Categories containing low numbers of genes are under-weighted so that the EASE score is more robust than the Fisher exact test. The EASE score is a significance level with smaller EASE scores indicating increasing confidence in over-representation. We selected GO categories that have EASE scores of 0.05 or lower as significantly over-represented.

3.9 Biological Evaluation and Pathway Analysis

Differentially expressed genes between HM and WT were further analyzed using Ingenuity® Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) software. Prior to
uploading data into IPA, a statistical analysis of RNA-seq data was performed. For a gene to be considered differentially expressed, the adjusted p-value must be less than or equal to 0.05. Resulting RNA-Seq data Entrez IDs and fragments per kilobase per million mapped (FPKM) values were entered into IPA. IPA is based on the manual curation of scientific literature to identify pathways, networks, and functional categories that are significantly represented in the input gene list.

3.10 Confirmation of Dysregulated Genes with Real-Time PCR

To confirm dysregulated genes between WT and HM samples, quantitative real-time PCR (qPCR) was performed. Palatal tissues were extracted from embryos at 14.5 and 16.5dpc and RNA extraction was conducted as previously described using PicoPure™ RNA Isolation Kit (ThermoFisher Scientific, San Francisco, CA) with DNase Treatment (Qiagen, Hilden, Germany) used to extract high-quality RNA consistently from very few cells (Zhu et al., 2012a, LaGamba et al., 2005). RNA was converted to cDNA using Superscript™ IV VILO™ Master Mix (ThermoFisher Scientific, San Francisco, CA) providing a highly efficient and thermostable reverse transcriptase allowing significant cDNA yield at high temperatures in less time. An additional pre-amplification step was conducted using TaqMan™ PreAmp Master Mix (ThermoFisher Scientific, San Francisco, CA) with a custom pre-amplification pool of 32 selected genes of interest to amplify small amounts of cDNA without introducing amplification bias—specifically for small, precious mRNA samples—allowing more qPCR reactions. Samples underwent 14 preamplification cycles were with thermal cycling conditions of 95°C for 15 seconds and 60°C for 4 minutes followed by immediate placement on ice. Next, samples underwent 1:20 dilution diluted with TE buffer. Finally, samples were placed on 96-well custom array plates in technical triplicate and qPCR executed with TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, San Francisco, CA) reagents. PCR conditions were 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

3.11 Statistical Analysis of Real-Time PCR
Technical repeat’s normalized gene-specific $\Delta^{CTCt}$ value were subtracted from the housekeeping gene $\Delta^{CtCt}$ value. Then, data were analyzed using analysis of variance (ANOVA) on the repeat-normalized $\Delta\Delta^{CtCt}$ values, including the control group and translate effects estimated from the ANOVA onto the multiplicative scale. The values of tested 29 genes were normalized by adjusting for the concentration of abundant known house-keeping genes, like 18SrRNA, GAPDH and $\beta$-actin, and the $\Delta^{Ct}$ values of naive/vehicle group. PCR assays detecting the reference genes, 18SrRNA, GAPDH or $\beta$-actin calculated alongside those for the gene of interest and the resulting dataset were analyzed. 18SrRNA, GAPDH, and $\beta$-actin are constitutively expressed and were therefore used as controls in the quantitative analyses.
CHAPTER 4: RESULTS

4.1 RNA Sequencing Quality Analysis

The bioinformatics analysis of RNA-Sequencing showed raw RNAseq average read counts of 65.5 million paired-end reads (130.1 million total reads) per sample for the eight sample profiles (Figure 7a). Average read-length started as 101 base pairs (bp) in raw reads, subsequently reduced to an average of 95.86bp following trimming and filtering (Figure 7b). Overall average read quality score increased from 35.84 to 36.79 and the percentage of high quality bases—bases with a quality score greater than 20—per sample increased from 96.28% to 99.20% following trimming and filtering (Figure 7c and d). Both the total number of reads and the average read length parameters showed small changes in quantity, resulting in significant quality improvement after trimming and filtering.
Figure 7: (a) The total number of paired-end reads, (b) average read length in base pairs (bp), (c) average read quality, and (d) % of bp in the ensemble of reads that exceed a quality score of 20.
4.2 RNA Sequencing Transcript Quantification

RNA-seq analysis generated expression data for 103,215 transcripts. The transcripts with a TPM value less than 1.0 in all eight samples (WT14.5a, WT14.5b, WT16.5a, WT16.5b, HM14.5a, HM 14.5b, HM16.5a, HM16.5b) were eliminated from downstream analysis leaving 52,475 transcripts. Hierarchical clustering of all transcripts reveals that the samples are clearly separated by time with little effect of genotype warranting a need for supervised analysis methods to identify the differences in gene expression due to genotypic variance (Figure 8). Further, the close proximity of branching at 14.5dpc in HM and WT compared to a more distant relationship at 16.5dpc indicates that the samples are more similar at 14.5dpc with growing disparity over time. Therefore, the effects of Tgf-β3 knockout are more pronounced at 16.5dpc.
Figure 8: Hierarchical clustering of all the samples using all 52,475 transcripts with TPM>1 in at least one sample group.
4.3 Differential Expression Analysis

Differential expression analysis showed a significant temporal difference in gene expression between samples; there were over 4115 significantly differentially expressed genes (SDEG) between 14.5 and 16.5dpc in WT samples and 5304 SDEGs between 14.5 and 16.5dpc in HM samples (Table 1). From our transcriptome analysis, a majority of differentially expressed genes were upregulated (WT = 2421; HM = 3153) compared to downregulated (WT = 1694; HM = 2151) from 14.5 to 16.5dpc demonstrating that transcriptional induction overshadows transcriptional silencing between these time points. A fold-change (FC) cut-off of 2.0 was applied to further define significant differential expression; downregulated differentially expressed genes decreased dramatically (WT=134, HM=191) compared to differentially expressed upregulated genes (WT=1675, HM=1936) again emphasizing the difference in upregulated versus downregulated genes. Venn diagrams were created to illustrate the number of differentially expressed genes dysregulated from 14.5 to 16.5dpc in WT and HM (FC>2.0) (Figure 9a). By comparison, the difference in gene expression between genotypes at the same time point was minimal (HM14.5 vs. WT 14.5 = 13; HM16.5 vs. WT16.5 = 38). Again following trend, the genotype differences at 14.5dpc are minimal with no gene dysregulated with a FC>2.0 and a greater difference between genotypes at 16.5dpc and 13 genes dysregulated with FC>2.0. With the difference in genotype minimal, we defined the effects of Tgf-β3 knockout by comparing the temporal gene expression of WT and HM. Genes significantly dysregulated within each genotype were termed “WT Specific” (WS, 479+72) and “HM Specific” (HS, 690+129) as depicted in Figure 9a.

Further analysis to demonstrate the functional mechanisms associated with dysregulated genes between samples from 14.5 to 16.5dpc are highlighted in GO and KEGG pathways that are statistically significantly enriched among the samples (Figure 9c).
| Time Comparison | Genotype Comparison | # of SDEG (|FC| > 2.0) |
|-----------------|---------------------|------------------|
| WT              | Up in WT 16.5 vs. WT 14.5 | 2421           |
|                 | Down in WT 16.5 vs. WT 14.5 | 1694           |
| HM              | Up in HM 16.5 vs. HM 14.5 | 3153           |
|                 | Down in HM 16.5 vs. HM 14.5 | 2151           |
| Genotype        | 14.5                | 8               |
| Comparison      | Up in HM 14.5 vs. WT 14.5 | 0               |
|                 | Down in HM 14.5 vs. WT 14.5 | 5               |
|                 | 16.5                | 30              |
|                 | Up in HM 16.5 vs. WT 16.5 | 10              |
|                 | Down in HM 16.5 vs. WT 16.5 | 3               |

Table 1: Significantly Differentially Expressed Genes (SDEG, multiple hypothesis testing corrected p-value < 0.05) across time and genotype points separately listed for up-/down-regulation (FC: fold change).
Figure 9: (a) Comparison of significantly differentially expressed genes (SDEGs) between E16.5 and E14.5 in the WT and HM groups. WT Specific-Up (WSU): Genes uniquely upregulated in the WT group at E16.5; WT Specific-Down (WSD): Genes uniquely downregulated in the WT group at E16.5; HM Specific-Up (HMU): Genes uniquely upregulated in the HM group at E16.5; HM Specific-Down (HMD): Genes uniquely downregulated in the HM group at E16.5. (b) Hierarchical clustering of 501 (429+72) WT specific and 819 (690+129) HM specific genes. (c) Significantly enriched Gene Ontology categories in the WT specific and HM specific gene lists. Groups shown.
4.4 Real-Time PCR Confirmation of Dysregulated Genes

Our qPCR data of selected WT and HM genes (Table 2 and 3) supports RNAseq results using normalized fold-change ratio to our reference control genes—18sRNA, GAPDH, and β-actin (Appendix A). The qPCR data shown in Figure 10 confirms the results of our RNAseq analysis, demonstrating at 16.5dpc WT palates have a significantly increased expression of Cdh1, Ocln and F2rl1 and significantly decreased expression of Tnfrsf11b, Fndc3c1, Dlx1, and Gas2 compared to the endogenous control set (p<0.01). Similarly, in HM palates as shown in Figure 11, qPCR data confirms RNAseq results showing a significantly increased expression of Chrng, Col2a1, Coll1a1, Coll1a2, L1cam, Adam12, Fas, Hspg2, Lox, Itgb4, Klf5, Cldn1 and Nrcam and significantly decreased expression of Wnt9b, Alx4, Pax1, Kcp, Msx1, Ppp1r17, Pdgfc, Twist1, and Wnt5a at 16.5dpc compared to the controls (p<0.01).
<table>
<thead>
<tr>
<th>ENSEMBLE Gene ID</th>
<th>Gene Name</th>
<th>Adjusted p-value</th>
<th>Fold Change (WT16.5 / WT 14.5)</th>
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<td>00303</td>
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<tr>
<td>21638</td>
<td>Ocln</td>
<td>5.78E-10</td>
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<td>42228</td>
<td>Lyn</td>
<td>1.37E-07</td>
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<td>F2rl1</td>
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<td>Tnfrsf11b</td>
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<td>30498</td>
<td>Gas2</td>
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<td>Fndc3c1</td>
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<td>-4.58</td>
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**Table 2.** Selected dysregulated genes in WT with FC>2.0 (p<0.05).
<table>
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<tr>
<th>ENSEMBLE Gene ID</th>
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<th>Adjusted p-value</th>
<th>Fold Change (HM16.5 / HM 14.5)</th>
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Table 3. Selected dysregulated genes in WT with FC>2.0 (p<0.05).
Figure 10. Fold change (log2) expression of WT gene mRNA relative to reference control genes (18rSRNA, GAPDH and β-actin). Bar heights indicate mean expression of the genes in samples. Error bars indicate 95% confidence interval estimates of the mean expressions. One asterisk indicates statistically significant difference between the means of a sample set compared to the mean of the control sample set to 5% (correspond to a p-value <0.05); two asterisks indicate statistically significant difference to 1% (correspond to a p-value <0.01).
Figure 11. Fold change (log2) expression of HM gene mRNA relative to reference control genes (18rSRNA, GAPDH and β-actin). Bar heights indicate mean expression of the genes in samples. Error bars indicate 95% confidence interval estimates of the mean expressions. One asterisk indicates statistically significant difference between the means of a sample set compared to the mean of the control sample set to 5% (correspond to a p-value <0.05); two asterisks indicate statistically significant difference to 1% (correspond to a p-value <0.01).
CHAPTER 5: DISCUSSION

Orofacial clefting is the most commonly treated craniofacial anomaly in the pediatric hospital setting (Jugessur et al., 2009). The identification of genetic and environmental causes and risk factors continues to be at the forefront of current research. Scientific advances allowing more thorough, sensitive means of gene exploration help identify new mechanisms for embryonic development and cleft causation with the ultimate goal of converting scientific findings to clinical care (Jugessur et al., 2009). The development of high-throughput DNA sequencing methods, RNA-seq, allows the entire transcriptome to be surveyed quantitatively (Wang et al., 2009). Bioinformatics analysis was applied to RNA-seq data to identify differentially expressed genes and functional pathways that may elucidate the biological mechanisms underlying palate fusion.

5.1 Known Cleft Palate Genes

Based on the OMIM and MGI database, current knowledge demonstrates over 300 genes that, when mutated, cause CP in mice and humans. The role of TGF-β3 gene has been overwhelmingly established as a crucial molecule that is necessary for normal palate development—in its absence CP occurs (Ozturk et al., 2013). In this study we focus on the role of Tgf-β3 in the murine model and its associated molecules that are fundamental in normal palatogenesis. Our study identifies genes that are functionally regulated by Tgf-β3; in WT mice normal palatogenesis sustains whereas, in HM mice, CP results due to altered genes under control of Tgf-β3. Our data reveals that several significantly differentially expressed genes have been previously identified as CP genes. WT mice showed two specific genes (Twist1, Wnt5a) to be downregulated significantly at 16.5dpc. HM mice, on the contrary, showed an increased expression of Chrng, Col2a1, Col11a1, Col11a2, and L1cam genes at 16.5dpc, that play crucial roles in palatogenesis. We analyzed the specific cellular function, role in palatogenesis, and association with TGF-β signaling pathway of suspected genes using IPA and GeneCards.
Twist acts as a basic helix-loop-helix transcription factor essential for EMT in both embryonic development and cancer. Twist is a well-established E-cadherin repressor (Yu et al., 2008). It has been shown convincingly that EMT is a major mechanism of palatal seam disintegration – the critical final stage of palate fusion (Nawshad, 2008). Twist is present in the zone of palatal shelf fusion—indicating its likely importance to palatogenesis. Further, in experiments where Twist expression is downregulated, palate fusion is hindered or delayed (Yu et al., 2008). Conversely, in chicken palates, the addition of Tgf-β3 upregulated Twist mRNA indicating it is downstream of Tgf-β signaling (Sun et al., 1998). Our data confirms that downregulation of Twist gene in HM inhibits palate fusion resulting in a cleft at 16.5dpc.

Genetic screenings have implicated several isoforms of WNTs—WNT3A, WNT5A, WNT11—with non-syndromic CL/P (Meng et al., 2009). The WNT signaling pathways are essential for cell proliferation, differentiation, and survival (Brugmann et al., 2007). Furthermore, these genes are known to regulate mid-face development, upper lip fusion and are likely associated with the etiology of orofacial clefts (Brugmann et al., 2007). In situ hybridization studies have shown Wnt5A expression in the frontonasal prominences and maxillary process which fuse to form the primary palate (Yamaguchi et al., 1999). Gene interaction studies suggest a variation in WNT5A regulating neural crest cell differentiation may predispose to an orofacial cleft (Chiquet et al., 2008). Specifically, Wnt5a directs cell migration in a graded manner along the anteroposterior axis of the palate; loss of Wnt5a in mice leads to a complete cleft of the secondary palate (He et al., 2008). Overall, substantial data demonstrates an essential role of WNT family members in orofacial growth allowing immaculate fusion of facial primordia. The HM palate showed a decreased expression of Wnt5a from 14.5dpc to 16.5dpc showing a lack of directional palatal cell migration and proliferation necessary for both mesenchymal and epithelial homeostasis resulting in a cleft.
CHRNG (Cholinergic receptor, nicotinic, γ) is a transmembrane receptor for acetylcholine with five different subunits—two α, one β, one δ and one γ. Binding of acetylcholine activates voltage-gated sodium channels resulting in an action potential in muscle (Hoffmann et al., 2006). During fetal development, the γ subunit is commonly expressed (Kariminejad et al., 2016). The γ subunit is essential for neuromuscular signal transduction connecting axon and muscle; γ-knockout is lethal in mice (Hoffmann et al., 2006). In humans, mutations in CHRNG cause Escobar syndrome and a spectrum of Multiple Pterygium syndromes characterized by several craniofacial deformities including, but not limited to, cleft palate, joint contractures, pterygia and micrognathia (Vogt et al., 2012). Current literature shows pterygium is also associated with IRF6 and p63 mutations in humans, which are both known to be associated with TGF-β3 pathway (Lihua et al., 2015) (Ozturk et al., 2013). Chrng is upregulated in the HM mice at 16.5dpc indicating that in absence of Tgf-β3 in HM, Irf6 and p63 proteins are differentially expressed resulting in the persistence of palatal periderm resulting in palatal cleft.

Several types of collagen are variably expressed in the developing palate and essential for ECM metabolism. Collagen fibers have been shown to contribute to palatal shelf elevation, shelf adhesion, and ECM formation. COL2A1 mutations are present in Stickler Syndrome and chondrodysplasias (Vandenber et al., 1991, Ahmad et al., 1995). Further, defects in several collagens—Col1a2, Col2a1, Col11a22, and ColXla1—are linked to cleft palate (Meng et al., 2009, Schutte & Murray, 1999). In the HM palate, Col2a1, Col11a1, and Col11a2 were all significantly upregulated from 14.5 to 16.5dpc suggesting their increased presence in the basement membranes of persistent, intact palatal epithelia and periderm. Persistence of palatal periderm ultimately impedes palatal fusion resulting in palatal cleft.

The L1 cell adhesion molecule (L1CAM) is a member of the immunoglobulin gene superfamily and is associated with a spectrum of disorders collectively known as L1 syndrome. X-linked hydrocephalus (XLH), Hirschsprung’s disease, fetal alcohol spectrum disorders, and
cancerous are some of the variable presentations of L1 malfunction (Schäfer & Altevogt, 2010). Abnormalities of L1CAM are characterized by severe mental retardation, hydrocephalus, spastic tetraplegia, bilateral adducted thumbs, and orofacial clefts. Previous literature reports have shown individuals with XLH who have cleft palate suggesting that L1CAM may contribute to both phenotypes (Okamoto et al., 2004). While the mechanisms underlying L1cam function are still elusive, it is generally involved in cell proliferation, adhesion and migration as well as critical in the development of carcinomas. Based on these data, we propose that L1cam in HM is involved in maintaining palatal epithelia and periderm attachment and integrity which results in cleft palate.

5.2 Genes Upregulated at 16.5dpc vs. 14.5dpc in Homozygous Palates

The TGF-β family members are integral to palatogenesis and responsible for an array of functions required for palate fusion—cell migration, EMT, ECM synthesis and deposition, degradation of basement membrane, cell proliferation and apoptosis. In HM mice, Tgf-β1 and Tgf-β2 are expressed in MEE cells and mesenchymal cells respectively (Nawshad et al., 2004). Several upregulated genes in HM mice are under control of other TGF isoforms: Adam12, Clu, Fas, Hspg2, Cldn1, Lox, Itgb4, and Klf5. These genes, regulated by Tgf-β1,2 in HM may regulate multiple cellular functions essential to proper palatal growth in HM but are unrelated to palatal fusion, which is a unique characteristic of cleft palate in Tgf-β3 knockouts.

Disintegrin metalloproteases, ADAMs, regulate key cellular processes such as apoptosis, proliferation, and cell adhesion. ADAMs family members have been shown to be under the control of TGF-β signaling showing changes in regulation at the gene expression level (Ramdas et al., 2013). In the developing HM mouse palate, Tgf-β1 and Tgf-β2 are expressed in both palatal epithelia and mesenchyme (Nawshad et al., 2004). The upregulation of Adam12 in the HM palate at 16.5dpc is indicative of its control by different isoforms of Tgf-β necessary for the attainment of palatal growth and elevation, but not fusion, which is regulated by Tgf-β3.
Clusterin (Clu) is a multifunctional glycoprotein that has a role in epithelial cell differentiation, cell-cell adhesion, and regulation of apoptosis (Itahana et al., 2007). Clu is synthesized by cells of epithelial and mesenchymal origin; synthesis is upregulated in tissues undergoing remodeling or injury exerting a protective function in a stressed environment. Tgf-β1 was shown to increase expression of Clu in various cell types: nervous tissue, astrocytes, fibroblasts, lung epithelial cells, aortic endothelial cells and in HeLa cells in culture (Wegrowski et al., 1999). The upregulation of Clu at 16.5dpc in HM palatal tissue is a result of induced expression by isoform TGF-β1 suggesting Clu plays no role in palatal fusion resulting in cleft palate.

Apoptosis is essential for embryogenesis, particularly in tissue and organ development and tissue homeostasis. Fas is a member of the TNF receptor superfamily and a well-known mediator of apoptosis (Goldthorpe et al., 2015). It has been suggested that Tgf-β induces resistance to apoptosis in lung fibroblasts through suppression of Fas via miR-29 (Matsushima & Ishiyama, 2016). In the HM palate, Fas is upregulated at 16.5dpc induced by other Tgf-β isoforms or pro-apoptotic factors contributing to palatal cell death that is essential in palatogenesis.

Perlecain (Hspg2) is a proteoglycan that is a key component of basement membranes and ECM. An absence of Hspg2 in mice and humans causes lethal chondrodysplasia (Hara et al., 2017). Hspg2 has a roles in cell adhesion, proliferation, and angiogenesis. Tgf-β1-induced Hspg2 deposition has been demonstrated in COPD airway smooth muscle (Ichimaru et al., 2012). We propose that Hspg2 is functional in palatal mesenchyme under the control of Tgf-β1 regulating palatal mesenchymal ECM necessary for palatal growth in HM mice, but without any implication in palatal fusion.

Claudin-1 (Cldn1), a transmembrane protein localized to the surface of epithelial cells, is crucial for formation and function of tight junctions (Zhang et al., 2016). Disruption of tight junctions has been shown to lead to the induction of EMT in cancers with a subsequent loss of cell-
cell contacts (Katayama et al., 2017). It has also been demonstrated that Tgf-β1 exposure leads to EMT and decreased Cldn1 in MDCKII cells (Medici et al., 2006). We suggest in the HM mouse palate, the increased Cldn1 expression from 14.5 to 16.5dpc indicates a persistence of cell-cell adhesion and therefore a persistence of the MEE and periderm ultimately hindering palate fusion.

*Lysyl oxidase (Lox)* is an enzyme essential for basement membrane development and maturation. Active Lox modifies collagen formation to help stabilize a functional ECM; in excess, Lox can lead to compromised basement membrane function promoting abnormal ECM accumulation and fibrotic diseases. Lox has also been shown to promote apoptosis and act as a tumor suppressor (Kim et al., 2017). In lung cancer, TGF-β is shown to increase LOX contributing to cancer metastasis (Araz et al., 2014). In the HM palate, Lox may be upregulated as a result of Tgf-β1 and Tgf-β2 maintaining ECM homeostasis necessary for palatal growth with no role in palate fusion seen in WT mice.

*Integrin β4 (Itgb4)* is a member of the integrin family of cell adhesion receptors essential for cell migration in embryonic development, wound healing, inflammatory responses, and tumor metastasis. Expressed in epithelial cells, Itgb4 is a component of hemidesmosomes that provides attachment to the basement membrane (Miyazaki et al., 2015). Tgf-β1 is known to regulate expression of several integrins. The addition of Tgf-β1 to various cell types resulted in cytoskeletal reorganization of the β4 integrin subunit (Scardigli et al., 1996) suggesting the upregulation of Itgb4 in the HM palate is under control of Tgf-β1; this upregulation is important for palatal growth and elevation by modulating basement membrane with palatal epithelia. Further, Itgb4 seems to have no role in palate fusion which is strictly regulated by Tgf-β3 in WT palates.

Transcription factor Klf5 belongs to the zinc-finger protein family and acts downstream of multiple signaling pathways including TGF-β. Klf5 is a known modulator of proliferation, differentiation, cell cycle, and apoptosis and has been associated with different cancers and
cardiovascular disease (Li et al., 2015). In HM palatal epithelia, Klf5 is independent of regulatory Tgf-β3, however it continues to be regulated by both Tgf-β1 and Tgf-β2 showing its increased presence at 16.5dpc. Such upregulation of Klf5 gene in palatal epithelia in HM mice indicates palatal epithelial growth, but no relationship with fusion.

5.3 Genes Downregulated at 16.5dpc vs. 14.5dpc in Homozygous Palates

As previously mentioned, Wnt family members are critical to orofacial development. Further, Wnt signaling pathways regulate a variety of developmental processes, including cell proliferation, differentiation and cell polarity that are fundamental in palate development (Cadigan & Nusse, 1997, Wodarz & Nusse, 1998). Both Wnt3 and Wnt9b are expressed in the developing facial ectoderm and the canonical Wnt signaling pathway is activated during facial outgrowth and fusion (Lan et al., 2006). Thus, in mice, reduced Wnt9b expression or lack of Wnt signaling pathways, as seen in HM embryos, contributes to failure in palatal morphogenesis resulting in palatal cleft.

Alx4 is a paired-like homeodomain transcription factor that is mainly expressed in the mesenchymal tissues of developing bone, teeth, limbs and mammary tissue. It plays a pivotal role in craniofacial development and epithelial-mesenchymal interaction. Several studies have revealed mutations of Alx4 cause craniofacial anomalies, including facial clefting (Beverdam et al., 2001). During the development of the palate, as the palatal shelves adhere at the midline, two layers of intervening MEE cells form the MES which must be subsequently dissolved to allow successful palate fusion. EMT is thought to be an important mechanism required for MES disintegration (Nawshad, 2008). Deletion of Alx4 was demonstrated to induce reversion of EMT (Yuan et al., 2015, Hudson et al., 1998). Therefore, loss of Alx4 in HM palates may impair EMT during palatal fusion and impede breakdown of MES resulting in a palatal cleft.
*Paxl* is a member of the paired box (PAX) family of transcription factors. Members of PAX family play critical roles in pattern formation during embryogenesis and may be essential for development of the vertebral column (Sonnesen et al., 2008). High DNA methylation rates of *Paxl* are detected in tissues of several types of cancers, suggesting that *Paxl* acts as a tumor suppressor gene (Guerrero-Preston et al., 2014). Inactivation of *Paxl* gene may result in enhanced resistance to apoptosis and repression of terminal differentiation (Su et al., 2009, Kan et al., 2014, Cheng et al., 2016, Hassan et al., 2017). In our RNA-seq data, downregulated expression of *Paxl* in HM palates may be responsible for persistence of periderm cells which bolsters the persistence of the MES and ultimately leads to failure of fusion in palatal development.

*Kcp* is a secreted cysteine-rich domain protein and acts as a regulator of the TGF-β superfamily pathways which enhances BMP signaling while inhibiting both the activin-A and TGF-β1-mediated signaling pathways (Soofi et al., 2017). BMP signaling is essential during organ development, including palatogenesis. Specifically, BMP2, BMP4, and BMP5 are expressed in both epithelia and mesenchyme throughout palatogenesis (Nie et al., 2006). Lu et al. showed the importance of BMP signaling during palatogenesis by demonstrating a decrease expression of *Bmp2*, -4, and -5 in mice exhibiting cleft palate (Lu et al., 2000). In our HM samples, deficiency of *Kcp* may alter the levels of *Bmp* signaling, leading to aberrant cell proliferation and cell death during palatal development, resulting in palatal cleft.

*Msxl*, like the *Kcp* gene, is also known to regulate BMP signaling; BMP signaling has been proposed to be downstream of *Msxl* during palatal development (Zhang et al., 2002). *Msxl*, a member of the Muscle Segment Homeobox gene family, acts as a transcriptional repressor and functions in diverse cell types regulating proliferation, differentiation and angiogenesis (Medio et al., 2012). *Msxl* is primarily expressed in the growing edges of maxillary prominences and the anterior part of the palatal shelves as palatogenesis enters later stages (Hilliard et al., 2005, Zhang et al., 2002). Mice with null mutation of *Msxl* develop cleft palate—clearly demonstrating a
critical role of Msx1 in palate development (Satokata & Maas, 1994). As shown in our HM mice, reduced expression of Msx1 at 16.5dpc is indicative of cleft palate.

*Ppp1r17* is a substrate for cGMP-dependent protein kinase and is involved in central nervous system development and intracellular signal transduction. *Ppp1r17* implements protein serine/threonine phosphatase inhibitor activity and inhibits phosphatase activities of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) complexes (Endo et al., 1999). During embryonic development, cellular and molecular activities are regulated by phosphorylation and dephosphorylation by protein kinases and phosphatases, respectively. Palatogenesis relies on a multitude of orchestrated signal transduction pathways, such as TGF-β, requiring protein phosphorylation and enzymatic dephosphorylation. In embryonic murine palates, *Pp1* and *Pp2a* were reported to account for virtually all detectable serine/threonine protein phosphatase activity during palatogenesis (Weston et al., 2002). Therefore, decreased expression of *Pp1* or *Pp2a* due to impaired function of *Ppp1r17* may cause disruption in orchestration of *Tgf-β* signaling pathways and ultimately cell proliferation, epithelial differentiation, and apoptosis in palatal development of HM mice. As demonstrated in the HM mouse at 16.5dpc, decreased *Ppp1r17* and resulting dysfunction of *Tgf-β* pathways results in cleft palate.

*Pdgfc* is a member of the platelet-derived growth factor (PDGF) family and plays an essential role in the regulation of multiple biological processes including embryonic development, cell proliferation, cell migration and survival. The role of *Pdgfc* as a mitogenic factor in regulating proliferation of mouse embryonic palatal mesenchymal cells has been well established (Ding et al., 2004, Han et al., 2006). *Pdgfc* deficiency induces retardation of mesenchymal proliferation and differentiation in palatal shelves, which accounts for failure of palate fusion and cleft formation in *Pdgfc* (−/−) embryos (Ding et al., 2004). Although retardation of palatal growth in *Tgfβ-3* knockout mice is not observed, this does not exclude a disruption in palatal mesenchymal homeostasis due
to the reduction of *Pdgfc*. As seen in the HM mouse at 16.5dpc, a decrease in *Pdgfc* could potentially result in inadequate EMT—a fundamental process in successful palate fusion.

5.4 Genes Upregulated at 16.5dpc vs. 14.5dpc in Wild-type Palates

In WT palates, several genes under the control of *Tgf-β3* are significantly differentially expressed compared to HM; consequent lack of these temporal gene changes in HM results in palatal cleft. It is important to reiterate that all isoforms of TGF-β ligand (1, 2 and 3) are crucial for normal palate development. These isoforms act in tandem in a well-orchestrated time and location dependent manner. Although mutants of these isoforms result in CP phenotype with differing degrees of penetrance (*Tgf-β1* is embryonically lethal, 20% for *Tgf-β2*); it is *Tgf-β3* that causes 100% penetrance with CP as the only phenotype (Zhu et al., 2012b). However, the compromise of cellular and morphological functions of these isoforms vary significantly. Our data reveals that upregulated WT genes—*Cdh1*, *Ocln*, *Lyn*, *F2rl1*, *Tspan2*, and *Tnfrsf11B*—may contribute to normal palate fusion.

The cell-cell adhesion protein *E-cadherin* (*Cdh1*) is a member of the cadherin family of calcium-dependent cell adhesion glycoproteins (Becker et al., 1994). Just like all epithelia, the palatal shelf epithelium expresses *Cdh1* during palatogenesis. Mutation of the *Cdh1* gene has been shown to cause CL/P (Meng et al., 2009). In WT mice *Cdh1* is upregulated from 14.5 to 16.5dpc. At 14.5dpc *Cdh1* is essential for integrity of MEE and overlying periderm of the palatal shelves. As the palate fuses we expect dissolution of MEE and periderm with a localized decrease in epithelial cells and therefore *Cdh1* (Nawshad et al., 2007). However, at 16.5dpc we expect a significant increase of stratified squamous epithelial cells lining the oral and nasal sides of the palate resulting in an overall increase in *Cdh1*.

Similar to *Cdh1*, the transmembrane protein *Occludin* (*Ocln*) plays a role in tight junction assembly of different epithelia, which is also upregulated at 16.5dpc in WT palate (Mir et al.,
2016b). *Ocln* is essential for homeostasis of epithelia; any decrease in *Ocln* decreases cell-cell adhesion and reduces apoptosis (Mir et al., 2016a). With the increase in quantity of epithelial cells in WT palates, we also expect an increase in occludin. Both of these genes, *Cdh1* and *Ocln*, enforce palatal epithelial adhesion as well as maintain epithelial architectural and functional integrity—hence their increase in WT is expected and justified.

The *lyn* gene belongs to the protein kinase superfamily and is localized to the cell surface (Roberts et al., 2014). *Lyn* plays an important role in immune response, hematopoiesis, response to growth factors and cytokines, and integrin signaling (Lim et al., 2015). Studies show that Tgf-β is involved in phosphorylation of *Lyn* and specifically have shown a link between *Lyn* and Tgf-β in chronic myeloid leukemia (CML) (Li et al., 2013). Using MYL cells from a CML patient, it was found that TGFβ drives LYN ubiquitination and turnover via c-cbl transcription and expression (Smith et al., 2012). Similarly, *Tgf-β* control may be contributing to the increased presence of *Lyn* in WT palates at 16.5dpc to facilitate integrin signaling as well as maintaining normal palatal immune response and homeostasis.

*F2R* like trypsin receptor 1 (*F2rl1*), also known as *Par2*, is a member of the G-protein coupled receptor family of proteins serving pleiotropic functions in vertebrate development and postnatal homeostasis (Sales et al., 2015). *Par2* is expressed in various cell types including gastrointestinal tract, skin, lung, kidneys, smooth muscle, endothelium, epithelium and fibroblasts. *Par2* has been found to induce colonic inflammation, kidney inflammation, and tissue fibrosis (Chung et al., 2013). G-protein coupled receptors are known to transactivate EGFR in various cell types. An *in vitro* study showed *Par2* transactivates EGF and TGF-β-receptors via PI3K and MAPKs ultimately leading to phosphorylation of Smad2 and -3. Smad2/3 phosphorylation is essential to TGF-β-dependent signaling and activation of transcription factors to facilitate palatal cell proliferation, differentiation, and apoptosis (Chung et al., 2013). Additional signaling
pathways and proteins, such as G-protein coupled receptors, play a critical role in palate development as demonstrated by its increase in WT palates at 16.5dpc (Cobourne, 2004).

*Tetrspanins (Tspans)* consist of a large family of 4-transmembrane domain proteins. *Tspans* have recently gained importance as regulators in cancer malignancy, immune response, fertilization, and infectious disease (Zhao et al., 2017). *Tspans* have been shown to play crucial roles in biologic processes including cell adhesion, proliferation, differentiation, and migration (Zhao et al., 2017). Recently *Tspan2* has been found to be the only *Tspan* family gene induced by TGF-β in vascular smooth muscle cells via class SMAD pathway (Zhao, Wu et al. 2017). While there is no direct link of *Tspan2* to palatogenesis, its implication with TGF-β/SMAD pathway and crucial role in cell adhesion, proliferation, and differentiation warrant further investigation into its role in palatogenesis. However, our data suggest that the role of *Tspan2* may be limited to cellular migration, proliferation and differention via Smad pathways during palatogenesis.

*Osteoprotegrin (Tnfrs11b)* is a key regulator of bone metabolism and is crucial for bone homeostasis; *Tnfrs11b* inhibits osteoclast activity allowing new bone formation by osteoblasts (Smane & Pilmane, 2016). *Tnfrs11b* knockout mice have severe osteoporosis while overexpression leads to osteopetrosis (Zehnder et al., 2006). As early as 14.5dpc, mesenchymal condensations are observed on both sides of the palatal midline which undergo chondrogenesis initially and ultimately membranous ossification that give rise to the hard palate (Martinez-Alvarez et al., 2004). We expect *Tnfrs11b* signaling to be upregulated from 14.5 to 16.5dpc in preparation for osteogenesis.

### 5.5 Genes Downregulated at 16.5dpc vs. 14.5dpc in Wild-type Palates

As previously described, downregulation of WT genes allow normal palate fusion. Our data show decreased expression of *Fndc3c1*, *Dlx1*, *Gas2*, *Bnc2* and *Vcan* facilitate successful palate fusion.
*Fndc3c1*, a member of a novel gene family, encodes a protein consisting of fibronectin module type III and is a component of ECM proteins functioning in cell adhesion, differentiation, migration and embryogenesis (Porcionatto, 2006, Potts & Campbell, 1996). Although *Fndc3c1* is mostly expressed in all embryonic germ layers except heart and yolk sac from E7.5-E8.5 (Hou et al., 2007). Further, *Fndc3c1* is shown to be expressed in trophoblast stem cells during extraembryonic ectoderm differentiation in mice (Pearton et al., 2014). During palate formation, ectoderm-derived MEE and periderm are created to form a protective shield against premature fusion, but must dislodge prior to shelf adherence to allow a confluent palate (Obholz et al., 2006, Nawshad, 2008, Hu et al., 2015b). Therefore, it’s logical to postulate that reduced expression of an ectoderm differentiation gene, *Fndc3c1*, may facilitate elimination of periderm from underlying epithelium allowing palate fusion.

**Distal-Less Homeobox 1 (Dlx1)** encodes a protein that functions as a transcriptional regulator of signaling from several TGF-β superfamily members. All six Dlx family members are present in the first pharyngeal arch in both mesenchyme and ectoderm (Jeong et al., 2012). Mice with mutations of both *Dlx1* and *Dlx2* exhibit cleft palate demonstrating the essential role of *Dlx* family in palatogenesis (Jeong et al., 2012). At early stages in palate formation, the defect in *Dlx* family genes results in a growth defect of palatal shelves. Later stages of palatogenesis are also affected via *Dlx1* and *Dlx2* regulation of downstream signaling molecules such as *Shh*, *Fgf10*, and *Bmp4* (Jeong et al., 2012). Our RNA-seq data show decreased activity of *Dlx1* at 16.5dpc in the WT palates. We postulate that the decrease in *Dlx1* prior to palatal fusion may allow Tgf-β signaling to properly guide dislodgment of periderm and subsequent fusion in palatogenesis.

*Growth arrest specific gene 2 (Gas2)* is a caspase-3 substrate that regulates cell cycle and apoptosis (Sgorbissa, Benetti et al. 1999). The cleaved form of *Gas2* is capable of modifying microfilament and cell shape during apoptosis (Brancolini et al., 1995). Cell death by apoptosis is a fundamental process maintaining normal development and homeostasis of multicellular
organisms. During palatogenesis, MEE cells in the MES are proposed to, in part, undergo apoptosis to facilitate mesenchymal confluence (Nawshad, 2008). *In vitro*, however, cultured murine keratinocyte cells displayed reduced Gas2 protein levels during growth arrest induced by TGF-β treatment, whereas significantly upregulated Gas2 activity was observed in rapidly proliferating cells (Manzow et al., 1996). Therefore, it is plausible to suggest that during palate fusion, lower levels of Gas2 is a reflection of quiescent epithelial cells undergoing apoptosis or EMT instead of robust proliferation. Future studies are necessary to investigate the role of Tgf-β in regulating expression of Gas2.

The *Bnc2* gene encodes a conserved zinc finger protein containing three pairs of zinc fingers and a nuclear localization signal (Vanhouwtteghem et al., 2009). According to reports, *Bnc2* plays a role in skin color saturation and skin cancer development (Jacobs et al., 2015). *Bnc2* is found mainly in keratinocytes of stratified squamous epithelium and is functionally involved in cell proliferation (Vanhouwtteghem et al., 2009). Observations of growth arrest of tumor cells induced by stable expression of *Bnc2* also supports *Bnc2* as a putative tumor suppressor gene (Akagi et al., 2009). In a recent study, *Bnc2* was shown in mesenchymal cells surrounding the palatal cleft in mice; while the exact role of *Bnc2* is unclear, it likely regulates expression of genes that encode transcription factors, genes essential for growth, and signaling molecules and receptors necessary for successful palate fusion (Vanhouwtteghem et al., 2009). The involvement of *Bnc2* in palatogenesis warrants further investigation.

*Versican (Vcan)* is a chondroitin sulfate proteoglycan and component of the extracellular matrix essential to embryogenesis; lack of *Vcan* is lethal in mice (Snyder et al., 2015). *Vcan* plays a crucial role in various cellular processes including cell adhesion, proliferation, differentiation and apoptosis; one isoform, V1, specifically promotes EMT (Snyder et al., 2015). Further, increasing evidence has demonstrated that V1 is capable of inducing apoptotic resistance in cultured cells (Sheng et al., 2005, LaPierre et al., 2007). *Tgf-β3*-mediated apoptosis and subsequent disintegration
of the MES is a critical event for successful palate fusion (Iordanskaia & Nawshad, 2011, Nawshad et al., 2004, Kaartinen et al., 1997). Thus, it’s plausible that suppressed expression of Vcan at 16.5dpc allows the breakdown of the MES. While we know it is a critical gene, further examination of Vcan activity throughout embryogenesis would be of benefit to our understanding as to how it contributes to palatogenesis.

5.6 Dysregulated Genes independent of TGF-β

The heat shock protein Crystallin-αB (Cryab) is a member of the heat shock protein family with an array of biological functions (Malin et al., 2016). Cryab is known to bind and stabilize cytoskeletal proteins and play a role in EMT in liver and lung fibrosis (Malin et al., 2016). An in vitro experiment in retinal pigment epithelial cells showed overexpression of Cryab siRNA decreased E-cadherin and increased Snail and Slug in protein and mRNA levels (Ishikawa et al., 2016). Snail family members have been implicated in triggering of EMT and cell survival (Kudo-Saito et al., 2009). Cryab, in relation to Snail, could be acting as an anti-apoptotic agent in persistent palatal shelf epithelium and periderm. In cleft mice, increased levels of Tgf-β1 in the palatal mesenchyme induces Snail expression and inhibition of cell death in the MEE (Martinez-Alvarez et al., 2004). Upregulated in HM mice at 16.5dpc, the relationship between Cryab and Snail may be related to apoptosis of palatal mesenchyme and epithelia that are necessary in palatogenesis requiring further investigation.

Neuronal cell adhesion molecule (Nr-cam) is a member of the immunoglobulin superfamily and was recently identified as a target of gene signaling in human melanoma and colon carcinoma cells and tissue (Conacci-Sorrell et al., 2005). Nr-cam protects cells from apoptosis via extracellular signal-regulated kinase and AKT signaling pathways (Conacci-Sorrell et al., 2005). The PI3K/Akt pathway suppresses apoptosis and promotes cell growth and proliferation (Bian et al., 2009). Nr-Cam is upregulated in the HM palate at 16.5dpc and may be contributing to
decreased apoptosis in persistent MEE and periderm of palatal shelves ultimately hindering palatal fusion.

5.7 Direct Comparison of Homozygous and Wild-type Palates at 14.5 and 16.5 dpc

The RNA sequencing data shows few differentially expressed genes when directly comparing genotypes at 14.5 and 16.5 dpc shown in Table 4. At 14.5, only 13 genes were differentially expressed between WT and HM ranging from FC -1.46 to 1.39—a very concentrated difference between genotype in comparison to the differential gene expression over time.

_Ephrins_ are a family of cell surface receptors involved in embryonic development including the palate (Risley et al., 2009b). _Ephrins_ are shown to be necessary for both cell proliferation and palatal adhesion; further, multiple ephrin/eph receptors are responsible for normal palate development (Risley et al., 2009a). Mutations in the _EphrinB1_ gene are seen in craniofrontonasal syndrome giving rise to cranial defects including cleft lip and palate in both humans and mice (Risley et al., 2009b). Similarly, _EphB2_ and _EphB3_ have been implicated in palate development and present in palatal mesenchyme and epithelium (Risley et al., 2009a). Knockout of both _EphB2_ and _EphB3_ causes cleft palate in mice (Orioli et al., 1996). Differentially expressed _Ephb3_ signaling directly affects palatogenesis, but the exact role of _Ephb3_ remains undetermined.

Transcription factor _Sox6_ belongs to the _Sox_ family and is expressed in various tissues; it serves many regulatory functions in the development of mesoderm, ectoderm and endodermal tissues. In the mouse model, _Sox6_ is expressed in the central nervous system, otic vesicle, somites branchial arches, thymus notochord, craniofacial mesenchyme, limb buds, and liver (Hagiwara, 2011). _Sox6_ is implicated in both activation and suppression of gene transcription affecting genes essential to palate development and known to contribute to cleft formation such as _Fgf3_ and _Col2a1_ (Küchler et al., 2014, Meng et al., 2009). _Sox6_ negatively regulates expression of _Fgf3_ and
activates Col2a1 (Hagiwara, 2011). Our current knowledge indicates Sox6 plays various roles in embryonic development, warranting further investigation of its involvement in palatogenesis.

Keratin family proteins comprise the intermediate filament system in epithelial cells having significant interactions with the ECM throughout key processes such as development, tissue remodeling and repair, and differentiation (Kurpakus et al., 1992). In the oral cavity, immunostaining shows keratin in the oral periderm covering the maxillary processes, palatal shelves, and future nasal cavity (Casey et al., 2006). Periderm has been previously characterized by keratin expression (Paul et al., 2017, Iwasaki et al., 2006). The oral periderm prevents the palatal shelves from abhorrently adhering to other oral structures during palatogenesis; further, periderm cell death is critical to facilitate palatal shelf adhesion (Casey et al., 2006). The genotypic difference in Krt5 expression, upregulated in HM at 14.5dpc, is likely due to persistent periderm and therefore increased expression in the palatal shelf epithelium.

Greater differences exist between genotypes at 16.5dpc; a total of 38 differentially expressed genes existed between WT and HM at 16.5dpc ranging from FC -2.75 to 16.75 as shown in Table 5.7. Epidermal growth factor receptor (Egfr) signaling is essential for normal craniofacial development. Egfr regulates development via downstream targets including matrix metalloproteinases (Mmp) (Miettinen et al., 1999). Studies have shown abnormal function of Egfr results in cleft palate (Meng et al., 2009). In Egfr deficient mice, it is postulated that palate adhesion fails due to decreased Mmp presence (Miettinen et al., 1999). Both Egfr and Mmp13 are dysregulated between WT and HM at 16.5dpc; Egfr is upregulated in HM at 16.5dpc while Mmp13 is downregulated in HM at 16.5dpc. The difference in expression between HM and WT demonstrates the involvement of both Egfr signaling and its target Mmp13 in palatogenesis.

As previously mentioned, the collagen fibers are known to contribute to palatal shelf elevation, shelf adhesion and ECM formation (Vandenberg et al., 1991). Increased presence of
Col6a6 in HM compared to WT at 16.5dpc is indicative of increased presence of basement membranes of persistent, intact palatal epithelia with periderm with a cleft.

Similar to its counterpart differentially expressed at 14.5, Krt4 is upregulated in HM at 16.5dpc. As previously discussed, the increased expression of Krt4 is indicative of persistent periderm which will hinder proper palatal fusion in HM.
Table 4: Selected genes that are uniquely significantly differentially expressed within genotypes at different time points (adjusted p-value < 0.05) in the HM or WT groups.

<table>
<thead>
<tr>
<th>Genotype Comparison</th>
<th># of SDEG</th>
<th>Selected Genes</th>
</tr>
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<tbody>
<tr>
<td>14.5</td>
<td>8</td>
<td>Ephb3, Krt5</td>
</tr>
<tr>
<td>Up in HM 14.5 vs. WT 14.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Down in HM 14.5 vs. WT 14.5</td>
<td>5</td>
<td>Sox6</td>
</tr>
<tr>
<td>16.5</td>
<td>30</td>
<td>Egfr, Col6a6, Krt4</td>
</tr>
<tr>
<td>Up in HM 16.5 vs. WT 16.5</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Down in HM 16.5 vs. WT 16.5</td>
<td>8</td>
<td>Mmp3</td>
</tr>
</tbody>
</table>
5.8 Significantly Dysregulated Gene Ontology Categories & KEGG Pathways

Significantly differentially expressed genes were found both inter- and intra- time point and genotype. In order to identify predominant biological processes that are significantly dysregulated, genes are grouped by common properties into GO categories, such as cell adhesion (Young et al., 2010). Another method of categorizing genes is by KEGG functional assignment. KEGG provides a link from a specific gene to pathway or complex, such as metabolism (Kanehisa & Goto, 2000b).

Identified functional categories that are uniquely or commonly overrepresented in our samples are listed in Figure 4.3c. In the HM palates, we see significantly upregulated functional categories including ECM-receptor interaction, cell adhesion, and focal adhesion. We expect the genes in these categories are likely contributing to the persistence of the periderm layer that essentially impedes palate fusion (Nawshad et al., 2004). Conversely, in WT palates, cell-differentiation and apoptotic process are significantly overrepresented. As we expect, proper palate fusion requires both cell-differentiation and apoptosis to remove the MES and allow proper fusion (Nawshad et al., 2007).

5.9 Functional Analysis of SDEGs in WT and HM at 16.5 vs. 14.5dpc

The RNA-seq data shows that in WT at 16.5dpc of palatal development, several pathways are active and functional including: TGF-β, ERK/MAPK, p38 MAPK, and PI3K/AKT. It is known that these pathways are under the control of TGF-β; however, they can also be regulated by other factors active in palate development at 16.5dpc. These pathways are shown to regulate genes displayed in Figure 5.1. The p38 MAPK pathway is a key mediator downstream of TGF-β pathway which synergistically causes downregulation of the transcriptional regulator Gsc as demonstrated in Figure 5.2. Gsc is reported to regulate cell migration and EMT during embryonic development suggesting inhibition of Gsc indicates completion of palate fusion at 16.5dpc (Xue et al., 2014).
Tgf-β1 conjointly with p38 MAPK and additional molecules shown in Figure 5.3 regulate cell morphology and differentiation of fibroblasts that lend to construction of ECM and EMT during palate development (Griffith & Hay, 1992, Kalluri & Weinberg, 2009).

Conversely in HM, the major pathways illustrate a different regulatory profile at 16.5dpc which results in cleft palate as shown in Figure 5.4. As in the Tgf-β3 knockout, Tgf-β3 signaling is inactive allowing PI3K/AKT and ERK/MAPK pathways to impair normal palate fusion by altering the expression of Tlx2 as shown in Figure 5.5. The Tlx2 gene plays a role in proliferation and differentiation of neural crest cell lines (Nelms & Labosky, 2010). Although the involvement of Tlx2 in palatogenesis is largely unexplored, Tlx2 has been reported in diseases associated with cleft palate (Puri & Shinkai, 2004). Genes illustrated in Figure 5.6, such as Tbx3 and Wnt9b, that participate in regulating crucial biological processes including cell morphology and embryonic morphogenesis show altered expression levels at 16.5dpc in HM and may be responsible for failure of palate fusion.
Figure 12. Upregulated or downregulated significantly differentially expressed genes (SDEG), fold change >2.0, and relevant pathways over time 14.5dpc to 16.5dpc in WT palates.
Figure 13. Illustration of SDEGs in TGF-β3 signaling pathway related to major pathways expressed in WT.
Figure 14. SDEGs from 14.5dpc to 16.5dpc in relation to cell morphology and differentiation of fibroblasts in WT.
Figure 15. Upregulated or downregulated SDEGs, fold change >2.0, and relevant pathways over time 14.5dpc to 16.5dpc in HM palates.
Figure 16. Illustration of SDEGs in TGF-β3 signaling pathway in relation to major pathways in HM.
Figure 17. SDEGs from 14.5dpc to 16.5dpc in relation to morphology of cells and morphogenesis of embryo in HM.
5.10 Real-time PCR confirmation of Gene Dysregulation

Our qPCR data is in agreement with our RNA-seq results, shown in Figure 4.4, demonstrating at 16.5dpc WT palates have an increased expression of the cell adhesion genes Cdh1, Ocln and receptors F2rl1 (Par2) which are essential in palatal MEE cell architecture and play a vital role in palatal shelf fusion allowing palatal confluency. On the contrary, we observe a downregulation of Tnfrsf11b, important for osteoclastogenesis, hence, its requirement in palate development is negligible (Naranjo et al., 2016). Similarly, Fndc3c1 is mostly expressed in embryonic endoderm, serving little role in palatal embryonic cell origin which are ectoderm and ectomesenchymes (Hou et al., 2007). Although Dlx1 gene is important for craniofacial and palatal vertical outgrowth, its role in palatal fusion as shown in our study has not been reported (Wu et al., 2015, Jeong et al., 2012). Similarly, Gas2, which has been implicated in skeletal development, has no implication in embryonic palate development (Diez-Roux et al., 2011). Accordingly, all genes unessential to palate fusion (Tnfrsf11b, Fndc3c1, Dlx1 and Gas2) were shown to be downregulated in our study.

In HM, qPCR data shown in Figure 4.41 confirms RNA-seq results showing an increased expression of Chrng, Col2a1, Coll11a1, Coll11a2, L1cam, Adam12, Fas, Hspg2, Lox, Itgb4, Klf5, Cldn1 and Nr-cam. First, several genes upregulated in HM palates have been confirmed as known CP genes including: Chrng, Col2a1, Coll11a1, Coll11a2, and L1cam (Vogt et al., 2012, Meng et al., 2009, Schutte & Murray, 1999, Okamoto et al., 2004, Dixon et al., 2011). Second, in the Tgf-β3 knockout mouse model, we expect other isoforms, Tgf-β1 and Tgf-β2 to maintain activity and continue control of Adam12, Fas, Hspg2, Lox, Itgb4, Klf5, and Cldn1 as previously discussed (Ramdas et al., 2013, Matsushima & Ishiyama, 2016, Ichimaru et al., 2012, Araz et al., 2014, Scardigli et al., 1996, Li et al., 2015, Medici et al., 2006). Finally, independent of Tgf-β control, Nr-cam uses AKT signaling pathway to protect cells from apoptosis—an essential component of
normal palate fusion (Nawshad, 2008, Conacci-Sorrell et al., 2005). We postulate this decreased apoptosis lends to cleft formation.

Genes downregulated at 16.5dpc in HM palates—Wnt9b, Alx4, Pax1, Kcp, Msx1, Ppp1r17, Pdgfc, Twist1, and Wnt5a—are also in agreement with RNA-seq results. Downregulation of genes essential for embryogenesis, craniofacial development, and orofacial patterning (Wnt9b, Alx4, Pax1, Kcp, Msx1, Ppp1r17, and Pdgfc) likely disrupts fundamental processes required for normal palate fusion as previously discussed (Cadigan & Nusse, 1997, Beverdam et al., 2001, Sonnesen et al., 2008, Soofi et al., 2017, Zhang et al., 2002, Weston et al., 2002, Ding et al., 2004). Additionally, we confirm downregulation of known CP genes, Twist1 and Wnt5a, in HM palates (Dixon et al., 2011, Meng et al., 2009).
CHAPTER 6: CONCLUSION

6.1 Conclusion

Our study is the first global gene analysis of Tgf-β knockout mice alleles using deep sequencing methods. While we know Tgf-β3 is essential for proper palate fusion, we do not know what downstream molecular mechanisms are altered in Tgf-β3 knockouts. In this study, we identified a multitude of significantly differentially expressed genes both temporally and between phenotypes that may play key regulatory roles during critical stages of palate development. The genes implicated in CP may lead to a better understanding of the underlying genetic mechanisms of palatogenesis and provide novel potential targets for gene therapy approaches in treating cleft palate.

6.2 Future Research and Study Limitations

Global transcriptome profiling provides a multitude of genetic information. We have provided various genes that warrant further investigation on their involvement in palatogenesis. Continued exploration of genes dysregulated in Tgf-β alleles will further contribute to our knowledge of downstream molecular activities and pathways controlled by Tgf-β, ultimately enhancing our understanding of CP deformity and providing new genetic targets for prevention and treatment.

First, limitations of this study include several difficulties with a murine model. Research animals are expensive and do not predictively reproduce. This specific strain also experienced a virus during the time of the study in which all breeding was halted. Further, the tissues from mouse embryo palates are very small, difficult to extract, and provide very few cells. Second, this study evaluates an animal model; therefore, we cannot assume the effect of TGF-β is identical in humans.


Appendix A: Calculation Fold Change (Ratio) Using $C_q$ Differences.

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