Role of Transforming Growth Factor (TGF) – β Signaling in Craniofacial Development, Malignancies and Regeneration

Jingpeng Liu
University of Nebraska Medical Center

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ROLE OF TRANSFORMING GROWTH FACTOR (TGF) – β SIGNALING IN CRANIOFACIAL DEVELOPMENT, MALIGNANCIES AND REGENERATION

By

Jingpeng Liu

A DISSERTATION

Presented to the Faculty of

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(Oral Biology)

Under the Supervision of Professor Ali Nawshad

University of Nebraska Medical Center
Omaha, Nebraska

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ABSTRACT

Jingpeng Liu, Ph.D.
University of Nebraska, 2017

Supervisor: Ali Nawshad, Ph.D.

The transforming growth factor (TGF) – β signaling pathway regulates a diversity of fundamental cellular processes such as cell growth and proliferation, cell differentiation, migration, apoptosis and other biological functions, both during embryogenesis and in adult tissue homeostasis. TGF-β is known to play a critical role in palatal development as in TGF-β knockout murine models, TGF-β deficiency causes cleft palate, a common craniofacial deformity in human due to abnormality of growth, elevation or fusion of the two palatal shelves. In order to investigate the mechanisms of how TGF-β regulates palatogenesis, we generated TGF-β3 knockout (−/−) murine models, performed systematic analysis of the transcriptomes of palatal tissue and identified dysregulated genes which are potentially responsible for occurrence of cleft palate. Moreover, we demonstrated that recombinant EphB2/Fc treatment-induced activation of ephrin reverse signaling was sufficient to rescue palatal fusion when TGF-β3 signaling was blocked. In addition, in oral squamous cell carcinoma, we revealed that TGF-β1 increased OSCC cell proliferation by upregulating the expression of ΔNp63 and c-Myc oncogenes and therefore promoted cancer progression. Furthermore, we established standard experimental periodontitis models in rats and by taking advantage of RNA-Seq technology, we successfully revealed that mechanisms of Simvastatin-induced periodontal bone regeneration. These data highlight the pivotal role of TGF-β signaling in craniofacial development, malignancies and tissue regeneration.
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<tbody>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>CLP</td>
<td>cleft lip/palate</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>CP</td>
<td>cleft palate</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine Triphosphate</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and Differentiation Factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head-and-neck squamous cell carcinoma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin growth factor 1</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IRF6</td>
<td>interferon regulatory factor 6</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MEE</td>
<td>medial edge epithelial</td>
</tr>
<tr>
<td>MES</td>
<td>midline epithelial seam</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>Nr-Cam</td>
<td>Neuronal cell adhesion molecule</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>PFKFB3</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>qPRC</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SDEG</td>
<td>significantly differentially expressed genes</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor- associated macrophages</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TPM</td>
<td>Transcripts Per Million</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factors</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XLH</td>
<td>X-linked hydrocephalus</td>
</tr>
</tbody>
</table>
INTRODUCTION

TGF-β superfamily of secreted factors contains over 40 members, including Activins, Inhibins, Nodals, Bone Morphogenetic Proteins (BMPs) and Growth and Differentiation Factors (GDFs) in mammals (Weiss and Attisano 2013). TGF-β signals are transmitted through heteromeric complexes, assembled by type I and II transmembrane serine/threonine kinase receptors, and transduced directly from cell-surface receptors to the nucleus through intracellular mediators, known as Smads (Wrana, Attisano et al. 1994, Feng and Derynck 2005, Massagué 2008, Moustakas and Heldin 2009). While Smad pathway controls TGF-β superfamily signaling transduction in a ubiquitous manner and functions universally in almost all cell types, TGF-β signaling can also be operated independently of Smad by a number of cascades, such as MAP kinase pathways and the Phosphatidylinositol 3-kinases (PI3K) pathway (Zhang 2009, Lamouille and Derynck 2011, Mu, Gudey et al. 2012). TGF-β superfamily members are involved in a wide range of cellular processes, including proliferation, differentiation, apoptosis and cellular homeostasis (Morikawa, Derynck et al. 2016). For an instance, in vertebrates, TGF-βs participate in the morphogenesis of most organs, and defects in signaling in this context result in severe human diseases (Wu and Hill 2009). In addition, TGF-β1 can also promote glioblastoma by inducing PFKFB3 gene expression (Rodríguez-García, Samsó et al. 2017), mediate malignant behavior of normal fibroblasts (Roberts, Anzano et al. 1981) and induce skeletal muscle regeneration (Delaney, Kasprzycka et al. 2016). Similarly, TGF-β2 is shown to behave as tumorigenic promoter in multiple kinds of cancer (Friess, Yamanaka et al. 1993, Sun, Chua et al. 2011) and be capable of inducing chondrocyte regeneration (Wang, Lou et al. 2003). TGF-β3 was also found to be closely associated with metastasis and prognosis of human breast carcinoma and prostate cancers (Auvinen, Lipponen et al. 1995, Djonov, Ball et al. 1997, Jin, Li et al. 2001, Parada, Arciniega et al. 2004). This part of introduction reviews mechanisms of how TGF-β regulates palatal development and oral squamous cell carcinoma progression as well as regeneration and inflammatory responses in
experimental periodontitis, followed by identification of gap in knowledge and outlining of thesis project.

**Palate development**

*Development of the mammalian secondary palate*

In humans and mammals, the palate forms the roof of the mouth, separating the oral cavity from the nasal cavity (Wingerd 2013). The palate can be divided into two sections: the primary palate which consists of the philtrum and the upper incisor region of the maxilla anterior to the incisive foramen and the secondary palate which contains the rest of the hard and soft palate (Bush and Jiang 2012). The presumptive primary and secondary palates arise from the oral side of the medial nasal and maxillary processes, separately, which are derived from the frontonasal prominence during the early stage of embryogenesis. Particularly, the secondary palate morphologically forms as paired structures, called palatal shelves. The palatal shelves are mainly composed of mesenchymal cells which are derived from the neural crest and covered by an epithelial layer, originated from ectoderm (Matsuyama, Iwadate et al. 2003). As the palatal shelves continuously grow from the maxillary processes, they initially project vertically flanking the developing tongue into the oral cavity and subsequently becomes horizontal above the dorsum of the tongue through a quick process known as elevation. The exact mechanism to explain the rapid reorientation of the palatal shelves is yet to be explored. Nevertheless, several hypotheses have been suggested and under consideration, including rapid rotation of the shelves and growth-based regression of the distal portion and outgrowth in the horizontal direction (Lazzaro 1940). Following elevation, the palatal shelves continue to expand toward the midline where they adhere and form a midline epithelial seam, whose disintegration is a prerequisite for complete fusion and the formation of the confluent roof of the oral cavity (Bush and Jiang 2012). Palatogenesis begins in the sixth week and palatal fusion is completed by 12 weeks of gestation in humans while in mice palatal shelves become morphologically detectable by 11.5dpc and completely fused by 17dpc.
Knockout mouse models for study of role of TGF-β in palatogenesis

Knockout mice have been serving ideal models to investigate the roles of genes whose sequences are explicit while functions are yet to be explored. By deactivating a particular gene in mice and analyzing differences from normal behavior or physiology, researchers are able to indicate the gene’s probable functions. In studies of palatal development, knockout mice have been a critical tool that is widely used.

TGF-β variants conserve high similarities in terms of both structure and functions. Nevertheless, knockout of these genes in mouse models lead to distinctive phenotypes. Previous studies reported that in mice, approximately 50% of the homozygous TGF-β1 (−/−) and 25% of heterozygous TGF-β1 (+/−) embryonic development is ceased by E10.5 due to defective hematopoiesis and endothelial differentiation of extraembryonic tissue (Dickson, Martin et al. 1995). Controversially, other studies suggested that TGF-β1 (−/−) mice encounter a severe preimplantation defect which occurs prior to the morula stage (Kallapur, Ormsby et al. 1999). TGF-β2 depleted mice exhibit a wide array of developmental defects, and malformations including lung, limb, cardiac, craniofacial and urogenital deformities, and therefore, prenatal lethal (Sanford, Ormsby et al. 1997). TGF-β2 seems to have a strain-dependent effect in palatogenesis. TGF-β2 mutant mice do not regularly display significant palate defects, except that small percentage of TGF-β2 knockout mice revealed cleft palate on a mixed 129/Black Swiss background (Shull, Ormsby et al. 1992, Jin and Ding 2014). Additionally, accumulating evidence indirectly implied that TGF-β1 and TGF-β2 may participate in regulating mesenchymal cells in conjunction with Wnt signaling (Sharpe, Foreman et al. 1992, Matsuyama, Iwadate et al. 2003). In contrast to TGF-β1 and TGF-β2, the function of TGF-β3 during palate development has been remarkably better explored. TGF-β3 signaling is known to regulate various cellular biofunctions through the orchestration of its downstream molecules (Ahmed, Liu et al. 2007). Researchers demonstrated that TGF-β3 knockout (−/−) mice exhibit cleft palate without any other deformities (Proetzel, Pawlowski
et al. 1995). Recent studies have shown that p63 and interferon regulatory factor 6 (IRF-6) regulate palatal adhesion (Gritli-Linde 2010). The major isoform, truncated p63 (ΔNp63), is expressed in basal epithelial cells and is essential for epidermal and palatal development (Koster, Kim et al. 2004, Thomason, Dixon et al. 2008). In addition, the spatial and temporal regulation of IRF-6/p63 are suggested to be key factors in the formation and maintenance of MEE differentiation and periderm desquamation (Richardson, Dixon et al. 2009). Ingraham et al. showed (Ingraham, Kinoshita et al. 2006) that IRF6-null mice lack periderm within the oral mucosa, resulting in premature fusion of the palate with the tongue epithelia, and formation of a cleft palate.

The expression pattern of TGF-β during palatogenesis

In mammals, palate, the roof the oral cavity, consists of the primary palate and secondary palate. Compared to the secondary palate which accounts for the majority of adult hard palate, the primary palate serves a much smaller part. The secondary palate is the origin of the hard and soft parts of the palate. The development of the secondary palate is orchestrated by multiple sequential events that involve palatal shelf growth, reorientation, adhesion and fusion of the palatal shelves in the midline and the disintegration of the midline epithelial seam (MES) (Chai and Maxson 2006). Mouse models are an ideal system to study and explore human palate development, thanks to their similarities (Figure 1) (Bush and Jiang 2012). Early researches have demonstrated all three TGF-β (TGF-β1, 2 and 3) isoforms to be expressed during palatogenesis. At embryonic day 10.5 (E10.5) in mouse embryos, which corresponds to early sixth week of human gestation, the paired medial nasal processes merge with each other, as well as with the bilateral maxillary processes to form the upper lip and the primary palate, respectively. Around E11.0 in mice, corresponding to human gestation week six, the secondary palate initially becomes morphologically detectable as outgrowths of the palatal shelves from the inner side of the maxillary processes. Next, from E11.5–E14.0, the palatal shelves continue to grow vertically flanking the developing tongue in the oral cavity. Expression of TGF-β1 and TGF-β2 are found in the both palatal epithelial and
mesenchymal cells between E12.0~E12.5, whereas TGF-β3 has restricted expression only in epithelial cells (Gehris, D'Angelo et al. 1991). When it comes to 14.5 DPC~E15.0 in mice, corresponding to 7th ~ 8th week in human gestation, the palatal shelves undergo a rapid change through which they are reoriented into horizontal position above the dorsum of the tongue. Expression of TGF-β3 at this phase is significantly increased in epithelial cells. Meanwhile, in mesenchymal cells, expression of TGF-β1 declines while TGF-β2 remains robust activity (Fitzpatrick, Denhez et al. 1990). Constant growth of mesenchymal cells elongates the palatal shelves and drives the shelves into approximation in the midline where transient structure, termed midline epithelial seam (MES) is formed by medial edge epithelial (MEE) cells from opposing palatal shelves. The subsequent degeneration of MES takes place in order to allow mesenchymal confluence of palatal shelves. Over the span of fusion, interestingly, MEE cells express both TGF-β1 and TGF-β3. TGF-β2, however, is limited to express only in the mesenchymal cells with very high intensity (Fitzpatrick, Denhez et al. 1990, Pelton, Hogan et al. 1990). In addition, palatal shelves also fuse with the primary palate anteriorly and the nasal septum posteriorly. Progressively, at E15.5 in mouse embryos, which corresponds to weeks 8~9 in human gestation, fusion of the paired palatal shelves is completed, during which, all three TGF-β isoform have distinct expression patterns. TGF-β1 is mainly expressed in the palatal and nasal spectrum ossification area; TGF-β2 is expressed in the mesenchymal cells in vicinity to the midline of the palate, whereas TGF-β3 expression is observed thought he mesenchyme post to fusion (Fitzpatrick, Denhez et al. 1990, Proetzel, Pawlowski et al. 1995). Completion of the palatogenesis separates the early oro-nasal cavity into an oral and a nasal cavity.
Figure 1. Palatogenesis in the mouse. (A) Timecourse of palate development in mice. (B-F) Scanning electron micrographs showing oral views of the secondary palate at representative developmental stages [reprinted from Kaufman (Kaufman, 1992) with permission]. Orange lines mark sites of fusion between the medial nasal processes and maxillary processes, white arrowheads point to initial outgrowths of the primary palate, white arrows point to the initial outgrowth of the secondary palatal shelves, red arrowheads mark the initial site of palatal adhesion and fusion, and the yellow arrowhead points to the gap between the primary and secondary palates that will disappear following fusion between these tissues. (G-U) Representative histological frontal sections from anterior (G-K), middle (L-P), and posterior (Q-U) regions of the developing palate at each indicated stage. The middle palate region is flanked by the developing upper molar tooth germs (black arrows in M-P) and corresponds to the palatine region of the future hard palate. The posterior palate region corresponds to the future soft palate. At E11.5 (G,L,Q), the palatal shelf outgrowths arise from the oral surface of the maxillary processes. At E13.5 (H,M,R), the palatal shelves exhibit distinct shapes along the AP axis. By 14.5 DPC (I,N,S), the palatal shelves have elevated to the horizontal position. At ~E15.0 (J,O,T), the palatal shelves make contact at the midline and initiate fusion by formation of the midline epithelial seam (MES) in the mid-anterior region (arrowhead in O). By E15.5 (K,P,U), palatal shelf fusion is evident in the middle and posterior regions, with complete removal of the MES (black arrowheads in P,U). Remnants of the MES can still be seen in the anterior region (K) at this stage and the palatal shelves also fuse superiorly with the nasal septum. Magnification is not equivalent between stages. MdbP, mandibular process; MNP, medial nasal process; MxP, maxillary process; NS, nasal septum; PP, primary palate; PS, palatal shelf; SP, secondary palate; T, tongue. Obtained from (Bush, Lan et al. 2002).
TGF-β signaling

*TGF-β superfamily of proteins*

TGF-β refers to the homodimeric products of a diversity of genes. The TGF-β superfamily contains more than 30 members, including TGF-βs, bone morphogenetic proteins (BMPs) activins, inhibins and other related proteins. Here we limit our study in TGF-βs. To date, among the TGF-βs, six distinct isoforms have been discovered with a variable degree of homology (Santibañez, Quintanilla et al. 2011). Most of the members in this family have been purified as dimers from natural or recombinant sources. TGF-β was initially revealed as activities mediated by retro-virally-transformed cells (Roberts, Anzano et al. 1981). TGF-β proteins are expressed in numerous normal cells and tissues and regulate a variety of key cell function, and have critical roles in both normal growth and development and carcinogenesis. The name of TGF-β was taken from the first member of this family to be identified from human platelets, which was TGF-β1 (Cheifetz, Weatherbee et al. 1987, Massague 1990). All various members of the TGF-β family are initially produced as larger precursor molecules which are subsequently cleaved to release mature carboxy-terminal fragment of around 110-140 amino acids. In contrast to poor conservation of the pro-domain of the precursors among different members, the mature region in much more highly conserved. In particular, at least seven cysteine residues within the mature region show little invariance across family members (Kingsley 1994). As for TGF-βs, the extend of identity between this five mature TGF-β sequences can be up to 82%, but individually, all five TGF-βs are extremely conserved. For an instance, the degree of identity between the mature TGF-β1, - β 2, - β 3 from avian and mammalian species is high than 97% (Derynck, Rhee et al. 1987, Jakowlew, Dillard et al. 1988, MADISEN, WEBB et al. 1988, Kondaiah, Sands et al. 1990). The high degree of conservation suggests common ancestor of TGF-β members while different TGF-β genes are mapped to separate chromosomes in both humans and mice (Massague 1990).
Various cell types have been discovered to express one or multiple forms of TGF-β, at least at mRNA level, in vitro (Derynck, Lindquist et al. 1988). Overall, expression patterns of different TGF-β proteins differ between cell types. In addition, descendent cells from same lineage don’t necessarily show uniformity in TGF-β expression (Massague 1990). All TGF-β proteins are actively expressed throughout embryonic development and into adulthood (Heine, Munoz et al. 1987, Rappolee, Brenner et al. 1988, Miller, Lee et al. 1989, Thompson, Flanders et al. 1989). In the mouse embryos, there is a discrepancy found between the immuno-histological localization and corresponding mRNA of a certain TGF-β form, which is proposed to result from diffusion and accumulation of the protein away from the synthesis sites (Assoian, Fleurdellys et al. 1987). Multiple known mechanisms control the expression and activity of TGF-β proteins, such as modification of TGF-β gene translation, production of TGF-β proteins in latent status and degradation of activated TGF-β proteins by ECM or circulation proteins. Phorbol esters, and TGF-β1 itself, are reported to be capable of triggering the translation of TGF-β genes through PKC pathway (Lawrence, Pircher et al. 1985, Akhurst, Fee et al. 1988, Van Obberghen-Schilling, Roche et al. 1988, Kontny, ZiÓLKowska et al. 1999).

*TGF-β superfamily receptors*

TGF-β superfamily ligands, including TGF-β, BMPs and activins, like many other hormonally active polypeptides, act on target cells by directly binding to membrane-bound proteins which are subsequently coupled to cytoplasmatic signal transducing segment. Indeed, TGF-β ligands mediate their effects by signaling though transmembrane serine/threonine kinase receptors, type I and type II receptors, (TβRI and TβRII, respectively) at the surface of target cells. TβRI and TβRII, two glycoproteins of 53-100 KD, are ubiquitously present at low levels in both avian and mammal cells and have very high affinity binding TGF-β ligands (Massague, Cheifetz et al. 1990). The role of TβRI and TβRII as TGF-β receptors was first discovered by the observation that TβRI and TβRII were the only TGF-β binding components in skeletal muscle myoblasts and murine
hematopoietic progenitor cells where TGF-β1 regulates cell differentiation and fibronectin expression, and suppresses cell proliferation, respectively (Massague, Cheifetz et al. 1986, Ohta, Greenberger et al. 1987). In humans, seven TβRI and five TβRII have been identified and individual ligands which preferably bind to TβRI and/or TβRII are listed in Figure 2. Besides, TGF-β may also interact with a third component of betaglycan, previously known as TβRIII (Shi and Massagué 2003, Bernabeu, Lopez-Novoa et al. 2009). Before bound to ligand, TβRI and TβRII exist in

![Schematic illustration of the selective binding of members of the transforming growth factor β (TGF-β) family to type I and type II serine/threonine kinase receptors (Carl-Henrik Heldin, and Aristidis Moustakas Cold Spring Harb Perspect Biol 2016;8:a022053).]
different forms, including monomers, homodimers and heterodimers. But ligand binding will stabilize a heterotetrameric structure (Chen and Derynck 1994, Henis, Moustakas et al. 1994, Ehrlich, Gutman et al. 2012). Upon binding of a TGF-β ligand, a heterotetrameric complex of two TβRI and TβRII is assembled. Characteristically, TGF-β1 and TGF-β3 possess higher affinity with TβRI than TβRII, therefore first binding to TβRII. Thereafter, TβRI is recruited to join the complex by recognizing a signature interface created by the TGF-β ligand-TβRII complex (Groppe, Hinck et al. 2008). Oligomerization of TβRI and TβRII leads to TβRII phosphorylation of the TβRI in a glycine and serine residues-rich region of the juxtamembrane domain, producing an active ligand-receptor complex. The activation of type I serine/threonine kinase then phosphorylates downstream effector, receptor-regulated Smads family members (therefore, named R-Smads) (Feng and Derynck 2005, Kang, Liu et al. 2009). Activated R-Smads in turn forms trimeric complexes with the common transducer, Smad4, which are then translocated to the nucleus where they bind DNA molecules, interact with transcriptional factors, coactivators and corepressors and regulate gene expression response. The activities of TGF-β receptors are regulated by several phosphorylation events. Activation of TβRII is accomplished by auto-phosphorylation at two residues, Ser213 and Ser409 while auto-phosphorylation leads to deactivation of TβRII (Luo and Lodish 1997). Similarly, TβRI can also undergo auto-phosphorylation on both serine and threonine residues, as well as tyrosine residues. The phosphorylation of TGF-β receptors, however, has been found to be counteracted by several phosphatases. For an example, protein phosphatase 1 was shown to bind Smad7, one of the inhibitory Smads (I-Smads), which blocks TGF-β signal transduction by competitively binding to TβRI and induce dephosphorylation of the receptor (Shi, Sun et al. 2004).
Figure 3. General mechanism of TGF-β receptor and Smad activation. At the cell surface, the ligand binds a complex of transmembrane receptor serine/threonine kinases (types I and II) and induces transphosphorylation of the GS segments (red) in the type I receptor by the type II receptor kinases. The consequently activated type I receptors phosphorylate selected Smads at C-terminal serines, and these receptor-activated Smads (R-Smads) then form a complex with a common Smad4. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes, through physical interaction and functional cooperation with DNA-binding transcription factors (X) and CBP or p300 coactivators. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7. R-Smads and Smad4 shuttle between nucleus and cytoplasm. The E3 ubiquitin ligases Smurf1 and Smurf2 mediate ubiquitination and consequent degradation of R-Smads, yet can also interact with Smad6/7 and thereby ubiquitinate the type I receptors. Obtained from (Derynck and Zhang 2003, Ahmed, Liu et al. 2007)
Mechanisms of Smad-dependent and Smad-independent signaling pathways

In canonical TGF-β signaling pathway, TGF-β utilizes Smads to regulate a wide array of cellular function. Smads are structurally related signaling effectors, the critical mediators of TGF-β signaling. By far, there are eight isoforms of Smad identified in vertebrates, Smad1 to Smad8. As illustrated in Figure 3, among the Smad proteins, Smad1 to Smad3, Smad5 and Smad8 are classified as receptor-activated Smads (R-Smads) as they are activated via phosphorylation by TGF-β receptors, such as TβRI, ALKs in response to TGF-β ligands (Itoh, Itoh et al. 2000, Massague 2000, Moustakas, Souchelnytskyi et al. 2001). Smad4 serves as the common mediator (co-Smad) in transducing TGF-β signals. Both R-Smads and co-Smads possess two highly conserved domains, N-terminal MH1 and C-terminal MH2. MH1 domain contains nuclear localization signals and DNA binding structure while MH2 domain comprise the L3 loop structure which characterizes the interaction between the R-Smads and type I receptors, which induce type I receptor to phosphorylate the C-terminal SSXS motif of R-Smads (Lo, Chen et al. 1998). Phosphorylation of the R-Smads triggers its combination with the MH2 domain of Smad4 and the formation of a heterotrimeric complex of two R-Smads and one Smad4. This complex then translocate into the nucleus and bind to DNA through MH2 domain (Massague and Xi 2012). The third class of Smad proteins are called inhibitory Smads, (I-Smads) which repress TGF-β signaling transduction by R-Smad and co-Smad.

I-Smads include Smad6 and Smad7. I-Smads antagonize the Smad signaling pathway in multiple manners. They can associate with type I receptor to prevent receptor-induced activation of R-Smads, competitively bind with phosphorylated R-Smads and interfere with their association with co-Smad, and even associate with DNA and the Smads complex in nucleus. Smad7 generally blocks signals from all TGF-β family proteins, whereas Smad6 mainly represses BMP signaling (Moustakas and Heldin 2009, Massague and Xi 2012, Weiss and Attisano 2013).
Upon ligand binding, TβRII phosphorylates the GS domain of the TβRI, which in turn, undergoes conformational changes that results in activation of the TβRI kinase and increase the binding affinity of the receptors for R-Smad (Huse, Chen et al. 1999, Huse, Muir et al. 2001), by forming a docking site in the MH2 domain of the R-Smads (Wu, Chen et al. 2000). TβRI then recruits and phosphorylates R-Smads at the last two serine residues at the C-terminal and by doing so, enables the association of R-Smads with co-Smad (Abdollah, Macias-Silva et al. 1997, Souchelnytskyi, Tamaki et al. 1997, Shi and Massague 2003). Post to C-terminal phosphorylation, the R-Smads also undergo conformational changed and are released from the TβRI. Free activated R-Smads then form oligomers with Smad4 by associating the C-terminal with the phosphoserine-binding pockets in the L3 loop structure of a neighboring Smad4 or R-Smads. Smad complexes may exist in multiple forms, heterodimers or heterotrimers, depending on transcriptional factors to interact with (Chacko, Qin et al. 2001, Wu, Hu et al. 2001).

The transportation of Smad proteins into the nucleus is fulfilled by importin and nuclear pore proteins. All Smads contain Lys-rich nuclear localization signal (NLS) motifs in the MH1 domain. They are, however, transported in different modes. R-Smad, in fact, doesn’t necessarily need to cooperate with co-Smad to enter nucleus, although co-Smad cotranslocates with R-Smads. Nuclear import of Smad1 and Smad3 is conducted by its NLS-motifs. Abovementioned C-terminal phosphorylation of the MH2 domain incites conformational changes which expose the NLS-motifs for importin-β to bind (Xu, Chen et al. 2000, Kurisaki, Kose et al. 2001). In contrast, Smad2 is imported into nucleus independent of importin-β. Instead, Smad2 interacts with nucleoporins CAN/Nup214 and Nup153 with its MH2 domain in order to shuttle between cytoplasm and nucleus (Inman, Nicolas et al. 2002, Xu, Kang et al. 2002). Smad4, unlike R-Smads, regardless of the presence or absence of ligands, constantly shuttle between the cytoplasm and the nucleus via activities of a constitutively active NLS motif in the MH1 domain and a nuclear export signal (NES) fragment in the linker region (Watanabe, Masuyama et al. 2000, Inman, Nicolas et al. 2002, Hata
and Chen 2016). In addition, Smad6 and Smad7 reside in the nucleus in the absence of TGF-β and are recently suggested as transcriptional factors that cooperate with TGF-β signaling in regulating expression of certain genes (Choy, Skillington et al. 2000, Hanyu, Ishidou et al. 2001, Itoh, Asao et al. 2001).

In addition to Smad-mediated signal transduction, TGF-β also exploits other signaling cascades, such as Mitogen-activated protein kinases (MAPK) pathways and Phosphoinositide 3-kinase (PI3K) pathway to reinforce, attenuate or modulate downstream cellular responses (Engel, McDonnell et al. 1999, Yu, Hebert et al. 2002). These non-canonical or Smad-independent pathways are triggered directly by ligand-receptor association (Zhang 2009). To begin with, TGF-β-induced activation of Erk/MAPK pathway was observed in epithelial cells, fibroblasts as well as breast cancer cells (Hartsough and Mulder 1995, Mucsi, Skorecki et al. 1996, Frey and Mulder 1997). Earlier findings reported a rapid activation of Ras by TGF-β, in which, TGF-β imposes rapid GTP loading on Ras, resulting in recruitment of Raf to the plasma membrane and activation of Erk through MEK1 (Mulder and Morris 1992, Yan, Winawer et al. 1994). Additionally, in the RTK/Ras/Erk signaling pathway, direct binding of growth factor to RTKs leads to activation of RTK via phosphorylation of multiple tyrosine residues in the cytoplasmic domain of RTK. Upon phosphorylation, signaling molecules that have Src homology 2 (SH2) or phosphor-tyrosine binding (PTB) domains, such as Src and Grb2, will be recruited to the RTK to activate Ras. Ras then binds Raf and activates MAPK cascade, including MEK and Erk (Yan, Winawer et al. 1994, Schlessinger 2000). Activation of Erk is critical for epithelial to mesenchymal transition (EMT), which is one of the major biological functions that regulated by TGF-β. EMT plays important roles in both physiological and pathological events, such as embryonic development and tumor metastasis, respectively (Thiery 2003, Lee, Dedhar et al. 2006).

Another well characterized non-Smad pathway is the JNK and P38 MAPK signaling pathway. TGF-β can rapidly activate JNK and p38 though MAP kinase kinases (Weston and Davis
Ligand-receptor association leads to TRAF proteins to interact with members of interleukin-1β receptor (IL-1R) or Toll-like receptors (TLRs) through its C-terminal TRAF domain, which causes lysine-63 (K63)-linked polyubiquitination of TRAF6 itself. Polyubiquitinated TRAF6 then recruits and activates TAK1 and triggers subsequent take-off of downstream JNK/p38 pathways (Wang, Deng et al. 2001, Lee, Chang et al. 2003). Similar to Erk cascade, JNK/p38 pathway also plays a very important role in TGF-β-induced EMT.

Indeed, Smad-mediated pathways and non-Samd pathways are not functioning independently. JNK/p38 cascade cooperates with Smad-dependent pathways in regulating certain cellular functions, such like TGF-β/BMP-induced. Similarly, Erk also phosphorylates R-Smads to regulate their activities. Besides, Erk substrates can also interact and function in cooperation with Smads to regulate gene expression (Kretzschmar, Doody et al. 1997, Hall, Young et al. 2003, Davies, Robinson et al. 2005, Matsuura, Wang et al. 2005). Mounting evidence has elaborated the conjunction between Smad-dependent and Smad-independent pathways in determining the outcomes of TGF-β signaling. Further characterization of both Smad-dependent and -independent TGF-β signaling and novel discoveries of molecular mechanisms will strengthen our understandings of how TGF-β regulates such wide array of biological processes.

Role of TGF-β in different stages of palatogenesis

TGF-βs play critical roles in coordinating palatal development by regulating cell proliferation, growth, differentiation and epithelia-mesenchymal transition (EMT) in mammals (Nawshad, LaGamba et al. 2004, Bush and Jiang 2012). Over the past decades, mounting research has significantly improved our understandings that TGF-β1 and TGF-β2 impel palatal growth by promoting DNA synthesis and cell proliferation during early phases, while TGF-β3 is dispensable for the dislodgement of MES by stimulating EMT and/or inducing apoptosis during the fusion process (Proetzel, Pawlowski et al. 1995, Taya, O’Kane et al. 1999, Nawshad, LaGamba et al. 2004). Furthermore, TGF-βs also contributes to guiding normal palate development by regulating
metabolism of the ECM, glycosaminoglycans and collagen during embryogenesis in mammal palates (Gehris, D'Angelo et al. 1991, D'Angelo, Chen et al. 1994). In humans, association of TGF-β3 and no-syndromic cleft lip/palate has been established in multiple populations (Jugessur, Lie et al. 2003, Kim, Kim et al. 2003, Vieira, Orioli et al. 2003). TGF-β3 knockout murine models exhibiting cleft palate due to failure of palatal fusion solidifies its pivotal function in ensuring complete palatal merging (Proetzel, Pawlowski et al. 1995). Moreover, reports have suggested that recovery of TGF-β3 signaling was sufficient to rescue the clefting, to a certain extend (Sun, Vanderburg et al. 1998, Cui, Shiomi et al. 2005, Yang and Kaartinen 2007). It is also worth noting that all birds naturally develop cleft palate as avian palates are physiologically null for TGF-β3 but can be induced to fuse by exogenous TGF-β3 treatment (Shah and Crawford 1979, Sun, Vanderburg et al. 1998, Yang and Lee 2001). Interestingly, ephrin-B reverse signaling is also required for palatal fusion in mice and is sufficient to cause fusion in chicken palates without the addition of TGF-β3 (San Miguel, Serrano et al. 2011).

*Ephrin reverse signaling*

Ephrins, also known as ephrin ligands or Eph family receptor interacting proteins, represent the largest family of receptor tyrosine kinase (RTKs). Ephs are subclassified into A and B groups depending on their binding preference to the glycosylphosphatidyl inositol-linked A ephrin or the transmembrane B ephrin ligands (Orioli and Klein 1997). One unique property that distinguishes from other RTK ligands is that they can also function as receptors while Ephrin receptors act as ligands, the signaling transduction though which is called “reverse signaling” (Murai and Pasquale 2003). However, the mechanisms by which "reverse" signaling occurs are poorly understood.

Reports that ephrin-B1 mutations are associated with craniofacial deformities, including cleft palate highlight a putative role for ephrin signaling in craniofacial development (Twigg, Kan et al. 2004, Wieland, Reardon et al. 2005, Davy and Soriano 2007, Torii, Izumi et al. 2007). Recently, a role for ephrin-B/EphB forward signaling has been suggested to regulate the
proliferation of palatal mesenchymal cells in a later stage (Risley, Garrod et al. 2009). In consistence with Risley’s findings, further investigation revealed that ephrin-B1 forward signaling contributes to promoting NCC-derived mesenchyme proliferation through MAKP pathways (Bush and Soriano 2010). In addition to this findings, Benson and his team found that ephrin reverse signaling is necessary and sufficient to induce palate fusion (San Miguel, Serrano et al. 2011). At 14.5 DPC, a stage when palatal shelves comes to approximation, ephrin-B2 was expressed in the palatal epithelial cells and exhibited a migrating tendency toward mesenchymal cells in the. Interestingly, in chicken palatal culture models which naturally develop cleft palate, exogenous activation of ephrin reverse signaling facilitated palatal fusion by activating PI3K signaling pathway.

Role of TGF-β in regeneration and inflammation

TGF-β/BMP signaling have established roles in bone homeostasis (Tan, Weng et al. 2007, Kamiya, Ye et al. 2008, Chen, Deng et al. 2012, Salazar, Zarkadis et al. 2013). TGF-β signaling is involved in osteoprogenitor proliferation, osteoblast differentiation, and bone formation (Chen, Deng et al. 2012). TGF-β can function through both canonical and non-canonical pathways to promote osteogenesis and bone remodeling (Lai and Cheng 2002, Lee, Choi et al. 2006, Kim, Kwak et al. 2007, Tan, Weng et al. 2007, Yasui, Kadono et al. 2011, Ishijima, Suzuki et al. 2012). Besides, TGF-β can also enhance cell proliferation and collagen formation by fibroblasts (Roberts, Sporn et al. 1986, Fine and Goldstein 1987, Bettinger, Yager et al. 1996). In addition, TGF-βs are known as certain anti-inflammatory cytokines and orchestrate vital events during initiation, progression and resolution of inflammatory responses (Wahl 1992). It’s reported that TGF-β can impose suppressive effects on activated macrophage by its ability to regulate the profile of active cytokines, such as reducing the expression of IFNγ and elevating the production of IL-1 receptor antagonist (Turner, Chantry et al. 1991, DELESPESSE, FARGEAS et al. 1992). Surprisingly, in our experimental periodontitis models in which simvastatin induced alveolar bone regeneration,
none TGF-β isoforms were identified to be significantly differentially expressed between experimental and unmanipulated control groups. Instead, another growth factor, insulin growth factor 1, (IGF-1) was shown by our data to be potential in the induction of bone regeneration. Growth factors control multiple biological processes, including cell survival, proliferation, differentiation, and mediate restoration of biological and physiological functions of impaired tissues (Lieberman, Daluiski et al. 2002, Gothard, Smith et al. 2014). IGF-1 is known to regulate cell growth and cell proliferation in a diverse types of tissues and to modulate the growing skeleton (Efstratiadis 2004). Earlier studies have shown that block of IGF-1 in mutant animals caused retarded bone formation, while on the other hand, overexpression of IGF-1 led to increase in bone volumes (Jiang, Lichtler et al. 2006). In addition, IGF-1 signaling is also critical in maintaining the proliferation of osteoblasts and in inducing ossification (Guntur and Rosen 2013).

IGF-1 binds to its receptor, IGF1R, a type II tyrosine kinase. Ligand-receptor binding triggers auto-phosphorylation of kinase domains of the receptor, leading to activation of downstream substrates insulin receptor substrate (IRS) proteins and Shc by tyrosine phosphorylation (Hernández-Sánchez, Blakesley et al. 1995). The IRS protein family is comprised of four members, IRS1, 2, 3 and 4. Of these four isomers, IRS1 and IRS2 have been demonstrated to be participating in maintaining bone turnover (Ogata, Chikazu et al. 2000, Akune, Ogata et al. 2002). Roles of IRS3 and IRS4, however, are yet to be explored with respect of bone.

IGF-1 signaling, through IRS1, activates PI3K and ERK/MAPK network, which can also be activated by TGF-β signaling (Baker, Liu et al. 1993, Ornitz and Marie 2002, Ling, Maile et al. 2005). Activated PI3K can phosphorylate and partially activate AKT, which in turn regulates a number of cellular processes, including bone development, through its downstream effectors, including transcriptional factors FoxO 1, 3 and 4, and mTOR (Peng, Xu et al. 2003, Laplante and Sabatini 2009, Ambrogini, Almeida et al. 2010, Kousteni 2012, Laplante and Sabatini 2013). In vitro, cultured PDL cells were observed to gain a dramatically enhanced proliferation rate and
increased osteogenic differentiation in response to IGF-1 stimulation. Transcript levels of O-cadherin (CDH11) and Osteocalcin were moderately increased (Reckenbeil, Kraus et al. 2017). Other studies have suggested that IGF-1 is able to enhance the osteogenic potential of BMP-2 (Kim, Kang et al. 2012, Choi, Lee et al. 2014) or other BMPs such as −7 or −9 (Chen, Jiang et al. 2010, Yang, Zhang et al. 2010) in vitro. In bone, IGF-1 stimulates differentiation of osteoblast and bone regeneration through the activation of the mammalian target of rapamycin (mTOR) pathway (Xian, Wu et al. 2012). Indeed, IGF-1 stimulates RUNX2 downstream gene expression, a critical step in osteogenesis, by up-regulating the protein levels of PI3K subunits, Akt, and increasing p70S6 kinase (p70 S6K) thus stimulating osteoblast differentiation, protein synthesis and cell growth (Fujita, Azuma et al. 2004). Moreover, IGF-1 is known to activate ERK pathway, which also phosphorylates RUNX2, thus enhancing RUNX2 dependent gene expression (Franceschi, Ge et al. 2007, Choi, Cho et al. 2008).

IGF-1 was also described to play anti-inflammatory actions (Andreassen, Frystyk et al. 2012). In recent studies, IGF-1 activities were observed to be reduced by inflammation in HIV patients (Suh, Lo et al. 2015), while higher levels of serum IGF-1 can suppress proinflammatory pattern in colitis by inducing expression of IL-10 in monocytes (Ge, Mo et al. 2015) and is also associated with lower inflammation status in obese adults (Fornari, Marocco et al. 2017). These findings are indicating a novel role of IGF-1 as anti-inflammatory molecule.

**Dual functions of TGF-β signaling in malignancies**

TGF-β regulates a wide range of biological functions including embryonic development, organogenesis, immune modulation, as well as caner progression. In normal, healthy tissue, basal release of TGF-β by local sources is sustained to maintain homeostasis. Upon stress or injury, blood platelets and diverse stromal components abundantly produce TGF-β to repress inflammation and regulate regeneration. Similarly, TGF-β is also present in tumor microenvironment, playing
different roles as malignancies advance (Massagué 2008). TGF-β in tumor may come from various sources. Coincidence of secretion accumulation of TGF-β with presence of tumor-infiltrating cells, such as leukocytes, macrophages, and bone marrow-derived endothelial, mesenchymal, and myeloid precursor cells implied suspected sources (Yang, Huang et al. 2008). In addition, TGF-β stored in bone marrow and in latent form can be released and activated during tumor metastasis (Kingsley, Fournier et al. 2007). Intriguingly, TGF-β exhibits dual activities in malignant disease; that is on one hand, it functions as an anti-tumor mediator while on the other hand, it promotes cancer invasion, metastasis and angiogenesis (Inman 2011, Principe, Doll et al. 2014). In benign epithelia and many early-phase tumors, TGF-β induces growth arrest to prevent premalignant progression and at late stages, however, TGF-β is switched to promote tumor growth and progression (Akhurst and Derynck 2001, Pasche 2001, Padua and Massagué 2009, Inman 2011). This phenomenon is known as TGF-β paradox (Morrison, Parvani et al. 2013). To date, the mechanisms behind the paradox remains elusive.

Tumor Suppression by TGF-β

The tumor suppressive roles of TGF-β are highly contextual, relying on progressive communications of multiple pathways. TGF-βRII and Smad4 are key components in TGF-β signaling, who’s normal functioning is vital in suppressing premalignant progression. Loss of TGF-βRII is commonly observed in human head-and-neck squamous cell carcinoma (HNSCC) (Lu, Herrington et al. 2006) and Smad4 deletion in mouse mammary glands causes spontaneous squamous cell carcinomas (Li, Qiao et al. 2003). Additionally, TGF-βRII and Smad4 deficiency strongly promotes the malignant progression of neoplastic lesions (Biswas, Chytil et al. 2004, Muñoz, Upton et al. 2006). Similarly, somatic annihilation of Smad4 and TGF-βRII accelerates adenoma to carcinoma transition in pancreatic and colorectal cancer (Jaffee, Hruban et al. 2002, Jones, Chen et al. 2008). TGF-β can also inhibit progression of cell cycle phase G1 through a number of mechanisms. To begin with, in benign epithelial cells, TGF-β can induce expression of
multiple CDK inhibitors, such as p15Ink4b and p21Cip, which inhibit cyclin-CDK complexes, through R-Smads-induced transcriptional activation of promoters (Seoane, Le et al. 2004, Gomis, Alarcón et al. 2006). In addition, c-Myc is a well-established transcriptional inducer of cell growth and proliferation (Dang 1999). TGF-β can downregulate c-Myc through Smad3/4, p107 E2F4/5, and C/EBPβ (Chen, Kang et al. 2002, Gomis, Alarcón et al. 2006). Moreover, TGF-β can exert tumor suppressive effect by regulating cell differentiation and lineage determination, such as inducing precursor cells into less proliferative state (Derynck and Akhurst 2007) and by triggering apoptosis to retain cell proliferation under control (reviewed in (Pardali, Kurisaki et al. 2000)).

**Tumorigenic Effects of TGF-β: Tumor Growth, Invasion, and Immune Evasion**

However, loss of tumor-suppressive shield of TGF-β accrues tumorigenic effects which promotes tumor growth and invasion. TGF-β-induced EMT is suggested to contribute to tumor propagation (Shipitsin, Campbell et al. 2007). TGF-β integrates both Smad-dependent and Smad-independent pathways to enhance EMT. Expression of high-mobility group A2 mediated by Smads upon TGF-β stimuli leads to expression of Snail, Slug and Twist, which are known as EMT markers (Thuault, Valcourt et al. 2006). Independent of Smad involvement, TGF-βRII can induce breakdown of cell junction complexes through phosphorylation of Par6 and consequently increase cell motility (Ozdamar, Bose et al. 2005). Moreover, TGF-β profits tumor development by elevating secretion of autocrine mitogenic factors, thereby promoting tumor cell proliferation. In glioma, TGF-β/Smad pathway can promote proliferation by inducing PDGF-B with an unmethylated PDGF-B gene and high phosphorylated Smads level seems to be a poor prognostic marker, supporting TGF-β’s role as an oncogenic factor in glioma (Bruna, Darken et al. 2007). In addition, TGF-β is also known to enable the tumors to escape immune surveillance, hence favoring tumor progression through several mechanisms (Byrne, Knox et al. 2008, Flavell, Sanjabi et al. 2010). In skin cancer, TGF-β-induced recruitment and retention of macrophages into tumor mass can trigger the progression of tumor. Tumor-associated macrophages (TAMs) are so robustly
phagocytic that they compete with dendritic cells and block presentation of tumor antigens to adaptive immune system by dendritic cells (Byrne, Knox et al. 2008). TGF-β can also directly inhibit functions of tumor antigen-specific cytotoxic T lymphocytes by repressing expression of cytolytic genes, such as IFNγ and Fas ligand (Thomas and Massagué 2005), and attenuates functions of effector T cells by suppressing IFNγ production (Ahmadzadeh and Rosenberg 2005) and blocking T cell receptor signaling of tumour infiltrating lymphocytes (Di Bari, Lutsiak et al. 2009).

**Gap in knowledge**

Based on our literature review and existing knowledge, it is likely that TGF-β may play important roles in palatal development, OSCC progression, as well as regeneration of periodontal ligament and alveolar bone. However, current literatures do not explain the exact mechanisms of TGF-β family proteins and their specific signaling mechanisms and downstream pathways to introduce multiple yet unique cellular/pathological processes, such as development, tumorigenesis and regeneration.

**Dissertation project**

In this project, we aimed to investigate the mechanisms of TGF-β regulating palatal development, promoting cancer cell proliferation and inducing alveolar bone regeneration in experimental periodontitis. The central hypotheses of this project is that: (i) knockout of TGF-β will alter expression profile of known CP-related and novel genes at critical stages during palatal development and the differential expression is specific for causing cleft palate; (ii) Ephrin is playing a novel role in regulating craniofacial development and inducing palatal fusion; (iii) TGF-β in tandem with its downstream pathway components ΔNp63 and c-Myc oncogenes, induces UMSSC cell proliferation, therefore promoting growth and invasion of OSCC; (iv) simvastatin treatment-induced inhibition of inflammation and alveolar bone regeneration in experimental periodontitis is
a reflection of deferential expression of transcriptomes that causes changes in protein production of known anti/pro-inflammatory and bone metabolic mediators as well as in behaviors of relevant pathways associated with these activities. This thesis is focused mainly on identifying potential genes and revealing underlying cell signaling mechanisms to explain corresponding cellular processes and biological events. Results present herein provide a comprehensive understanding of TGF-β signaling in craniofacial development, malignancies and regeneration. Findings in this work contribute to existing knowledge of TGF-β signaling and hopefully lay the foundation for future studies and clinical applications.
CHAPTER 1

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

1.1 Introduction

Orofacial clefting is the most common craniofacial anomaly treated in the pediatric hospital setting and is the second most common birth defect with a prevalence ranging from 1/500 to 1/2500 in humans (Schutte and Murray 1999, Jugessur, Farlie et al. 2009). Formation of a confluent palate is a precise orchestration of many processes including cellular movement, cell death, cell cycle progression, development, and growth and proliferation (Zhu, Ozturk et al. 2012). Proper palate formation requires multiple steps including: growth, elevation, adherence and fusion; failure or disruption of any step in the process can result in cleft palate (CP) (Zhu, Ozturk et al. 2012).


Many genes have been implicated in the etiology of cleft palate—a number of studies suggest transforming growth factor-beta 3 (TGF-β3) as a candidate gene for causing cleft palate (Lidral, Romitti et al. 1998, Sun, Vanderburg et al. 1998, Schutte and Murray 1999). TGF-β isoforms are essential for proper development, including palate fusion (Nawshad, Lagamba et al. 2005, Meng, Bian et al. 2009). The TGF-β3 knockout mouse model results in the phenotype of CP but lack other major anomalies. In chickens where the palate is normally cleft, exogenous TGF-β3 has been shown to induce palate fusion (Schutte and Murray 1999). Further, it has been reported
that TGF-β is a candidate gene for non-syndromic CP in humans (Lidral, Romitti et al. 1998, Ichikawa, Watanabe et al. 2006).

In our previous study (Ozturk, Li et al. 2013), we examined gene expression patterns of CP genes identified in human (OMIM) and mouse (MGI) genome databases characterizing transcripts that may play key regulatory roles through crucial stages of palatogenesis in wild-type (WT) and TGF-β3 knockout (KO) mouse model, also stated as TGF-β3 (-/-) homozygous (HM) in this study. With the advent of new genome sequencing technologies, it is possible to globally analyze the transcriptome changes in order to identify key molecular components and underlying mechanisms of palate formation during development. Failure of any key component during the stages of palatogenesis may result in CP. Next-generation sequencing (NGS) technologies, also known as RNA-Sequencing, have revolutionized our ability to discern gene activity with high-throughput with high levels of sensitivity and accuracy (Wang, Gerstein et al. 2009, Marguerat and Bahler 2010). Recent advances in RNA-Sequencing analysis provide higher quality, quantity, and degree of data authenticity that are reproducible with both technical and biological replicates (Patro, Duggal et al. 2017). The comparative analysis of TGF-β3 WT and HM mice by RNA-Sequencing provides detailed molecular information to further our understanding of palatogenesis.

As mentioned, TGF-β3 is paramount to proper palate formation, specifically during embryonic days (E) 14.5 dpc to 16.5 dpc (Nawshad, LaGamba et al. 2004). However, downstream molecular mechanisms directly and indirectly controlled by TGF-β3 signaling remain largely unexplored; a comparison of gene profiles of wild-type and TGF-β3 knockout mice will provide further understanding of genes functionally regulated by TGF-β3 during palatogenesis. Our objective in this study was to analyze the global transcriptome changes and their contribution to the development of CP among different gestational ages and TGF-β3 HM alleles. In this study, we analyzed the complete transcriptome of TGF-β3 mice by RNA-Sequencing at crucial stages of palatogenesis—shelf adhesion and fusion. We used palatal shelves extracted from 14.5 dpc and
16.5 dpc allelic mice to analyze differential expression patterns between phenotypes directly as well as the differentially expressed genes changing temporally between phenotypes from 14.5 dpc to 16.5 dpc conducting a novel inter- and intra-phenotype analysis.

The overall transcriptome analysis of TGF-β3 WT and KO mice revealed over 6000 significantly differentially expressed genes (SDEG) between 14.5 dpc and 16.5 dpc. To further define significance, a fold change (FC) cut-off value of 2.0, in addition to the adjusted p<0.05 cut-off) was applied. The resulting SDEGs over time were great (3936 genes) while the SDEGs between phenotype were minimal (51 genes) demonstrating the effects of TGF-β3 knockout are most significant temporally. Further, among temporally SDEGs, a greater number were up-regulated than downregulated in both phenotypes (WT Up in 16.5 vs. 14.5 = 1675, WT Down in 16.5 vs. 14.5 = 134; HM Up in 16.5 vs. 14.5 = 1936, HM Down in 16.5 vs. 14.5 = 191) suggesting transcriptional induction overshadows transcriptional silencing during development between these two time points. Using this data, we identified a multitude of genes that may contribute to cleft palate formation in TGF-β3-/- mice (Twist1, Wnt5a, Chrng, Col2a1, Adam12, Clu, Paxl, Mxs1, Cdh1, Lyn, Cldn1).

We then analyzed biological functions of these dysregulated genes and defined their molecular networks, and regulatory pathways, especially in relation to TGF-β signaling using Ingenuity Pathway Analysis (IPA). This data will allow a comprehensive analysis of TGF-β signaling during palatogenesis and provide insight on the temporal regulation of downstream TGF-β-regulated transcription factors that function in proper palate fusion.

1.2 Materials & Methods

1.2.1 Animal Selection and Breeding

TGF-β3 heterozygous (+/-) C57BL/6J breeder mice were obtained from Tom Doetschman (BIO5 Institute, University of Arizona, AZ). The reproduction and genotyping of TGF-β3-/- mice
was conducted as previously depicted (Proetzel, Pawlowski et al. 1995). Mice were accommodated and subject to procedures in University of Nebraska Medical Center (UNMC) College of Dentistry Animal Facilities under the approval of UNMC Institution Animal Care and Use Committee. Null mutant embryos were generated by intercrossing TGF-β3 heterozygous male and female mice in a Mendelian-fashion.

1.2.2 Genomic DNA purification and genotyping

Palatal tissues were dissected under the Nikon SMZ1000 stereomicroscope system (Nikon, Tokyo, Japan) from embryos collected at embryonic day (E) 14.5 and 16.5 dpc following the identification of vaginal plug, which is considered to be E 0.5. Palatal samples were stored in RNAlater Stabilization Reagent (Qiagen, Hilgen, Germany) to preserve the gene expression profile and individually labeled and corresponded to tail tissue used for genotyping. Genomic DNA from embryonic tails was isolated using Gentra Puregene Tissue Kit (Qiagen, Hilgen, Germany) following the manufacturer’s instructions. Primers used for genotyping were shown below:

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 400 bp and 1300 bp fragments for WT and mutated alleles, respectively. PCR conditions were initiated by 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. 15 ml of PCR production from each reaction was loaded to a homemade 1% agarose gel. Electrophoresis was run at constant 100V, 400mA and 400W for 1 hour and evaluated with Kodak Gel Logic 100 Imaging System (Kodak, Rochester, NY).

1.2.3 RNA Extraction, Construction of Small RNA Libraries and RNA-Seq

Total RNA was purified using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocols. Two biological and technical replicates from each
genotype and gestational stages were designed to ensure reproducibility and rule out possibilities of differences caused by technical procedures. Purity and concentration were measured by ultraviolet spectroscopy (NanoDrop, Wilmington, DE). RNA integrity evaluation, libraries construction and validation was performed as described in our precious study (Ozturk, Li et al. 2013). Briefly, RNA integrity numbers (RIN) (Fleige and Pfaffl 2006, Schroeder, Mueller et al. 2006), an algorithm for assigning integrity values to RNA measurements, were assayed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Construction of small RNA libraries was accomplished using the Illumina mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA) under the manufacturer’s guides. Basically, Poly (A) + RNA was extracted from 1 µg of total RNA using two steps of purification with oligo-dT-coated Sera-Mag magnetic beads. Then, the purified Poly (A) + RNA was subject to chemically fragmentation, followed by converting RNA fragments to cDNA using SuperScript II and random primers. Next, second strand synthesis was done using RNaseH and DNA polymerase I. The prepared cDNA was then treated with T4 DNA polymerase to render all of the termini perfectly blunt. After this treatment, adaptors were attached to both ends of the cDNA fragments mediated by T4 DNA ligase. Libraries were then validated by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) and prepared for sequencing through the Illumina Cluster Station. Finally, RNA-Sequencing was performed using Illumina HiSeq 2000 at UNMC Bioinformatics and Systems Biology Core.

1.2.4 RNA-Seq Analysis

RNA-Seq data was obtained for TGF-β3 +/- Homozygous (HM) and TGF-β3 +/+ wild type (WT) samples profiled at embryonic days (E) 14.5 and 16.5. Each genotype/time point is represented by two biological replicates resulting in 8 samples used for RNA-Seq, which is performed in 2x101bp paired-end mode on the Illumina HiSeq2000 next generation sequencer. Raw reads were analyzed with FASTQC (v. 0.11.5) for quality control (Andrews 2010). Overrepresented (e.g. adapter and similar technical) sequences remaining in the raw reads were
assessed and subsequently removed using Trimmomatic (v 0.36) in the palindrome mode based on default alignment detection and scoring parameters (Bolger, Lohse 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 filtered out. Transcript quantification was done based on the GRCm38.p5 reference genome using Salmon (v. 0.8.2) with default parameters (Patro, Duggal 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. Differential gene expression analysis was done using DESeq2 (Love, Huber et al. 2014). DESeq2 uses a negative binomial model to assess differential expression and employs the Benjamini Hochberg procedure (Benjamini and Hochberg 1995) for multiple hypotheses testing correction. 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.

Clustering of samples and/or genes was done using the Unweighted Pair Group Method with Arithmetic-mean method with 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, Sherman 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, Ball et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto 2000). Biologically relevant categories that are over-represented in the gene set and therefore may be of further interest were assessed using the 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 picked GO categories that have EASE scores of 0.05 or lower as significantly over-represented. We further analyzed the differentially expressed gene lists using the Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) software. 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.

1.2.5 Confirmation of Dysregulated Genes with PCR

To confirm dysregulated genes between wild-type and homozygous samples, quantitative real-time PCR (qPCR) was performed as described by previously (LaGamba, Nawshad et al. 2005, Zhu, Ozturk et al. 2012). Palatal tissues were extracted from embryos at E14.5 and E16.5 and RNA extraction was conducted as previously described using Arcturus® PicoPureTM RNA Isolation Kit (ThermoFisher Scientific, San Francisco, CA) used to extract high-quality RNA consistently from very few cells. RNA (500ng) was converted to cDNA using SuperscriptTM IV VILOTM 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 preamplification step was conducted using TaqManTM PreAmp Master Mix (ThermoFisher Scientific, San Francisco, CA) with a custom pre-amplification pool of genes of interest to amplify small amounts of cDNA without introducing amplification bias—specifically for small, precious mRNA samples—allow more qPCR reactions. Samples were pre-amplified for 14 cycles with thermal cycling conditions of 95°C for 15 seconds and 60°C for 4 minutes followed by immediate placement on ice. Finally, samples were diluted with TE buffer (Ph 8.0) to 1:20, and 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 second and 60°C for 60 seconds. Each technical repeat's gene-specific 
ΔCt value were subtracted from the housekeeping gene ΔCt value. Then, data were analyzed using 
analysis of variance (ANOVA) on the repeat-normalized ΔΔCt 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 β-actin and the ΔCt values of naive/vehicle group. PCR assays 
detecting the reference genes, 18SrRNA, GAPDH or β-actin calculated alongside those for the GOI 
and the resulting dataset were analyzed. 18SrRNA, GAPDH, and β-actin are constitutively 
expressed and were therefore used as controls in the quantitative analyses.

1.3 Results

Transcriptional profiling was performed using paired-end RNA-Seq for 4 sample groups 
(WT14.5, WT16.5, HM14.5, HM16.5), each run in duplicate (a,b) for a total of 8 samples. Raw 
RNAseq average read count was ~65.5M paired-end reads (i.e. ~130.1M total reads) per sample. 
After trimming and filtering, the number of average total reads per sample came down to ~128.1M 
(Figure 1.1a). The average read-length was 101 bp in the raw reads, which decreased to 95.86 bp 
following trimming and filtering (Fig 1.1b). On the other hand, the average read quality increased 
to 36.79 from 35.84 (Fig 1.1c) and the percentage of high quality bases (bases with a quality score 
> 20) per sample increased to 99.20% from 96.28% (Fig 1.1d) following trimming and filtering. 
Therefore, both the total number of reads and the average read length parameters showed small 
changes in quantity after trimming and filtering but this resulted in significant data quality 
 improvement. RNA-Seq analysis generated expression data for 103,215 transcripts. Transcripts 
that showed a TPM value less than 1 in both samples in all the four groups were eliminated from 
downstream analysis leaving 52,475 transcripts. In Figure 1.2, we show the hierarchical clustering 
of the samples using all 52,475 transcripts. This global unsupervised grouping reveals that the 
samples are separated clearly by time and the effect of genotype on the transcriptional profiling is
subtle. Hence, there is need for supervised analysis methods to identify the differences in gene expression due to genotypic variance. Furthermore, the distance of the branching points at days 14.5 dpc and 16.5 dpc implies that the similarity between WT and HM groups is higher at 14.5 dpc than it is at 16.5 dpc. Therefore, the effects of TGF-β3 knockout is more pronounced at 16.5 dpc.

Figure 1.1: Summery of reads. (a) 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.
Figure 1.2: Hierarchical clustering of all the samples using all 52,475 transcripts measured with TPM>1 in at least one sample group.
Differential expression analysis also showed the stark temporal difference in gene expression between samples. These results are summarized in Table 1.1. There were 4115 significantly differentially expressed genes (SDEG) between 16.5 dpc and 14.5 dpc time points in the WT samples. This number increased to 5304 when the same two-time points were compared in the HM samples. These results indicate that the transcriptional change in TGF-β3 knockout samples are more widespread than normal controls. Furthermore, in both genotypes the number of upregulated genes with time (going from 14.5 dpc to 16.5 dpc) is more than the number of downregulated genes showing that transcriptional induction overshadows transcriptional silencing within development between these two-time points. This difference in up/downregulation is further emphasized when we applied a 2.0-fold change (FC) cut-off (on top of the adjusted p < 0.05 cut-off) to define SDEGs. Most of the upregulated genes in 16.5 dpc compared to 14.5 dpc survived this FC cut-off but the number of SDEG that are downregulated in 16.5 dpc compared to 14.5 dpc decreased dramatically. This further underlined the trend in significant transcriptional induction with time.

### Table 1.1: Significantly Differentially Expressed Genes

| Genotype Comparison | Time Comparison          | # of SDEG | # of SDEG (|FC| > 2.0) |
|---------------------|--------------------------|-----------|-----------------|
|                     | WT                       |           |                 |
|                     | Up in WT16.5 vs. WT 14.5 | 2421      | 1675            |
|                     | Down in WT16.5 vs. WT 14.5 | 1694      | 134             |
|                     | HM                       |           |                 |
|                     | Up in HM16.5 vs. HM 14.5 | 3153      | 1936            |
|                     | Down in HM16.5 vs. HM 14.5 | 2151      | 191             |
|                     | Genotype Comparison      |           |                 |
|                     | 14.5                     |           |                 |
|                     | Up in HM 14.5 vs. WT 14.5 | 8         | 0               |
|                     | Down in HM 14.5 vs. WT 14.5 | 5         | 0               |
|                     | 16.5                     |           |                 |
|                     | Up in HM 14.5 vs. WT 14.5 | 30        | 10              |
|                     | Down in HM 14.5 vs. WT 14.5 | 8         | 3               |

(SDEG, multiple hypothesis testing corrected p-value < 0.05) across time and genotype points separately listed for up-/down-regulation (FC: fold change).
On the other hand, we observed very subtle differences between the two genotypes at a given time point. At days 14.5 dpc and 16.5 dpc, there were only 13 and 38 SDEGs between the WT and HM samples, respectively. In concordance with the unsupervised hierarchical clustering results, we see a greater difference between the genotypes at 16.5 dpc. This was further strengthened by observing 13 SDEGs with a FC greater than 2.0 at 16.5 dpc between the two genotypes while there were no SDEGs with an FC greater than 2.0 between the WT and HM samples at 14.5 dpc.

Figure 1.3: Summery of differentially expressed genes. (a) Comparison of significantly differentially expressed genes (SDEGs) between 16.5 DPC and 14.5 DPC in the WT and HM groups. WT Specific-Up (WS_U): Genes uniquely upregulated in the WT group at 16.5 DPC; WT Specific-Down (WS_D): Genes uniquely downregulated in the WT group at 16.5 DPC; HM Specific-Up (HM_U): Genes uniquely upregulated in the HM group at 16.5 DPC; HM Specific-Down (HM_D): Genes uniquely downregulated in the HM group at 16.5 DPC. (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 (sample genes in the groups are shown), (d) fold change of relevant WT and HM specific genes.
Since a direct comparison between the HM and WT groups yielded a very subtle difference, we defined the effect due to TGF-β3 knockout through comparing the temporal SDEGs in the two groups. Following our adjusted p < 0.05 and |FC|>2.0 cut-offs, we compared the 1675 and 1936 SDEGs that were upregulated in 16.5 dpc vs. 14.5 dpc in the WT and HM groups, respectively. Similarly, we compared the 134 SDEGs downregulated in 16.5 dpc vs. 14.5 dpc in the WT group with the 191 SDEGs downregulated in 16.5 dpc vs. 14.5 dpc in the HM group. These results are summarized in Figure 1.3a. We called the 501 (429+72) SDEGs uniquely up/downregulated in the WT group “WT specific” and similarly, we called the 819 (690+129) SDEGs uniquely up/downregulated in the HM group “HM specific”. In Figure 1.3b we show the hierarchical clustering of WT and HM specific SDEGs.

Our real-time PCR data (see Fig. 1.4) is in agreement with our RNA-Seq results demonstrating at 16.5 dpc WT palates have an increased expression cell adhesion genes Cdhl, Ocln and receptors F2rll (PAR2) which are essential in palatal MEE cells architecture and play a vital role in palatal shelves fusion when in contact in WT palates for palatal confluency. On the contrary, we observe all these other genes, including Tnfrsf11b, Fndc3c1, Dlx1 and Gas2, were shown to be downregulated in our study.

Similarly, consistency is also presented between our quantitative PRC results and RNA-Seq data (Fig. 1.5) in HM that is genes which are reported to be suppressed by TGF-β3, such as Fas, are dramatically upregulated in absence of TGF-β3. What’s more, genes that repress apoptosis (Col2a1, Col11a1, Col11a2 and L1cam) or fasten cell-cell adhesion (Cldn1) therefore plausibly causing persistence of periderm also showed increased expression. In addition, genes that are regulated by other TGF-β isoforms (Adam12, Hspg2, Lox and Klf5) to maintain ECM construction and stabilize homeostasis are also noticeably upregulated at 16.5dpc in HM palates as TGF-β1 and TGF-β2 are expressed and functional in developing palates when TGF-β3 is silenced. On the other hand, genes that plays essential roles in regulating cell proliferation to
drive mesenchymal growth (\textit{Wnt9b, Ppp1r17 and Pdgfc}), promoting cell differentiation and EMT to facilitate palatal fusion (\textit{Alx4, Pax1, Twist1 and Wnt5a}) and participating in TGF-\(\beta\)-related pathway to mediate TGF\(\beta\) signaling (\textit{Kcp and Msx1}), are downregulated in lack of TGF-\(\beta\), causing cleft palate.

In order to understand the genes and corresponding functional mechanisms that would explain the observed differences between HM and WT samples across E 16.5 and 14.5 dpc time points, we highlight the Gene Ontology functional categories and KEGG pathways that are statistically significantly enriched in the WT and HM specific gene lists (Figure 1.3c). Complete list of enriched GO categories and KEGG pathways can be found in following figures.

1.4 Discussion

1.4.1 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-\(\beta\) gene has been overwhelmingly established as a crucial molecule that is necessary for normal palate development—in its absence CP occurs (Ozturk, Li et al. 2013). In this study, we focus on the role/s of TGF-\(\beta\) and its associated molecules that are fundamental in normal palatogenesis. Therefore, our study identifies the genes that are regulated by TGF-\(\beta\), hence, WT normal palatogenesis sustains; whereas, in HM, CP results due to altered genes under control of TGF-\(\beta\). Our data reveals that seven significant differentially expressed genes between WT and HM from 14.5 to 16.5 dpc are known CP genes listed in Table 1.2. While, WT mice showed two specific genes (\textit{Twist1, Wnt5a}) to be upregulated significantly, HM mice, on the contrary, showed an increased expression of seven (\textit{Chrng, Col2a1, Col11a1, Col11a2, L1cam}) genes, that may play
Figure 1.4. Fold change (log2) expression of WT gene mRNA relative to reference control genes (18sRNA, 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).
crucial roles in palatogenesis also found in Table 1.2. We analyzed their specific cellular function, role in palatogenesis, and association with TGF-β signaling pathway using IPA and GeneCards. 

*Twist1* acts as a basic helix-loop-helix transcription factor essential for epithelial mesenchymal transition (EMT) in both embryonic development and cancer. *Twist1* is a well-established E-cadherin repressor (Yu, Kamara et al. 2008). It has been shown convincingly that EMT is major mechanism of palatal seam disintegration – a crucial final stage of palate development (Nawshad 2008). Our data, therefore, demonstrates that upregulation of *Twist1* gene in WT and its role in

**Figure 1.5.** 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)
<table>
<thead>
<tr>
<th>ENSEMBLE Gene ID</th>
<th>Gene Name</th>
<th>Adjusted p-value</th>
<th>Fold Change (HM16.5 / HM 14.5)</th>
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<tr>
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<td>Nrcam</td>
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</table>
palatal EMT is in agreement with existing knowledge as well as reiterates its role in palatal EMT. Prior to palate fusion, *Twist1* protein expression has been shown in palatal shelves and MEE both in vivo and in vitro (Yu, Kamara et al. 2008). In chicken palates, palatal fusion was incomplete when cultured palatal shelves were treated with 200bnM *Twist1* siRNA. Additionally, Twist decreased in palatal shelves treated with TGF-β3 neutralizing antibody (Katayama, Handa et al. 2017). In the WT palate, *Twist1* is downregulated at 16.5 confirming completion of seam EMT resulting in palatal mesenchymal confluence in fused palates shown in Table 1.2 and Figure 1.3c.

Genetic screenings have implicated several isoforms of *Wnts*—*Wnt3a, Wnt5a, Wnt11*—with non-syndromic cleft lip and/or palate (Meng, Bian et al. 2009). The Wnt signaling pathways

<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>F2rl1</td>
<td>1.73E-03</td>
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<td>Fndc3c1</td>
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Table 1.2: Selected genes that are uniquely significantly differentially expressed (adjusted p-value < 0.05) in the HM or WT groups.
are essential for cell proliferation, differentiation, and survival (Brugmann, Goodnough et al. 2007). Furthermore, these genes are known to regulate mid-face development and upper lip fusion and are likely associated with the etiology of orofacial clefts (Brugmann, Goodnough 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, Bradley et al. 1999).

Gene interaction studies suggest a variation in *Wnt5a* regulating neural crest cell differentiation may predispose to an orofacial cleft (Chiquet, Blanton et al. 2008). Specifically, *Wnt5a* directs cell migration in a graded manner along the AP axis of the palate (Tabora, Ferrera et al. 2008). Overall, substantial data demonstrates an essential role of WNT family in orofacial growth allowing immaculate fusion of facial primordia. The WT palate showed an increased expression of *Wnt5a* from 14.5 dpc to 16.5 dpc (Table 1.2, Figure 1.3c) allowing appropriate directional palatal cell migration and cell proliferation that are necessary for both palatal mesenchymal and epithelial homeostasis respectively, resulting in appropriate palate development and subsequent immaculate fusion.

*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, Müller et al. 2006). During fetal development, the γ subunit is commonly expressed (Kariminejad, Almadani et al. 2016). The γ subunit is essential for neuromuscular signal transduction connecting axon and muscle; γ-knockout is lethal in mice (Hoffmann, Müller 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 limited to, cleft palate, joint contractures, pterygia and micrognathia (Vogt, Morgan 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, Li et al. 2013). *Chrng* is upregulated in the HM palate at
16.5 dpc (Table 1.2) indicating that in absence of TGF-β3 in HM, IRF6 and p63 proteins are differentially expressed resulting persistent of palatal periderm that hinders palatal adherens, necessary for palatal fusion, resulting in palatal cleft.

Several types of collagen are variably expressed in the developing palate and essential for extracellular matrix (ECM) metabolism. Collagen fibers have been shown to contribute to palatal shelf elevation, shelf adhesion, and ECM formation. *Col2a1* mutations are present in Osteogenesis Imperfecta, Stickler Syndrome, and chondrodysplasias (Vandenberg, Khillan et al. 1991, Ahmad, Dimascio et al. 1995). Further, defects in several collagens—*Col1a2, Col2a1, Col11a2*, and *ColXla1*—are linked to cleft palate (Schutte and Murray 1999, Meng, Bian et al. 2009). In the HM palate, *Col2a1, Col11a1*, and *Col11a2* were significantly upregulated from 14.5 to 16.5 dpc (Table 1.2, Figure 1.3c) suggesting their increased presence in the basement membranes of persistent, intact palatal epithelia, over which palatal periderm adheres to. Persistence of palatal periderm impedes palatal fusion resulting in palatal cleft.

The *L1 cell adhesion molecule* (*L1cam*), shown in Table 1.2, 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 carcinomas are some of the variable presentations of L1 malfunction (Schäfer and Altevogt 2010). Abnormalities of *L1cam* are characterized by severe mental retardation, hydrocephalus, spastic tetraplegia, and bilateral adducted thumbs. Previous literature reports have shown individuals with XLH who have cleft palate and suggested that *L1cam* may contribute to both phenotypes (Okamoto, Del Maestro 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 epithelial and periderm attachment and integrity that allows inadequate palatal adherens resulting in cleft palate.
1.4.2 Genes under TGF-β Control

1.4.2.1 Genes Upregulated at 16.5 dpc vs. 14.5 dpc 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, extracellular matrix (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, LaGamba et al. 2004). Several upregulated genes in HM palate, shown in Table 1.2, are under control of other TGF-β isoforms: Adam12, Clu, Fas, Hspg2, Cldn1, Lox, Itgb4, and Klf5. These genes, regulated by TGF-β1 and 2 in HM, may potentially regulate multiple cellular functions of palatal mesenchyme such as extracellular matrix (ECM) synthesis and deposition, degradation of basement membrane, cell proliferation and apoptosis that are functional in palatal growth in HM but do not play any role in 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, McBride et al. 2013). In the developing HM mouse palate, TGF-β1 and TGF-β2 are expressed in both palatal epithelia and mesenchyme (Nawshad, LaGamba et al. 2004). The upregulation of Adam12 in the HM palate at 16.5 (Table 1.2) 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 is a multifunctional glycoprotein that has a role in epithelial cell differentiation, cell-cell adhesion, and regulation of apoptosis (Itahana, Piens et al. 2007). Clusterin 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 clusterin in various cell types: nervous tissue, astrocytes, fibroblasts, lung epithelial cells, aortic endothelial cells and in HeLa cells in culture (Wegrowski, Perreau et al. 1999). The upregulation of Clusterin at 16.5 dpc in HM palatal tissue (Table 1.2) is a result of induced expression by isoform TGF-β1 and plays no role in palatal fusion, therefore, results in cleft palate.

Apoptosis is essential for embryogenesis, particularly in tissue/organ development and tissue homeostasis. Fas is a member of the TNF receptor superfamily and a well-known mediator of apoptosis (Goldthorpe, Jiang et al. 2015). It has been suggested that TGF-β induces resistance to apoptosis in lung fibroblasts through suppression of Fas via miR-29 (Matsuhashima and Ishiyama 2016). In the HM palate, Fas is upregulated at 16.5 dpc (Table 1.2) induced by other TGF-β isoforms or other pro-apoptotic factors contributing to palatal cell death that is essential in palatogenesis until the palatal fusion phase begins, which is regulated by TGF-β3.

Perlecan (Hspg2) is a proteoglycan that is a key component of basement membranes and ECM. An absence of perlecan in mice and humans causes lethal chondrodysplasia (Hara, Yoshida et al. 2017). Perlecan has a role in cell adhesion, proliferation, and angiogenesis. TGF-β1-induced perlecan deposition has been demonstrated in COPD airway smooth muscle (Ichimaru, Krimmer et al. 2012). We propose that Hspg2 (Table 1.2) is functional in palatal mesenchyme under the control of TGF-β1 regulating palatal mesenchymal ECM necessary for palatal growth in HM without any implication in palatal fusion which results in palatal cleft in TGF-β3 Knockouts.

Claudin-1 (Cldn1), a transmembrane protein localized to the surface of epithelial cells, is crucial for formation and function of tight junctions (Zhang, Li 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, Handa et al. 2017). In the HM mouse palate, Cldn1 has increased expression from 14.5 to 16.5 dpc (Table 1.2) suggesting the consequence of overexpression of
**Cldn1** is persistence of cell-cell adhesion and therefore a persistence of the MEE and periderm hindering palatal 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 BM function promoting abnormal ECM accumulation and fibrotic diseases. Lox has also been shown to promote apoptosis and act as a tumor suppressor (Kim, Mecham et al. 2017). In lung cancer, TGF-β is shown to increase Lox contributing to cancer metastasis (Araz, Demirci et al. 2014). In the HM palate, Lox may be upregulated as a result of TGF-β1 and TGF-β2, as shown in Table 1.2, maintaining ECM homeostasis necessary for palatal growth, having no role in palatal fusion seen in WT palates.

*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, integrin β4 connects is a component of hemidesmosomes that allow attachment to the basement membrane (Miyazaki, Ohkubo 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 β4 integrin subunit (Scardigli, Soddu et al. 1996) suggesting the upregulation of Integrin β4 in the HM palate (Table 1.2, Figure 1.3c) is under control of TGF-β1 and this upregulation is important for palatal growth and elevation by modulating basement membrane with palatal epithelia. However, Itgb4 seems to have no role in palatal 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, Gu et al. 2015). In HM palatal epithelia, *Klf5* is independent of regulatory TGF-β3, however it can continue to be regulated by both TGF-β1 and TGF-β2 showing
its increased activation and presence at 16.5 dpc (Table 1.2). Such upregulation of Klf5 gene in palatal epithelia in HM indicates palatal epithelial growth that has no relationship with palatal fusion, in which, palatal adhesion, apoptosis and EMT are implicated and regulated strictly by TGF-β3 alone in WT.

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

Wnt family member 9b (Wnt9b) is a member of the WNT gene family (Table 1.2, Figure 1.3c). This gene is clustered with Wnt3, another family member, in the chromosome 17q21 region. Genetic analysis on a specific mouse strain of which newborns have spontaneous CLP has indicated two interacting loci. A recessive gene, clp1, critical for CLP development was genetically mapped to a chromosomal region homologous to the human 17q12 region (Juriloff, Harris et al. 2006). Interestingly, when removing exon 2, the homozygous mutants, Wnt9b−/Wnt9b−, exhibit CLP, strongly supporting an essential role of Wnt9b in CLP occurrence. Wnt signaling pathways regulate a variety of developmental processes, including cell proliferation, differentiation and cell polarity (Cadigan and Nusse 1997, Wodarz and Nusse 1998) that are fundamental in palate development. 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, Ryan et al. 2006). Thus, in mice, reduced Wnt9b expression or lack of Wnt signaling pathways, as seen in homozygous embryos, contribute to failure in palatal morphogenesis resulting in palatal cleft.

Alx4 is a paired-like homeodomain transcription factor that is mainly expressed in the mesenchymal of developing bone, teeth, limbs and mammary tissue and possesses a pivotal role in craniofacial development and epithelial-mesenchymal interaction. Several studies have revealed mutation of Alx4 causes craniofacial anomalies, including facial clefting (Beverdam, Brouwer et al. 2001). During the development of palate, upon adhesion of palatal shelves at the midline, two layers of intervening MEE cells from both shelves form the midline epithelia seam (MES) which has to be subsequently dissolved in order to complete palatal fusion. EMT is thought to be an
important mechanism for MES disintegration (Nawshad 2008). *Alx4* expression is reported to be restricted to sites of epithelia-mesenchymal interactions. Deletion of *Alx4* was demonstrated to induce reversion of EMT (Hudson, Taniguchi-Sidle et al. 1998, Yuan, Kajiyama et al. 2015). Therefore, loss of *Alx4* in TGF-β3-/- mutants (Table 1.2) may impair EMT during palatal fusion and impede breakdown of MES resulting in palatal cleft.

*Pax1* 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, Nolting et al. 2008). High DNA methylation rates of *Pax1* is detected in tissues of several types of cancers, suggesting that *Pax1* acts as a tumor suppressor gene (Lai, Lin et al. 2008, Huang, Lai et al. 2010, Chang, Huang et al. 2014). Inactivation of *Pax1* gene may result in enhanced apoptosis resistance and repression of terminal differentiation (Su, Lai et al. 2009, Cheng, Chang et al. 2016, Hassan, Hafez et al. 2017). In our RNA data, downregulated expression of *Pax1* (Table 1.2) in homozygous palates may be responsible for persistence of periderm cells which hampers the formation of MES and finally 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 inhibits both the activin-A and TGF-β1-mediated signaling pathways (Soofi, Wolf et al. 2017). The BMP signaling is essential during organ development, including pathogenesis. Specifically, BMP-2, BMP-4, and BMP-5 are expressed in both epithelia and mesenchyme through palatogenesis (Nie, Luukko et al. 2006). Lu et al. revealed importance of BMP signaling in palatogenesis by a mice model with cleft palate in which decreased expression of BMP-2, -4 and -5 was observed (Lu, Jin et al. 2000). In our TGF-β3 (−/−) mice, deficiency of *Kcp* (Table 1.2) may alter the levels of BMP signaling, leading to aberrant cell proliferation and cell death during the palatal development, consequently, result in palatal cleft.
Msx1, like Kcp gene, is also known to regulate BMP signaling during palatal development as BMP signaling has been proposed to be downstream of Msx1 during palatal development (Zhang, Song et al. 2002). Msx1 is 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, Yeh et al. 2012). The fact that mice carrying null mutation of Msx1 develop complete cleft palate clearly demonstrates the critical role of Msx1 in palatal development (Satokata and Maas 1994). Msx1 was primarily expressed in the growing edges of maxillary prominences and mainly restricted to the anterior part of the palatal shelves as palatogenesis enters to the later stages (Zhang, Song et al. 2002, Hilliard, Yu et al. 2005). Msx1 is essential to maintain normal cell proliferation and outgrowth of the maxillary prominences. Msx1 and Msx2 were found to have extensive functions in atrioventricular cushions and myocardium during EMT and mutation of Msx1/Msx2 resulted in impaired EMT (Chen, Ishii et al. 2008). Based on the fact that no differential expression of Msx2 was identified in our RNA-Seq data, it is logical to speculate a compensatory mechanism by Msx2 in absence of Msx1. However, redundancy of Msx2 was insufficient to compensate for compromised Msx1. In accordance with previous findings that expression of Msx1 was down regulated in TGF-βr2fl/fl:Wnt1-Cre models, our RNA-Seq data supports that lack of TGF-β-3 leads to reduced expression of Msx1 (Table 1.2) in mice which may be responsible for occurrence of cleft palate.

Ppp1r17, shown in Table 1.2, is a substrate for cGMP-dependent protein kinase and is involved in central nervous system development and intracellular signal transduction (Endo, Suzuki et al. 1999). It implements protein serine/threonine phosphatase inhibitor activity and inhibits phosphatase activities of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) complexes. Enzymatic phosphorylation by protein kinase and dephosphorylation by protein phosphatase is an extremely effective means by which cellular signals, such as TGF-β-3 (a strict serine/threonine) signaling pathway can be integrated and transduced into multiple biological
effects in developing organs. Normal palatogenesis critically depends on spatial and temporal functioning of distinct signaling pathways at the cellular and molecular level which, however, are controlled by activities of intracellular protein kinase and phosphatase. PP1 and PP2A were reported by Weston et al. (Weston, Freeman et al. 2002) to account for virtually all detectable serine/threonine protein phosphatase activity during the development of embryonic palate. Therefore, decreased expression of PP1 or PP2A due to impaired function of *Ppp1r17* may cause disruption in orchestration of TGF-β signaling pathways which regulate cell proliferation, epithelial differentiation, and apoptosis in palatal development resulting in cleft palate.

*Pdgfc* (Table 1.2, Figure 1.3c) is a member of the platelet-derived growth factor (PDGF) family that plays an essential role in the regulation of multiple biological processes, including embryonic development, cell proliferation, cell migration and survival. The importance of *Pdgfc* as a mitogenic factor in regulating proliferation of mouse embryonic palatal mesenchymal (MEPM) cells has been established in recent studies (Ding, Wu et al. 2004, Han, Xiao et al. 2006). *Pdgfc* deficiency induces retardation of mesenchymal proliferation and differentiation in palatal shelves, which accounts for failure of palatal fusion and causes cleft palate in *Pdgfc−/−* embryos (Ding, Wu et al. 2004, Choi, Marazita et al. 2009). Although, retardation of palatal growth in TGF-β3 knockouts is not observed, that, however, does not exclude other palatal mesenchymal homeostasis disruption due to the absence of *Pdgfc* that can potentially result in inadequate epithelial mesenchymal interaction (a fundamental process in palate development) resulting in cleft palate.

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

In the WT palates, several genes under the control of TGF-β3 are significantly dysregulated, while this is not the case in the HM mouse and consequently 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 time and location dependent manner, like
an orchestra. 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, Ozturk et al. 2012). However, the compromise of cellular and morphological functions of these isoforms vary significantly. Our data reveals that upregulated genes listed in Table 1.2—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, Atkinson et al. 1994). Just like all epithelia, the palatal shelf epithelium expresses E-cadherin during palatogenesis. Mutation of the Cdh1 gene has been shown to cause cleft lip and/or palate (Meng, Bian et al. 2009). In WT mice Cdh1 is upregulated from 14.5 to 16.5 dpc (Table 1.2, Figure 1.3c). At 14.5dpc Cdh1 is essential for integrity of medial edge epithelium (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, Medici 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 a persistent increase in Cdh1. Similar to E-cadherin, the transmembrane protein Occludin (Ocln) plays a role in tight junction assembly of different epithelia, which is also upregulated at 16.5 dpc in WT palate (Mir, Meena et al. 2016). Similarly, occludin is a transmembrane protein of tight junctions contributing to cell-cell adhesion. Occludin is essential for homeostasis of epithelia. Any decrease in occludin decreases cell-cell adhesion and reduces apoptosis (Mir, Meena et al. 2016). With the increase in quantity of epithelial cells in WT palates, we expect also an increase occludin (Table 1.2, Figure 1.3c). Both of these genes enforce palatal epithelial adhesion as well maintaining epithelial architectural and functional integrity—hence their increase in WT is expected and justified.
The *Lyn* gene belongs to the protein kinase superfamily localized to the cell surface (Roberts, Bishop et al. 2014). *Lyn* plays an important role in immune response, hematopoiesis, response to growth factors and cytokines, and integrin signaling (Lim, Koo 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, Fox et al. 2013). Using MYL cells from a CML patient, it was found that TGF-β drives Lyn ubiquitination and turnover via c-cbl transcription and expression (Smith, Tanaka et al. 2012). Similar TGF-β control may be contributing to the increased presence of *Lyn* in the WT palate at 16.5 (Table 1.2) to facilitate integrin signaling as well maintain 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 listed in Table 1.2 (Sales, Friis 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, Ramachandran 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, Ramachandran et al. 2013). Additional signaling pathways and proteins, such as G-protein coupled receptors, play a critical role in palate development (Cobourne 2004). TGF-β can stimulate the transcription of genes in response to EGF while EGF also regulates production of various growth factors in embryonic palatal tissues (Meng, Bian et al. 2009).
Tetraspanins (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, Wu et al. 2017). Tspans have been shown to play crucial roles in biologic processes including cell adhesion, proliferation, differentiation, and migration (Zhao, Wu 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 Tspan may be limited to cellular migration, proliferation and differention via Smad pathways during palatogenesis.

Osteoprotegrin (Tnfrsf11b) is a key regulator of bone metabolism and is crucial for bone homeostasis; osteoprotegrin inhibits osteoclast activity allowing new bone formation by osteoblasts (Smane and Pilmane 2016). Osteoprotegrin knockout mice have severe osteoporosis while overexpression leads to osteopetrosis (Zehnder, Kristiansen et al. 2006). As early as 14.5 dpc, 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, Blanco et al. 2004). We expect osteoprotegrin signaling to be upregulated from 14.5 to 16.5 dpc (Table 1.2) in preparation for osteogenesis.

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

Fndc3c1, a member of a novel gene family, encodes a protein consisting Fibronectin module type III, which functions in cell adhesion, differentiation, migration and embryogenesis (Potts and Campbell 1996, Porcionatto 2006). Fndc3c1 was found redundantly expressed in several tissues, including dental pulp and testis (Carrouel, Couble et al. 2008). In palate, periderm is created during embryonic development, forms a protective shielding against premature fusion and must dislodge prior to MEE fusion to allow a confluent palate (Obholz, Akopyan et al. 2006, Nawshad
2008, Hu, Liu et al. 2015). Therefore, it’s logical to postulate that reduced expression of *Fndc3c1* (Table 1.2) may participate in facilitating sloughing off of periderm from underlying epithelium causing immaculate fusion.

*Dlx1* is a member of a homeobox transcription factor and encodes a protein that functions as a transcriptional regulator of signaling from several TGF-β superfamily members in nucleus. Murine *Dlx1* genes have been demonstrated to play an essential role in controlling the development and function of inhibitory neurons in forebrain and craniofacial patterning (Stock, Ellies et al. 1996, Qiu, Bulfone et al. 1997, Anderson, Mione et al. 1999, Zerucha, Stühmer et al. 2000). At molecular level, accumulating evidence have reported in their study that *Dlx1* can induce inhibition of TGF-β/Smads signaling pathway via FLT3 activation and altered expression level of *Dlx1* seems to be functionally significant (Chiba, Takeshita et al. 2003, Starkova, Gadgil et al. 2011). In accordance with our RNA-Seq data which show decreased activity of *Dlx1* at later stages in palatogenesis, previous study has implicated *Dlx1* was might be involved in palatal shelves outgrowth and elevation but unlikely to participate in later steps during palatal development (Jeong, Cesario et al. 2012). We further postulate that *Dlx1* diminishment (Table 1.2) prior to palatal fusion may account for unlocking of TGF-β signaling that properly guides dislodgment of periderm and subsequent fusion in palatogenesis.

*Growth arrest specific gene 2* (*Gas2*) is a caspade-3 substrate therefore regulating cell cycle and apoptosis (Sgorbissa, Benetti et al. 1999). Cleaved form of *Gas2* is capable of modifying microfilament and cell shape during apoptosis (Brancolini, Benedetti et al. 1995). Cell death by apoptosis is a fundamental process maintaining normal development and homeostasis of multicellular organisms. During palatogenesis, MEE cells in 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, Brancolini et al. 1996). Thus, it is plausible to suggest that during palatal fusion, lower levels of Gas2 (Table 1.2) 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 (Table 1.2) is located on human chromosome 9 and encodes a conserved zinc finger protein which presumably functions as a regulatory protein of DNA transcript (Vanhoutrtemhem and Djian 2004). According to reports, Bnc2 plays a role in skin color saturation and skin cancer development (Jacobs, Hamer et al. 2015). Observations of growth arrest of tumor cells induced by stable expression of Bnc2 also implied Bnc2 as a putative tumor suppressor gene (Akagi, Ito et al. 2009). It was recently reported that Bnc2 was specifically expressed in mesenchymal cell but absent in epithelium in developing palate (Vanhoutrtemhem, Maciejewski-Duval et al. 2009). However, little was known regarding Bnc2 functions in regulating palatal morphogenesis. Thus, we can’t rule out possibilities that Bnc2 determines epithelial fate via mesenchymal-epithelial communication.

Versican gene encodes a large chondroitin sulfate proteoglycan, a major component of the extracellular matrix. Amongst the four known isoforms of versican, V0 and V1 are reported to be distinctly expressed in embryonic development and play important roles in cell proliferation, adhesion and apoptosis (Zimmermann, Dours-Zimmermann et al. 1994, Landolt, Vaughan et al. 1995, Wu, Sheng et al. 2004). Furthermore, increasing evidence have demonstrated that V1 is capable of inducing apoptotic resistance in cultured cells (Sheng, Wang et al. 2005, LaPierre, Lee et al. 2007). TGF-β3-mediated apoptosis and subsequent disintegration of midline epithelial seam is a critical event for successful palatal fusion (Kaartinen, Cui et al. 1997, Nawshad, LaGamba et al. 2004, Iordanskaia and Nawshad 2011). Thus, it’s plausible that suppressed expression of versican at 16.5 DPC (Table 1.2) facilitates breakdown of midline epithelial seam. Further
investigation of the versican’s signaling network should be of benefit to our understanding how versican contributes to palatogenesis.

1.4.3 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, Petrovic et al. 2016). Crystallin-αB (Table 1.2) is known to bind and stabilize cytoskeletal proteins and play a role in EMT in liver and lung fibrosis (Malin, Petrovic et al. 2016). An in vitro experiment in retinal pigment epithelial cells showed overexpression of Crystallin-αB siRNA decreased E-cadherin and increased SNAIL and SLUG in protein and mRNA levels (Ishikawa, Sreekumar et al. 2016). Snail family members have been implicated in triggering of EMT and cell survival (Kudo-Saito, Shirako et al. 2009). Crystallin-αB, 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, Blanco et al. 2004). Upregulated in HM mice at 16.5 dpc, the relationship between Crystallin-αB and Snail may be related to apoptosis of palatal mesenchyme and epithelia that are necessary in palatogenesis, but unrelated to palatal fusion that is regulated by TGF-β3 in WT palates.

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, Kaplan et al. 2005). Nr-Cam protects cells from apoptosis via extracellular signal-regulated kinase and AKT signaling pathways (Conacci-Sorrell, Kaplan et al. 2005). The PI3K/Akt pathway suppresses apoptosis and promotes cell growth and proliferation (Bian, Terse et al. 2009). As shown in Table 1.2, Nr-Cam is upregulated in the HM palate at 16.5 dpc and may be contributing to decreased apoptosis in persistent MEE and periderm of palatal shelves ultimately hindering palatal fusion.
1.4.4 Direct Comparison of Homozygous and Wild-Type Palates at 14.5dpc and 16.5dpc

The RNA-Sequencing data shows few differentially expressed genes when directly comparing genotypes at 14.5 and 16.5 dpc. At 14.5, only 13 genes were differentially expressed between WT and HM ranging from FC -1.46 to 1.39 (Table 1.2)—a very concentrated difference between genotype in comparison to the differential gene expression over time. A total of 38 differentially expressed genes existed between WT and HM at 16.5 dpc ranging from FC -2.75 to 16.75 as shown in Table 1.2.

*Ephrins* are a family of cell surface receptors involved in embryonic development including the palate (Risley, Garrod et al. 2009). *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, Garrod et al. 2009). 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, Garrod et al. 2009). Similarly, *Ephb2* and *Ephb3* (Table 1.2 and Figure 1.3c) have been implicated in palate development and present in palatal mesenchyme and epithelium (Risley, Garrod et al. 2009). Knockout of both *Ephb2* and *Ephb3* causes cleft palate in mice (Orioli, Henkemeyer 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—*Fgf3* and *Col2a1* (Meng, Bian et al. 2009, Küchler, Sabóia et al. 2014). *Sox6* negatively regulates expression of
Fgf3 and activates Col2a1 (Hagiwara 2011). Sox6 plays various roles in embryonic development, warranting further investigation on its involvement in palatogenesis.

Keratin family proteins comprise the intermediate filament system in epithelial cells having significant interactions with the extracellular matrix throughout key processes such as development, tissue remodeling and repair, and differentiation (Kurpakus, Stock 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, Lan et al. 2006). Periderm has been previously characterized by keratin expression (Iwasaki, Aoyagi et al. 2006, Paul, Palmer et al. 2017). 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, Lan et al. 2006). The genotypic difference in Krt5 expression, upregulated in HM at 14.5 dpc, is likely due to persistent periderm and therefore increased keratin expression in the palatal shelf epithelium. Similar to its counterpart differentially expressed at 14.5, Krt4 is upregulated in HM at 16.5. As previously discussed, the increased expression of keratin is indicative of persistent periderm which will hinder proper palatal fusion in HM.

Epidermal growth factor receptor (Egfr) signaling is essential for normal craniofacial development. Egfr regulates development via downstream targets including matrix metalloproteinases (MMPs) (Miettinen, Chin et al. 1999). Studies have shown abnormal function of Egfr results in cleft palate (Meng, Bian et al. 2009). In mice deficient of Egfr, it is postulated that palate adhesion fails due to decreased secretion of MMPs (Miettinen, Chin et al. 1999). Further, in TGF-β3 knockout mice, the loss of TGF-β-3 function leads to changes in MMP13 expression and loss of cell surface filopodia (Taya, O'Kane et al. 1999).

Both Egfr and Mmp13 are dysregulated between WT and HM at 16.5 dpc; Egfr is upregulated in HM at 16.5 dpc while Mmp13 is downregulated in HM at 16.5 dpc. 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, Khillan et al. 1991). Increased presence of Col6a6 (Figure 1.3c) in HM compared to WT at 16.5 dpc is indicative of increased presence of basement membranes of persistent, intact palatal epithelia with periderm—resulting in a cleft.

1.4.5 Functional analysis of SDEG in WT and HM across 16.5dpc vs. 14.5dpc

Our data reveals that in WT, at 16.5pdc of palatal development, several pathways are active and functional such as TGF-β, ERK/MAPK, p38MAPK and PI3K/AKT. Although these kinase pathways are also known to be regulated by TGF-β, but they can also be regulated by other factors which could well be active in palate development at 16.5 dpc. These pathways are shown to be regulating genes displayed in Figure 1.6. However, p38MAPK is a key mediator downstream of TGF-β pathway which synergistically causes downregulation of transcriptional regulator GSC (see Figure 1.7). Inhibition of GSC indicates completion of palatal fusion at 16.5dpc as GSC is reported to regulate cell migration and EMT during embryonic development (Xue, Ge et al. 2014). TGF-β1, in tandem with p38MAPK and many other molecules that are highlighted in Figure 1.8 regulates cell morphology and differentiation of fibroblasts that potentially account for construction of ECM (Kalluri and Weinberg 2009) and EMT (Griffith and Hay 1992) during palatal development.

In HM, however, a different regulatory profile is drawn by the major pathways at 16.5dpc, depicted in Figure 1.9, which results in occurrence of cleft palate as observed in our animal models. In agreement with TGF-β3 knockout, TGF-β3 signaling is not active and functional as seen in WT. Instead, PI3K/AKT and ERK/MAPK pathways impair normal palatogenesis process by altering expression of Tlx2 (Figure 1.10). Tlx2 plays a role in the
proliferation or differentiation of neural crest cell lines (Nelms and Labosky 2010). Although little is known how Tlx2 contributes to palatal development, dysfunction of Tlx2 was reported in diseases associated with cleft palate (Puri and Shinkai 2004). Genes (e.g. Tbx3, Wbt9b, Psdha4, Prkcb, et al) that participate in regulating crucial biological processes, such as cell morphology and embryonic morphogenesis show altered expression levels at 16.5 in HM that is considered responsible for failure of palatal fusion (Figure 1.11).

1.5 Conclusion

Identifying transcripts that play key roles in regulating palatal development in critical stages has been a powerful approach to answer the question that how TGF-β3 controls normal palatogenesis and how does the lack of TGFβ3 signaling cause cleft palate. In the previous study, we evaluated expression patterns of known CP genes in human and mice throughout different stages of palatogenesis. This study employed a different perspective in identifying potential CP genes based on differentially expressed genes between genotypes and gestational ages. Our data presented in this work provide a strengthened understanding of the complex genetic mechanism of TGFβ3-regulated palatogenesis. In addition, we discussed those genes that may play pivotal role in both mediating normal palatal development and causing cleft palate and elucidated their functions in relation to TGF-β canonical pathways. In general, our results represent state-of-the-art research in studying cleft palate and may shed light on future relevant studies and prenatal medical intervention of cleft palate.
Figure 1.6. Genes that are upregulated or downregulated over 2.0-fold change (SDEG) across 16.5dpc vs. 14.5dpc and relevant pathways in WT palates.
Figure 1.7. Illustration of SDEG in TGF-β3 signaling pathway in relation to major pathways in WT.
Figure 1.8. SDEG across 16.5dpc vs. 14.5 dpc in relation to cell morphology and differentiation of fibroblasts in WT.
Figure 1.9. Genes that are upregulated or downregulated over 2.0-fold change (SDEG) across 16.5dpc vs. 14.5dpc and relevant pathways in HM palates.
Figure 1.10. Illustration of SDEGs in TGF-β3 signaling pathway in relation to major pathways in HM.
Figure 1.11. SDEG across 16.5dpc vs. 14.5 dpc in relation to morphology of cells and morphogenesis of embryo in HM.
CHAPTER 2

EPHRIN REVERSE SIGNALING MEDIATES PALATAL FUSION AND EPITHELIAL-TO-MESENCHYMAL TRANSITION INDEPENDENTLY OF TGF-B3

2.1 Introduction

The secondary palate in humans and mice forms from shelves of mesenchyme covered by epithelium. These shelves grow out bilaterally from the internal surfaces of the maxillary processes, elongate on each side of the tongue and become horizontal above the tongue as it descends. As soon as the opposing shelves reach each other, the lateral surfaces of the medial edge epithelia (MEE) cells form the midline epithelial seam (MES) (Murray and Schutte 2004). Complete disintegration of the MES is essential to form a confluent structure, and failure of palatal fusion causes cleft palate, one of the most common birth defects (Croen, Shaw et al. 1998). Thus, understanding the mechanism of fusion is an important goal of craniofacial biology.

Palatal fusion has been thought to require Transforming Growth Factor β-3 (TGF-β3) because TGF-β3 knockout mice, as well as naturally TGF-β3-null avian systems, display cleft palate, and treatment of either with exogenous TGF-β3 rescues palatal fusion (Martinez-Alvarez, O'Kane et al. 1996, Sun, Vanderburg et al. 1998, Taya, O'Kane et al. 1999). Genetic and pharmacological studies have shown that the TGF-β3 signal, acting through serine/threonine kinase TGF-β receptors (TGF-βR) on MEE cells, activates Smad, p38 mitogen-activated protein kinase (MAPK), and phosphotidyl inositol 3 kinase (PI3K) pathways in palate epithelium (Kang and Svoboda 2002, Xu, Han et al. 2008). Fusion requires PI3K and either (but not necessarily both) the Smad or p38 pathways (Xu, Han et al. 2008). However, the mechanism of MES degradation is still in question. Numerous studies suggest that the epithelial cells undergo epithelial-to-mesenchymal transition (EMT), apoptosis, or both (thoroughly reviewed in (Nawshad 2008). Recent work on cultured primary MEE cells indicates that TGF-β3 causes these cells to shift gene expression
patterns away from epithelial markers to fibroblastic ones, while assuming a migratory phenotype. They then initiate caspase-dependent apoptosis. This entire process occurs in culture over the same 72 hour time frame as does fusion in the mouse embryo, consistent with a mechanism that is reflective of the actual process in vivo (Ahmed, Liu et al. 2007).

We recently reported a role for ephrin signaling in palatal fusion. The Ephs are the largest family of receptor tyrosine kinases. They are classified as A or B based on sequence homology and on their binding preference for the transmembrane B ephrin or the glycosyl phosphotidyl inositol linked A ephrin ligands (Orioli and Klein 1997). Eph-ephrin systems control a number of contact-dependent processes in development, including cell migration, boundary formation, and proliferation (Davy, Aubin et al. 2004, Davy and Soriano 2005, Davy and Soriano 2007). Ephs function as traditional receptor tyrosine kinases when bound by their ephrin ligands, but they can also act as ligands that activate signaling downstream of the ephrin, which assumes the role of receptor in what is called “reverse signaling” (Murai and Pasquale 2004). We reported EphB and ephrin-B expression in the MEE during fusion, and we found that ephrin-B reverse signaling is required for palatal fusion in mice and is sufficient to cause fusion in chicken palates without the addition of TGF-β3 (San Miguel, Serrano et al. 2011). This finding was supported by a report of cleft palate in ephrin-B2 reverse signaling-deficient mutant mice (Dravis and Henkemeyer 2011). Interestingly, we discovered that the ephrin reverse signal passes through PI3K, a signaling pathway not previously associated with reverse signaling (San Miguel, Serrano et al. 2011).

Here we report our most recent study of the cellular mechanism of ephrin reverse signaling in palatal fusion. We found that activation of reverse signaling in mouse palates is sufficient to cause fusion independently of TGF-βR, and that the ephrin signal activates an EMT-like program in palatal epithelial cells, but does not cause apoptosis in these cells. Our data describe a novel role for ephrins in craniofacial development, and clarify their role in palatal fusion.
2.2 Materials and Methods

2.2.1 Chemicals and reagents

Anti-TGF-β3 (Cat#AF-243-NA) and anti-EphB2 (Cat#AF467) were obtained from R&D Systems (Minneapolis, MN). The TGF-βRI Kinase Inhibitor VI (SB431542) was from Calbiochem (EMD Millipore Cat#616465) (Billerica, MA). EphB2 ectodomain Fc fusion protein was from R&D Systems (Cat #467-B2) (Minneapolis, MN). IgG Fc protein was from Calbiochem (EMD Millipore Cat #401104) (Billerica, MA). Recombinant TGF-β3 was purchased from R&D systems, CA. For Immunofluorescence, primary antibodies used (and the ir source) included the following: E-Cadherin, Desmoplakin, and Plakoglobin (kindly provided by Dr. James Wahl, University of Nebraska Medical Center), Vimentin (Sigma-Aldrich, MO), Fibronectin (Abcam, MA), ZO-1 (Invitrogen, CA). All antibodies and inhibitors were used at the concentration and time point recommended by the respective manufacturer/provider.

2.2.2 Embryonic palate culture

All animal care and experiments were performed under protocols approved by the Institutional Animal Care and Use Committees of the Baylor College of Dentistry and the University of Nebraska Medical Center. Mouse palate culture was performed as previously (Kang and Svoboda 2002, Yu, Kamara et al. 2008, San Miguel, Serrano et al. 2011). In brief: Palatal shelves were dissected from e13.5 CD1 mouse embryos and placed nasal side down on polycarbonate membranes (Nucleopore Corp.) with their medial edges in contact. The tissues were cultured with BGJb medium (Gibco) for 72 h. Medium was replaced every 24 h with fresh treatments. Anti-TGF-β3 was used at concentration of 10 µM. TGF-βRI Kinase Inhibitor VI (SB431542) was used at a concentration of 25 µM. Based on our initial dose-response experiments (not shown), this was the concentration of kinase inhibitor that abolished MES degradation in cultured palates while showing no signs of altered cell morphology. EphB2/Fc and control IgG Fc proteins were used at 5 µg/mL, as in our previously published studies. Fc proteins were clustered
by mixing with anti-human Fc in a 4 to 1 w/w ratio and incubated at 22°C for 1 h or overnight at 4°C. This treatment allows the soluble Fc proteins to mimic the clustering that occurs on cell membranes and is required to initiate biologically relevant signaling.

2.2.3 Histological analysis

Cultured palates were fixed in 4% formaldehyde/phosphate buffered saline, stabilized in low melting point agarose, and processed for paraffin embedding. Serial 6-µm sections were collected in the coronal orientation from anterior to posterior. Sections were stained with hematoxylin and eosin (H&E) and scored for fusion by at least two independent blinded observers using the previously described scale as follows (Kang and Svoboda 2002): A score of 5 denotes complete fusion with no epithelia persisting in the midline. A score of 4 means epithelial triangle or islands remain, but they are less than 1/3 the total width of the palatal shelf interface. A score of 3 signifies mesenchymal confluence was achieved in places, but over 1/3 or less of the palatal shelf interface, with large epithelial islands or triangles remaining. A score of 2 means that a continuous epithelial seam persisted in the midline. Palatal shelves that were not touching each other in the midline received a score of 1. Palatal shelves with a score of 1 were cultured in contact with each other but came apart during processing and embedding due to lack of adhesion. Any palates that were not in contact for the entire culture period were discarded and not scored.

2.2.4 Statistical analysis

2.2.4.1 Palate culture experiments

All palate fusion experiments were performed at least three times for a total n = 12–18 for each treatment group. Fusion scores reported are the mean ± standard error of the mean (SEM) of the pooled scores across all experiments. Statistical analyses were made using SPSS software. Mean Fusion Scores were analyzed using Kruskal–Wallis test with the Mann-Whitney U test used to analyze specific sample pairs for significant differences. Differences in fusion score between
groups with $P < 0.01$ were considered to be statistically significant. The statistical power of the samples in experiments was evaluated by G*POWER software (Version 3.1). The power with respect to the seriousness of types I and type II errors rate was calculated with the settings type I error, $\alpha = 0.01$ and type II error, $\beta = 0.05$. We expected that the power analysis under these settings and with a sample size large enough would yield a statistically significant effect.

### 2.2.4.2 Cell culture experiments

Data from at least three replicates for each parameter were evaluated and analyzed for significance by SPSS 14.0. The treatment groups included TGF-β3, EphB2/Fc and the control groups (IgG Fc). The observation times were collapsed due to the convenience of the study, and one-way ANOVA was conducted. The significance level was set as 0.05. AP-value of $\leq 0.05$ was considered significant. The one-way ANOVA indicated that the values differ significantly across the treatment groups. Bonferroni post-hoc comparisons of the treatment groups indicated that the negative control control group significantly differ from each other ($P \leq 0.005$). The comparison of each treatment group (time and dose) showed EphB2/Fc treatments groups also differed significantly from the negative control groups, ($P \leq 0.005$).

### 2.2.5 Culture of isolated primary MEE cells

Embryonic MEE cell culture was performed as previously described (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012). The single cell thick periderm covering on each shelf was removed by incubating the shelves with Proteinase K for 1hr at 37°C. The shelves were then cultured at 37°C for 12 h to allow brief adherence to the corresponding opposite shelf (adhered). Adhered shelves in organ culture were then cut close to the seam to ensure limited or no mesenchymal tissues attached to isolated seam. The shelves were then separated and treated with Dispase II for 30 min to allow the primary MES cells to separate from the underlying basement membrane so that epithelial cells could be collected without any mesenchymal
contamination. Cells were then cultured in flasks and harvested at the exponential growth stage (~80% confluence) before any exogenous treatment began.

2.2.6 Apoptosis assay

MEE cells were treated in culture with clustered IgG/Fc (negative control), EphB2/Fc, or Cisplatin (positive control) for 24 or 48 h. Cells were then fixed and underwent in situ terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the free 3′-OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein conjugated avidin (avidin-FITC) (TUNEL Apoptosis Detection Kit, Millipore, MA # 17-141f). The same samples underwent a second step of immuno-labeling for tubulin (Cell Signal, MA# 2148) with Alex Flour 488 conjugated secondary Antibody (Invitrogen, CA), followed by mounting with with DAPI (Vectashield, CA, H1200).

2.2.7 FACS analysis

MEE cells were grown in 10% FBS containing DMEM in T-25 flasks. Approximately 60% confluent cells were treated with 6.0 µM Aphidicholin for 16 h, washed with HBSS and released into complete medium for 30 min. Cells were then treated with complete medium containing clustered IgG/Fc, EphB2/Fc, or cisplatin. Cells were collected every 24 h for live and dead cell stain analysis with a BD FACSArray Bioanalyzer. Vibrant cell metabolic assay kit and Sytox red dead cell stain were purchased from Invitrogen. Cells were stained according to the manufacturer's protocol. In brief, floating cells were collected and resuspended in PBS with 2 µM C12-resazurin, followed by incubation for 15 min at 37°C. Cells were then detached by trypsin, pelleted, resuspended in 5 nM Sytox Red stain/mL, and incubated for a minimum 15 min at room temperature in the dark. The stained cells were analyzed on a BD FACSArray Bioanalyzer using a green laser at 532 nm to detect C12-resazurin and a red laser at 635 nm to detect Sytox Red stain.
2.2.8 Scratch-wound assay

The scratch-wound assay was conducted as previously described (Nawshad, Medici et al. 2007). MES cells were grown to 80% confluency in 6-well culture plates, and a uniform straight-line scratch was made with a sterile pipette tip. Scratches in EphB2/Fc (2, 5 and 10 µg/mL) treated and IgG Fc (control) wells were examined for 48h. The migration of cells (or gap filling) was monitored every 12h with phase contrast microscopy where cells were morphologically assessed for the migratory phenotype.

2.2.9 Cell Motility Assay

The Cell Motility Assay was conducted as reported (Nawshad et al., 2007). 8 µm pore size Transwell migration chambers of a 6-well plate (BD BioCoat, MA) were used for migration analyses. 5 × 105 MES cells were seeded in the presence of 5 mg/mL EphB2/Fc in 8 µm pore size Transwell migration upper chambers of a 6-well plate. Treated and control (Ig Fc) MES cells were allowed to migrate through the filter toward media containing serum (10%) for 24–48 h at 37°C. Cells that did not migrate through the filter were removed with a cotton swab from inside the upper chamber. Each filter was fixed in 4% Paraformaldehyde for 10 min, washed three times, each time for 5 min with 1x PBS, placed in Hematoxylin stain (Dako, Mayer's hematoxylin) for 20 min, rinsed with water, and placed in bluing reagent (alkaline solution such as a weak ammonia solution, 0.08% in water) until the stain turned blue. Subsequently, the filters were washed again using deionized water. Migrating MES cells on the lower side of the filter were randomly counted at 10 areas per field by phase-contrast microscopy. The mean of the 10 areas was determined and is represented in the bar graph in Figure. 2.6B.

2.2.10 Immunohistochemistry, immunofluorescence, and immunoblotting

The MES cells and embryonic palates from 14.0 to 16.5 dpc underwent Immunohistochemistry, Immunofluorescence and Immunoblotting techniques as described by us
previously (Ahmed et al., 2007; Nawshad et al., 2007; Iordanskaia and Nawshad, 2011; Jalali et al., 2012). For protein expression of MES cells by western blot, the cells were grown to confluence in 10% FBS and serum starved in 1% FBS for 24 h, followed by treatment with TGF-β3 (2 and 5 ng/mL) and EphB2 (2 and 5 µg/mL) in 1.0% FBS DMEM for 0–24 h for total protein extraction. For total proteins, we used the nuclear extraction kit from Chemicon total protein Extraction Kit (Millipore) as done by us previously (Ahmed et al., 2007; Iordanskaia & Nawshad 2011). The concentration of total protein was obtained with the Genesys 10 UV scanner (Thermoscientific) at 595 nm. 25 µg of protein extract was electrophoresed on a 10% denaturing gel and transferred onto a nitrocellulose membrane. The membranes were blocked with gelatin, washed with PBS-Tween, incubated with the EphB2 and TGF-β3 antibodies and reacted with anti-goat (1:1000) and anti-rabbit (1:2000) secondary antibodies (Cell Signaling). The bands were then visualized by using an odyssey scanner (Li-Cor). Intensity of the band was measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester). To perform a t-test analysis of mean intensity measurements, an ROI analysis was done from the data to Microsoft Excel software from the exported “.txt” files. Data points for all samples are paired by spatial arrangement on gel and compared pairwise to minimize the impact of subtle background artifacts on image analysis. MES cells or 8µm sections of 14.5 dpc palates from WT and TGF-β3 knockout mice underwent Immunofluorescence or immnohistochemistry, respectively, as described by us previously (Nawshad and Hay 2003, Ahmed, Liu et al. 2007, Nawshad, Medici et al. 2007). Immunofluorescence secondary antibodies were obtained from Invitrogen (Rhodamine, 1:100) and Jackson Immunoresearch (FITC, 1:200).

2.2.11 Gene expression

As described previously, (LaGamba, Nawshad et al. 2005, Xu, Han et al. 2008) RNA from MEE cells treated with clustered EphB2/Fc (1, 2, or 5 µg/mL) for 48 h, was harvested using the RNeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. RNA integrity was
assessed using formaldehyde gels in 1XTAE buffer, and RNA purity and concentration were
determined by the 260/280 ratio on a Nanodrop 2000C (ThermoScientific, MA). The Ct values
were exported into a Microsoft Excel Spreadsheet and analysed according to the ΔCt system. The
−ΔΔCt (Snail, Sip1, Twist and E-Cadherin/ vs IgG Fc control) values were plotted to show the genes
that are up or downregulated in fold/s increase.

The sequences of primers were obtained from the Invitrogen online PCR primer design
site, and were synthesized at the Molecular Biology Core Facility, UNMC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Snail</td>
<td>5’- GAGGTACAACAGACTATGCAATAGTTTC-3’</td>
<td>5’-CCTGCTGAGGCATGGTTACA-3’</td>
</tr>
<tr>
<td>Mouse Twist</td>
<td>5’- TCCGCGTCCCCACTAGCA -3’</td>
<td>5’- TTCTCTGGAAACAATGACATCTAGGT -3’</td>
</tr>
<tr>
<td>Mouse Sip1</td>
<td>5’- TTGTGCCCATACGAAAAAG -3’</td>
<td>5’- GTGCACAGTGTGACAATTTAATTGAA -3’</td>
</tr>
<tr>
<td>Mouse E-cadherin</td>
<td>5’-AAGTGACCGATGATGATGCC-3’</td>
<td>5’-CTTCTCTGTCCATCTCAGCG-3’</td>
</tr>
</tbody>
</table>

Gene expression was determined by normalization with the control gene, GAPDH. Each
RT-PCR experiment was performed in triplicate.

2.3 Results

2.3.1 Ephrin reverse signaling mediates mammalian palatal fusion independently of TGF-β3 and
TGF-βR kinase

We previously reported that exogenous ephrin activation causes fusion in chicken palates
without the need for TGF-β3 (San Miguel, Serrano et al. 2011). The chicken palate has long been
used as a convenient and naïve system to examine TGF-β3 signaling because it does not produce
endogenous TGF-β3. However, the chicken palate does not fuse naturally in development. We
therefore asked whether activation of the ephrin signal would also cause fusion of mammalian palates in the absence of their endogenous TGF-β signal. We answered this question in two ways using our mouse palate culture system. We performed these experiments by placing embryonic mouse palatal shelves in contact on a support, and observing MES degradation and fusion over 72 h. After histological processing, each palate was scored for fusion on a one to five scale to generate a mean fusion score (MFS) for anterior, middle, and posterior regions. First, we cultured a set of embryonic mouse palates in the presence of a blocking antibody against TGF-β3 with or without clustered EphB2/Fc protein to activate ephrin-B reverse signaling (preclustering with anti-Fc is necessary to induce signaling, whereas unclustered Eph/Fc acts as a competitive inhibitor of signaling). The use of neutralizing antibodies has long been an accepted way to effectively block TGF-β action in tissue culture (Martinez-Alvarez, O'Kane et al. 1996, Neptune, Frischmeyer et al. 2003) and we chose this method as more practical than generating, culturing, and treating large numbers of TGF-β3 knockout embryos. Second, we cultured another set of palates with a chemical inhibitor of the TGF-βR kinase (SB 431542), again with or without EphB2/Fc. We evaluated the palates for fusion in the anterior, middle, and posterior region of each using a 1–5 scale in which a score of 1 or 2 indicates failure to degrade the MES, while a score of 3 or above indicates significant epithelial degradation and mesenchymal confluence (see Materials and Methods and Figure 2.1A). Control palates in the anti-TGF-β3 experiment fused normally over the three-day time window of these experiments. Fusion was incomplete in the posterior region of these palates (the last part to fuse developmentally), averaging a 3.0 that nevertheless indicates substantial fusion. The anterior and middle regions had average score above 4, signifying near complete fusion (Table 2.1 and Figure 2.1B and C). Antibody treatment abolished MES degradation and palatal fusion such that the epithelial layers in the MES remained almost entirely intact, and no area averaged above a MFS of 2 (Table 2.1 and Figure 2.1B and C). This result validated our use of neutralizing antibody to block TGF-β3 activity. Kinase inhibitor treatments to block TGF-βR kinase-based signaling also abrogated fusion (Table 2.1 and Figure 2.1D and E). Addition of recombinant EphB2/Fc restored
wide-spread seam degradation and largely rescued fusion in antibody-treated palates. Anterior and posterior regions had average scores of 2.6, meaning that, although several of the palates showed significant mesenchymal confluence in these areas, the epithelial layers remained intact on average. However, the middle portion averaged an MFS of 3.9, indicating near complete fusion (Table 2.1 and Figure 2.1B and C). EphB2/Fc addition to inhibitor-treated palates largely rescued fusion in the middle and posterior regions with MFS of 3.8 and 3.0, respectively, but the anterior remained essentially unfused with MFS of 1.9 (Table 2.1 and Figure 2.1D and E). The fact that EphB2/Fc treatment restored fusion in the presence of SB431542 demonstrates that the kinase inhibitor did not impair fusion through non-specific or toxic effects on the tissue. Therefore, activation of ephrin reverse signaling rescued overall palatal fusion in the absence of TGF-β signaling, but the anterior palate was particularly resistant to this rescue.

One concern with our use of clustered EphB2/Fc to activate reverse signaling is that this reagent could also block forward signaling by binding to B ephrins and blocking them from binding

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Anterior</th>
<th>Middle</th>
<th>Posterior</th>
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<tbody>
<tr>
<td>IgG Fc</td>
<td>4.5 ± 0.08</td>
<td>4.6 ± 0.09</td>
<td>3.0 ± 0.24</td>
</tr>
<tr>
<td>IgG + anti-TGF-β3</td>
<td>1.4 ± 0.08</td>
<td>2.0 ± 0.10</td>
<td>1.3 ± 0.23</td>
</tr>
<tr>
<td>EphB2/Fc + anti-TGF-β3</td>
<td>2.6 ± 0.17</td>
<td>3.9 ± 0.11</td>
<td>2.6 ± 0.08</td>
</tr>
<tr>
<td>IgG Fc + SB431542</td>
<td>3.5 ± 0.17</td>
<td>4.7 ± 0.22</td>
<td>3.4 ± 0.10</td>
</tr>
<tr>
<td>EphB2/Fc + SB431542</td>
<td>1.2 ± 0.13</td>
<td>1.7 ± 0.20</td>
<td>1.1 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>1.9 ± 0.08</td>
<td>3.8 ± 0.11</td>
<td>3.0 ± 0.20</td>
</tr>
</tbody>
</table>
to endogenous Eph receptors. Two pieces of data rule this possibility out as an explanation for our results. First, we previously cultured mouse palates with an unclustered EphA4/Fc. This reagent inhibits reverse signaling because it binds B ephrins without activating signaling while acting as a competitive inhibitor to prevent binding of endogenous Ephs. We here with clustered EphB2/Fc. Second, we showed that treatment with clustered ephrin-B2/Fc to activate forward signaling was unable to cause fusion in chicken palates, even though clustered EphB2/Fc did cause fusion (San Miguel, Serrano et al. 2011). Together, these data demonstrate that ephrin reverse signaling is required for mouse palatal fusion and that exogenous activation of this ephrin signal is capable of causing MES degradation and fusion in the absence of a TGF-β signal.

Our results indicate that ephrins are downstream of TGF-β3 in palatal fusion, and so we investigated the possibility that TGF-β3 may simply activate Eph expression in the MEE to cause the fusion signal. Because we know that EphB2, at least, is capable of acting as a ligand to induce fusion, we examined its expression in the palatal MEE in the absence of TGF-β3. We found that EphB2 protein expression levels in the palates of TGF-β3 knock out mice were comparable to those in wild type mice, as assessed by immunohistochemical stain (Figure 2.2A). Further, when we cultured primary palatal MEE cells in the presence of either TGF-β3 or clustered EphB2/Fc, we found that TGF-β3 did appreciably increase EphB2 levels on Western blot (Figure 2.2B). Interestingly, EphB2/Fc did not cause a noticeable increase in TGF-β3 protein, suggesting that the ephrin signal may feedback to stimulate TGF-β-related pathways during fusion. From these data, we conclude that it is likely not the role of TGF-β3 to simply induce EphB expression and thereby initiate fusion.

2.3.2 Ephrin reverse signaling does not cause apoptosis in palatal MEE cells

Many studies support the theory that elimination of the palatal MES occurs by programmed cell death, and that the apoptotic signal comes, at least in part, from TGF-β3 (Glucksmann 1965,

Because ephrins are known to signal apoptosis in other mammalian systems during development...
Figure 2.1. Ephrin reverse signaling induces palatal fusion without TGF-β3. Mouse e13.5 palatal shelves were dissected and grown with their medial edges in contact for 72h in the presence of treatments as indicated. All samples received either EphB2/Fc or IgG Fc protein at 5 µg/mL. Tissues were then fixed, paraffin-embedded and sectioned in the coronal orientation from anterior to posterior for histological analysis. Anterior, medial, and posterior regions were scored for fusion based on our one to five scale (see Materials and Methods). Values shown are mean ± SEM with n = 12 to 18 palates for each group pooled from three independent experiments. (A) Diagram of palate scoring. (B) Control palates were treated with IgG Fc control protein and fused normally, with a slight decrease in posterior score indicative of the incomplete fusion commonly observed in some embryos during the 72 h experimental period (MFS = 4.5 anterior, 4.6 middle, 3.0 posterior). Palates treated with 10 µM anti-TGF-β3 failed to fuse (MFS = 1.4 anterior, 2 middle, 1.3 posterior) and displayed intact MES. Palates treated with anti-TGF-β3 antibody + EphB2/Fc fused substantially better, especially in the middle region, displaying significant MES degradation (MFS = 2.6 anterior, 3.9 middle, 2.6 posterior). (C) Example H&E stained sections from each experimental group in A. (D) Experimental conditions were the same as in A, except that the SB431542 inhibitor of the TGF-βR kinase was used at 25 µM instead of anti-TGF-β3. IgG Fc control palates fused normally (MFS = 3.5 anterior, 4.7 middle, 3.4 posterior), and SB431542 abolished fusion (MFS = 1.3 anterior, 1.7 middle, 1.1 posterior). EphB2/Fc largely rescued fusion in the presence of kinase inhibitor (MFS = 2.0 anterior, 3.8 middle, 3.1 posterior). (E) Example H&E stained sections from each experimental group in C. Differences between antibody or inhibitor treated groups and their corresponding EphB2/Fc treated groups were statistically significant as determined by Mann Whitney U Test (**P < 0.001). Arrows denote midline epithelial cells. Scale bars = 100 µm.
Figure 2.2. TGF-β3 is not required for EphB2 expression. (A) Sections of palates from wild type and TGF-β3 knockout mice were stained with antibody against EphB2. Staining (reddish-brown, DAB) is apparent in the MEE with both genotypes. (B) Mouse palatal MEE cells were grown in the presence of the indicated doses of either 10 μM TGF-β3 or 5 μg/mL EphB2/Fc for 48 h before being harvested for Western analysis with anti-TGF-β3 or anti-EphB2. UN = untreated; IgG = IgG Fc treated control. TGF-β3 treatment did not increase EphB2 levels while EphB2 treatment increased TGF-β3 levels modestly. Thus, the ability of TGF-β3 to cause palatal fusion cannot be explained by simple stimulation of EphB expression.
(Depaepe, Suarez-Gonzalez et al. 2005), we hypothesized that the role of ephrin reverse signaling in the palate is to initiate apoptosis in MEE cells. To test this hypothesis, we cultured primary mouse MEE cells in the presence of EphB2/Fc over 48 h and then looked for apoptosis using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Even though TGF-β3 typically causes widespread cell death in these cultures by 48 h (Ahmed, Liu et al. 2007), we were surprised to find no evidence of apoptosis in EphB2/Fc-treated cells compared to IgG Fc negative controls at either 24 or 48 h (Figure 2.3). To confirm this result, we quantified apoptosis in cultured MEE cells using a fluorescent live/dead assay with fluorescence activated cell sorting (FACS). Treatment with cisplatin over 48 h (as a positive control) activated apoptosis in 30% of cells (Figure 2.4C). By contrast, treatment with clustered EphB2/Fc over the same period generated less than 0.5% apoptotic cells (Figure 2.4B), and no more than in the IgG Fc negative control (Figure 2.4A).

Figure 2.3. Apoptosis assay by TUNEL. MEE cells were isolated from the mouse palatal MES and grown to 80% confluence, then treated with 5 µg/mL IgG Fc (−ve control) or clustered EphB2/Fc. At 24 or 48 h, cells were labeled with anti-tubulin (Alexa Fluor 488, red), avidin-FITC for TUNEL (green), and DAPI for nuclei (blue). IgG Fc-treated MEE showed strong tubulin expression without any TUNEL signal throughout the experiment. EphB2/Fc-treated cells showed no substantial change in Tubulin expression, and very few cells undergoing apoptosis at either 24 or 48 h. Cisplatin treated MEE cells (+ve control) underwent apoptosis within 24 h with reduced tubulin on average, and showing a characteristic rounded and clumped morphology.
Figure 2.4. Apoptosis assay by FACS. Activation of ephrin reverse signaling in does not increase cell death in FACS-sorted MEE cells. MEE cells grown in culture to 80% confluence were treated for 48 h with IgG Fc (−ve control), EphB2/Fc, or cisplatin (+ve control), then incubated with the nucleic acid dyes C12-resazurin (excitation at 488nm) and Sytox Red (at 633nm) to label live and dead cells, respectively, and analyzed by flow cytometry. Live cells are shown in green (Q1). Dead cells are shown in red (Q4). Cell cycle-arrested cells are shown in pink (Q2) and cellular debris in blue (Q3).

(A) 87.7% of IgG Fc-treated cell were alive and viable with only 0.6% intact cells dead. (B) EphB2/Fc treatment showed no difference in the number of dead cells compared to the negative control (86.5% viable and 0.4% dead cells). (C) Cisplatin treatment caused cycle arrest in 19.1% and death in 30.2% of total cells.
2.3.3 Ephrin reverse signaling induces mesenchymal traits in MEE cells

We previously observed some ephrin-B2-expressing MEE cells in the lateral mesenchyme of fusing palates, suggesting that they may have migrated away from the midline (San Miguel, Serrano et al. 2011). This is reminiscent of the report by Jin and Ding that found genetically labeled MEE cells in similar positions and interpreted them as evidence of EMT and migration (Jin and Ding 2006). Other studies have also reported evidence that these cells undergo EMT as part of the mechanism of MES degradation, possibly migrating into the adjacent mesenchyme prior to undergoing apoptosis (Fitchett and Hay 1989, Shuler, Halpern et al. 1992, Nawshad, LaGamba et al. 2004, Jalali, Zhu et al. 2012). In support of this theory, TGF-β3 added to MEE cells in culture causes EMT-like phenotypic changes, cell migration, and gene expression before initiating apoptosis (Ahmed et al., 2007). We therefore used the MEE culture system to test the hypothesis that ephrin reverse signaling causes EMT-like changes in these cells. Cultured MEE cells grown to confluence exhibit the hallmarks of epithelial cells: tightly packed, cuboidal cells joined in a sheet by desmosomes and tight junctions. E-cadherin, desmoplakin, plakoglobin, and zona occludens-1 (ZO-1) are among the proteins that are conspicuously and highly expressed in these epithelia-specific junctions. We observed that the expression of these proteins, while maintained in IgG Fc-treated control cells, was markedly diminished in MEE cells after 24 h of exposure to EphB2/Fc, and largely disappeared by 48 h (Figure 2.5A and B). At the same time, expression of the fibroblast markers fibronectin and vimentin increased in these cells (Figure 2.5B). These data indicate the disassembly of desmosomes and tight junctions in favor of the assembly of the focal adhesions more suited for mobility. Consistent with this shift, EphB2/Fc-treated cells also lost their tight packing over this time period and assumed a looser, mesenchymal shape (Figure 2.5A and B).
Figure 2.5 Ephrin reverse signaling causes EMT-like marker changes in mouse palatal MEE cells.

Embryonic mouse MEE cells were cultured for 48 h in either IgG Fc or EphB2/Fc protein at 5 ng/mL, then fixed and processed for immunofluorescent detection of epithelial or mesenchymal markers. (A) Expression of the epithelia-specific cell junction markers E-cadherin, demoplakin, and plakoglobin (green) virtually disappeared after 48 h of EphB2/Fc treatment. (B) Expression of the mesenchymal markers fibronectin (green) and vimentin (red) increased dramatically after 48 h of EphB2/Fc exposure while expression of epithelia-associated proteins E-cadherin (red) and Z01 (green) essentially disappeared.
We tested whether Eph/B2/Fc treatment caused MEE cells to become more motile, as their marker expression suggested, using a scratch-wound assay. Monolayers of MEE cells were scratched with a pipet to create a cell-free zone, and then treated with EphB2/Fc or control IgG Fc for 48 h. Substantial numbers of EphB2/Fc treated cells moved into the cleared scratch area over this period, whereas control cells moved very little (Figure 2.6A). We quantified the effect of EphB2 on motility with a transwell assay. MEE cells were placed in the transwell chambers and allowed to cross a filter in the presence of either IgG Fc or EphB2/Fc. After 48 h, the number of cells migrating through the filter was six-fold higher in EphB2/Fc cultures over that observed in controls (Figure 2.6B). From our immunofluorescence and scratch-wound data, we concluded that activation of ephrin reverse signaling in MEE cells causes them to assume a phenotype indicative of EMT.

2.3.4 Ephrin reverse signaling induces EMT-associated gene expression in MEE cells

EMT requires a shift in gene expression, and so we examined the levels of some key transcription factors associated with gene expression profile changes in EMT. Both the basic helix-loop-helix (bHLH) transcription factor Snail and the zinc-finger Smad-interacting protein 1 (Sip1) are upregulated during developmental EMT and have been shown to repress E-cadherin expression (Jalali, Zhu et al. 2012). The EMT-associated bHLH factor Twist1 is also upregulated during palatal fusion and plays a role in MES degradation (Yu, Kamara et al. 2008, Yu, Ruest et al. 2009, Micalizzi, Farabaugh et al. 2010). We quantified the mRNA levels of these three genes in MEE cells after 48 hours of EphB2/Fc treatment using real-time PCR. The messages for these genes increased in a dose-dependent manner. Snail mRNA doubled at the 5 µg/mL dose of EphB2/Fc that was used for all of our palate and MEE culture experiments, and Sip1 increased more than five-fold versus control at the same dose. Although Twist1 mRNA increased only
Figure 2.6. Ephrin reverse signaling induces migration of mouse palatal MEE cells. (A)

Embryonic MEE cells were grown to confluence and then scratched with a needle to create a cleared area with uniform borders. The cells were treated with IgG Fc or EphB2/Fc for 48 h. (B) The number of cells that migrated across an 8 μm membrane in a transwell chamber was counted at 24 and 48 h. The change in the number of migrating cells was determined by comparison to control (IgG Fc) and plotted as numbers of migrating cells (mean ± SD.; n = 3; *P < 0.005 compared with controls AP-value of ≤0.05 was considered significant. The one-way ANOVA indicated that the values differ significantly across the treatment groups. All EphB2 treatment (time dependent) differed significantly (*P ≤ 0.005) from the control groups (IgG Fc).
The results in this study, along with our published data (San Miguel, Serrano et al. 2011), show that ephrin reverse signaling is necessary and sufficient to cause mouse palatal fusion, even
in the absence of TGF-β3, a growth factor that was previously considered indispensible for fusion. Further, we show that the ephrin signal causes an EMT-like change in palatal epithelial cells, but does not cause them to undergo apoptosis. Our findings are significant for three reasons. First, the fact that ephrins cause EMT in palatal epithelial cells adds weight to the argument that palatal fusion proceeds through an EMT mechanism. Second, the discovery that ephrin signaling during fusion is separate from, and can supersede, TGF-β3 shifts the focus of intracellular signaling away from purely those pathway intermediates affiliated with the TGF-βR serine/threonine kinase receptor. Third, the association of ephrin reverse signaling with EMT reveals a previously unknown role for ephrins in activation of a gene expression program.

There are two prevailing theories of the mechanism of MES degradation in palatal fusion. One argues that the MEE cells proceed through EMT to achieve mesenchymal confluence in the palate (Shuler, Guo et al. 1991, Shuler, Halpern et al. 1992, Sun, Vanderburg et al. 1998, Kang and Svoboda 2005, Nawshad, Lagamba et al. 2005, Yu, Ruest et al. 2009). The other says that these cells are removed by apoptosis to allow the mesenchyme to join (Glucksmann 1965, Martinez-Alvarez, Tudela et al. 2000, Cuervo and Covarrubias 2004). Both of these views have been supported with strong evidence. Recent data suggest that these theories are not mutually exclusive. Ahmed et al. reported that MEE cells in culture exposed to TGF-β3 undergo EMT, with appropriate changes in morphology and gene expression, followed by apoptosis (Ahmed, Liu et al. 2007). Their studies are consistent with genetic evidence from mouse studies of palatogenesis. Jin and Ding showed that Apaf1 knockout mice, while deficient in apoptosis, developed fused palates, indicating that fusion does not rely on apoptosis alone. However, histological examination revealed that the triangles of epithelial cells normally found at the oral and nasal edges of fusing palates persisted in Apaf1 knockouts, whereas they eventually disappear in wild type animals (Jin and Ding 2006). Ahmed et al observed these same triangles in cultured palates treated with a caspase inhibitor. Of course, caspase-independent apoptosis may still be involved in the process (Dormann and Bauer
On balance however, the data suggest that both EMT and apoptosis combine to remove the MEE cells and complete palatal fusion. Ephrin-B signaling has been shown to induce apoptosis in other systems (Depaepe, Suarez-Gonzalez et al. 2005, Park, Kim et al. 2013), but we were unable to demonstrate such a role in palatal epithelial cells. Our finding that EphB2 treatment both induces fusion and initiates EMT in MEE cells independently of TGF-β3 supports a hypothesis in which ephrin induction of EMT is a part of the fusion mechanism, but perhaps leaves the job of programmed cell death to TGF-β3. Our EphB2 treatments did not completely rescue fusion in TGF-β-blocked palates, and this observation could be explained by an insufficiency of ephrin reverse signaling to activate a specific part of the fusion program, such as a TGF-βR-dependent apoptotic activity that removes remaining MEE cells. Alternatively, it could be that there is a TGF-βR-specific signal (e.g: one that is Smad-associated) that, while not formally required for fusion, combines with the ephrin signal to complete fusion in the observed time window. In either model, ephrin and TGF-β signals would collaborate to complete the fusion process, with some signaling branches in common and some unique to each.

The B ephrin cytodomain contains docking sites for a number of signaling proteins. Conserved tyrosines can be phosphorylated and function as SH2 domain binding sites (Holland, Gale et al. 1996, Brückner, Pasquale et al. 1997). The SH2/SRC3 adaptor protein Grb4/Nckβ was shown to bind to activated ephrin-B1 and signal the disassembly of actin cytoskeletal elements (Cowan and Henkemeyer 2001). The C-terminal end also carries a PDZ domain binding motif (Lu, Sun et al. 2001). Any of these signaling motifs may participate in signaling fusion in the palate. However, the Henkemeyer group demonstrated that mutation in mice of all known conserved ephrin-B2 tyrosines and the PDZ binding domain does not produce cleft palate, even though homozygous deletion of the entire cytodomain in ephrin-B2/LacZ mice does (Dravis and Henkemeyer 2011). This means that ephrin-B2 contains an as yet unidentified signaling domain that is crucial for palatal seam degradation. We previously published that PI3K signaling is required
for ephrin-mediated fusion (San Miguel, Serrano et al. 2011). This pathway has not previously been associated with reverse signaling and represents uncharted territory in the ephrin field. We are focusing our efforts on identification of the ephrin-B domain responsible for the PI3K signal and its binding proteins.

PI3K phosphorylates Akt, which in turn activates mTor complexes to induce cell migration (Gulhati, Bowen et al. 2011). Activation of mTor is associated with carcinoma EMT and metastasis, and so the connection of ephrin-Bs to PI3K provides an explanation for why Eph/ephrin signal activation is so often associated with tumor metastases. The PI3K/Akt/mTor axis also connects to the EMT transcriptional program. The mTor kinase phosphorylates the transcriptional activator Stat3 (Yokogami, Wakisaka et al. 2000, Zhou, Wulfkuhle et al. 2007), which in turn activates expression of Twist1 and Snail as part of the EMT transcriptional program (Yamashita, Miyagi et al. 2004, Qin, Xu et al. 2012), and both Twist1 and Snail are important for palatal fusion (Yu, Kamara et al. 2008, Yu, Zhang et al. 2013). Although ephrin-B reverse signaling was previously shown to associate with both Stat3 and the Groucho repressor of Stat3 (Bong, Lee et al. 2007, Kamata, Bong et al. 2011), very little is known about the potential for reverse signaling to access a gene expression program. The connection of ephrin-B signals to the PI3K pathway in our previous work showed that a connection to transcriptional activation in EMT is plausible. Our data presented here indicate that such a connection exists and is functional during the developmental process of palatal fusion. It also implies that the same connection functions in cases of metastatic EMT, and suggests that ephrin-mediated pathways may be valid targets for cancer therapies.
CHAPTER 3

TRANSFORMING GROWTH FACTOR-B1 ACTIVATES ΔNP63/C-MYC TO
PROMOTE ORAL SQUAMOUS CELL CARCINOMA

3.1 Introduction

The incidence of malignancies of the oral cavity is estimated at 42,000 cases in the United States annually, most of which are oral squamous cell carcinomas (OSCCs) (Siegel, Ma et al. 2014). An increasing body of evidence demonstrates that transforming growth factor (TGF)-β1, acting in concert with its downstream effectors, plays a pivotal role in regulating the initiation and progression of OSCC. To prevent tumorigenesis, during the early stage of tumor development, TGF-β1 acts as a tumor suppressor, relying on its ability to inhibit cell proliferation by inducing cell cycle G1 arrest (Davies, Robinson et al. 2005). However, compared with normal cells, most OSCC cells are defective in their response to inhibiting the TGF-β1 signaling mechanism. This is one of the key mechanisms that promotes the oncogenic development of epithelial-derived tumors (Ichijo, Momose et al. 1990).

TGF-β1 functions in a variety of cellular processes, including cell proliferation, differentiation, apoptosis, epithelial mesenchymal transition (EMT) and invasion, matrix synthesis, and immune responses (Massague and Wotton 2000). TGF-β1 initiates these processes by activating Smad-dependent and/or Smad-independent pathways, such as the phosphoinositide-3 kinase (PI3K) and MAPK signaling pathways (Bakin, Tomlinson et al. 2000, Shi and Massague 2003, Moustakas and Heldin 2005). Paradoxically, TGF-β1 also functions as a key oncogenic promoter during the later stages of epithelial-derived tumors (Paterson, Patel et al. 1995, Lu, Reh et al. 2004). Its prosurvival function can be attributed to Smad-independent pathway effectors, such as PI3K and AKT pathways (Akhurst and Derynck 2001, Muraoka-Cook, Shin et al. 2006).
One of the mechanisms by which TGF-β1 can stimulate differentiation is by inducing p63, located on chromosome 3 q27-29. In fact, p63 was shown to be elevated in up to 80% of OSCC cases (Sniezek, Matheny et al. 2004). The p63 gene encodes two major protein isoforms, TAp63 and ΔNp63, both of which have transactivating and transcriptional repressing activities regulating a wide range of target genes (Koster, Kim et al. 2004). In most basal epithelial cells, the predominant isoforms expressed at the protein level are of the ΔNp63 type and are believed to be master regulators of epithelial differentiation (Candi, Cipollone et al. 2008). Surprisingly, c-Myc can also be activated to induce cell proliferation, which is mediated via the TGF-β/Smad pathway in several malignancies (Frederick, Liberati et al. 2004, Zhu, Ozturk et al. 2012), including OSCC (Paterson, Patel et al. 1995).

Characterization of the response to TGF-β1 in OSCC should be of clinical significance, as elucidating its role may contribute to our understanding with regard to how signaling pathways are switched, ultimately altering the course of OSCC differentiation. On the basis of our results, we conclude that although TGF-β1 has distinct cellular functions, including stimulation of OSCC cell survival or basal cell proliferation and EMT by ΔNp63, the switch to upregulating the cell cycle, therefore supporting cancer growth, is mediated via the induction of c-Myc in UMSCC38 cells.

3.2 Material and Methods

3.2.1 Reagents and antibodies

All the antibodies and reagents were used according to the conditions recommended by the manufacturers or providers and confirmed by us previously (Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012). The sources are listed in Table 3.5.
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3.2.2 Tissues and cell lines

Human normal oral gingival mucosa (control) and OSCC tissue samples were provided by Dr. Peter J. Giannini (University of Nebraska Medical Center). The protocol for the use of human samples was reviewed and approved by the institutional review board (#113-15-NH) and conducted in full compliance with federal regulations and University of Nebraska Medical Center Institutional Review Board policies. We stained 8-µm sections of tumor tissue with hematoxylin and eosin, according to our established protocols (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012). We utilized 10 samples of normal oral gingival mucosa (as control) as well each category of tumor differentiation stage (well-differentiated, moderately differentiated, and poorly differentiated OSCC).

For the human head and neck squamous cell carcinomas (SCC) cell lines, we used six OSCC cell lines established at the University of Michigan (UMSCC10B, UMSCC11B, UMSCC17B, UMSCC23, UMSCC38, and UMSCC74B; generously provided by Dr. Thomas E. Carey, University of Michigan, Ann Arbor, MI). However, only two cell lines (UMSCC38 and UMSCC11B) responded to TGF-β1, and the others did not demonstrate any cellular changes (morphologically, phenotypically, or biochemically). Hence, we chose to focus strictly on the cell lines that responded to dissect the signaling pathway and limit our study to explore how TGF-β1 activates downstream signaling pathways to induce cellular changes (proliferation and EMT). We felt that it would be much more constructive to focus on these two cell lines and investigate their mechanisms in OSCC progression.

UMSCC38 was obtained from OSCC from the primary site of origin, whereas UMSCC11B was obtained from a secondary OSCC resulting from relapse and recurrence at the primary site. The nature and characteristics of these cell lines have been described by Lin et al (Lin, Grandis et al. 2007). Cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO2.
Human oral/laryngeal SCC cell lines were obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) in 2010 and 2011. These cell lines had been previously characterized genetically and morphologically21-28 and have been retested and authenticated in the present study. Table 3.1 from Lin et al (Lin, Grandis et al. 2007) shows genotyping data obtained with 10 common tetranucleotide repeat sequences on 73 of the most commonly used University of Michigan Squamous Cell Carcinoma (UMSCC) head and neck cell lines. Representative photographs from each of the genetically characterized cell lines are also illustrated in their paper (Lin, Grandis et al. 2007), as well as on the University of Michigan Head and Neck Cancer SPORE web page (http://www.med.umich.edu/cancer/hn-spore/) for easy access for other investigators who have these lines in their laboratory.

3.2.3 Immunofluorescence analysis

We stained 8-µm normal oral gingival mucosa and OSCC sections with immunofluorescence, according to our established protocols.(Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012) Sections were mounted with DAPI (4′,6-diamidino-2-phenylindole)-containing Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and imaged with the Nikon A1 confocal system (Nikon Instruments Inc., Melville, NY) on a Nikon 90i upright fluorescence microscope.

2.5 × 104 UMSCC38 and UMSCC11B cells/well were cultured in 10% FBS/DMEM on glass coverslips until they reached 40% to ~60% confluence. Cells were washed with sterilized phosphate-buffered saline (PBS) twice before being applied to a 48-hour serum-free starvation (the synchronization) in DMEM, and then treated with TGF-β1 in 0.2% FBS/DMEM. The subsequent fixation and staining of cells were carried out as previously described by us (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012). Stained cells were mounted with Vectashield mounting medium containing DAPI. All images were obtained on an inverted motorized microscope (Axiovert 200 M, Zeiss, Jena, Germany) using
SlideBook 5.0 image acquisition software (Intelligent Imaging Innovations Inc., Ringsby Ct, Denver). Five microscopic fields were selected randomly (×40 magnification), in which both immunofluorescence- and DAPI-stained cells were counted manually as the number of positive stained cells and total cells, respectively; the ratio of the positively stained cells was then calculated.

3.2.4 Cell proliferations and viability assays

Trypan blue exclusion assay was undertaken to evaluate cell number using trypan blue Solution (Life Technologies, Grand Island, NY) manufacturer protocol. Briefly, UMSCC 38 and 11 B cells were plated onto six-well cell culture plates at 2.5 × 10^4 UMSCC cells/well in 2 mL of culture medium with FBS/DMEM (0.2% and/or 10%) with or without 2, 5, and 10 ng/mL of recombinant TGF-β1 treatment for 24, 48, and 72 hours at 37°C. The cells were harvested by suspension in 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3. Cell counts were performed in triplicates using hemocytometer, with trypan blue exclusion for identification.

We also undertook both MTT and neutral red (NR) assays using manufacturer protocol as detailed in Vybrant MTT cell proliferation assay kit and Neutral Red stain (Life Technologies, Grand Island, NY). Both assays measure metabolic function/activity in mitochondria (MTT) and lysosomes (NR). Briefly, 2.5 × 10^4 UMSCC38 and UMSCC11B cells underwent treatment with TGF-β1 (2, 5, and 10 ng/mL) for 24, 48, and 72 hours (with 0.2% and/or 10% FBS/DMEM). Treatments were terminated with the addition of NR stock solutions to the cells in 96-well microtiter plates for 4 hours at 37°C. For NT, added solution was aspirated and wells were rinsed with 4% formaldehyde containing 1% calcium chloride, followed by 1 mL of glacial acetic acid in 100 mL of 50% ethanol for 15 minutes. For MTT, cell treatments were terminated with 10 µL of MTT solution for 2 hours at 37°C until the purple formazan crystal developed. For both, the comparative analyses of the absorbances were plotted at 550-nm optical density, and for each treatment three replicates were examined.
3.2.5 Western blot analysis

UMSCC cells were cultured in 10% FBS/DMEM until they reached 50% confluence. Then, the medium was replaced with FBS-free DMEM for a 48-hour serum starvation before TGF-β1 treatments. For protein expression of UMSCC cells by Western blot, total protein was extracted. For nuclear and cytoplasmic proteins, we used the nuclear extraction kit from Chemicon Nuclear Extraction Kit (Millipore, MA) as performed previously (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012). The concentrations of the nuclear, cytoplasmic, and total proteins were obtained using NanoDrop 2000c spectrophotometer (ThermoScientific, Rockford, IL). According to the standard Western blot protocol, 30-µg protein samples were electrophoresized in sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed by various antibodies.

The intensity of the band was measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester, NY). To perform a t test analysis of mean intensity measurements, a region of interest analysis was conducted from data converted to Microsoft Excel (Redmond, WA) from the exported “.txt” files. Data points for all samples are paired by spatial arrangement on gel and compared pairwise to minimize the impact of subtle background artifacts on image analysis.

3.2.6 Flow cytometry assay

We cultured 2.5 × 104 UMSCC cells/well in 10% FBS medium until they reached 40% to ∼60% confluence in six-well plates. After serum starvation, cells were cultured in 0.2% FBS/DMEM with recombinant TGF-β1. Cells without TGF-β1 treatment served as a negative control, and those grown in 10% FBS represented a positive control. Cells were then harvested and prepared for subsequent cell cycle analysis, including EdU (5-ethynyl-2′-deoxyuridine) incorporation and PI (propidium iodide) staining, which have been detailed in our published protocols (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012). Flow
cytometry was conducted on a BD FACSArray Bioanalyzer (BD Biosciences, San Jose, CA) and analyzed with FlowJo_V10 software.

3.2.7 Transwell cell migration assay

The cell migration assay was performed three times as described in Nawshad et al (Nawshad, Medici et al. 2007). Using Innocyte Cell Migration Assay (EMD Biosciences, San Diego, CA). For the cell migration assay, UMSCC38 and 11 B cell lines were treated with TGF-β1 (5 ng/mL) for 24 and 48 hours. The endogenous c-Myc and Smad4 were blocked 24 hours before TGF-β1 and continued for an additional 24 and 48 hours in the presence of TGF-β1, with the medium changed every 24 hours. PI3K was blocked 60 minutes before TGF-β1 treatment.

Briefly, 8-µm pore size transwell migration chambers in 96-well plates were used for migration analyses. Both treated and untreated cells were allowed to migrate across the membrane insert toward media in the presence of serum for 48 hours at 37°C (chemotactic migration). Cells that migrated through the membrane and attached to the lower side of the cell culture insert (per field) were subsequently detached using a cell detachment buffer containing Calcein-AM fluorescent dye (excitation max.: 485 nm; emission max.: 520 nm). The mean data of 10 areas were determined using a BD Bioanalyzer fluorescent plate reader (BD Biosciences, San Jose, CA).

3.2.8 Cell treatment

UMSCC38 and UMSCC11B cells were treated with, (1) TGF-β1 (2, 5, and 10 ng/mL) for 6, 12, 24, 36, and 48 hours; (2) 0.2% FBS/DMEM, negative “untreated” (UnTr) control; (3) 10% FBS/DMEM, “positive” control; and (4) inhibition (with short hairpin RNA, and small synthetic chemical inhibitor) and activation (with full-length cDNA) as confirmed by us previously (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012).

To inhibit ΔNp63, c-Myc and Smad4 (Smad dependent pathway), cells were both transiently transfected with small hairpin RNA (shRNA) for ΔNp63 (pRetrosuper-sh-ΔNp63), c-
Myc (pRetrosuper-sh-c-Myc) and Smad4 (pRetrosuper-sh-Smad4) (Addgene, Cambridge, MA), for 24 hours by themselves followed by an additional 24 hours in the presence of TGF-β1 treatment, which specifically targets the coding region of murine ΔNp63, c-Myc, and Smad4. To activate, we transfected cells with ΔNp63 and c-Myc, full-length ΔNp63 (pCMV-Entry-p63; Origene, MD) and c-Myc cDNAs (pcDNA3.3 c-Myc; Addgene, Cambridge, MA) for 24 hours by themselves followed by an additional 24 hours with TGF-β1. The pRetrosuper-sh-ΔNp63, pRetrosuper-sh-c-Myc, pRetrosuper-shSmad4 and pCMV-Entry-p63, pcDNA3.3 c-Myc were transfected into both cells by gently adding solution containing 10 µL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 300 µL of Opti-MEM medium for 24 hours, as described previously (Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012). The Smad-independent pathway via PI3K was blocked by using commercially available small synthetic chemical inhibitors, LY294002 (20 µM) (Cell Signaling Technology, Danvers, MA) for 60 minutes. Following all inhibitions (pRetrosuper-shSmad4 and chemical inhibitors), TGF-β1 (5 ng/mL) was added in the presence of these molecules for another 24 hours before mRNA extraction. All inhibitors/activators were used at the concentration and time points recommended by the respective manufacturer/provider as well as confirmed by us previously (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Jalali, Zhu et al. 2012, Zhu, Ozturk et al. 2012). The medium was changed every 24 hours. Details of these plasmids/constructs are described in Table 3.2.

3.2.9 Real-time quantitative polymerase chain reaction (qRT-PCR)

As described previously (Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012), UMSCC38 and UMSCC11B cells were serum starved for 48 hours, followed by treatment with TGF-β1 (5 ng/mL) for an additional 24 hours. RNA was harvested by using the RNeasy Mini Kit (Qiagen, Alameda, CA) according to the manufacturer's instructions. RNA integrity was assessed using formaldehyde gels in 1×TAE buffer, and RNA purity and concentration were determined by a 260/280 ratio on a Nanodrop 2000 C (ThermoScientific, Chelmsford, MA). The sequences of
primers for cyclins, c-Myc, and GAPDH (detailed in Tables 3.3 and 3.4) were obtained from the Invitrogen online PCR primer design site, and were synthesized at the Molecular Biology Core Facility, UNMC. Gene expression was determined by normalization with the control gene, GAPDH. Each real-time polymerase chain reaction (RT-PCR) experiment was performed in triplicate.

3.2.10 Transfection and luciferase assay

The TGF-β-mediated effect on ΔNp63 and c-Myc promoter activity was measured through firefly luciferase assays in both cell lines as previously shown by us (Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012). UMSCC38 and UMSCC11B cells were transiently transfected with ΔNp63 promoter: ΔNp63 promoter region (pGL3-2 kΔNp63; −1848 to +152), generously provided by Dr. Iyoto Katoh (University of Yamanashi, Japan). We also employed several deletions/mutations of Smad binding sites on the c-Myc promoter, as detailed in Table 3.2. UMSCC38 and UMSCC11B cells were transiently transfected with pBV-Luc plasmid harboring the c-Myc promoter (2500 bp; pBV-Luc-Myc-WT plasmid) or derivative plasmids containing mutations in TBE1 (pBV-Luc-TBE1 mut) or TBE2 (pBV-Luc-TBE2 mut) or both sites (pBV-Luc-TBE1/2 mut) (obtained from Addgene, Cambridge, MA). Additionally, we used c-Myc promoter region deletion constructs: PBV-Luc-DEL1 (WT, with intact TBE1, TBE2, and TIE sites), pBV-Luc-DEL3 (without TBE1, but with intact TBE2 and TIE), pBV-Luc-DEL4 (without TBE1 and TBE2 but with intact TIE) also obtained from Addgene (Cambridge, MA). All these plasmids have been used by us previously and details of c-Myc luciferase protocol have been reported previously (Ahmed, Liu et al. 2007, Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012).

For the pGL3-2 kΔNp63 luciferase assay, we followed the protocol provided by Dr. Katoh (Fukunishi, Katoh et al. 2010). Lipofectamine 2000 transfection reagent was used according to the manufacturer's protocol. Transfection of UMSCC38 and UMSCC11B cells with pGL3-empty vector and pBV-Luc vector without the c-Myc promoter region (empty vectors) were performed as
negative controls. Recombinant TGF-β1 (5 ng/mL) was added to the transfected cells for 24 hours, and luciferase activity was detected using the Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). Results are reported as mean ± SD of at least three independent experiments.

3.2.11 Chromatin immunoprecipitation assay

As described previously (Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012), equal quantities (4 × 10^7 cells) of the untreated control (0.2% FBS), TGF-β1 (5 ng/mL) stimulated cells

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<td>LY294002 (20µM)</td>
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<td>Other Reagents</td>
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<tr>
<td>Fetal Bovine Serum (FBS) (#SH30910.03)</td>
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<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM) (#10-013-CV)</td>
<td>Corning Cellgro, Manassas, VA</td>
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<td>Penicillin-Streptomycin Solution, 50X (#30-001-Cl)</td>
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<tr>
<td>Trypsin 0.25% (1x) Solution (#SH30042.01)</td>
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<tr>
<td>Recombinant Human TGF-β1 (rhTGFβ1) (#240-B)</td>
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<td>Protease Inhibitor Cocktail, 50x (#G6521)</td>
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<tr>
<td>DC Protein Assay Kit I (#500-0111)</td>
<td>Bio-Rad Laboratories, Hercules, CA</td>
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<tr>
<td>Immobilon-P Membrane (#IPVH00010)</td>
<td>Millipore, Bellerica, MA</td>
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<tr>
<td>Vectashield mounting medium containing DAPI (#H-1200)</td>
<td>Vector Laboratories, Burlingame, CA</td>
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Table 3.2. List of all shRNA, plasmids, constructs and other reagents used in experiments
Table 3.3. List and sequences of primers for cyclin D, E, A, B and GAPDH

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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CyclinD</td>
<td>5’-TTTCTTGTAGCGGCTGTTGT-3’</td>
<td>5’-CGGAACACTAGAACAATCCAAGATGCAAATG-3’</td>
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<tr>
<td>CyclinE</td>
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<td>5’-TTGATAGACATAGCAGAGACATGCTG-3’</td>
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<td>CyclinA</td>
<td>5’-CTGTAAGATTCCTCCGGGCTCCTTG-3’</td>
<td>5’-GTAAGGCGAGGAGCCGCGAG-3’</td>
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<td>CyclinB</td>
<td>5’-GTTAGGGTGTCTTCTCGAATCGG-3’</td>
<td>5’-TTCTCGTGAATTCCTCTGTGTTCT-3’</td>
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<td>GAPDH</td>
<td>5’-ACATCGCTCAGACACCAGT-3’</td>
<td>5’-TGATGTTGAGGTCAATGAAGGG-3’</td>
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Table 3.4. List and sequences of primers to mutate Smad binding sites on c-Myc promoter

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<th>Site</th>
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<th>Reverse</th>
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<tr>
<td>Human c-Myc (TBE1)</td>
<td>5’-TCTCCACTTGCCCCTTTTA-3’</td>
<td>5’-CGGAGTTCCCAATTTCAG-3’</td>
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<tr>
<td>Human c-Myc (TBE2)</td>
<td>5’-GCGCCCATATTACCCTTC-3’</td>
<td>5’-GAAAGGCGCGCTTTT-3’</td>
</tr>
<tr>
<td>Human c-Myc (TIE)</td>
<td>5’-CTTTATAATGCGGAGGTCTTGACG-3’</td>
<td>5’-GCTATGGGCAAAGGTTCGTTGGATG-3’</td>
</tr>
<tr>
<td>ShRNAs/cDNAs/Plasmids/Other Reagents</td>
<td>Source</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------------------------------</td>
<td>----------------------------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>Small hairpin RNAs (ShRNAs)</td>
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<tr>
<td>ShRNA c-Myc (pRetrosuper-sh-c-Myc)</td>
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<tr>
<td>ShRNA Smad4 (pRetrosuper-shSmad4)</td>
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<tr>
<td>Full length cDNAs</td>
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<tr>
<td>c-Myc (pcDNA3.3 c-Myc)</td>
<td>Addgene</td>
<td></td>
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<tr>
<td>Plasmids/Constructs</td>
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<tr>
<td>TBE1 mutations: (pBV-Luc-TBE1mut)</td>
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<tr>
<td>TBE2 mutation: (pBV-Luc-TBE2mut)</td>
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<tr>
<td>TBE1 and 2 mutations (pBV-Luc-TBE1/2mut)</td>
<td>Addgene</td>
<td></td>
</tr>
<tr>
<td>WT c-Myc promoter pBV-Luc-DEL1 (WT, with intact TBE1, TBE2 and TIE sites)</td>
<td>Cambridge, MA</td>
<td></td>
</tr>
<tr>
<td>TBE1 deletion: pBV-Luc-DEL3 (without TBE1, but with intact TBE2 and TIE)</td>
<td>Cambridge, MA</td>
<td></td>
</tr>
<tr>
<td>TBE1 and 2 deletion: pBV-Luc-DEL4 (without TBE1 and TBE2 but with intact TIE)</td>
<td>Cambridge, MA</td>
<td></td>
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<tr>
<td>ΔNp63 promoter region (pGL3-2kΔNp63; -1848 to +152)</td>
<td>Dr. Iyoto Katoh</td>
<td>(Fukunishi, Katoh et al. 2010)</td>
</tr>
<tr>
<td>PI3 kinase inhibitor</td>
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in 80% to 90% monolayers were fixed with 1% formaldehyde for 10 minutes at real time for chromatin cross-linking. The reaction was stopped by adding glycine to a final concentration of 0.125 M, and the cells were immediately washed twice with ice-cold PBS and harvested by scraping in ice-cold 1×PBS+PMSF. The Simple ChIP Enzymatic Chromatin IP kit (Magnetic beads) (Cell Signaling Technology) was used according to the method successfully used by us previously (Iordanskaia and Nawshad 2011, Zhu, Ozturk et al. 2012).

Briefly, cross-linked chromatin was isolated from the lysates by sonication three times for 6 seconds each, using the Microson XL 2000 sonicator. Immune precipitation of the cross-linked chromatin with rabbit anti-Smad4 antibody (Cell Signaling Technology) was subsequently performed. For negative control, rabbit IgG (Cell Signaling Technology) and for positive control, Histone H3 antibody were used. The 100-µL chromatin samples in binding buffer from the Chromatin IP kit (Cell Signaling Technology), containing 15-µg chromatin DNA were incubated overnight with antibodies at 4°C. The subsequent binding with magnetic beads, immunocomplex washing, and DNA extraction were carried out following the manufacturer's protocol. Purified DNA was analyzed by polymerase chain reaction (PCR with primers specific for Smad4-binding elements in the c-Myc promoter. The primer design is detailed in Table 3.4.

Resulting DNA fragments were detected by electrophoresis in 1.5% agarose gel. To quantitatively analyze Smad4 binding with three potential Smad binding sites in c-Myc promoter following TGF-β1 treatment, RT-PCR analysis of DNA, purified from the immunocomplexes with anti-Smad4 antibody, was performed. DNA samples were subjected to quantitation by RT-PCR using RealMasterMix Sybr Rox (5 Prime, Hamburg, Germany). Reactions were performed in a 20-µL volume, according to the manufacturer's recommendations. The mean ± SD obtained from three independent experiments were compared.
3.2.12 Statistical analysis

Data from at least three replicates for each parameter were evaluated and analyzed for significance by SPSS version 14.0 (SPSS Inc., Chicago, IL), and one-way analysis of variance was conducted. qRT-PCR data were analyzed by two-way analysis of variance (OriginPro v. 8.0) and Student's t test (Microsoft Excel). The significance level was set as 0.05. A P value of ≤0.05 was considered significant.

3.3 Results

3.3.1 Characterization of proliferation status of various grades of OSCCs

To assess the actively cycling status of OSCC, proliferating cell nuclear antigen (PCNA), Ki67, cyclin A, ΔNp63, and TGF-β1 were immunofluorescence stained and compared against E-cadherin (Figure 3.1, green) to demonstrate the epithelial architecture, in addition to cytokeratin 14 (Figure 3.3, green; readers are referred to cytokeratin 14 data [which is related to E-Cadherin], please see Figs 3.2, 3.4 and 3.5) to reveal that the malignant epithelial tissue in the extracellular matrix (ECM) is of basal epithelial origin.

In the gingival mucosa, PCNA expression was confined to cell nuclei within the basal, parabasal, and suprabasal layers (see Figure 3.1, red, A), Ki67 was limited to mostly basal and few parabasal epithelial nuclei (see Figure 3.1, red, E), cyclin A expressed in epithelial cells and ECM (see Figure 3.1, red, I), while ΔNp63 revealed nuclear positivity in basal and parabasal keratinocytes (see Figure 3.1, red, M). Their expression showed a very similar pattern in well-differentiated and moderately differentiated OSCC, demonstrating positivity in basal, parabasal, and/or suprabasal layers of the tumor nests. However, their expression decreased in cells toward the center of the tumor nest (see Figure 3.1, red, B-O). Nevertheless, in the tumor nest of poorly differentiated OSCC, where the cancer cells lost their epithelial features, the markers were expressed in only a small portion of cancer cells (see Figure 3.1, red, D, H, L, and P), showing a significantly decreased
Figure 3.1 Immunofluorescence stain of PCNA, Ki67, cyclin A, ΔNp63, and TGF-β1. Different grades of oral squamous cell carcinoma (OSCC) express proliferating cell nuclear antigen (PCNA), Ki67, cyclin A, ΔNp63, and transforming growth factor-β1 (TGF-β1) in comparison to E-cadherin. 

(A, E, I, M): Proteins expression in normal oral gingival mucosa (red): PCNA, Ki67, and ΔNp63 revealed nuclear positivity staining in epithelia cells, and cyclin A expressed in both the epithelial cells and extracellular matrix (ECM). 

(B, C, F, G, J, K, N, O): Proteins expression in well- and moderately differentiated OSCC (red): All of them showed a very similar pattern as they were in normal oral gingival mucosa, but their expression decreased in the cells toward the center of the tumor nest. 

(D, H, L, P): Proteins expression in poorly differentiated OSCC (red): In the tumor nest, these proteins expressed only in a small portion of cancer cells (red), showing a significantly decreased expression fashion except for Ki67. 

(Q-T): TGF-β1 expression in OSCC (red): TGF-β1 showed both nuclear and cytoplasmic staining in the epithelium as well as in the ECM (red). 

(A-T): E-cadherin expression in OSCC (green): E-cadherin expression was strictly within the epithelial membrane of the squamous layers, except in poorly differentiated OSCC, it was reduced and discontinuous (green, D, H, L, P, and T). In all slides, nuclei were stained with DAPI (blue). Scale Bar at 400 µm (p) for low magnification; 100 µm (P, inset) for higher magnification.
Figure 3.2. Histological analysis of normal oral gingival mucosa and cancer tissues from well-, moderately- and poorly-differentiated OSCC patients. All the tissue samples were stained with hematoxylin and eosin (H&E) for histopathological examination. A, The oral epithelium consists of a basal layer (stratum basale, SB), a spinous layer (stratum spinosum, SS), a granular layer (stratum granulosum, SG) and a cornified layer (stratum corneum, SC). B, The neoplastic squamous cells are still similar to the normal squamous cells, but are less orderly. This is a well-differentiated squamous cell carcinoma, and the keratin pearl (KP) is surrounding with several layers of neoplastic squamous cells. C, The cells in the cancer nest are still stratified, but not orderly in moderately differentiated OSCC. D, In poorly differentiated OSCC, it is obviously that the tumor cells are throughout the full thickness of the tumor nest and random arranged.
Figure 3.3. Different grades of OSCC express Ki67, cyclin A, ΔNp63 and TGFβ1 in comparison to cytokeratin14. When, cytokeratin14 (green), a specific marker for basal keratinocytes 46 were used in comparison, PCNA, Ki67, cyclin A, ΔNp63, and TGFβ1 expression (red, A-P) were consistent with those shown in Fig 3.1A. Cytokeratin14 expressed in all epithelial cells (expressing E-Cadherin in Figure 3.1A). In all slides, nuclei were stained with DAPI (blue). Scale Bar at 400µm (P) for low magnification; 100µm (P, inset) for higher magnification.
Figure 3.4. Different grades of OSCC express PCNA, Ki67, cyclin A, ΔNp63 and TGFβ1 in comparison to E-Cadherin in higher magnification as shown in Figure 3.1 insets.
Figure 3.5. Westernblot assay of expression of ΔNp63 and c-Myc in UMSCC38 cells. A, Westernblot was performed on homogenous UMSCC38 cells that were treated with 10% FBS (positive control), small hairpin RNA (shRNA) for ΔNp63 and c-Myc as well as full length cDNA for ΔNp63 and c-Myc (Addgene, MA) for 48 h to inhibit and activate ΔNp63 and c-Myc respectively. B, To abolish the effect of endogenous Smad4 and PI3K, westernblot was performed on homogenous UMSCC38 and 11B cells treated with pRetrosuper-shSmad4 for 48 h and small synthetic chemical inhibitors, LY294002 for 60 min, respectively. All short hairpins (sh) target the coding region of ΔNp63, c-Myc and Smad4. Empty vector and scrambled shRNA vector were also used as negative controls. All treatment showed significant loss of the targeted protein. Intensity of the band was measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester, NY).
expression profile. TGF-β1 revealed both nuclear and cytoplasmic expression in the epithelium as well as in the ECM (see Figure 3.1, red, Q-T).

In well-differentiated and moderately differentiated OSCC, E-cadherin (green) expression was strictly confined within the epithelial membrane of the squamous layers. However, in poorly differentiated OSCC, its expression was noticeably reduced, demonstrating a discontinuous pattern (see Figure 3.1, green, D, H, L, P, and T), whereas cancer cells dispersed throughout the full thickness of the tumor nest revealed loss of characteristic epithelial features. Our results suggest that in early-stage OSCC, there is preservation of normal epithelial architecture and morphology, which, however, were lost as oral carcinomas progressed to the later stages (see Figure 3.1A, E, I, M, and Q). The expression pattern of these proteins was similar, indicating a generalized, high-proliferative activity in the epithelia. Cytokeratin14 showed strong cytoplasmic expression in all epithelial cells, further confirming that the epithelial cells invading in the ECM originate from the squamous epithelial lining (see Figure 3.3).

3.3.2 TGF-β1 effects on the population of UMSCC cells expressing proliferation proteins

UMSCC cells were assessed for their cell proliferation status by evaluating PCNA, Ki67, cyclin E2, and ΔNp63 in response to TGF-β1 treatment. Our results demonstrate that TGF-β1 exerts both dose-dependent and time-dependent inductive effects on the numbers of cells positively stained with these key proliferation effectors in both cell lines up until 24 hours (Figures 3.6A and 3.6B). However, unlike the increased cell proliferation at 48 hours in UMSCC38 (see Figures 3.6A, f-h; and Figure 3.6C), UMSCC11B resulted in two distinctive observations: (1) The numbers of UMSCC11B cells positively stained decreased sharply; (2) most UMSCC11B cells lost their cobblestone morphology, developing an elongated, fibroblast-like morphology, implying that they were undergoing
Figure 3.6. Transforming growth factor-β1 (TGF-β1) regulates proliferating cell nuclear antigen (PCNA), Ki67, cyclin E2, ΔNp63, and E-cadherin expression in UMSCC38 cells (A). Column (a): PCNA, Ki67, cyclin E2, and ΔNp63 revealed negative staining in untreated UMSCC38 cells. Column (b): All the cells grown in 10% FBS/DMEM (positive control) were nuclear positive stained by PCNA, Ki67, cyclin E2, or ΔNp63. Columns (c-h): PCNA, Ki67, cyclin E2, and ΔNp63 expression in TGF-β1-treated cells. The number of nuclei positively stained cells increased in a time and dose-dependent manner, compared with untreated groups. Furthermore, as indicated by E-cadherin staining along the cell membrane (red), the cobblestone morphology was preserved in all treated UMSCC38 cells. TGF-β1 regulates PCNA, Ki67, cyclin E2, ΔNp63, and E-cadherin expression in UMSCC11B cells. Columns (a-b): PCNA, Ki67, cyclin E2, and ΔNp63 expressed very similarly as that in UMSCC38 untreated and positive control cells. Columns (c-e): 24 h TGFβ1 treatment increased the number and intensity of PCNA, Ki67, cyclin E2, and ΔNp63 (green) expression in UMSCC11B cells (B) compared to untreated control groups. Also, the expression pattern of the epithelial marker, E-cadherin (red), was concentrated on the cell membrane, forming a continuous membranous, similar to that in the untreated cells. Columns (f-h): When treated with TGF-β1 for 48 hours, the number of positive cells decreased significantly, and the membranous expression of E-cadherin (red) was reduced and interrupted thereafter. (C): Percentage of immunofluorescence positively stained UMSCC38 and UMSCC11B cells. For both UMSCC38 and UMSCC11B cells, only very limited untreated cells express PCNA, Ki67, cyclin E2, or ΔNp63. (a-d): Percentage of positively stained UMSCC38 cells for PCNA (a), ΔNp63 (b), Ki67 (c), and cyclin E2 (d). Over 90% of UMSCC38 cells grown in 10% fetal bovine serum (FBS) were positively stained for these proteins. When cells were treated with TGF-β1 for 24 hours, the percentages increased as the dilutions of TGF-β1 were raised. At 48 hours, the percentages raised to very similar level with that of positive control (10% FBS) groups. (e-h): Percentage of positively stained UMSCC11B cells for PCNA (e), ΔNp63 (f), Ki67 (g), and cyclin E2 (h). Almost 95% of cells grown in 10% FBS were positive stained for PCNA, ΔNp63, Ki67, and cyclin E2. When cells were treated with TGF-β1 for 24 hours, the percentages increased obviously as the dilutions of TGF-β1 were raised. But at 48 hours, the percentages of positively stained cells reduced obviously, very similar to that of untreated cells.
EMT (see Figure 3.6B, f-h). These data indicate that TGF-β1 is a facilitator of cell proliferation in UMSCC38, but to a lesser degree in UMSCC11B, inducing EMT-like changes instead.

The number of cells expressing those proteins in both cell lines is plotted in Figure 3.6C, confirming the protein expression findings and pattern in Figures 3.6A and 3.6B. The actual numerical value is shown in Table 3.6.

3.3.3 TGF-β1 effects on DNA synthesis rate of UMSCC cell lines

To investigate the role of TGF-β1 in UMSCC cell cycle and proliferation, we used the EdU incorporation assay to assess the ability of TGF-β1 to promote DNA synthesis in those cell lines. Only EdU positive cells in S phase are shown in Figures 3.7A and 3.7B.

For UMSCC38 cells, uptake of EdU, indicative of new DNA synthesis and cell proliferation, increased in a time-dependent and dose-dependent fashion by TGF-β1 treatment compared with untreated cells. Only 4.43% of untreated UMSCC38 cells were incorporated with EdU, but for positive control groups of 6, 12, 24, 36, and 48 hours, the percentages were 19%, 30.2%, 43.7%, 53.4%, and 75.1%, respectively. All TGF-β1 treated groups differed significantly from control groups; the difference was also significant between the positive controls at different time points. For 2 ng/mL TGF-β1 treated cells, percentages of cells incorporated with EdU were 8.76, 13.23, 13.7, 15.5, and 23.2 for 6, 12, 24, 36, and 48 hours, respectively. The percentages were 12.2%, 17.9%, 21.1%, 23.7%, and 31.8% for 5 ng/mL TGF-β1 treated cells, and 9.39%, 13.7%, 18.7%, 20.6%, and 24.3% for 10 ng/mL TGF-β1 groups, respectively.

Our data demonstrate that the percentage of EdU incorporation increased upon TGF-β1 treatment in a dose-dependent and time-dependent manner. It is obvious that the percentage of S phase cells increase chronologically. Among TGF-β1 treatment groups,
Table 3.6. List of the percentages of positively IF stained UMSC38 and UMSCC11B cells

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<tr>
<th></th>
<th>0 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
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<tbody>
<tr>
<td></td>
<td>Un treated</td>
<td>Un treated</td>
<td>10% FBS</td>
</tr>
<tr>
<td>PCNA</td>
<td>9.0 9</td>
<td>8.4 6</td>
<td>95.8 9</td>
</tr>
<tr>
<td>ΔNp63</td>
<td>12. 46</td>
<td>10. 0</td>
<td>79.8 5</td>
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<td>Ki67</td>
<td>11. 67</td>
<td>9.2 4</td>
<td>100. 0</td>
</tr>
<tr>
<td>Cyclin E2</td>
<td>16. 77</td>
<td>14. 04</td>
<td>100. 0</td>
</tr>
<tr>
<td>PCNA</td>
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<td>17. 78</td>
<td>94.0 5</td>
</tr>
<tr>
<td>ΔNp63</td>
<td>13. 11</td>
<td>8.3 3</td>
<td>91.2 4</td>
</tr>
<tr>
<td>Ki67</td>
<td>13. 5</td>
<td>10. 81</td>
<td>96.3 3</td>
</tr>
<tr>
<td>Cyclin E2</td>
<td>6.3 6</td>
<td>5.3 2</td>
<td>97.0 6</td>
</tr>
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</table>
(i) Untreated, +10% FBS, 24h, +10% FBS, 48h, +5ng/ml TGFβ1, 12h, +5ng/ml TGFβ1, 24h, +5ng/ml TGFβ1, 36h, +5ng/ml TGFβ1, 48h

(ii) % UMSCC 11B cells in different phases of cell cycle progression

- Untreated
- 10% FBS, 24h
- 10% FBS, 48h
- 5ng/ml TGFβ1, 6h
- 5ng/ml TGFβ1, 12h
- 5ng/ml TGFβ1, 24h
- 5ng/ml TGFβ1, 36h
- 5ng/ml TGFβ1, 48h
**Figure 3.7. EdU incorporation assay in UMSCC cells and quantification.** A, The effect of transforming growth factor-β1 (TGF-β1) on DNA synthesis in UMSCC38 cells. (i) Using BD Biosciences FACSArray, a significant proportion of cells were found to occupy distinct cell cycle phases, including G0/G1, S, and G2/M, within the selected boxed region. Larger boxed region identifies all proliferating cells as determined by their levels of DNA synthesis as assessed by PI within the G0/G1, S, and G2/M phases of the cycle. The smaller inset box includes the cells that are incorporated with EdU or S phase cells. Only results of 5 ng/mL TGF-β1 treatment groups and control groups were shown in this figure. (ii) In the untreated (0.2% fetal bovine serum [FBS]) control group, few untreated UMSCC38 cells were incorporated with EdU. The percentages were raised in a time-dependent manner when cells were grown in 10% FBS (positive control). All the TGF-β1 treatment groups differed significantly from the control groups, whereas between the treated cells at different time points, the difference was also significant. Among TGF-β1 treatment groups, 5 ng/mL TGF-β1 had the greatest effect on DNA synthesis in UMSCC38 cells. A P value of ≤ .05 was considered significant (as indicated by *). The one-way analysis of variance (ANOVA) indicated that the values differ significantly across the treatment groups. Here, we only show the EdU positive cells in the S phase. (iii) In response to TGF-β1, UMSCC38 cells showed progressive increased in the number of cells in the span of 0 to 72 hours compared with negative control, untreated cells as determined by healthy log phase culture with 0.4% trypan blue stain. All the TGF-β1 treatment groups differed significantly from the control groups, whereas between the treated cells at different time points, the difference was also significant. Among TGF-β1 treatment groups, 5 ng/mL TGF-β1 had the greatest effect on cell proliferation, as determined by cell number in UMSCC38 cells. A P value ≤ .05 and ≤ .005 were considered significant (as indicated by * and **, respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups. (iv) Similarly, both MTT (mitochondria) and NT (lysosome) metabolic function that determines cell viability and proliferation shown to be increased progressively in UMSCC38 cells in 0 to 72 hours compared with negative control, untreated cells, in response to TGF-β1.
All the TGF-β1 treatment groups differed significantly from the control groups, whereas between the treated cells at different time points, the difference was also significant. A $P$ value $\leq .05$ and $\leq .005$ were considered significant (as indicated by * and **, respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups. 

**B.** The effect of TGF-β1 on DNA synthesis in UMSCC11B cells. (i) In UMSCC11B cells, TGF-β1 treated for 6, 12, and 24 hours increased EdU incorporation compared with the untreated group, but thereafter, EdU incorporation was reduced, and there was no significant difference between them and the untreated control groups. Only results of the 5 ng/mL TGF-β1 treatment groups and control groups are shown in this figure. (ii) For untreated cells (0.2% FBS), few were in the S phase. However, 10.4% to 42% UMSCC11B cells were EdU positive when cultured in 10% FBS (positive control) for 6 to 48 hours. When cells were treated with TGF-β1, at the very beginning, the portion of S-phase cells increased to some extent, but when treated for a longer time, this portion decreased sharply, showing no significant difference from untreated cells. All the TGF-β1 treatment groups differed significantly from positive controls, and between the positive controls, the difference was also significant. Also, cells treated with 5 ng/mL of TGF-β1 showed the greatest effect, compared with 2 and 10 ng/mL TGF-β1 treatment groups. A $P$ value of $\leq .05$ was considered significant (as indicated by *), and $P$ value $\leq .005$ is indicated by **. (iii) In response to TGF-β1, UMSCC11B cells showed increased in the number of cells in the first 24 hours, followed by cessation of cell proliferation, compared with negative control, untreated cells as determined by opacity density of MTT/NT. But the progression ceased following 24 hours of TGF-β1 treatment. The treatment groups differed significantly from the control groups, and between the treated cells at different time points, the difference was also significant. A $P$ value $\leq .05$ and $\leq .005$ were considered significant (as indicated by * and **, respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups. (iv) MTT/NT assays show increased in cell viability by all the TGF-β1 treatment groups that differed significantly from the control groups, and between the treated cells at different time point, the difference was also significant.
A $P$ value $\leq .05$ and $\leq .005$ were considered significant (as indicated by * and **, respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups. C, Induction of cell cycle progression by TGF-β1 in UMSCC38 cells. (i) DNA content and cell cycle distribution of UMSCC38 cells treated with TGF-β1. Only results of the 5 ng/mL TGFβ1–treated cells and control groups are shown. Cells cultured in 10% FBS for 24 and 48 hours were set as positive control. TGF-β1 treatment for 6, 12, 24, 36, and 48 hours enhanced UMSCC38 cell cycle progression, as shown by the reduction in the number of G₀/G₁ phase cells, with a subsequent increase in the number of cells in the S and G₂/M phases compared with the untreated control group (0.2% FBS). (ii) The percentage of UMSCC38 cells in each phase of cell cycle. Only data from the 5 ng/mL TGF-β1 treatment groups and control groups are shown in this figure. TGF-β1 treatment induced progression through the cell cycle, and the induction was time dependent. Also, there was significant difference on the cell cycle stages among all the TGF-β1 treatment groups. The one-way ANOVA indicated that the values differed significantly across the treatment groups. A $P$ value of $\leq .05$ was considered significant (as indicated by *), and $P$ value $\leq .005$ is indicated by **. D, Induction of cell cycle progression by TGF-β1 in UMSCC11B cells. (i) DNA content and cell cycle distribution of UMSCC11B cells treated with TGF-β1 (5 ng/mL) for 0, 6, 12, 24, 36, and 48 hours were assessed by PI staining. Only the data of cells treated with 5 ng/mL TGF-β1 and the control groups are shown in this figure. TGF-β1 treatment did not enhance UMSCC11B cell cycle progression, as shown by no obvious changes in the number of cells in different phases, compared with the untreated groups (0.2% FBS). (ii) The percentage of UMSCC11B cells in each phase of the cell cycle. TGF-β1 treatment did not induce progression through the cell cycle. The percentages of UMSCC11B cells in each cell cycle stage for TGF-β1 treatment groups differed significantly from the positive control (10% FBS for 24 and 48 hours) groups. However, there was no significant difference on the percentage of cells in the G₀/G₁ and G₂/M phases among different TGF-β1 treatment groups.
After TGF-β1 treated for 6, 12, and 24 hours, the amount of cells in the S and G₂/M phases increased significantly compared with those in untreated groups, whereas for cells treated for 36 and 48 hours, the ratio of S-phase cells decreased obviously, similar to that of untreated groups. The one-way ANOVA indicated that the values had no significant difference among the treatment groups. A P value of ≤0.05 was considered significant (as indicated by *).

5 ng/mL TGF-β1 had the greatest effect on DNA synthesis in UMSCC38 cells (see Figure 3.7A, ii). Similarly, our data also confirmed that UMSCC 38 cells are progressively proliferating as detected by trypan blue assay, clearly demonstrating an increase in cell counts by the TGF-β1 treatment groups (see Figure 3.7A, iii). The treated cells were viable as confirmed by MTT/NT ratio (see Figure 3.7A, iv).

However, for UMSCC11B cells, TGF-β1 treatment did not stimulate DNA synthesis progression in a similar manner as that for UMSCC38 cells. Of the untreated cells, 3.33% were in S phase, whereas 10.4%, 17.4%, 30.6%, 38%, and 42% of positive control UMSCC11B cells were EdU positive for 6, 12, 24, 36, and 48 hours, respectively. As for cells treated with TGF-β1 for 6, 12, and 24 hours, there were 7.42%, 6.44%, and 5% of cells positive for EdU in the 2 ng/mL TGF-β1 treatment group; 8.37%, 6.41%, and 5.87% of EdU positive cells in the 5 ng/mL groups; and 8.21%, 8.54%, and 4.63% of cells were in S phase in the 10 ng/mL group. However, when cells were treated for a longer time, the portion of S phase cells decreased sharply. The percentages of EdU-positive cells in 2, 5, and 10 ng/mL TGF-β1 treatment groups were 4.79, 2.39, and 4.05 at 36 hours and 3.74, 3.34, and 9.25 at 48 hours, respectively, similar to what was observed with untreated cells. All TGF-β1 treatment groups differed significantly from positive control groups, whereas among them, the difference was also significant at different time points. Similarly, UMSCC38 cells treated with 5 ng/mL TGF-β1 showed the greatest effect compared with the other groups. In agreement with EdU incorporation results, our data confirmed that unlike UMSCC 38 cells, UMSCC11B cells were not progressively proliferating beyond 24 hours, as detected by the
trypan blue assay, clearly demonstrating an absence of persistent growth as indicated by cell counts obtained from different TGF-β1 treatment groups (see Figure 3.7B, iii) after 24 hours. However, these treated cells were viable, as confirmed by the MTT/NT ratio (see Figure 3.7B, iv) after 24 hours.

The seemingly unintuitive results can be attributed to the dichotomous nature of the TGF-β1 cytokine, which is known to exert multiple cellular outcomes depending on dose and time. Our results suggest that TGF-β1 promotes DNA synthesis in UMSCC38 cells and acts as a potent oncogenic factor (see Figure 3.7A). In UMSCC11B cells, TGF-β1 treatment resulted in a negligible increase in EdU incorporation compared with controls. TGF-β1 seemed to moderately promote DNA synthesis in the initial 24 hours only, after which, it lost its proproliferative effect for prolonged treatments beyond 24 hours (see Figure 3.7B). These data suggest that UMSCC11B cells demonstrate significantly less proliferative activity and are largely refractory to the pro-proliferative TGF-β1 signaling compared with their counterpart UMSCC38 cell.

3.3.4 Effect of TGF-β1 on cell cycle progression in UMSCC cell lines

To demonstrate the effect of TGF-β1 on distribution of cell cycle phases, synchronized UMSCC38 and 11 B cells were measured for DNA content with PI.

TGF-β1 treatment induced progression through the cell cycle of UMSCC38 cells, and the induction was time dependent. The percentages of untreated cells in the G0/G1, S, and G2/M phases were 86.3, 5.41, and 5.81, respectively. However, for positive control cells, there were 46.5%, 43.7%, and 8.01% of cells in three phases at 24 hours, whereas at 48 hours, 17%, 75.1%, and 2.35% of cells were in these phases. The distribution of cell cycle stages of TGF-β1 treatment groups differed significantly from control groups. Also, there was a significant difference in the cell cycle stages among all treated groups. Cells in the G0/G1 phase decreased obviously when treated with TGF-β1; 78.3%, 73.7%, 66.8%, 65.7%, and 62.5%, respectively, for 5 ng/mL TGF-β1 treated for 6, 12, 24, 36, and 48 hours. However, cells in S phase increased significantly chronologically,
supported by 12.2%, 17.9%, 21.1%, 23.7%, and 31.8% at 6, 12, 24, 36, and 48 hours; and those in G2/M phase were 6.33%, 6.81%, 8.79%, 6.95%, and 7.61%, separately.

For UMSCC11B cells, TGF-β1 treatment did not induce progression through the cell cycle. The percentages of untreated UMSCC11B cells in the G0/G1, S, and G2/M phases were 79.2%, 3.33%, and 3%, respectively. All cell cycle stages of treated groups differed significantly from positive controls. For positive control cells, there were 51.1%, 30.6%, and 15.5% of cells in these phase at 24 hours, whereas at 48 hours, 39%, 42%, and 13.4% of cells were in the G0/G1, S, and G2/M phases. However, there was no significant difference on the percentage of cells in the G0/G1 and G2/M phases among different TGF-β1 treatment groups. As for cells treated with 5 ng/mL TGF-β1 for 6, 12, 24, 36, and 48 hours, there were 77.5%, 78.8%, 81.5%, 86.1%, and 81.8% of cells in the G0/G1 phase; 8.37%, 6.41%, 5.87%, 2.39%, and 3.34% of cells in the S phase; and 11.5%, 12.3%, 10%, 8.65%, and 9.67% of cells in the G2/M phase. After being treated with TGF-β1 for 6, 12, and 24 hours, the amount of cells in the S and G2/M phases increased significantly compared with untreated cells. For cells treated for 36 and 48 hours, the ratio of S-phase cells decreased obviously, showing no significant difference with that of untreated groups.

Our results suggest that TGF-β1 induces progression of successive cell cycles and is a fundamental cytokine that propagates UMSCC38 cell proliferation (Figure 3.7C). Unlike UMSCC38, UMSCC11B cell cycle progression was not enhanced by TGF-β1 treatment and no significant change was observed compared with the untreated cells (Figure 3.7D, ii). These results suggest that although TGF-β1 is a potent inducer of the cell cycle in UMSCC38 cells as late as 48 hours, it cannot stimulate progression of successive cell cycles in UMSCC11B cells. The reason may be that, at this stage, UMSCC11B cells are programmed for cellular differentiation, such as EMT, during which persistence of the cell cycle will negatively affect such phenotypical changes. These UMSCC cell lines revealed a different degree of proliferation, and FACSArray data revealed no cell death by PI in the sub-G1 population segment where it shows dead cells or debris.
3.3.5 TGF-β1 activate both Smad-dependent and Smad-independent pathways in UMSCC cells

To evaluate the pathways TGF-β1 uses to induce cell cycle progression, protein expression by Western blot analysis of phosphor-Smad, phospho-AKT, and ΔNp63 were undertaken. Cells were treated with TGF-β1 (2, 5, and 10 ng/mL) for 30 minutes for phospho-Smad2 and AKT proteins and 24 hours for ΔNp63 detection.

Phosphorylation of Smad2 is a clear indication of an active and functional Smad pathway in UMSCC38 cells but in limited fashion in UMSCC11B cells. Our results indicate that unlike UMSCC11B, TGF-β1 activates the Smad-dependent signaling pathway in UMSCC38 cells. However, the results (Figure 3.8A) also showed increased phosphorylation level of pAKT, by TGF-β1 treatments in UMSCC11B cells, unlike UMSCC38 cells, indicating that PI3K was activated by TGF-β1 in UMSCC11B. Intriguingly, the level of ΔNp63 protein increased in response to TGF-β1 treatments equally in both cell lines. These data suggest that activation of the Smad-independent pathway, PI3K (AKT), is regulated by TGF-β1 in UMSCC11B cells, whereas UMSCC38 cells predominately use the Smad pathway. The current results support the notion that TGF-β1 stimulates both Smad-dependent and Smad-independent pathways in UMSCC38 and UMSCC11B autonomously (see Figure 3.8A).

3.3.6 Induction of cyclins by TGF-β1 in the UMSCC cells

Complete assessment of cyclin mRNA levels show that the mRNA levels of cyclin A and B were higher than cyclin D and E in both cell lines. The data support the hypothesis that TGF-β1 induces the cell cycle by increasing mRNA levels of cyclins, with cyclin mRNA levels falling sharply after 24 hours in UMSCC11B cells, unlike in UMSCC38 cells, which continue to escalate all cyclin mRNA levels (Figure 3.8C). These results indicate that cell
Figure 3.8. Pathways used by transforming growth factor-β1 (TGF-β1) during UMSCC cell proliferation. A, TGF-β1 uses both Smad-dependent and Smad-independent pathways during UMSCC cell proliferation. pSmad2 was expressed in UMSCC38 cells, but the expression was indifferent with TGF-β1 treatment of different dosages; however, its expression in the UMSCC11B was negligible in 2 and 5 ng/mL treatments and very low in the 10 ng/mL treatment. Subsequently, Western blot analysis of phosphorylated proteins was performed to determine the effects of TGF-β1 on Smad-independent pathways. pAKT showed no expression in UMSCC38 cells with any doses of TGF-β1 treatments. UMSCC11B showed similarly increased expression of pAKT with no difference in the TGF-β1 treatment conditions. ΔNp63 expression in both UMSCC38 and UMSCC11B were comparable and showed increased expression in a dose-dependent manner in comparison to control (actin). B, All three doses of TGF-β1 treatment groups differed significantly among themselves and between two cell lines (P ≤ .05, as indicated by *) from the untreated control cells (0.2% fetal bovine serum [FBS]) (P ≤ .005, as indicated by **). Results from the blots (see Figure 3.4A) and the intensity of the bands were measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester, NY). To perform a t test analysis of mean intensity measurements, a region of interest analysis was done from the data to Microsoft Excel software. Data points for all samples are paired by spatial arrangement on gel and compared pairwise to minimize the impact of subtle background artifacts on image analysis. C, Regulation of cyclin mRNA by TGF-β1. Cyclin D, E, A, and B mRNA expressions were determined by RT-PCR in TGF-β1 (5 ng/mL)–treated UMSCC38 and UMSCC11B cells every 12 hours for 48 hours. TGF-β1–treated cells had higher cyclins (D, E, A, and B) mRNA expression levels compared with the untreated control (0.2% FBS). Compared with UMSCC11B, TGF-β1 had chronologically higher cyclins (D, E, A, and B) mRNA levels in UMSCC38 cells in a time-dependent fashion till 48 hours. However, the levels sharply dropped after 24 hours in the UMSCC11B cell lines and continued to decline until 48 hours. D, Regulation of cyclin D mRNA by TGF-β1 in UMSCC cells: The levels of cyclin D mRNAs in response to TGF-β1, when ΔNp63 and c-Myc are either
cycle status of UMSCC38 and UMSCC11B are uniquely different and UMSCC11B cease to proliferate after 24 hours of TGF-β1 treatment.

Since cyclin D is the only member of the cyclin protein family involved in regulating cell cycle progression in all phases of the cell cycle, we investigated whether ΔNp63 and c-Myc are interdependent in activating cyclin D and, therefore, the cell cycle. We showed that induction of c-Myc and ΔNp63 (by cDNA) resulted in an increase in cyclin D mRNA levels in UMSCC38 cells, but with very limited effect on UMSCC11B cells. However, such effect on cyclin D was significantly increased with c-Myc induction, compared with ΔNp63. Similarly, absence of ΔNp63 and c-Myc (by shRNA) reduced mRNA levels of cyclin D with pronounced effect by c-Myc inhibition compared to ΔNp63 (Figure 3.8D). These outcomes of inhibition and activation of ΔNp63 and c-Myc had a more noticeable effect on UMSCC38 cells while having a restricted influence on UMSCC11B cell lines. These data suggest that enhanced induction of the cell cycle
in UMSCC38 cells is regulated by both ΔNp63 and c-Myc, but the basal level cell cycle seen in UMSCC11B cells is caused by its failure to induce c-Myc significantly.

3.3.7 TGF-β1 activates ΔNp63 gene activity

Interestingly, the effect on ΔNp63 gene activity, as measured by luciferase 2 K ΔNp63 promoter region, was more balanced in both UMSCC38 and UMSCC11B cell lines. Although gene activity was lower in the UMSCC11B cell line, the trend of increased ΔNp63 gene activity was symmetric (Figure 3.8E). However, blocking both Smad4 and PI3K significantly reduced the ΔNp63 gene activity, indicating that both UMSCC38 and UMSCC11B utilize Smad-dependent and Smad-independent pathways, respectively, to stimulate ΔNp63, which seems to be required for inducing basal cell cycle and survival.

3.3.8 c-Myc promoter activity and Smad-binding elements

In earlier studies by us16 and others,15 it has been shown that c-Myc has several potential Smad-binding elements (SBEs), namely, TBE1, TBE2, and TIE. A schematic diagram of the 2.5-kb c-Myc promoter showing the three potential SBEs is shown in Figure 3.9A. The transfected cells were treated with TGF-β1 (5 ng/mL) for 24 hours, and luciferase assays demonstrated increased transactivation of the c-Myc promoter in response to TGF-β1 (pBV-Luc-DEL1-WT). Since the SBEs are functional and important in the activation of c-Myc promoter, as shown in Figure 3.9B, we wanted to determine if these SBEs harbor Smad proteins on the c-Myc promoter. We performed a chromatin immunoprecipitation assay to determine if Smad4 was bound to the SBEs (TBE1, TBE2, and TIE). Our data suggest that although TGF-β1 induces increased c-Myc promoter activity in the UMSCC38 cell lines, UMSCC11B had limited c-Myc promoter activity (Figure 3.9C). Both functional SBEs (TBE1 and TBE2) are necessary to induce optimum c-Myc gene activity, since mutations of these (TBE1 and TBE2) regions drastically reduced the effect of TGF-β1 on c-Myc promoter activity (see Figure 3.9B). These data, combined with the observation that mutation of TBE1 and TBE2 decreased luciferase expression by the greatest amount (see
Figure 3.9B), suggest that TBE1 and TBE2 are the most important regions in the c-Myc promoter for Smad4-mediated transactivation induced by TGF-β1 signaling in UMSCC38 cells but not in UMSCC11B cells.

3.3.9 TGF-β1 facilitates cell migration in UMSCC11B cells but not in UMSCC38 cells

On the basis of the data showing a dramatic inhibition of the cell cycle in UMSCC11B cells after 24 hours of TGF-β1 treatment (see Figure 3.8C), as well as limited c-Myc promoter activity and no Smad binding to c-Myc promoter (see Figures 3.9B and 3.9C), unlike in UMSCC38 cells, we explored the role of TGF-β1 on cell migration in both cell lines. We undertook a transwell cell migration assay (Figure 3.9D) at 24 and 48 hours of TGF-β1 treatment by using a combination
Figure 3.9. Transforming growth factor-β1 (TGF-β1) induces c-Myc gene activity by stimulating Smad promoter binding. A, Schematic diagram of the human c-Myc promoter: ~2.5 kb, based on previous studies, showing three potential Smad-binding elements (SBEs): TBE1 (between −359 and −329 bp), TBE2 (between −95 and 46 bp) and TIE. On the basis of these findings, we predicted that Smad-dependent transcriptional regulation is mediated by these SBEs, which might suggest that c-Myc is a direct downstream TGF-β1 target. B, SBEs are essential for c-Myc gene activity: TGF-β1 treatment resulted in an approximate 130-fold increase of luciferase activity in cells transfected by pBV-Luc-DEL1-WT compared with an empty vector (control) in UMSCC38 cells, whereas c-Myc gene activity increased by approximately 25-fold in UMSCC11B cells, attesting that TGF-β1 is more potent in activating the c-Myc promoter in UMSCC38 cells compared with UMSCC11B cells. However, in response to TGF-β1 transactivation, deletions of the SBE (TBE1) in the c-Myc promoter (pBV-Luc-DEL3) decreased the promoter activity proportionately in both UMSCC38 and UMSCC11B, but the levels were dramatically reduced when both TBE1 and TBE2 were deleted (pBV-Luc-DEL4). When the TBE1 region was mutated (pBV-Luc-TBE1 mut) and with TBE2 (pBV-Luc-TBE1/2 mut), c-Myc gene activity was reduced significantly in both UMSCC38 and UMSCC11B cell lines. Background luciferase activity (empty vector) was subtracted from all data. Error bars indicate standard deviation of three independent preparations. The results are shown as a mean ± SD obtained from three luciferase values (P ≤ .05 as indicated by * and P ≤ .005 as indicated by **). C, Smad, activated by TGF-β1, binds to the c-Myc promoter: The real-time polymerase chain reaction (RT-PCR) analysis displayed that Smad4 is strongly bound to the TBE1 and TBE2 sites in UMSCC38 cells, instead of the TIE site. In contrast, UMSCC11B did not show any significant binding of Smad4 to any of the SBEs of c-Myc promoter in response to TGF-β1. Moreover, in UMSCC38 cells, among the three binding sites, TBE2 showed highest binding to Smad4. The specificity of the bindings was confirmed by the positive (Histone H3) and negative (Rabbit IgG) controls (data not shown). The results are shown as a mean ± SD obtained from three independent chromatin preparations (P ≤ .05 as indicated by * and
of treatment conditions (blocking c-Myc, Smad4, and PI3K). The results demonstrate that TGF-β1 causes increased cell migration in UMSCC11B cell lines compared with UMSCC38 cell lines. Furthermore, the addition of TGF-β1 for up to 48 hours results in a dramatic increase in UMSCC11B cell migration. In addition, UMSCC11B cell migration can be halted significantly when PI3 kinase is blocked.

3.4. Discussion

OSCC is the most common intraoral malignancy, often with a dismal outcome (Mignogna, Fedele et al. 2004, Gourin, Conger et al. 2008). Defining the exact signal cascades that may be implicated in various epithelial-derived carcinomas cells is essential for developing effective targeted therapeutic regimens. In this study, using different grades of OSCC and primary (UMSCC38) and secondary tumor (UMSCC11B) cell lines, we investigated the role of TGF-β1 in squamous cell proliferation and differentiation.
We demonstrated that TGF-β1 had a more pronounced effect in UMSCC38 cell proliferation compared with UMSCC11B cell proliferation. We also demonstrated that TGF-β1 activated both Smad-dependent and Smad-independent pathways in UMSCC38 and UMSCC11B cells lines, respectively, to induce cell survival/basal cell proliferation via ΔNp63. Our results demonstrate that TGF-β1 is capable of facilitating OSCC cell proliferation via ΔNp63/c-Myc working in tandem in the initial phase, whereas in the later phase, TGF-β1 induces EMT in the absence of c-Myc. This dual role is not unique, as TGF-β1 is known to stimulate multiple cellular functions and phenotypes based on dose, time, and the microenvironment.

It is important to point out that these UMSCC cell lines are extremely heterogeneous (origin, etiology, genetic alterations, location, degree/phase of cancer progression, and secondary metastasis vary from one cell line to the other) like most cancer cell lines and they are destined to act differently as a result of TGF-β1 treatments. Genetic drift over time in cultured cell lines is bound to show diverse cellular changes using established cell lines. Because of the critical diversity in native and in vitro nature of UMSCC cell lines as model systems, it is more than likely that they ought to behave differently under the same treatment condition. Therefore, here, we emphasize that each measurement or test accurately determines important variations in our techniques for two UMSCC cell lines in response to TGF-β1 in a time-dependent and dose-dependent manner, establishing validity, reliability, and replicability and achieving appropriate levels of statistical power and sensitivity. The data also demonstrate that it is quite plausible for TGF-β1 to cause EMT in one cancer cell line and proliferation in another, and the purpose of this paper is to describe the mechanism of TGF-β1 signaling that causes these two separate cellular phenomena, which are not a rare but a typical response to those two cell lines.

The nuclear localization of PCNA and Ki67 is an indication of cell proliferation during the late G1 and S phases of the cell cycle (Tsurimoto 1998). First, we established that the degree of differentiation and progression of OSCC and UMSCC cell lines were directly related to the
chronologic progression of the cell cycle, in addition to increased expression of well-established cell proliferation proteins, including PCNA, Ki67, cyclins, and ΔNp63. Interestingly, TGF-β1 was co-expressed in the same cells that also harbor the different cell proliferation proteins present at different stages of cancer progression and differentiation, indicating the decisive role that TGF-β1 plays in regulating cancer cell cycle and proliferation involving ΔNp63. The uniform expression of E-cadherin in the early stages and the significantly decreased expression in the later stages attest to the loss of cell–cell adhesion, thus promoting EMT in poorly differentiated SCCs. Our data suggest that when comparing UMSCC38 and UMSCC11B cells, UMSCC38 cells tend to maintain increased cell proliferation in response to TGF-β1. Conversely, UMSCC11-B cells cease their upregulation of the cell cycle beyond 24 hours, ultimately undergoing EMT at 48 hours.

We demonstrated that although TGF-β1 stimulated the cell cycle via ΔNp63 and c-Myc in varying degrees in UMSCC38 and UMSCC11B cell lines, UMSCC38 cells were affected to a greater extent. Our results demonstrate that TGF-β1 has an effect on the UMSCC38 cell cycle that is in accordance with the chronologic progression through different phases of the cell cycle; however, such induction is limited in UMSCC11B cells. Moreover, termination of the cell cycle in UMSCC11B beyond 24 hours after TGF-β1 induction implies that UMSCC11B cells might undergo cellular cytoskeletal rearrangement for migratory phenotype, during which cessation of the cell cycle is very common (Oft, Heider et al. 1998).

It is well established that TGF-β1 can induce signals using either Smad-dependent or Smad-independent pathways, or both (Massague and Wotton 2000). However, Smad2/Smad4 is a functional regulator of the Smad pathway in UMSCC38 cells lines, whereas UMSCC11B cell lines independently utilize the PI3K/AKT pathway. Given the uniqueness of UMSCC38 and UMSCC11B cells in terms of their cell cycle response to TGF-β1, utilization of separate pathways by UMSCC38 and UMSCC11B would appear to be expected. This divergence of signaling mechanisms may be the deciding factor for continuation of the cell cycle in UMSCC38, as opposed
to cessation in UMSCC11B cells. These combined results indicate that TGF-β1 initiates signals via both Smad-dependent and Smad-independent pathways in UMSSC38 and UMSSC11B cells respectively.

ΔNp63 is preferentially expressed in proliferative keratinocytes (Yip and Tsao 2008). Its expression is restricted to the nuclei of cells in the basal and parabasal layers of the stratified squamous epithelium, suggesting its involvement in the development and maintenance of epithelial structures (Okuyama, Ogawa et al. 2007) and a regulatory role in epithelial proliferation and differentiation (Pellegrini, Dellambra et al. 2001). Accordingly, our ΔNp63 luciferase assay data demonstrate that TGF-β1 signals via both Smad-dependent and Smad-independent pathways in UMSSC38 and UMSSC11B cell lines, respectively, induces ΔNp63 gene activity. An increased in ΔNp63 expression was detected in up to 80% of primary OSCC tumors (Weber, Bellmann et al. 2002, Sniezek, Matheny et al. 2004). However, since ΔNp63 expression falls in UMSSC11B cells at 48 hours in response to TGF-β1, this may be indicative of that they are undergoing EMT. This switch, as seen in UMSSC11B cellular behavior and phenotype, facilitates invasion at this stage of cancer progression. This is in contrast to the ΔNp63-dependent proproliferation, a prosurvival pathway that seems to exist in invasive SCCs. The signaling mechanisms that trigger the shift in UMSSC11B but not in UMSSC38 cells may be the result of different mechanisms in Smad-dependent and Smad-independent pathways. Moreover, as shown in the UMSSC38 cells, enhanced ΔNp63 expression appeared to be a prerequisite for well-differentiated OSCCs to maintain their noninvasive phenotype (Fukunishi, Katoh et al. 2010). These findings suggest that loss of ΔNp63 confers the invasive behavior that is observed in poorly differentiated OSCCs, as ΔNp63 is a well-known antimetastatic protein (Moergel, Abt et al. 2010).

It has been well established that c-Myc is an oncogene, whose role in cell proliferation has been demonstrated in various systems (Feng, Liang et al. 2002, Oster, Ho et al. 2002, Yagi, Furuhashi et al. 2002, Pelengaris and Khan 2003). We demonstrated that TGF-β1 is capable of
inducing cell cycle progression, and we also showed that UMSCC38 cell proliferation is achieved by the induction of c-Myc gene activity, which requires functional Smad signaling. However, such signaling is limited in UMSCC11B cells, thus downregulating the cell cycle as well. Our data also indicate that TGF-β1 mediates its effect on c-Myc via binding of Smad to the TBE1 and TBE2 elements, particularly in UMSCC38 cells, but not in UMSCC11B cells. In addition, we demonstrated that cessation of a robust cell cycle (via cyclins/c-Myc in UMSCC11B), resulted after 24 hours of TGF-β1 treatment and that only UMSCC11B cells undergo EMT, but not UMSCC38 cells. Interestingly, the signaling pathway that TGF-β1 utilizes to induce EMT in UMSCC11B cells is via non-Smad, PI3K/AKT pathways, whereas UMSCC38 cells maintain Smad pathways to activate c-Myc-dependent cell proliferation. TGF-β1 promotes the growth, progression, and migration of established tumors (Elliott and Blobe 2005). Furthermore, TGF-β1 is known to promote the transcription of Snail and SIP-1, two repressors of E-cadherin expression, thereby diminishing E-cadherin and inducing EMT, which is critical for cancer cell motility, invasion, and metastasis (Jakowlew 2006).

To correlate the degree of differentiation in vitro (consistent with most studies and generally accepted protocol), we demonstrated that oral cancer cell lines undergo cell proliferation and transformation (which are phases of differentiation in vivo) in relation to changes in cell proliferation and EMT proteins in response to TGF-β1. Overall our results demonstrate that induction of the cell cycle is a key phase that takes place during OSCC progression and tumor growth and is dependent on c-Myc induction. On the basis of our solid data, we postulated the following (Figure 3.10): (1) the TGF-β/Smads/ΔNp63/c-Myc pathway remains active in the noninvasive primary but higher proliferative attributes of OSCCs, particularly UMSCC38, and (2) the TGFβ/PI3 K/AKT/ΔNp63 pathway is functional (in the secondary tumor that resulted from relapse and recurrence at the primary site) at the inception stage of invasive OSCC from the secondary, as seen in the UMSCC11B cell lines. We further conclude that although TGF-β1 has
distinct functions in cancer progression, its downstream signaling partners and transcription factors regulate the ultimate cancer cell fate and promote the transition from dysplasia to invasive SCC.

Figure 3.10. Schematic diagram of the proposed mechanism of oral squamous cell carcinoma (OSCC) proliferation and invasion. On the basis of our data, we hypothesized that in the initial stage, UMSCC cell proliferation (both in the primary tumor, UMSCC38, and the secondary tumors, UMSCC11B cell lines) is achieved by TGF-β1/Smads/ΔNp63/c-Myc pathway with higher proliferative attributes in UMSCC38. However, subsequently, TGF-β1 switches its signaling via the PI3K/AKT/ΔNp63 pathway at the inception stage for EMT/invasive, primarily in the secondary tumor, as seen in the UMSCC11B cell lines (that resulted from relapse and recurrence at the primary site). We further conclude that although TGF-β1 has distinct functions in cancer progression, its downstream signaling partners and transcription factors regulate ultimate cancer cell fate and achieve switch from tumor growth to invasion.
CHAPTER 4

MECHANISMS FOR SIMVASTATIN-INDUCED PERIODONTAL BONE REGENERATION

4.1. Introduction

Periodontitis is an inflammatory disease causing bone resorption around teeth, and is a primary cause of tooth loss in humans (Albandar, Brunelle et al. 1999). Periodontitis affects approximately 47% of the adult population in the United States, the equivalent to 65 million people (Eke, Dye et al. 2012, Eke, Dye et al. 2012). While reduction of the bacterial biofilm around teeth is a cornerstone of therapy, management of the host inflammatory response and stimulation of bone regeneration is required in more advanced cases. The bone regeneration in periodontitis is difficult because it must be attempted in a highly-inflamed microenvironment, therefore, requiring both stimulation of bone growth and resolution of local inflammation. The conventional therapy entails a surgical procedure exposing and debriding the bony defect, then treating the bone surface with synthetic bone matrix and growth factors (Hoffmann, Al-Machot et al. 2016). However, recent clinical evidence suggests that nonsurgical debridement of the tooth root and injection of statin formulations, particularly simvastatin, can regenerate a significant portion of the bone lost to periodontitis (Pradeep and Thorat 2010, Pradeep, Priyanka et al. 2012), thereby reducing patient morbidity and cost.

While simvastatin was originally designed to suppress cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, it also has been shown to have bone anabolic and anti-inflammatory properties (Mundy 2001). However, the mechanisms for periodontal bone regeneration and inflammation control remain poorly understood.

Naturally-occurring periodontitis causes bone resorption on a highly-variable time table over decades, so experimental periodontitis in the rat model is often used to cause measurable
periodontal bone loss over a one-week period, and local simvastatin injections have been shown to prevent this bone loss or regenerate bone around teeth (Fig. 4.1 (Bradley, Zhang et al. 2016)). The purpose of the current study was to explore the genes and signaling pathways associated with bone turnover and inflammation that are either up-regulated or down-regulated by the creation of experimental periodontitis, then altered by local injection of a water-soluble simvastatin prodrug. Since the cellular mechanisms maintained and regulated by simvastatin-induced signaling have not been extensively explored to enable an understanding of those genes functioning in repression of periodontitis and in stimulation of bone regeneration, our objective in this study was to analyze transcriptome changes and their contribution in periodontal regeneration. In this study, we analyzed the transcriptome of simvastatin prodrug capable of locally delivering high doses to the periodontitis inflammatory lesion and bone in experimental periodontitis bone loss and inflammation. This study evaluates and characterizes some crucial transcripts that may play key regulatory roles in periodontitis-induced bone resorption and simvastatin-induced regeneration.

The hypothesis of this investigation is that experimental periodontitis enhances gene activation (and protein production) of known pro-inflammatory and bone catabolic mediators. Furthermore, locally applied simvastatin will decrease inflammatory mediators and stimulate bone anabolic genes and protein production. Finally, relevant pathways associated with these activities will be suggested.

4.2. Materials and methods

4.2.1 Animal Procedures

Twenty (20) mature female Sprague Dawley rats were housed and treated in the University of Nebraska Medical Center (UNMC) College of Dentistry Animal Facility under the approval of UNMC Institutional Animal Care and Use Committee (IACUC #13-006-03). Rats were acclimated one week before oral interventions. An induction chamber connected to an isoflurane anesthetic vaporizer initiated anesthesia with 1% to 4% isoflurane/100% O2 (1 to 3 L/minute), which was
subsequently maintained by application of a nose cone with 0.5% to 2% isoflurane/100% O2 (0.5 to 1 L/minute) over experimental procedures. Animals’ weight was recorded after anesthesia induction and before all procedures to monitor weight gain/loss. Rats were randomly divided into two groups of ten rats each (Table 4.1). The maxillary second molars had 4-0 silk ligatures placed subgingivally to induce experimental periodontitis (ExP). All ligatures were removed one week later and three local injections of 1.5 mg simvastatin-pyrophosphate prodrug (SIM-PPi) were delivered into the palatal gingiva at the mesial, mid-palatal and distal aspects of the second molars to the depth of 2mm using 26G x ½ sterile syringes. Rats were euthanized 3 days later by CO2 asphyxiation. Animals were weighed after euthanasia and weight changes between groups were compared by t-test. Palatal gingiva tissues of the second molars on both sides were collected from rats using sterile #15 blades and evenly pooled into 3 samples within each group for subsequent RNA extraction. The palates with intact interproximal gingiva were fixed in 10% buffered formalin solution for subsequent micro-CT and histological evaluation.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 11</th>
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<tr>
<td>1</td>
<td>10</td>
<td>Left: untreated</td>
<td>Remove ligatures</td>
<td>Euthanize</td>
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<td>2</td>
<td>10</td>
<td>Left: ligatures</td>
<td>Remove ligatures</td>
<td>Euthanize</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Right: ligatures</td>
<td>Inject SIM/PPi</td>
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</tr>
</tbody>
</table>

Table 4.1. Experimental Groups

4.2.2 RNA Extraction, Construction of Small RNA Libraries and RNA-Seq

Total RNA was isolated using the RNeasy Protect Mini Kit (Cat # 74124; Qiagen, CA) following the manufacturer’s instructions and measured for purity and concentration by ultraviolet spectroscopy (NanoDrop 2000c, Wilmington, DE). RNA integrity evaluation, libraries construction and validation were performed as previously reported (Ozturk, Li et al. 2013). RNA-
Sequencing was performed using Illumina HiSeq 2500 at the UNMC Bioinformatics and Systems Biology Core.

4.2.3 RNA-Seq analysis

RNA-Seq data were obtained for unmanipulated controls (denoted as C), samples with ligature only (ExP, denoted as L), samples treated with the carrier only following ligature removal (ExP+PPI, denoted as P), and samples treated with SIM and the carrier following ligature removal (ExP+SIM/PPI, denoted as S). Each group was represented by three biological replicates resulting in 12 samples used for RNA-Seq, which was performed in 75bp single-end mode. Raw reads were analyzed with FASTQC (v. 0.11.5) for quality control (Andrews 2010). Overrepresented (e.g. adapter and similar technical) sequences remaining in the raw reads were assessed and subsequently removed using Trimmomatic (v 0.36) in the palindrome mode based on default alignment detection and scoring parameters (Bolger, Lohse et al. 2014). Trimmomatic also was 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 filtered out. Transcript quantification was done based on the Rnor_6.0 reference genome using Salmon (v. 0.8.2) with default parameter (Patro, Duggal et al. 2017). Salmon uses sample-specific models such as correction for GC-content bias that improves the accuracy of transcription abundance estimates. Transcripts Per Million (TPM) in Salmon’s output was used as the relative abundance measure employed in our downstream analysis. Differential gene expression analysis was done using DESeq2 (Love, Huber et al. 2014). DESeq2 uses a negative binomial model to assess differential expression and employs the Benjamini Hochberg procedure (Benjamini and Hochberg 1995) for multiple hypotheses testing correction. 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.
Clustering of samples and/or genes was done using the Unweighted Pair Group Method with Arithmetic-mean (UPGMA) method utilizing 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, Sherman 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, Ball et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa, Araki et al. 2008). Biologically relevant categories that were over-represented in the gene set and, therefore, may be of further interest were assessed using the 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 were 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. GO categories that had EASE scores of 0.05 or lower were picked as significantly over-represented. The differentially expressed gene lists was further analyzed using the Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) software. 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. Raw RNA-Seq data is available at NCBI-SRA database under the BioProject PRJNA417128

4.2.4 Micro-CT (μCT) Measurements

The micro-computational tomography (μCT) measurements were performed on intact molar areas using a high-resolution Skyscan 1172 micro-CT system as described in our previous study (Bradley, Zhang et al. 2016). Briefly, maxillae were scanned by the micro-CT, followed by 3D reconstructions using internal software and reorientation of 3D models to standard position for
future evaluation. The distance from the molar cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured in sagittal interproximal views by one masked examiner to confirm ExP bone loss compared to control using t-test (Fig. 4.1).

4.2.5 Histology and Immunofluorescence

The palates fixed in 10% buffered formalin solution were decalcified in 5% formic acid solution for 2 weeks, and processed and embedded in paraffin. Eight (8) µm thick serial sagittal sections were collected and stained with hematoxylin and eosin to show crestal interproximal bone and gingival landmarks between first and second molars (Fig. 4.1). Immunofluorescence techniques as described by us previously (Hu, Liu et al. 2015) were used to analyze the distribution of selected bone anabolic and inflammatory proteins suggested by RNA-Seq data. Briefly, above mentioned sections were blocked with 10% normal goat serum (NGS) in PBS for 1 hour at room temperature in a humidity chamber and incubated with primary antibodies (see below) in PBS containing 10% NGS at 4°C overnight, followed by incubation with 5% NGS-diluted secondary antibody for 1 hour at room temperature. Normal rabbit serum (Abcam, ab166640) at the same concentrations was used in negative controls, respectively. The fluorescence was observed with a fluorescence confocal laser scanning microscopy with a Nikon A1 CLSM (Nikon Inc., Japan). Measurement of staining intensity was performed in Adobe Photoshop CC 2017 (Adobe, CA). Three representative areas were selected from each image for measurement. Intensity of staining was determined as gray value (mean). Statistical analysis was performed using one-way ANOVA in Graphpad Prism 7 (GraphPad Software, CA). Average with 95% IC were shown in quantification bar graph. Significant differences were summarized with *.

Primary antibodies used in this study include: Rabbit anti-MMP-9 antibody (Abcam, ab76003, 1:200), Rabbit anti-IGF-1 antibody (Biorbyt, orb10886, 1: 200), Rabbit anti-TNF-α antibody (GeneTex, GTX110520, 1:100). Secondary antibodies used in this study include: Goat anti-Rabbit

4.3. Results

No rat groups showed significant change in weight during the experimental period. ExP caused a significant increase in periodontal bone distance from the CEJ to ABC (1.10±0.3mm) compared to unmanipulated controls (0.67±0.22mm, p<0.0005), confirming establishing ExP.

Raw RNAseq average read count was ~19.0M single-end reads per sample, which decreased to ~18.6M following trimming and filtering. Similarly, the average read-length decreased to 70.0 bp in the processed reads from 74.5 bp in the raw reads. The quality preprocessing increased the average read quality to 34.08 from 32.28 and the percentage of high quality bases (with a quality score > 20) per sample increased to 95.85% from 92.48%. These results indicate that read quality was increased significantly at a marginal cost of decreased total number of reads and average read length (see Fig. 4.6). RNA-Seq analysis generated expression data for 31,202 transcripts. Transcripts that showed a TPM value more than 1 in at least 2/3 biological replicates in at least one sample group (C, L, P, or S) were carried on for downstream analysis rendering a total of 17,093 transcripts. In Fig. 4.2a, the number of genes that are significantly differentially expressed between each pair of sample groups are shown. Experimental periodontitis in the rat model up-regulated 1,743 genes and down-regulated 1,133 genes compared to unmanipulated contralateral controls (Fig. 4.2a). Both the SIM-PPi and PPi altered a similar number of genes, yet PPi (carrier) alone had almost no effect beyond the ligature-induced periodontitis (no up-regulated genes, one down-regulated gene). Therefore, PPi was considered an inert carrier and all subsequent
comparisons were conducted among ExP, ExP+SIM-PPi, and unmanipulated controls (C). For the remaining three groups, unsupervised hierarchical clustering analysis was performed using all the transcripts (Fig. 4.2b). The clustering results showed clear separation of all three groups from each other, further separating the unmanipulated controls from the ExP and the ExP+SIM-PPi groups. This blind clustering shows that from a global transcriptional profiling perspective, the application of SIM-PPi perturbs the ExP group enough to form a distinct group but not merged with the control, possibly due to the dominant effect of the inflammatory response.

**Fig. 4.1 Confirmation of establishment of experimental periodontitis.** Micro-computed tomography images of normal interproximal bone height between maxillary first and second molars (white bar, Control). Experimental periodontitis caused bone loss (red bar, ExP), while ExP followed by local simvastatin injections caused bone preservation/regeneration (shorter red bar, ExP + SIM). Histologic image of ExP shows gingiva next to the tooth root (T) and bone (B). White box indicates the area of interest for the immunofluorescence evaluations in the current study.
Genes up-regulated at least 2-fold by ExP compared to control included many proinflammatory markers, including matrix metalloproteinases (2,8,9), interleukin (IL) 1 beta, 17 and tumor necrosis factor superfamily and receptor, complement components C1q and C5a receptor, LPS binding protein, and toll-like receptor 2. Bone turnover factors also were up-regulated, including collagen type 1. ExP showed 2-fold down-regulated odontogenic ameloblast associated protein (mediates junctional epithelial attachment to teeth) and insulin-like growth factor (IGF) binding protein 6 (that enhances IGF signaling).

To focus on the unique effect of simvastatin on gene expression, the overlap between genes dysregulated due to ExP and ExP+SIM-P Pi models were calculated (Fig. 4.3). Although the two dysregulated gene lists showed a high overlap indicating the shared effects of the ligature model in both groups, there were a significant number of genes that were dysregulated uniquely because of simvastatin. Genes up-regulated 2-fold by ExP+SIM-P Pi versus control, but not ExP alone versus control, included anti-inflammatory IL-10 and IL-1 receptor like 1 (IL1rl1), and bone anabolic osteocrin (stimulates osteoblast phenotype) and IGF-1 (Fig. 4.3). The figure highlights significantly enriched functional/pathway categories. We also provide in the figures the full list of up/down

**Fig. 4.2: Summary of differentially expressed genes.** Statistically significantly differentially expressed (adjusted p-value < 0.05) genes between each pair of sample groups (a). Unsupervised hierarchical clustering analysis of the three sample groups (Control (C), ExP (L), ExP+SIM-P Pi (S)) using all the
regulated genes along with genes uniquely dysregulated in the ExP+SIM-PPi model and the functional/pathway categories that are significantly enriched in this unique gene list.

![Image of bar graph and table showing gene expression and pathways](image)

**Fig. 4.3:** Overlap of genes that are dysregulated in ExP (L) and ExP+SIM/PPi (S) models compared to the unmanipulated controls (C). Hierarchical clustering of and functional groups and pathways overrepresented by the 596+702 genes uniquely dysregulated in the ExP+SIM/PPi (S) model.

The data showed that simvastatin directly activated IGF-1 ligand and subsequently it also increased the expression of several other downstream molecules such Ras, AKT and decreased the expression of SOS, SOCS, PDK1, PKA, 14-3-3. These fine balances between up/down regulated genes are necessary to trigger: a) activation of periodontal ligament growth, b) induction of alveolar bone regeneration as well as c) repression of periodontitis (Figs. 4.7, 4.8 and 4.9). In addition to IGF-1, simvastatin also activates FGF7 ligand which upregulates AKT and HGF genes and downregulates SOS and CREB genes, which in tandem, cause periodontal fibroblast growth and maintain periodontal fibers homeostasis (Fig. 4.10 and 4.11). Simvastatin also induced activation of IL1rl1 that is necessary for immune response and repression of periodontitis by upregulating PKD, MAPK, JNK, ACVRL1, PDGF, CXCL3, SCL, GORASP2, and KLF2 and down regulating...
IL1, MGEA5, GSK3B, ERBB2, AP1, ATXN3 (Fig. 4.15). We also observed that simvastatin treatment caused repression of Wnt/β-catenin signaling. And loss of Wnt/β-catenin activates ILK, AKT, TCF4/LEF1 and Frizzled but also simultaneously represses TGFβr, PP2A, Groucho, CBP, CX43 and Dsh genes. Our data suggest that simultaneously activation and suppression of these genes (by repression of Wnt/β-catenin signaling) are necessary for pro-osteogenesis by enhancing osteoblast differentiation as well activation of periodontal fibroblast proliferation and morphogenesis. By regulating these genes, Wnt/β-catenin signaling can induce both bone and fibroblast differentiation and morphology to maintain a pro-osteogenic and fibrogenic homeostasis (Fig. 4.5, 4.12-1.14). In addition, we also show several other genes (GRK3, ARG1, SIX1, IL-10, MITF and OSTN) which are indirectly regulated by IGF-1, FGF7, Wnt/β-catenin and IL1rl1 and play decisive roles in a) activating periodontal ligament/fibroblast growth and homeostasis; b) induction of alveolar bone repair and regeneration and c) immune response and repression of periodontitis. These genes, induced by simvastatin, are important for periodontal regeneration and in tandem with IGF-1, FGF7, Wnt/β-catenin and IL1rl1, and play a vital role in improving and treating periodontitis (Fig. 4.5).

To investigate how simvastatin activation of inflammatory genes MMP-9 and TNF-α and bone regeneration genes IGF-1 translate into in situ at protein levels, immunofluorescence of these proteins in the periodontium was analyzed. The protein expression of IGF-1 was substantially upregulated after simvastatin treatment and mainly localized along the surface of alveolar bone. In contrast, expression of inflammatory markers, MMP-9 and TNF-α, were predominately within gingiva. MMP-9 was significantly upregulated by ExP and drug carrier PPI alone. TNF-α gained noticeable upregulation in periodontitis samples compared to unmanipulated controls. Both were reduced in response to simvastatin treatment (Fig. 4.4).
Fig. 4. Immunostaining analysis of protein expression of IGF-1, MMP-9 and TNF-α in different sample groups. (A) IGF-1 showed significantly elevated expression in response to simvastatin treatment(s) compared to experimental periodontitis (L and P) and unmanipulated controls (C). Unmanipulated controls have limited expression of MMP-9 and TNF-α, whereas in experimental periodontitis group without SIM, MMP-9 and TNF-α expression was significantly upregulated. However, simvastatin treatment remarkably reduced the expression of MMP-9 and TNF-α. Each bar value, as stated, represents the size and magnification of the image. (B) Quantification of staining intensity of IGF-1, MMP-9 and TNF-α in different sample groups. Statistical analysis was performed by on-way ANOVA. Error bars represent 95% confidence interval. * p < 0.05; ** p < 0.01; *** p < 0.001. C, Control; L, ExP; P, Exp+PPI; S, ExP+SIM/PPI.
4.4. Discussion

Placement of the ligature around the rat maxillary second molar is a standard model for inducing interproximal inflammation and bone loss (experimental periodontitis: ExP) and testing pharmacotherapeutic interventions (Bradley, Zhang et al. 2016, Struillou, Boutigny et al. 2010). Within 11 days of ligature placement, a large number of proinflammatory genes associated with both rat and human periodontitis were up-regulated. These included two cytokines most often associated with periodontitis, IL-1 beta and tumor necrosis factor (Preshaw and Taylor 2011, Spolidorio, Ramalho Lucas et al. 2014). In addition, IL-17 is a potent pro-osteoclast activator linked to periodontitis pathogenesis (Zenobia and Hajishengallis 2015). Matrix metalloproteinases, specifically 2, 8 and 9, are key drivers of collagen and bone destruction in periodontitis, as well as...
regulators of periodontal inflammation (Franco, Patricia et al. 2017). Complement, particularly C5a receptor, has been increasingly implicated in periodontitis etiology, since blocking this receptor in rats inhibits periodontal breakdown (Damgaard, Holmstrup et al. 2015). The major initiator of periodontitis is the lipopolysaccharide (LPS) components of the bacterial biofilm accumulated in the gingival crevice, and increased LPS-binding protein (LBP) is correlated to the amount of ligature-induced periodontitis in baboons (Ebersole, Steffen et al. 2010). The expression of Toll-like receptor 2 has been shown to increase in periodontal disease. Therefore, up-regulation of these genes is consistent with induction of periodontitis inflammation.

Bone loss associated with ExP was radiographically evident after one week of ligature placement in the current and previous studies (Bradley, Zhang et al. 2016). Therefore, regulation of genes involved in bone turnover should be evident when the surrounding gingiva was sampled 3 days after ligature removal. The up-regulation of collagen type 1 gene corresponds to the synthesis and release of collagen type 1 as the primary bone metabolism event during periodontitis bone turnover, and has promoted its use as a marker of disease activity (Giannobile, Al-Shammari et al. 2003). The down-regulation of genes responsible for periodontal homeostasis and bone growth also occurred with periodontitis bone loss. Odontogenic ameloblast associated protein has been shown to mediate junctional epithelial attachment to teeth, is present in a healthy periodontium, and is absent in the pathologic periodontitis pocket (Lee, Ji et al. 2015) as was created in ExP. Decreasing insulin-like growth factor 1 (IGF-1) binding proteins are associated with variable tissue outcomes, ranging from periodontal attachment loss to decreased IGF-1 (Zapf, Schmid et al. 1990, Harb, Holtfreter et al. 2012). IGF-1 has been shown to be significantly underexpressed in periodontitis lesions compared to healthy periodontal sites (Choi, Kim et al. 2014).

MMP-9 is a well-established inflammatory marker in periodontitis and elevated expression of MMP-9 was demonstrated in close relationship to chronic periodontal disease (Makela, Salo et
al. 1994, Rai, Kharb et al. 2008, Marcaccini, Novaes et al. 2009). Similarly, TNF-α is a known potent inflammation-induced osteoclastogenic cytokine (Noorloos, Meer et al. 1990, Page 1991, Birkedal-Hansen 1993, Lam, Takeshita et al. 2000). TNF-α strongly promotes inflammatory osteolysis by stimulating activation of macrophages and osteoclast precursors and enhancing expression of osteoclastogenic cytokines (Horwood, Elliott et al. 1998, Lam, Takeshita et al. 2000). Our immunoassay findings, in agreement with previous studies (Lazăr, Loghin et al. 2015), revealed that MMP-9 showed higher expression in periodontitis but was significantly reduced by simvastatin injections. In similarity, we also observed that simvastatin treatment substantially repressed activity of TNF-α. Together, our data clearly suggest that while ligature induces upregulation of inflammation agents and causes bone resorption, simvastatin exerts both anti-inflammatory and anti-osteoclastogenic effects by antagonizing expression of MMP-9 and TNF-α. These results are consistent with the gene expression pattern.

Interestingly, we report for the first time a novel association between SIM treatment upregulation IGF-1 in experimental periodontitis in rat molars. IGF-1 has long been thought to be one of the major anabolic factors responsible for limiting or reversing periodontal bone destruction (Okada and Murakami 1998). To further investigate the SIM-delivery and activation of IGF-1 in experimental periodontitis, protein expression confirmed that IGF-1 expression was low in ExP samples and SIM-Ppi injected into the periodontitis lesion quickly increased IGF-1 protein expression (Fig. 4.4). When compared to reparative and regenerative roles of simvastatin as shown in Fig. 4.1, it is understandable that simvastatin-induced activation of IGF-1 may play a crucial role in reversing rat periodontitis. However, the question remains as to how does IGF-1 ligand, induced by simvastatin, causes either of suppression proinflammatory molecules and/or activates anti-inflammatory molecules. Does IGF-1 also play role in osteogenesis induction and/or suppression of osteoclastogenesis? And finally, does IGF-1 concurrently play a role in “alveolar bone regeneration” and “suppression of periodontitis” in tandem or only does one or the other to restore
normal periodontal homeostasis? Although the premises of our study are not to explore those mechanisms in detail, our data seems to suggest that IGF-1 functions as pro-fibrogenesis, pro-osteogenesis as well as an anti-inflammatory molecule and is capable of activating pathways to ensure all these three periodontal mechanisms are active, making IGF-1 a key regulator of periodontitis repair that is activated by simvastatin. It has been shown that IGF-1 is capable of stimulating PDL fiber bundles cell proliferation and local osteoblast precursor proliferation, differentiation and mineralization of new bone (Han and Amar 2003). And the major signaling pathways that IGF-1 uses are both MAPK and PI3K pathways to induce osteogenesis (Wang, Bikle et al. 2013). Interestingly, IGF-1 modulates AKT/GSK3β pathways to induce its anti-inflammatory effect in rats (Wang, Li et al. 2016). Mice lacking IGF-1 (KO) are born small with delayed mineralization and reduced chondrocytes (Bikle, Majumdar et al. 2001). Mice with mutated IGF-1 gene (IGF-1 -/-) have decreased expression of early retinal inflammation (Arroba, Rodriguez-de la Rosa et al. 2016). However, for normal periodontal homeostasis and regeneration, simvastatin-induced activation IGF-1 alone may not be sufficient and necessitate activation of FGF7 and IL1rl1 and suppressing Wnt/β-catenin signaling to induce all three phases (anti-periodontitis, pro-periodontal fibrogenesis and pro-alveolar osteogenesis) of periodontal repair and regeneration (Fig. 4.5). Here we explain the genes that are regulated by these multiple pathways (IGF-1, FGF7, Wnt /β-catenin and IL1rl1) causing a) activating periodontal ligament/fibroblast growth & homeostasis; b) induction of Alveolar bone repair and regeneration and c) immune response and repression of periodontitis.

Our data show an induction of interleukin 1 receptor-like 1 (IL1rl1), also known as receptor suppression of tumorigenicity (ST) 2 gene, in response to simvastatin treatment. ST2 is a crucial binding receptor for IL33 (Raggatt and Partridge 2010). The cytokine IL-33 has been recently linked in physiological bone remodeling (Miller 2011). IL-33 cytokine falls within to the IL-1 family (Miller 2011) and is constitutively expressed in several organs and tissues (Schmitz,
Owyang et al. 2005, Carriere, Roussel et al. 2007, Chackerian, Oldham et al. 2007, Ohno, Oboki et al. 2009) and it functions are linked with type 2 helper T-cell immune reaction (Schmitz, Owyang et al. 2005, Chackerian, Oldham et al. 2007, Ohno, Oboki et al. 2009), initiation of cell cycle (Carriere, Roussel et al. 2007), apoptosis (Na, Hudson et al. 2012), and control of bone resorption (Saidi, Bouri et al. 2011, Schett 2011, Schulze, Bickert et al. 2011, Zaiss, Kurowska-Stolarska et al. 2011). IL-33 and its receptor ST2 (Tominaga 1989) are expressed by osteoclasts (Ohno, Oboki et al. 2009, Mun, Ko et al. 2010, Schett 2011, Schulze, Bickert et al. 2011, Zaiss, Kurowska-Stolarska et al. 2011), osteoblasts (Werenskiold, Rössler et al. 1995, Saidi and Magne 2011), and osteocytes (Saidi and Magne 2011, Díaz-Jiménez, Núñez et al. 2017). The role of IL-33/ST2 in the relation to bone physiology is debatable. It may function as dual role, either to suppress bone resorption (Saleh, Eeles et al. 2011, Schett 2011, Schulze, Bickert et al. 2011, Zaiss, Kurowska-Stolarska et al. 2011) or stimulate osteoclast formation or both, independent of the receptor activator of NF-κB (RANK) and the RANK ligand (RANKL) system. It has been shown that upregulated levels of IL-33 in gingival crevicular fluid (Buduneli, Özçaka et al. 2012) and periodontal tissues (Köseoğlu, Hatipoğlu et al. 2015) in inflammatory conditions are associated with alveolar bone loss. Mechanically stressed cells in the periodontium release IL-33, which halts osteoclastogenesis, implying that IL-33/ST2 have anti-osteoclastogenic effects and reduce osteoclast formation (Lima, Macari et al. 2015). Our data, in relation to periodontitis showed that simvastatin upregulates IL33 and ST2, implying that activated IL-33/ST2 signaling can induce anti-osteogenic activity and contribute in periodontal alveolar bone regeneration. Moreover, signaling via receptor (sST2) can also induces anti-inflammatory properties (Díaz-Jiménez, Núñez et al. 2017) in periodontitis, implying dual roles (anti-inflammatory and pro-osteogenesis) of IL33/ST2 signaling by simvastatin that facilitates periodontal regeneration. It has been shown that an increase in the expression of osteoclast markers and bone resorption in periodontium occurs in St2−/− mice subjected to mechanical loading. In contrast, there was greater expression of MMP-9 in St2−/− than WT mice. Our data showed that while ligature induces MMP-9 expression causing periodontitis,
simvastatin treatment, on the contrary represses MMP-9 (Fig. 4.4) in the rat periodontium is in agreement with the St2−/− data. These results imply that simvastatin which induced increased expression of IL1rl1 is necessary to repress MMP-9 to sustain anti-inflammatory effect.

Our data shows that simvastatin induces an increased expression of FGF7 gene (Fig. 4.3) that can potentially augment mineralization. The local delivery of FGF7 increases the expression of osteogenic markers, mineralization with enhanced osteogenesis and chemo-attraction in mandibular bone formation (Poudel, Bhattarai et al. 2017). Although the exact mechanism by which simvastatin-induced FGF7 facilitated osteogenesis is largely unknown, it has been demonstrated that FGF7 activates dexamethasone, ascorbic acid, and β-glycerophosphate (DAG)-induced increases in bone-like nodule formation and calcium accumulation (Jeon, Kook et al. 2013). FGF7 augmented mRNA expression of RUNX, osterix, bone sialoprotein (BSP), and osteocalcin in the presence of DAG suggests that FGF7 stimulates osteogenic differentiation (Jeon, Kook et al. 2013). Similarly, our data have also shown that simvastatin can increase the expression of RUNX3. However, whether RUNX3 is a direct target of simvastatin or activated via FGF7 remains to be explored. Interestingly, it has been shown that RUNX3 deficient mice have significant decreased osteoblast numbers as well as a lower mineral deposition volume (Saito, Ohba et al. 2015). Therefore, it is postulated that simvastatin-induced increased expression of RUNX3 might be involved in rat alveolar bone formation, regeneration and mineralization (Bauer, Sharir et al. 2015).

Our data reveals that IL-10 is upregulated in simvastatin-treated rat molar periodontal tissues. IL-10 is a potent anti-inflammatory cytokine which also inhibits osteoclastic bone resorption and regulates osteoblastic bone formation (Zhang, Chen et al. 2014). Subgingivally-delivered SIM in humans has been shown to stimulate IL-10 in fluid around periodontitis pockets and improve periodontal attachment (Grover, Kapoor et al. 2016). Other studies showed that IL-10 knockout endothelial cells had lost the reparative capacity for altered blood vessels, suggesting that
IL-10 is required for the formation of blood vessels around damaged bone and is a prerequisite for the restoration of the integrity of that bone (Yue, Garikipati et al. 2017). Importantly, IL-10 KO-EPC were highly enriched in microRNAs and proteins that promote inflammation and apoptosis and inhibit angiogenesis (Yue, Garikipati et al. 2017). Another study showed that IL-10 (−/−) mice are highly susceptible to bone loss induced by the periodontal pathogen, Porphyromonas gingivitis (Sasaki, Okamatsu et al. 2004). Therefore, it is suggested that simvastatin-induced increased expression of IL-10 probably facilitates alveolar bone regeneration and limits periodontitis.

Simvastatin also causes upregulation of osteocrin (Ostn) gene that has been recently discovered as a secreted protein produced by cells of the osteoblast lineage, and plays an important role in modulating bone formation and growth (Moffatt, Thomas et al. 2007). Osteocrin is a soluble osteoblast regulator (Thomas, Moffatt et al. 2003) and is also expressed in osteoblasts in developing bone (Bord, Ireland et al. 2005). It has not previously been associated with simvastatin application and periodontitis. It has been shown that Ostn expression has intense immunoreactivity in osteoblasts on bone-forming surfaces and also in newly incorporated osteocytes (Bord, Ireland et al. 2005). Therefore, in our model, we predict that increased expression Ostn in response to simvastatin treatment induces osteogenesis and modulates alveolar bone formation and growth.

Gene activation during ExP in the rat reflects many of the key pro-inflammatory components seen in human periodontitis, including IL-1β, TNF-α, IL-17, MMP-9 and complement c5a. Local injection of simvastatin into ExP caused down regulation of MMP-9 and TNF-α, and upregulation of anti-inflammatory genes and proteins IL-10 and IL 1r11 in ExP lesions. Simvastatin also stimulates of the potent bone anabolic modulator IGF-1. The signaling pathways involve in the process that are activated, are IGF-1, FGF7, while repressed pathways is Wnt/ β-catenin. A better understanding of these signaling mechanisms will help identifying enhanced pharmacotherapeutic approach to limit or regenerate periodontitis bone loss.
Fig. 4.6: Summary of raw reads. Number of Reads (a), Average Read Length (b), Average Read Quality (c), and percentage of base pairs above a base quality score of 20 (d) are shown separately for raw reads and reads following trimming and filtering.
Fig. 4.7. Effect of IGF-1 signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to proliferation of connective tissue cells in periodontal tissues
Fig. 4.8. Effect of IGF-1 signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to immune and inflammatory response in periodontal tissues
Fig. 4.9. Effect of IGF-1 signaling and its downstream gene expression changes in Exp-SIM/PPI in relation to proliferation of fibroblast cells in periodontal tissues.
Fig. 4.10. Effect of FGF signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to proliferation of connective tissue cells in the periodontal tissues.
Fig. 4.11. Effect of FGF signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to proliferation of fibroblast cells in the periodontal tissues
Fig. 4.12. Effect of Wnt/β-catenin signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to alveolar bone cell differentiation.
Fig. 4.13. Effect of Wnt/β-catenin signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to alveolar bone cell morphology.
Fig. 4.14. Effect of Wnt/ß-catenin signaling and its downstream gene expression changes in Exp-SIM/PPi in relation to periodontal fibroblast proliferation.
Fig. 4.15. Effect of IL1r1 signaling and its network gene expression changes in Exp-SIM/PPi in relation to periodontal inflammatory response
CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 Summary

TGF-β signaling has extensive functions in both the developing embryo and the adult organism, including regulating cell proliferation, cell differentiation, apoptosis, regeneration and cellular homeostasis. In this dissertation, the role of TGF-β in palatal development, oral squamous cell carcinoma and periodontal bone regeneration was studied. As elaborated in Chapter 1, knockout of TGF-β3 altered expression profile of transcriptome in TGFb-3 (−/−) animals, causing cleft palate by inducing up/down-regulation of certain genes. We identified numerous genes with potential roles in the palatal development in both WT and HM mice. In Chapter 2, we reported a novel mechanism that drives palatal fusion independent of TGF-β3, which is Ephrin reverse signaling, through mediating EMT but not apoptosis. PI3K/AKT/mTOR pathway cascade also incite EMT in connection with Ephrin reverse signaling. Our findings presented in this project indicate that this complex network is functional during palatal development and may establish platforms for future studies in defining targets for cancer treatment. Furthermore, despite that the mysteries of TGF-β paradox in benign and malignant cells remains elusive, we successfully validated that TGF-β1 can induce cancer progression partially by promoting cell proliferation and migration in oral malignance via activating Np63/c-Myc and postulate that downstream signaling components, including PI3K/AKT, Np63 and Smads carry out decisive regulations that determine the terminal fate of cancer cells, in spite of TGF-β1’s distinctive function in cancer progression. Finally, in Chapter 4, although it was surprising to notice that TGF-β didn’t play a dominant role in regulating bone regeneration in experimental periodontitis, we explored the mechanism of how simvastatin exerts both anti-inflammatory and pro-osteogenic functions by systematically analyzing genetic profiles using RNA-Seq technology. Identification of strong potent bone anabolic modulators, IGF-1,
strengthens our understanding of signaling mechanisms in bone regeneration and will help direct future research in efforts to develop pharmacotherapeutic strategies to treat periodontal diseases.

Overall, our data contribute to expand our basic knowledge about the diverse functions of TGF-β in a variety of biological processes and hopefully shed light on further studies in utilizing current information for better diagnosis, clinical intervention and treatment in the future.

5.2 Future studies

5.2.1 Take a step forward to eliminating cleft palate.

Cleft lip and palate is a common human birth defect. Causes and developing mechanisms of cleft palate have been extensively studied in animal models and human populations over the past decades. Thanks to these elegant work, we now have gained huge progress in identifying the genes and pathways that play a role in palate development, as well as in recognizing the environmental risk factors that increase possibility of cleft palate. We, by taking advantage of studies in animal models and bioinformatics technologies, further clarified the missing knowledge about the role that TGF-β plays in regulating palate development. However, it remains elusive how ALK5, IRF6, ΔNp63 and many other genes suggested by our RNA-Seq data control craniofacial morphogenesis during embryogenesis, either independently or in assistance to TGF-β. We have successfully created ALK5^{fl/fl} and IRF6 conditional knockout (IRF6 CKO) mice strains and future studies will be carried out by crossing ALK5^{fl/fl} and IRF6 CKO to Krt14:Cre mice to reveal more comprehensive mechanisms.

Meanwhile, there has also been an increasingly clearer picture of diagnoses, medical interventions to improve clinical outcomes, and preventative strategies for human birth defects, including cleft palate. It’s strikingly exciting that clinical attempts have been made to restore the
appropriate flux of relevant pathways by administrating either ligands or antagonists (Shin, Lee et al. 2012). Our findings may also lay the foundation and shed light on future therapeutic strategies in rescuing palatal clefting at early stage of embryogenesis.

5.2.2 Clinical trials of simvastatin in patients with periodontal diseases in future studies

It has become an increasing public concern that periodontal diseases are showing high prevalence in adolescents, adults, and older individuals both in developed and developing countries, affecting 20-50% of global population. Periodontal diseases comprise a diversity of inflammatory conditions that compromise the supporting tissues of the teeth, including the gingiva, alveolar bone and periodontal ligament. Untreated periodontal diseases could lead to alveolar bone resorption, tooth loss and even contribute to systematic inflammation, such as cardiovascular disease and diabetes, as reviewed in (Nazir 2017). Surgical and nonsurgical therapies have proved effectiveness in treating periodontal diseases. Based on our findings in this work that local injection of simvastatin effectively improves inflammatory condition and induces bone regeneration, future studies may focus on designing clinical trials and developing pharmacotherapeutic approaches to limit or rescue periodontal diseases. Although clinical studies aiming to evaluate *in situ* application of simvastatin in chronic periodontitis patients (Ranjan, Patil et al. 2017, Vemanaradhya, Emani et al. 2017) have been carried out, further investigations are urgently needed to extensively appraise optimal dosage and administrations, as well as potential side-effects on a larger scale.
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