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Cell type-specific transforming growth factor-β (TGF-β) signaling in the regulation of salivary gland fibrosis and regeneration

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ABSTRACT

Salivary gland damage and hypofunction result from various disorders, including autoimmune Sjögren's disease (SjD) and IgG4-related disease (IgG4-RD), as well as a side effect of radiotherapy for treating head and neck cancers. There are no therapeutic strategies to prevent the loss of salivary gland function in these disorders nor facilitate functional salivary gland regeneration. However, ongoing aquaporin-1 gene therapy trials to restore saliva flow show promise. To identify and develop novel therapeutic targets, we must better understand the cell-specific signaling processes involved in salivary gland regeneration. Transforming growth factor-β (TGF-β) signaling is essential to tissue fibrosis, a major endpoint in salivary gland degeneration, which develops in the salivary glands of patients with SjD, IgG4-RD, and radiation-induced damage. Though the deposition and remodeling of extracellular matrix proteins are essential to repair salivary gland damage, pathological fibrosis results in tissue hardening and chronic salivary gland dysfunction orchestrated by multiple cell types, including fibroblasts, myofibroblasts, endothelial cells, stromal cells, and lymphocytes, macrophages, and other immune cell populations. This review is focused on the role of TGF-β signaling in the development of salivary gland fibrosis and the potential for targeting TGF-β as a novel therapeutic approach to regenerate functional salivary glands. The studies presented highlight the divergent roles of TGF-β signaling in salivary gland development and dysfunction and illuminate specific cell populations in damaged or diseased salivary glands that mediate the effects of TGF-β. Overall, these studies strongly support the premise that blocking TGF-β signaling holds promise for the regeneration of functional salivary glands.

1. Introduction

Hyposalivation, the objective and pathological loss of saliva production resulting from salivary gland damage, can arise due to autoimmune Sjögren's disease (SjD), IgG4-related disease (IgG4-RD), and collateral glandular damage caused by head and neck radiotherapy.[1–3] Viral infections and other systemic autoimmune conditions, such as rheumatoid arthritis, can also result in salivary gland dysfunction.[4,5] Additionally, medications such as antidepressants and anxiolytics, when used long term, can lead to chronic hyposalivation.[6,7] Hyposalivation may lead to debilitating second-order effects such as problems with speech, decreased taste sensation, malnutrition, irritation of the oral mucosa, tooth enamel deterioration, and increased incidence of oral infections and gingivitis.[8] Though promising approaches to promote salivary gland regeneration and restore saliva secretion continue to be investigated, including gene therapies,[9,10] cell-based strategies,[11–17] bioengineering approaches,[18–20] and drug-based strategies that target regenerative signaling pathways,[21,22] there remains no approved curative therapeutic strategy to restore salivary gland function in these disease processes.

Our understanding of tissue regeneration is heavily informed by our understanding of organogenesis. As will be discussed in this review, more than twenty years of research have demonstrated that transforming growth factor-β (TGF-β) signaling is required for overall development and is temporally critical to every stage of salivary gland development.[23–26] In addition to inflammation and acinar atrophy, fibrosis develops in all the salivary gland disorders discussed in this review. Though extracellular matrix remodeling is essential to wound healing and regeneration, fibrosis results in tissue hardening and loss of function that is often irreversible. TGF-β signaling is essential to fibrosis. Therefore, the TGF-β superfamily of ligands, their cognate receptors, and their complex regulatory network may offer numerous therapeutic targets to block glandular fibrosis and promote salivary gland regeneration. To better understand how to utilize this signaling network to promote

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regeneration, we must first understand the roles of TGF-β signaling in salivary gland injury and regenerative processes, as well as its roles in salivary gland development. In this review, we discuss evidence of a role for TGF-β in salivary gland fibrosis and regeneration, along with recent findings on the cellular specificity of the TGF-β-mediated responses involved and the potential that these studies will lead to a therapeutic approach to promote salivary gland regeneration in humans.

2. Salivary disorders

Many disorders impact the structure and function of the salivary glands. Three causes of hyposalivation that lead to tissue degeneration, and for which therapeutic regenerative therapies do not currently exist, include autoimmune Sjögren's disease and IgG4-related disease, and collateral salivary gland damage caused by radiotherapy for the treatment of head and neck cancers. This section introduces these salivary gland disorders and provides the context for discussing studies on how TGF-β regulates salivary gland regeneration.

2.1. Sjögren's disease (SjD)

Sjögren's disease (SjD) is a common autoimmune disease characterized by dry eye and dry mouth and lymphocytic infiltration of the lacrimal and salivary glands that leads to chronic inflammation. However, SjD is a systemic disease that affects other exocrine and non-exocrine tissues.[27–30] B cells play an essential role in the pathogenesis of SjD. Indicators of B cell hyperactivity in SjD include the production of autoantibodies (*e.g.*, anti-Ro/SSA, anti-La/SSB), hypergammaglobulinemia, and the development of B cell lymphomas.[31,32] SjD is highly sexually dimorphic, with an estimated ratio of affected women to men ranging from 9:1 to 20:1, and it has been demonstrated that SjD is X chromosome dose-dependent.[33] Further, epigenetic modifications have been shown to alter transcription factor expression that enhances the expression of X chromosome-linked genes implicated in SjD.[34] In addition to genetic factors,[35] SjD is triggered by environmental factors, including various viral infections.[36–40] Treatment options for oral and ocular pathologies in SjD primarily focus on symptom management, emphasizing the pressing need for alternative therapeutic approaches.

2.2. IgG4-related disease (IgG4-RD)

IgG4-RD is an autoimmune disorder characterized by mass formation in many tissues, including lacrimal and salivary glands, that can lead to permanent organ damage.[41,42] The elevated presence of $IgG4^+$ plasma cells and fibrosis in affected tissues are hallmarks of IgG4-RD.[43] Not only does IgG4-RD's multi-organ nature confound diagnosis and treatment, but it is also an outlier among autoimmune disorders in that it manifests more often in males than females.[42,44] IgG4-RD is believed to follow a biphasic progression, with the first stage characterized by inflammation followed by fibrosis. During the inflammatory phase, target tissues are infiltrated by antigen-experienced B and T lymphocytes that drive the secretion of pro-fibrotic cytokines such as IL-1β, IL-6, IFNγ, TGF-β, and others.[45–48]

2.3. Radiation-induced hyposalivation

Globally, head and neck cancers account for almost 1 million new cancer cases annually, and radiotherapy (RT), along with surgical resection, are the current treatments of choice.[49] RT for head and neck cancers is commonly delivered using a fractionated dosing strategy, where small daily doses (generally 2 Gy) to a total of 70 Gy are delivered, which damages surrounding tissues, including salivary glands.[50] Hyposalivation is a common side effect of RT of the head and neck, with up to 60% loss in saliva output observed within days of treatment in nearly all patients.[51] This RT-induced hyposalivation may last for weeks to months or persist indefinitely, leading to a significant decrease in the quality of life.[52–54] Studies have demonstrated that the various major salivary glands, and even different regions of the same gland, are differentially radiosensitive.[55,56] The causes of RT-induced hyposalivation are multifactorial and include the depletion of acinar cells and failure to regenerate secretory acini, chronic inflammation, and the development of fibrosis.[57–59]

3. TGF-β superfamily

The TGF-β superfamily is composed of numerous cytokines that can be divided into two major families. In mammals, the TGF-β/Nodal family includes TGF-β1, 2, and 3, Nodal, Activins (A, B, C, and E), six growth differentiation factors (GDFs; GDF1, 3, 8, 9, 10, and 11), Activin-inhibiting inhibins, and Lefty, which inhibits Nodal co-receptors. The bone morphogenetic protein (BMP) family includes BMPs, including inhibitory BMP3, along with three GDFs (GDF5, 6, and 7) and the anti-muellerian hormone (AMH) (Table 1).[60–73] TGF-β subfamily members are pleiotropic cytokines secreted by fibroblasts and epithelial cells, whose function is tissue-specific and environment-dependent.[74] TGF-β was originally discovered alongside transforming growth factor-α (TGF-α), which together were known as sarcoma growth factor (SGF).[75] In mammals, there are three known isoforms of titular TGF-β (TGF-β1, TGF-β2, and TGF-β3) that have been shown to form hetero- and homodimers that bind to the same receptor complex.[76,77] TGF-β1 has been the most extensively studied isoform, and although all three isoforms serve similar functions, TGF-β3 has shown opposing roles in certain tissue contexts.[78] TGF-β isoforms are synthesized as pre-pro-proteins and rapidly dimerize after signal sequence cleavage. Post-translationally, sugar moieties are added to the amino-terminal pro-domain of TGF-βs that comprises latency-associated peptide (LAP). LAPs non-covalently bind to TGF-β via their carboxy-terminal growth factor domain and are required for proper folding and dimerization of TGF-β.[79] Once dimerized, proteolytic cleavage at the LAP-binding domain produces a mature TGF-β dimer. Once formed, the LAPs remain non-covalently bound to the mature TGF-β dimer, forming the small latent complex (SLC). In the extracellular milieu, SLCs bind to various high molecular weight proteins called latent TGF-β binding proteins (LTBPs), forming large latent complexes (LLCs; *i.e.*, TGF-β:LAP:LTBP complexes).[80,81] LTBPs regulate the availability of TGF-β by sequestering TGF-β in LLCs, which retains TGF-β in the extracellular matrix.[82] Although activation of mature TGF-β likely involves proteolytic cleavage of the LLCs mediated by thrombospondin, which subsequently releases TGF-β:LAPs from LTBPs, TGF-β activation also requires dissociation of the LAPs, which may occur via proteolysis by plasmin, matrix metalloproteases or bone morphogenic protein-1, or by physical disruption via integrin-mediated contraction.[83,84]

TGF-β signaling is initiated by TGF-β ligands binding to cell surface TGF-β receptors (TGFβRs). There are seven type I (TGFβRI; ALK1-ALK7), five type II (TGFβRII; TGFβR2, BMPR2, ACVR2A, ACVR2B, and AMHR2), and one type III (TGFβRIII; β-glycan) receptors,[85,86] each with varying affinities for TGF-β ligands (Table 1). Type I and type II TGFβRs contain a dual-specificity cytoplasmic kinase domain capable of both serine/threonine and tyrosine kinase activity.[87] Downstream signaling is initiated upon binding of a mature TGF-β dimer to a heterotetrameric TGFβR complex formed between two type I and type II TGFβRs.[88] Although TGFβRIII is the most widely expressed TGFβR, it does not possess kinase activity and serves as a coreceptor for type I and type II TGFβRs.[89] Additionally, endoglin, a disulfide-linked dimeric glycoprotein, is a coreceptor for TGFβRI.[90] As we will discuss in detail in this review, TGF-β superfamily members are essential for salivary gland development, injury, and regeneration. In the next section, we introduce canonical and non-canonical TGF-β signaling pathways before discussing the roles of TGF-β superfamily members in salivary gland fibrosis and regeneration in the subsequent sections.

Table 1
TGF-β superfamily ligands and receptors.

Ligands	Type I receptor	Type II receptors	Co-receptors	R-SMADs
TGF-β1, TGF-β2, TGF-β3	TGFBR1/TβRI/ALK5	TGFBR2/TβRII	TGFBRIII/Betaglycan	SMAD 2/3
Activin A, B, AB, AC	ACVR1B/ActR1B/ALK4, ACVR1C/ActR1C/ALK7	ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 2/3
Activin C, E	ACVR1C/ActR1C/ALK7	ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 2/3
Nodal	ACVR1B/ActR1B/ALK4, ACVR1C/ActR1C/ALK7	ACVR2A/ActRII, ACVR2B/ActRIIb	Cripto/Tdgf1, Cryptic	SMAD 2/3
GDF1	ACVR1B/ActR1B/ALK4, ACVR1C/ActR1C/ALK7	ACVR2A/ActRII, ACVR2B/ActRIIb	Cripto/Tdgf1, Cryptic	SMAD 2/3
GDF3	ACVR1B/ActR1B/ALK4, ACVR1C/ActR1C/ALK7	ACVR2A/ActRII, ACVR2B/ActRIIb	Cripto/Tdgf1, Cryptic	SMAD 2/3
GDF8/Myostatin	ACVR1B/ActR1B/ALK4, TGFBR1/TβRI/ALK5	ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 2/3
GDF9	TGFBR1/TβRI/ALK5	BMPR2		SMAD 2/3
GDF10	ACVR1B/ActR1B/ALK4	ACVR2B/ActRIIb		SMAD 2/3
GDF11	ACVR1B/ActR1B/ALK4, TGFBR1/TβRI/ALK5, ACVR1C/ActR1C/ALK7	ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 2/3
BMP2	BMPR1A/ALK3, BMPR1B/ALK6	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb	repulsive guidance molecule (RGM) family	SMAD 1/5/8
BMP4	BMPR1A/ALK3, BMPR1B/ALK6	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb	RGMs	SMAD 1/5/8
BMP5	BMPR1A/ALK3, BMPR1B/ALK6, ACVR1A/ActR1A/ALK2	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb	RGMs	SMAD 1/5/8
BMP6	BMPR1A/ALK3, BMPR1B/ALK6, ACVR1A/ActR1A/ALK2	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb	RGMs	SMAD 1/5/8
BMP7	BMPR1A/ALK3, BMPR1B/ALK6, ACVR1A/ActR1A/ALK2	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 1/5/8
BMP8	BMPR1A/ALK3, BMPR1B/ALK6, ACVR1A/ActR1A/ALK2	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 1/5/8
BMP8B	BMPR1A/ALK3, BMPR1B/ALK6, ACVR1A/ActR1A/ALK2	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 1/5/8
BMP9/GDF2	ACVRL1/ALK1	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb	endoglin	SMAD 1/5/8
BMP10	ACVRL1/ALK1	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb	endoglin	SMAD 1/5/8
BMP15	BMPR1B/ALK6	BMPR2		SMAD 1/5/8
GDF5/BMP14	BMPR1B/ALK6	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 1/5/8
GDF6/BMP13	BMPR1B/ALK6	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 1/5/8
GDF7/BMP12	BMPR1B/ALK6	BMPR2, ACVR2A/ActRII, ACVR2B/ActRIIb		SMAD 1/5/8
AMH		AMHR2		SMAD 1/5/8
Inhibin A or B		ACVR2A/ActRII, ACVR2B/ActRIIb	TGFBRIII/Betaglycan	Inhibits Activins
Lefty			Cripto/Tdgf1, Cryptic	Inhibits Nodal, SMAD2 and SMAD5
BMP3	BMPR1A/ALK3, ACVR1B/ActR1B/ALK4	ACVR2B/ActRIIb		Inhibits BMPs

4. TGF-β signaling

4.1. Canonical TGF-β signaling

The binding of TGF-βs to TGFβRs to activate downstream signaling pathways occurs in a stepwise manner, *e.g.*, dimeric TGF-β binding induces a conformational change in TGFβRII that activates the receptor's kinase domain, which subsequently catalyzes the transphosphorylation of TGFβRI. This facilitates the binding of phospho-TGFβRI to the TGF-β:TGFβRII complex, thereby forming a tetrameric receptor:ligand complex capable of downstream signaling[91–93] (Fig. 1). TGF-β signaling is also regulated at the receptor level by ubiquitylation and ubiquitin-mediated degradation of TGFβRs[94] and sumoylation.[95] In addition, TGF-β:TGFβR signaling is transduced by SMAD proteins (SMADs), transcription factors that are classified into three groups based on their function, *i.e.*, receptor-regulated SMADs (R-SMADs; SMAD1, SMAD2, SMAD3, SMAD5, SMAD8), common SMAD (Co-SMAD; SMAD4), and inhibitory SMADs (I-SMADs; SMAD6 and SMAD7).[96] Type I TGF-β receptors can be divided based on the R-SMAD they phosphorylate.[85] In most cases, induction of the TGFβRI signaling pathway by TGF-β, or nodal and activin ligands, leads to phosphorylation of SMAD2 and SMAD3 (henceforth termed TGF-β/SMADs).[85] In contrast, BMP ligand binding to TGFβRI induces the phosphorylation of SMAD1, SMAD5, and SMAD8 (henceforth termed BMP/SMADs).[85] Both I-SMADs, SMAD6 and SMAD7, have been shown to inhibit phosphorylation of R-SMADs by forming complexes with TGFβRI[97,98] as well as recruiting E3 ubiquitin ligases to induce degradation of TGFβRI.[99–102]

R-SMADs and Co-SMAD consist of two globular domains called Mad-Homology-1 and -2 (MH1 and MH2) at their N-terminal and C-terminal, respectively.[103] The MH1 domain contains a hairpin structure and DNA-binding capabilities, and the MH2 domain contains multiple hydrophobic patches and dictates interactions with adaptors and regulatory proteins. The only documented Co-SMAD in mammals is SMAD4.[104] R-SMADs are activated upon phosphorylation by TGFβRI, leading to SMAD4 oligomerization with active R-SMADs. I-SMADs are activated by TGF-β family members and inhibit R-SMADs phosphorylation by competing for TGFβRI binding.[105] Scaffold protein interactions have been shown to facilitate SMAD activation. SARA (SMAD anchor for receptor activation) has been demonstrated to bind to and retain un-phosphorylated (inactive) R-SMADs at the plasma membrane, which

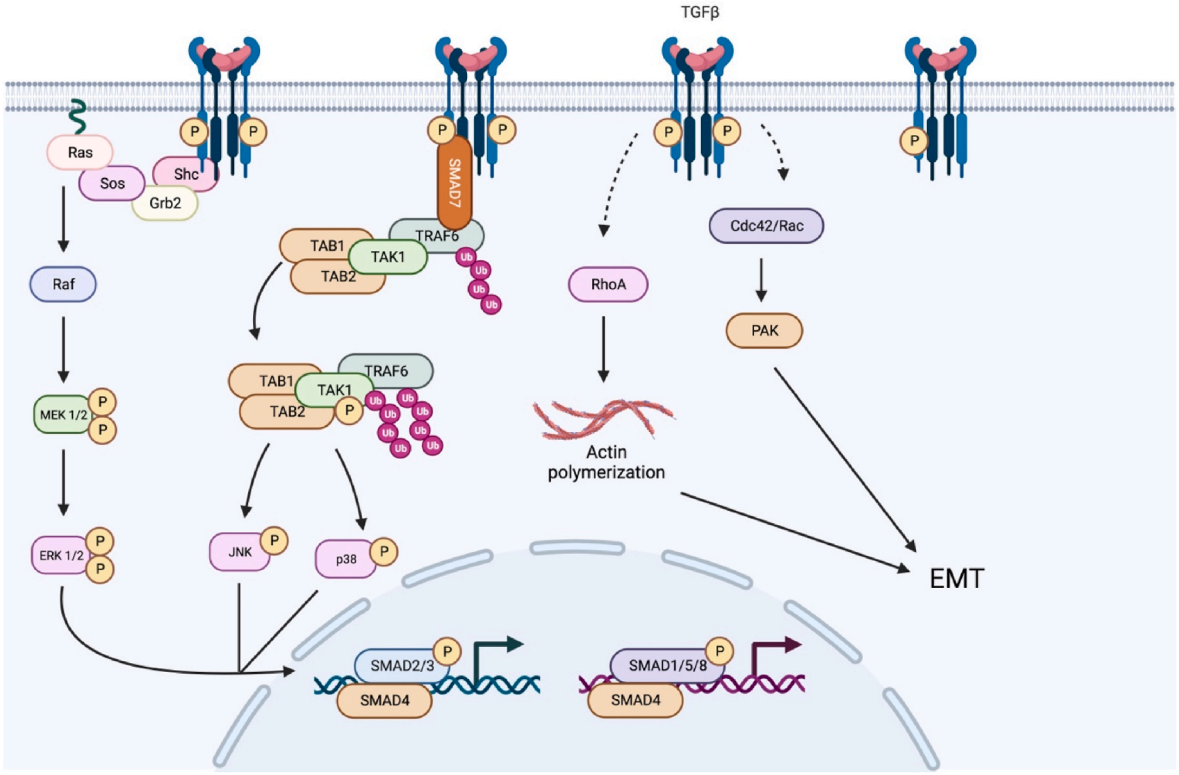

Fig. 1. Intracellular TGF-β Signaling Cascade Upon ligand (TGF-β or BMP) binding, an active tetrameric receptor:ligand complex forms (*i.e.*, $(P\text{-}TGF\beta RI)_2$:$(TGF\beta RII)_2$; TGF-β/BMP) which binds SARA and/or R-SMAD proteins in ligand-specific or SMAD subtype-specific manners. R-SMAD proteins interact with the kinase domains of the active receptor:ligand complex, leading to SMAD activation via its phosphorylation and dissociation from the complexes. Once activated, R-SMADs localize to the nucleus by binding SMAD4, which binds to SMAD-binding elements to regulate the transcription of target genes. Also, SMAD6 and SMAD7 with Smurf2 act as accessory proteins to regulate the indicated steps in SMAD activation.
Figure created with Biorender.com.

serves as a bridge between TGFβRI and R-SMADs, specifically SMAD2 and SMAD3.[106] Other scaffold proteins, such as microtubules, filamin, and caveolin 1, have been shown to regulate R-SMAD signaling non-specifically.[107,108] The Co-SMAD SMAD4, however, can be regulated in a specific manner by TGFβRI associated protein-1 (TRAP-1), which has been proposed to anchor SMAD4 to the plasma membrane proximal to the TGFβR heterotetramer, thereby facilitating its interaction with R-SMADs.[109] Trimers of two R-SMADs and SMAD4 have been shown to be the functional units responsible for downstream transcriptional activation despite SMAD4 not being essential for nuclear localization.[110,111] The I-SMADs, SMAD6 and SMAD7, serve inhibitory roles where SMAD6 predominantly inhibits BMP/SMADs signaling, and SMAD7 inhibits both BMP/SMADs and TGF-β/SMADs signaling. SMAD6 acts as a competitive antagonist of SMAD4, forming an inactive SMAD1/SMAD6 complex,[112,113] whereas SMAD7 inhibits TGFβR-mediated phosphorylation as well as phosphorylation of SMAD2 and SMAD3.[98,114,115] Interestingly, activation of TGFβRs by TGF-β1 has been shown to upregulate SMAD7 mRNA, hinting at a possible negative feedback loop.[114]

In their inactive state, monomeric SMADs are constitutively shuttled between the cytoplasm and the nucleus via nucleopores.[116] However, in their active oligomeric form, SMAD complexes require nuclear import/export factors for transport.[117] Once localized to the nucleus, active SMADs can negatively or positively regulate gene transcription. A conserved hairpin in the MH1 domains of SMADs 1, 3, 4, 5, and 8 binds with low affinity to palindromic repeats in SMAD-binding elements (SBEs) with a minimum sequence of 5′-AGAC-3’.[48] Due to a difference in the positioning of the MH1 hairpin within SMAD1 compared to other SMADs, SMAD1 can also bind to GC-rich regions.[118] With SBEs being found throughout the genome, as are GC-rich regions, one would expect to see many genes regulated by SMADs; however, SMAD-dependent transcriptional activity alone is insufficient for target gene expression due to the low binding affinity of SMAD complexes for SBEs. Additionally, since the MH1 DNA-binding hairpin is highly conserved among R-SMADs, there is a lack of selectivity among SMADs for SBE binding, with SMADs 1, 3, and 4 having similar affinities for SBEs.[119,120] Thus, TGF-β-induced SMAD-mediated transcriptional regulation heavily depends on transcriptional coregulators that interact with nuclear-localized SMAD complexes. Indeed, lineage-specific coregulators have been shown to direct TGF-β- and BMP-induced transcriptional regulation in B cells, myeloid and erythroid precursors, and myoblasts.[121,122]

4.2. *Non-canonical TGF-β signaling*

Despite the elegance of the SMAD signaling system, SMADs alone do not account for the plethora of biochemical processes that can be regulated by TGF-β signaling. Indeed, it is now recognized that TGF-β signaling can crosstalk with other signaling pathways and modulate diverse cell signaling events (Fig. 2). TGF-β signaling activates the mitogen-activated protein kinases (MAPKs) ERK1/2 in multiple cell types.[123–125] TGFβRII can be phosphorylated on Y284 by Src, which serves as a docking site for Grb2 and ShcA.[126] Additionally, activated TGFβRI can recruit and directly phosphorylate ShcA, thus promoting the formation of a ShcA/Grb2/Sos/Ras complex capable of activating Raf and serving as a scaffold for MAPK/ERK signaling.[127] Through this pathway, rapid accumulation of phosphorylated p21/Ras occurs in rat intestines within minutes of treatment with TGF-β.[128] Ras, a MAP4K, acts in this MAPK/ERK cascade to activate Raf, a MAP3K. Activated Raf localizes to the plasma membrane, where it activates MEK1/2, a

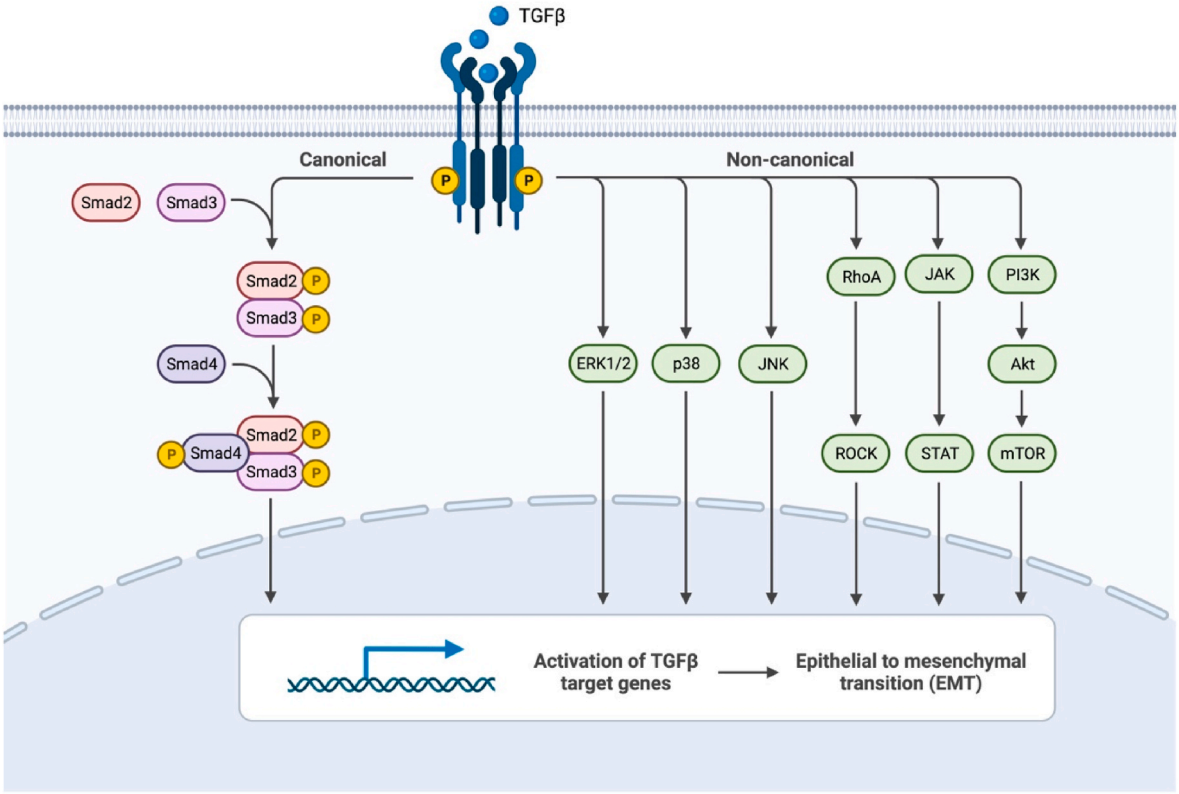

Fig. 2. Non-canonical TGF-**β Signaling**. In addition to canonical SMAD-mediated signaling, TGF-β can activate diverse non-canonical signaling cascades in a cell type-specific manner. Figure created with Biorender.com.

MAP2K, and MEK1/2 subsequently activates the MAPK ERK1/2. In addition to this rapid response to TGF-β, latent ERK1/2 activation occurs on the order of hours in some cell lines and is SMAD4-dependent.[129] This latent response to TGF-β promotes *de novo* synthesis of proteins in the MAPK/ERK phosphorylation cascade. Activation of ERK1/2 is a key regulator of the epithelial to mesenchyme transition (EMT).[130–132] Although a normal part of embryonic development, EMT is associated with tumor metastasis and fibrosis.[133] During EMT, cells lose their epithelial characteristics and acquire those of mesenchymal cells, including downregulation of adhesion proteins and increased matrix metalloprotease (MMP) levels. Additionally, ERK1/2 can phosphorylate SMADs 1, 2, and 3, indicating that TGF-β-MAPK crosstalk is bidirectional.[134–137]

In addition to the ERK1/2 MAPK cascade, TGF-β signaling can activate the JNK and p38 MAPK cascades. JNK and p38 are MAPKs downstream of MAP2K, MKK4, and MKK3/6, which can be regulated by the MAP3K TGF-β-activated kinase (TAK1).[138] Studies utilizing dominant-negative SMAD4 demonstrated that SMAD4 is dispensable for TGF-β-mediated JNK activation, indicating that this crosstalk occurs independently of SMAD signaling.[139,140] Embryos of TAK1-deficient mice exhibit a similar phenotype to TGFβRI or TGFβRIII loss-of-function mutants that show defects in vasculature formation.[141] Studies on TRAF6, which plays a role in the interleukin (IL)-1R- and toll-like receptor (TLR)-mediated activation of TAK1, demonstrated that TRAF6 is indispensable for TAK1-mediated activation of the JNK and p38 MAPK cascades.[142,143] Lysine-63-linked polyubiquitylation of TRAF6 was necessary to recruit and activate TAK1, which enables TAK1-mediated activation of JNK and p38.[144] Further studies demonstrated that TRAF6 directly interacts with TGFβRII and that binding of TRAF6 to the activated TGFβR tetramer induces lysine-63-linked polyubiquitylation of TRAF6 and its association with TAK1.[142,143] It was also shown that TGF-β-induced phosphorylation of p38 was dependent on SMAD7 expression, where SMAD7 is believed to act as an adapter protein, indicating that SMAD7's role in TGF-β signaling extends beyond its inhibition of R-SMAD phosphorylation.[145]

Alternative pathways for TGF-β signaling include the regulation of Rho-like GTPases and the PI3K/Akt and JAK/STAT pathways. Like the MAPK pathways, TGF-β-induced activation of RhoA is a critical regulator of EMT.[146] Like TGF-β-induced activation of ERK1/2, some cells exhibit a latent activation of RhoA, indicating a biphasic response to TGF-β in part due to SMAD-mediated transcriptional activation.[147] In addition to RhoA, TGF-β can activate PAK2 via Cdc42 GTPase activation.[148] Physical interaction between Cdc42 and the TGFβR complex and associations with other members of the Cdc42-PAK2 signaling pathway have been demonstrated.[149] Physical interactions between TGFβRI, TGFβRII, and the p85 regulatory subunit of PI3K, as well as TGF-β-induced activation of PI3K/Akt, indicate that TGF-β induces EMT via activation of the PI3K/Akt pathway.[150–152]

The JAK/STAT pathway is ubiquitous among vertebrates and is found in some lower-order organisms.[153,154] The binding of TGF-β and subsequent activation of TGFβRs bring multiple JAKs in close proximity, enabling TGFβR-mediated transphosphorylation of key tyrosine residues in JAKs that promote their activation.[155] Activated JAKs, in turn, phosphorylate key tyrosine residues in STATs, leading to their dimerization and nuclear localization. The JAK/STAT pathway provides transcription factors for genes containing consensus interferon-γ-activated sequence (GAS) recognition motifs, which regulate GAS-containing gene transcription. Additionally, crosstalk between the TGF-β and JAK/STAT pathways has been shown to negatively regulate canonical SMAD signaling via the upregulation of SMAD7.[156]

5. Salivary gland development

Our collective understanding of salivary gland development has provided a framework for investigating mechanisms that facilitate glandular regeneration. TGF-β1, TGF-β2, and TGF-β3 along with their cognate receptors, TGFβRI and TGFβRII, are all expressed during

salivary gland development, suggesting their involvement in organogenesis.[25] Indeed, TGF-β signaling is critical to salivary gland development,[23] including ductal differentiation[24] and branching morphogenesis.[24–26] Expression of TGF-β signaling molecules is cell- and developmental stage-specific.[25] For example, TGF-β1, TGF-β2, and TGF-β3 are expressed in the mesenchyme during the initial bud stage, but TGF-β3 is the sole isoform present in the mesenchyme during the pseudoglandular, canalicular, and terminal bud stages.[25] Studies using global knockout of TGFβR signaling components have demonstrated that TGF-β is required for overall development, as embryonic or perinatal lethality was observed following deletion of TGF-β2,[157] TGF-β3,[158] TGFβRI,[159] or TGFβRII.[160] Interestingly, E16.5 and newborn mice lacking either TGF-β2 or TGF-β3 developed normal submandibular glands, suggesting that other TGFβR signaling components regulate TGF-β-dependent salivary gland development.[25] To address this possibility, it was shown that global knockout of TGF-β1 in mice did not cause embryonic or perinatal lethality, and these mice developed normal salivary glands. However, the TGF-β1 knockout mice developed multiorgan focal lymphocytic infiltration, including in salivary glands, which resulted in death by four weeks of age.[161,162] This lymphocytic accumulation in the salivary glands of TGF-β1 knockout mice mirrors the chronic inflammatory response observed in human SjD patients and mouse models of SjD.[161,163] A later study using conditional Cre recombinase-mediated knockout of TGFβRI in mice (TGFβRI-coko mice) driven by mouse mammary tumor virus (MMTV) restricted TGFβRI knockout to the salivary and mammary glands. Results indicated that TGFβRI-coko mice were born normally with normal salivary gland development. Still, female TGFβRI-coko mice developed focal inflammation of the salivary and mammary glands and the heart, which proved lethal by 4–5 weeks of age.[164] These results were similar to earlier findings in global TGF-β1 knockout mice.[161,162] Female TGFβRI-coko mice also had increased levels of proinflammatory cytokines in the salivary glands, including IL-1, IL-2, IL-12, and IFNγ, indicative of a Th1-specific response.[164] The female TGFβRI-coko mice also exhibited disruption of the wild-type aquaporin 5 distribution in submandibular glands, suggesting impairment of secretory function. However, male TGFβRI-coko mice did not develop inflammation and had a lifespan similar to wild-type mice. TGFβRI deletion using adenoviral vector-mediated Cre recombinase delivery directly into the submandibular gland (SMG) via the Wharton's excretory duct of adult TGFβRI-floxed mice ($TGF\beta RI^{fl/fl}$ mice) further demonstrated that female, but not male, $TGF\beta RI^{fl/fl}$ mice develop inflammatory foci in the SMG.[164] These data support the conclusion that female mice are more susceptible to salivary gland inflammation and glandular dysfunction caused by impaired TGF-β signaling. Thus, TGF-β signaling during salivary gland development is sex- and tissue-dependent.

Though essential for organogenesis, elevated expression of TGF-β signaling components is also problematic for salivary gland development.[165] The conditional overexpression of TGF-β1 in the mammary and salivary glands impeded normal salivary gland development in mice, with apparent disruption of proper branching, atrophy of granular convoluted ducts (GCDs) and acini, severe fibrosis, and salivary gland hypofunction.[166] *In vitro* studies using a mixed salivary gland cell culture system to study epithelial-mesenchymal interactions have demonstrated that exogenous addition of TGF-βI results in acinar cell loss, inhibition of acinar differentiation, and increased expression of collagen, effects that could be abolished by inhibition of TGFβRI with SB525334.[167] Therefore, proper TGF-β signaling regulation is essential for salivary gland development.

6. TGF-β in salivary gland disease

TGF-β signaling is involved in the pathogenesis of multiple diseases, including inflammatory diseases, cancer, and fibrosis.[168–173] TGF-β signaling is essential for the regulation of the fibrotic pathway, which has been studied in chronic kidney fibrosis, idiopathic pulmonary fibrosis, cystic fibrosis of the lung, and other tissues and fibrotic disorders.[170–173] TGF-β, secreted by senescent cells, can also mediate normal aging-related fibrosis.[170] Further, TGF-β signaling is upregulated in salivary gland pathologies and disease models.[174,175] For example, TGF-β is upregulated in the sera and salivary glands of patients with SjD, and SMAD-mediated mechanisms are active in SjD.[175–177] TGF-β is also expressed in fibroblasts and lymphocytic foci in fibrotic lesions of labial salivary glands in SjD patients.[178] Recent evidence shows that TGF-β induces EMT-dependent salivary gland fibrosis in primary SjD.[176] TGF-β is also elevated in the parotid and submandibular glands of patients receiving radiotherapy for head and neck carcinomas[179] compared with control glands. TGF-β staining in the collateral irradiated glands was concentrated in epithelial cells and myofibroblasts. As we discuss in the following sections, TGF-β also contributes to salivary gland fibrosis in a mouse salivary duct ligation-induced injury model.[166,180]

The regeneration of healthy salivary gland tissue following damage caused by inflammation or radiation exposure is often impeded by abundant extracellular matrix (ECM) deposition and fibrosis. TGF-β1 regulates ECM deposition by promoting the biosynthesis of collagen and fibronectin and the expression of protease inhibitors that reduce ECM degradation.[166,181] TGF-β1 also enhances ECM deposition by inducing myofibroblast differentiation.[182,183] Indeed, TGF-β signaling is essential to salivary gland fibrosis.[166,180] The following two sections concern TGF-β signaling in salivary gland disease and tissue regeneration models.

7. Salivary gland injury and regeneration models

7.1. Salivary gland ductal obstruction and ligation models

Ligation followed by deligation of the main excretory ducts of salivary glands is a widely used model of salivary gland inflammation and subsequent regeneration, respectively, as deligated glands fully recover from the ligation-induced atrophic state. Experimental models of salivary gland ductal obstruction have been employed in mice, rats, cats, rabbits, and pigs to study the pathogenesis of sialopathies caused by obstruction, such as salivary gland inflammation (sialadenitis) and salivary gland stones (sialithiasis).[174,184–189] To obstruct ducts by ligation and investigate initiation of salivary gland dysfunction and degeneration, surgical sutures or clips are used, which enables easy deligation of the duct to monitor responses associated with recovery of normal salivary gland function. This approach has advantages over other models, such as radiation-induced salivary gland damage or sialoadenectomy, where full recovery is not obtainable. For the submandibular gland, ductal ligation-induced atrophy develops quickly with changes in epithelial acinar and ductal cell morphology and immune cell infiltration observable within 24 h. Upon ductal deligation, acinar and ductal epithelia recover within three days.[190,191] Without ductal deligation, many saliva-secreting acinar cells succumb to apoptosis within seven days, and their debris becomes phagocytosed by intraepithelial macrophages.[192] Another study demonstrates a progressive loss of approximately 85% of the salivary acini in rats, resulting in epithelial atrophy at two weeks post-ligation.[193] Notably, ductal ligation has been employed to treat excessive salivation (sialorrhea).[194–196]

Using a ductal ligation model where the Wharton's SMG excretory duct was temporarily ligated, our laboratory was the first to report the upregulation of TGF-β signaling components following SMG ductal ligation in C57BL/6 mice that correlated with glandular fibrosis and acinar atrophy.[174] Following a seven-day ligation, the ligature was removed, and gland regeneration was observed at 28 days following ductal deligation. In addition to functional restoration, histological changes, including loss of acini and fibrosis, were reversed following deligation. The elevated expression of both canonical and non-canonical TGF-β signaling components (TGF-β1, TGF-β3, TGFβRI, SMAD2/3, TAK1, TAB1, Snail, Slug) observed following ductal ligation was also reversed upon deligation. The fibrosis markers, collagen I and

fibronectin, were highly expressed with ductal ligation and returned to near baseline levels following deligation. Significantly, in this study, *in vivo* treatment with the TGFβRI inhibitors, SB431542 and GW788388, blocked collagen I and fibronectin upregulation caused by ligation, supporting the notion that TGF-β signaling through TGFβRI is essential for fibrosis development observed in the ligation model. Woods et al. also noted that TGFβRI expression was restricted to acinar cell populations within ligated and control SMGs.[174]

Further studies reported similar findings in a rat model. Yasumitsu et al. utilized the rat SMG ligation model where the excretory duct was ligated for seven days, followed by removal of the ligature and collection of glands at 0, 1, 3, 7, and 14 days following deligation.[197] Whereas in healthy glands, TGF-β1 expression was restricted to granular convoluted tubules and striated ducts and absent in acinar cell populations within SMGs, TGF-β1 staining was observed in connective tissue and ductal cell populations from 0 to 7 days following deligation, and returned to a non-ligated control staining pattern by 14 days.[197]

These studies demonstrate the involvement of TGF-β signaling in rodent salivary ductal ligation models of glandular fibrosis and the regeneration of normal salivary gland morphology and function upon ductal deligation. Further, these studies reveal cell-specific variation in the expression patterns and function of TGF-β signaling components in the ductal ligation model. Studies using single-cell omic technologies (*e.g.,* transcriptomics, proteomics) to resolve the cell type-specific contributions to glandular fibrosis are needed. Some of the recent advances in this area are discussed in the next section.

7.2. Sialoadenectomy/resection models

Sialoadenectomy is a procedure that involves the partial or total surgical removal of salivary glands. Clinically, sialoadenectomy has been used to treat salivary gland inflammation, tumors, and cysts (sialoceles). However, it also serves as an approach to studying the effects of salivary gland injury on the submandibular, parotid, and sublingual glands. Sialoadenectomy of the submandibular gland (SMG), which produces 70% of human resting saliva, is a more commonly used murine model than sialoadenectomy of other salivary glands.[198] This is likely because the SMG is easily accessible in mice, compared to the parotid and sublingual glands, and has a compact structure that makes the SMG easy to locate and remove. Sialoadenectomy of SMGs has been utilized in small rodent models such as mice,[199] rats,[200,201] and hamsters[202]and larger animal models such as dogs to study tissue regeneration after partial sialoadenectomy, infection, and tumor development. Additionally, sialoadenectomy has been used to investigate odontogenic responses beyond the salivary glands, such as the role of epidermal growth factor (EGF) in rodent saliva. Morris-Wiman et al. studied the role of EGF in the morphology and maintenance of taste buds using sialoadenectomized rats lacking both submandibular and sublingual glands.[203] Their findings indicate that EGF secretion from these glands induces the formation and maintenance of fungiform taste buds and papillae. In contrast, EGF was dispensable for circumvallate papillae taste buds. Extending beyond the oral cavity, Helmrath et al. utilized sialoadenectomy of SMGs in mice to demonstrate that salivary gland-derived EGF was involved in bowel adaptation after small bowel resection.[204]

Only a few studies have utilized partial sialoadenectomy to investigate cell signaling responses to this injury. Instead, partial sialoadenectomy (alternatively referred to as partial resection or biopsy punch) is often used as a model for testing bioengineering approaches to salivary gland regeneration.[205–208] Using a partial salivary gland resection model, O'Keefe et al. demonstrated increased macrophage presence in the gland and concomitant elevated expression of both TGF-β1 and TGF-β3.[198] A study utilizing a hamster sialoadenectomy model of the submandibular and sublingual glands determined that after sialoadenectomy, TGF-β1-expressing eosinophils infiltrated the wound during repair.[209] Additional work is needed to better understand the cell signaling pathways involved in tissue regeneration following partial salivary gland resection.

7.3. Cancer and irradiation models

Despite a general decrease in tobacco smoking and the adoption of electronic cigarettes, data from the SEER program of the National Cancer Institute of the NIH shows an upward trend in the age-adjusted incidence of oral cancers between 2000 and 2019. The SEER program estimates over 54,000 new cases of oral cancer in 2023, representing 2.8% of all new cancer diagnoses. Notwithstanding the remarkable positive effects of immunotherapeutics such as pembrolizumab and nivolumab, surgical resection and radiotherapy remain first-line treatments for oral cancers.[210] Radiotherapy for head and neck cancers causes collateral damage to adjacent odontogenic tissues, including salivary glands and gustatory tissue, leading to a significant decline in the patient's quality of life.[211,212]

Radiation-induced injury models have been used to study salivary gland regeneration, to identify the molecular and cellular processes governing acute and chronic damage caused by irradiation, and to identify therapeutics that may offer radioprotection and stimulate glandular regeneration following injury. Mouse models of radiation-induced injury utilize single (1–15 Gy) or fractionated (5 fractions of 1–6 Gy) dosing regimens targeted at the head and neck region, either using image-guided irradiation or by shielding the remainder of the body with lead shields.[213–217] For a detailed review of the spatiotemporal effects of salivary gland irradiation in animal models, we refer the readers to the Jasmer et al. publication.[214]

It has been shown that following irradiation in humans, TGF-β1 expression is quickly elevated,[218] and a study of patients receiving radiotherapy for the treatment of head and neck squamous cell carcinoma, demonstrated that post-irradiation plasma levels of TGF-β1 were predictive of late morbidities.[219] Another study showed a 10-fold increase in TGF-β protein levels in parotid and submandibular gland samples from patients experiencing radiation-induced hyposalivation.[179] These findings are consistent with results from other models of radiation-induced tissue damage, such as pulmonary pneumonitis due to thoracic radiotherapy. One study involving patients receiving pulmonary radiotherapy for the treatment of lung cancers revealed that though all patients had elevated plasma TGF-β levels following treatment, those that returned to baseline levels by the end of radiotherapy did not develop pulmonary pneumonitis. In contrast, those with sustained elevation of plasma TGF-β levels developed radiation-induced pneumonitis.[220]

8. Cell-specific TGF-β signaling in salivary gland regeneration

Extracellular matrix deposition and remodeling are essential to wound healing and regeneration, whereas fibrosis can result in tissue hardening and dysfunction.[221] The development of fibrosis is mediated by several cell types, including tissue-resident fibroblasts, endothelial cells, myofibroblasts and their stromal precursors, and immune cells, including lymphocytes and macrophages.[222–224] However, prior studies offer little insight into the contributions of specific cell types to salivary gland injury and regeneration in experimental models of glandular fibrosis, such as the ductal ligation model. This section will discuss different cell populations influencing salivary gland injury and regeneration through TGF-β signaling (Table 2).

8.1. Stromal cells

Altreith et al. utilized ligation of Wharton's and Bartholin's ducts of the submandibular and sublingual glands, respectively, and observed glandular changes 7 and 14 days post-ligation.[225,226] Consistent with previous reports, these studies showed that ductal ligation induced acinar cell atrophy, extracellular matrix accumulation, and collagen remodeling within seven days, which was significantly more advanced

Table 2
Cell-specific Roles and Evidence of TGF-β signaling in salivary gland regeneration.

Cell Type	Role(s)	Evidence	Reference
PDGFRα^{+}/β^{+} stromal cells	Fibrosis: ECM deposition, collagen remodeling	Elevated expression (scRNA-seq data) of latent TGF-β binding protein-2 (*Ltbp2*) was found following a 14-day ductal ligation of SMGs and sublingual glands.	226
Stromal Cells	Not determined in study	Irradiated patient submandibular and parotid gland samples revealed upregulation of TGF-β specifically in stromal cells and myofibroblasts.	179
Macrophages (M2-like)	Fibrosis	Increased macrophage numbers and elevated expression of both TGF-β1 and TGF-β3 were found following a 14-day ductal ligation of SMGs and sublingual glands.	239
ΔNp63^{+} basal and myoepithelial progenitor cells	Proliferation and differentiation of salivary gland epithelial cells, primarily acini	By ablating ΔNp63^{+} progenitor cells in the salivary gland, it was determined that loss of ΔNp63 activates TGF-β signaling, and that Fst/activin/Smad signaling is responsible for the maintenance of the salivary gland stem/progenitor cell population.	223
Myofibroblasts	Transdifferentiation of fibroblasts into myofibroblasts	Irradiated patient submandibular and parotid gland samples revealed upregulation of TGF-β specifically in stromal cells and myofibroblasts.	179

at 14 days following ductal ligation. Utilizing immunohistochemical analysis, Altreith et al. detected a three-fold expansion of PDGFRα^{+} stromal cells with ductal ligation. This response was previously reported to promote proacinar cell differentiation via a BMP-dependent mechanism and expression of multiple ECM genes in embryonic salivary gland organoids.[227] These authors also used a lineage tracing mouse (Gli1--CreER^{T2};R26^{TdT}) and demonstrated that the number of a subset of PDGFRα^{+}/β^{+} stromal cells expressing glioma-associated oncogene 1 (Gli1) was significantly enhanced at 14-days post-ligation. Gli1 has been implicated in fibrosis development in multiple tissue types,[228–230] and a population of Gli1+ cells has previously been identified in developing SMGs on embryonic day 16[227]. Following ductal ligation, the Gli1^{+} cells were shown to be located in the neurovascular regions of the SMG, where they contributed to ECM deposition and active collagen remodeling.[225]

With the advent of single-cell transcriptomics, we can now resolve cell type-specific gene expression changes caused by perturbations such as ductal ligation in an unbiased manner. Altreith et al. performed single-cell transcriptomic analyses of the submandibular and sublingual glands following a 14-day ligation of their main excretory ducts.[225] This analysis revealed a limited number of Gli1^{+} cells but identified a subpopulation of fibrogenic PDGFRα^{+}/β^{+} stromal cells that were expanded following ductal ligation. The differentially expressed genes in PDGFRα^{+}/β^{+} stromal cells in the duct-ligated glands included several genes known to be essential for fibrosis development, including periostin (*Postn*), secreted phosphoprotein 1 (*Spp1*), and latent TGF-β binding protein-2 (*Ltbp2*).[225] LTBPs interact with fibrillin microfibrils within the extracellular matrix,[231] and, as discussed earlier, LAP, LTBP, and TGF-β combine to form a large latent complex (LLC). Other studies show that LTBP2 is secreted from lung myofibroblasts and is involved in idiopathic pulmonary fibrosis,[232] while LTBP4 has been implicated in renal fibrosis.[233] Thus, fibrogenic PDGFRα^{+}/β^{+} stromal cells that are expanded by ductal ligation may be mediators of salivary gland fibrosis, partly by contributing to pro-fibrotic TGF-β signaling.

8.2. Macrophages

Altreith et al. also reported a 49- and 69-fold increase in F4/80^{+} macrophage staining in the SMG at 7- and 14-days post-ductal ligation, respectively. Macrophages, particularly those that are pro-fibrotic, known as M2-like macrophages, can influence the recruitment and proliferation of fibroblasts and their differentiation into myofibroblasts through the secretion of several factors, including TGF-β1.[234–236] Macrophages can also indirectly contribute to TGF-β signaling by secretion of factors necessary to activate latent TGF-β1, such as metalloproteases.[237] As mentioned earlier, using a partial salivary gland resection model that results in fibrosis, O'Keefe et al. reported increased macrophage presence in the gland and elevated expression of TGF-β1 and TGF-β3, along with several other markers associated with M2-like macrophages.[198] Importantly, this study showed regional differences, with F4/80^{+} macrophage staining elevated globally across the gland at three days post-partial sialoadenectomy, but these macrophages remained elevated within fibrotic foci throughout the study duration of 56 days. Taken together, these studies indicate that macrophages may be an essential regulator of TGF-β-dependent salivary gland fibrosis.

8.3. Progenitor cells

In the submandibular gland, ΔNp63 is expressed in basal and myoepithelial cells that serve as progenitors capable of giving rise to all mature epithelial cell subtypes in the SMG.[238] Using a ΔNp63 floxed (ΔNp63$^{fl/fl}$) mouse crossed with a tamoxifen-inducible UBC^{CreERT2} mouse, Min et al. ablated ΔNp63 in adult mice by administering tamoxifen at 10–12 weeks of age.[239] The SMGs of these global ΔNp63KO mice were smaller than wild-type mice, with reduced ductal structures and enlarged acini, decreased saliva production, and diminished K19 expression. Restricting the ablation of ΔNp63 to p63-expressing cells by crossing ΔNp63$^{fl/fl}$ mice with Trp63^{CreERT2} mice yielded SMG phenotypic changes that were similar between mice with global and restricted ablation of ΔNp63. Using bulk RNA-seq, multiple differentially expressed genes were identified between wild-type and ΔNp63KO mouse SMGs, including genes involved in TGF-β, Notch, MAPK, Hippo, and Wnt signaling.[239] The elevated mRNA expression of multiple genes involved in TGF-β signaling in the ΔNp63KO mice suggests that loss of ΔNp63 can activate TGF-β signaling. Single-cell transcriptomic analysis was used to further evaluate progenitor cell-specific contributions in ΔNp63KO mice, which further demonstrated that ΔNp63 was essential for retaining the stem/progenitor cell populations in the SMG as basal/progenitor cells were absent in ΔNp63KO glands. Strikingly, ΔNp63KO glands had fewer acinar cell clusters, suggesting that overall gland maintenance, particularly of acini, was disrupted by the loss of ΔNp63-expressing progenitor cells. It was also shown that follistatin (*Fst*), which was highly expressed and co-expressed within the progenitor cell cluster with *Trp63* in control SMGs, was highly downregulated in ΔNp63KO glands. Follistatin binds to and inhibits activin-mediated activation of SMAD2/3. *In vitro* studies also demonstrated that Fst was

Table 3
Targeting TGF-β in models of salivary gland damage.

Model/Cell Source	Inhibitor	Target(s)	Result	Reference
In Vitro				
Embryonic murine salivary gland epidermis	RepSox	TGFβRI	RepSox increased the proliferation of $p63^+$ epidermal progenitor cells.	250
Heterogeneous adult mouse SMG preparations were grown on Matrigel	SB525334	TGFβRI	SB525334 increased the expression of acinar cell markers (*e.g.*, *amy1a*, *aqp5*, and *zo1*), and increased formation of acinar-like structures, irrespective of their proximity to mesenchymal cells.	167
Homogenous SMG epithelial organoids	LDN19318	BMP receptors, ALK2 and ALK3.	LDN19318 inhibited proacinar (AQP5) marker expression.	227
2D and 3D cultures of human salivary gland progenitor cells	A83-01	TGFβRI	A83-01 reduced expression of the ductal marker keratin 7.	252
Human salivary gland epithelial cultures from healthy subjects	SB431542	TGFβRI	SB431542 reduced TGF-β1-induced expression of the fibrosis markers vimentin and collagen type I compared to cells treated with exogenous TGF-β1 alone.	176
In Vivo				
SMG Wharton's duct ligation	SB431542 and GW788388	TGFβRI	SB431542 and GW788388 blocked upregulation of the fibrosis markers, collagen I and fibronectin.	174
SMG Wharton's duct ligation	SIS3	SMAD3	SIS3 reduced fibrosis, improved salivary function, reduced release of IL-1β and IL-6, reduced acinar atrophy, and preserved normal gland morphology.	253

a key regulator of activin in salivary gland epithelial progenitor cells (SGEPCs) and that Fst/activin/SMAD signaling was responsible for the maintenance of salivary gland stem/progenitor cells.[239] These studies revealed that TGF-β signaling, downstream of Fst and activin activation, governs the proliferation and differentiation of salivary gland epithelial cell populations, consistent with results using other models. For instance, Mou et al. found that in airway epithelium, TGF-β/BMP/SMAD signaling was suppressed in $p63^+$ basal cells and that SMAD signaling promoted epithelial differentiation. In addition, inhibition of SMAD signaling resulted in an expansion of stem cell populations.[240] A recent study in airway epithelium indicated that basal cells promote tissue regeneration via TGF-β following SO_2-induced airway injury.[241]

Importantly, these studies reveal the requirement for ΔNp63 in SMG homeostasis in the absence of inflammation or salivary gland injury resulting from ductal ligation or irradiation. A recent publication from Song et al. utilized a unilateral ductal ligation model where the main excretory duct of the SMG was ligated for 14 days and deligated for 14 days, during which the SMG regenerated.[242] These studies utilized *Acta2*^{CreERT2};*Δ Np63*$^{fl/fl}$;*Rosa26-TdTomato* knockout (ΔNp63MECcKORFP) mice, which enabled visualization of myoepithelial cells in which ΔNp63 was ablated. Results determined that while ΔNp63 was essential for SMG homeostasis,[239] its expression in SMG myoepithelial cells was not required for SMG regeneration following ductal ligation, as SMA^+ myoepithelial cells compensated for the loss of ΔNp63 cells by contributing to the regeneration of both ductal and acinar cell populations.[242] The contribution of TGF-β signaling in ΔNp63 salivary gland progenitor cells to salivary gland regeneration remains to be investigated. Further analysis of scRNA-seq data made publicly available by Altreith et al.[225] may shed more light on the cell type-specific expression of TGF-β family genes following ductal ligation. However, the published data evaluated gene expression following ductal ligation, not during the regenerative process that follows deligation. As discussed in the discussion section, a subsequent preprint from Altreith et al.[243] includes single-cell transcriptomic data from deligated samples, though the analysis is limited to endothelial cell populations.

8.4. Myofibroblasts

Generating bioactive TGF-β from latent stores requires localized coordination of proteases and integrins within fibrotic lesions.[244] TGF-β targets various cell types, including fibroblasts, macrophages, epithelial cells, and vascular cells.[244] TGF-β1 is critical for myofibroblast activation,[245] though specific evidence for the involvement of myofibroblast activation in salivary gland fibrosis is not well documented. However, following radiotherapy, patient submandibular and parotid gland samples revealed that TGF-β upregulation is not uniformly distributed across specimens. TGF-β upregulation was not observed in parotid and submandibular gland acinar and ductal epithelial cells of radiotherapy patients, but rather, TGF-β expression was significantly elevated in stromal cells and myofibroblasts.[179] In addition, the co-expression of α-SMA and TGF-β in myofibroblast populations was indicative of fibroblast transdifferentiation into myofibroblasts.[179]

9. Targeting TGF-β signaling to promote salivary gland regeneration

TGF-β superfamily members are pleiotropic, regulating diverse immune responses,[246] development,[23,247,248] wound healing and fibrosis,[172,174] cellular maturation, and differentiation.[249] Several studies have employed pharmacological inhibitors to target TGF-β signaling *in vitro* using standard and organoid cultures of salivary gland cells and *in vivo* using various glandular injury and disease models (Table 3). Targeting TGF-β signaling is complicated by the fact that both elevated and depleted TGF-β signaling are problematic for normal development, regeneration, and function of salivary glands.

Using a crude preparation of embryonic murine salivary gland epidermis, it was shown that the TGFβRI inhibitor, RepSox, increased the proliferation of $p63^+$ epidermal progenitor cells without disrupting their ability to respond to Ca^{2+} addition.[250] In another study, heterogeneous adult mouse SMG preparations grown in culture demonstrated cell-specific and temporal expression differences, as well as differential localization of TGF-β family members.[167] Early passage epithelial cells, but not mesenchymal cells, showed high expression of TGF-β1 and TGFβRII localized to the cytoplasm, while TGFβRI was robustly expressed in both epithelial cytoplasm and nuclear area. Alternatively, both late-passage mesenchymal and epithelial cells highly expressed TGF-β1, TGFβRI, and TGFβRII. They also grew heterogeneous adult mouse SMG preparations on Matrigel with or without TGF-β1 and TGFβRI inhibitor, SB525334, to assess the role of TGF-β1 in salivary gland epithelial and mesenchymal differentiation.[167] Acinar-like structures did not form near mesenchymal cells in the presence of TGF-β1 but did in Matrigel-only or cultures additionally treated with SB525334,[167] revealing that TGFβRI inhibition promoted acinar-like structure formation. This study further showed differential effects of TGF-β1 signaling on epithelial and mesenchymal populations. TGF-β1 alone resulted in elevated collagen expression and inhibited the formation of acinar-like structures when epithelial cells were located near mesenchymal cells. In the presence of TGF-β1, the addition of SB525334 increased the expression of acinar cell markers (*e.g.*, *amy1a*, *aqp5*, and *zo1*) and the formation of acinar-like structures, irrespective of proximity to

mesenchymal cells. As addressed by Piraino et al., though both RepSox and SB525334 inhibit TGFβRI, comparing published results with these inhibitors is difficult, given differences in cell sources and preparations, culture conditions, and inhibitor affinities.[251] Another *in vitro* study using the selective BMP receptor inhibitor, LDN19318, revealed that BMP signaling was necessary for proacinar marker (*i.e.*, AQP5) expression in homogenous salivary gland epithelial organoid cultures.[227] In both 2D and 3D cultures of human salivary gland progenitor cells, the TGFβRI inhibitor, A83-01, reduced expression of the ductal marker keratin 7 (K7).[252] Studies with salivary gland progenitor cell cultures indicate that K7 expression is often a marker of cellular stress, which impedes hyaluronic acid (HA)-based hydrogel tissue engineering approaches to promote salivary gland regeneration.[252] A study that treated human salivary gland epithelial cell cultures from healthy subjects with either TGF-β1 or TGF-β1 plus the TGFβRI inhibitor, SB-431542, demonstrated that TGFβRI inhibition causes a significant reduction in levels of the fibrosis markers vimentin, collagen type I, and E-cadherin, comparable to levels seen in untreated salivary gland epithelial cells from healthy subjects.[176]

The first *in vivo* evidence of a role for TGF-β signaling in salivary gland damage was provided by Woods et al., who utilized a 7-day SMG ductal ligation model (*i.e.*, ligation of the Wharton's duct) to evaluate the effect of blocking TGF-β signaling with the TGFβRI inhibitors, SB431542 and GW788388.[174] Results showed that collagen I and fibronectin mRNA levels were elevated at seven days following duct ligation and that the addition of the TGFβRI inhibitors decreased collagen I and fibronectin gene expression.[174] A recent study demonstrated that the specific SMAD3 inhibitor, SIS3, reduced SMG fibrosis and dysfunction caused by a 7-day Wharton's duct ligation.[253] This study further showed that SIS3 inhibited the release of the proinflammatory cytokines IL-1β and IL-6, reduced acinar atrophy, and preserved normal gland morphology.[253] These *in vivo* studies clearly indicate that salivary gland damage due to ductal ligation is mediated by TGF-β signaling, though the role of TGF-β in gland regeneration post-deligation needs further investigation.

10. Further discussion

More than thirty years ago, TGF-β was identified as a key factor in the development of fibrosis, and subsequently, this role has been substantiated by many studies in multiple tissue and disease settings.[171,172,180,254] In addition to the essential role of TGF-β signaling in salivary gland development,[23] the role of TGF-β in promoting salivary gland damage, specifically fibrosis, has only been recently identified.[174,253] As described in this review, convincing evidence for the cell-specific roles of TGF-β signaling in salivary gland regeneration following tissue damage is limited to investigations with a small number of cell types with scant data available on salivary glands. However, cell populations from other tissues where regeneration of cells damaged by fibrosis has been investigated provide insights into signaling pathways that may regulate salivary gland regeneration. In particular, we hypothesize that specific immune cell populations and endothelial cells play a role in the fibrosis of salivary epithelia and suggest that potential pathways discussed in this review may represent effective targets to regenerate salivary glands damaged by disease or other causes (*e.g.*, cancer radiotherapy).

TGF-β signaling is known to regulate T cell differentiation, proliferation, and function.[246,255,256] T cells have been implicated in the development of fibrosis, as has been supported by studies using various tissue fibrosis models and disease contexts.[257] For example, studies of pulmonary fibrosis following thoracic irradiation have revealed a shift in $CD4^+$ T lymphocytes from TH1 and TH17 in the early inflammatory (*i.e.*, pneumonitic) phase to pro-fibrotic TH2 and T_{REG} cells in the fibrotic phase.[258] Whether this shift in $CD4^+$ T cells is recapitulated in salivary gland fibrosis models remains to be determined. In addition to adaptive immune cell populations, innate lymphoid cells (ILCs) are key regulators of fibrosis.[259–261] ILC classes (*i.e.*, ILC1, ILC2, and ILC3) are either anti- or pro-fibrotic,[259–261] and previous studies have identified a unique subset of ILCs present in mouse submandibular and sublingual but not parotid glands that are similar to anti-fibrotic, IFN-γ-producing ILC1s but don't produce significant amounts of IFN-γ.[262,263] In a systemic sclerosis model, it was demonstrated that TGF-β, which is elevated in systemic sclerosis, indirectly promotes fibrosis through ILC2s by decreasing their expression of anti-fibrotic interleukin 10 (IL-10) and stimulating myofibroblast differentiation.[264] As we work to fully understand the cellular landscape most appropriate for promoting salivary gland regeneration and define suitable pharmacological targets in the TGF-β signaling pathway for restoring salivary gland function, T lymphocytes and ILCs represent immune cell populations of significant interest.

The endothelial-specific role of TGF-β in fibrosis development has been reported in multiple tissues, including skin, lung, kidney, and liver.[244] One compelling study in mice showed that TGF-β activation in endothelial cells resulted in skin, lung, and microvascular fibrosis development, associated with myofibroblast infiltration and endothelial-mesenchymal transition.[265] In a chronic kidney disease mouse model, heterozygous knockout of TGFβRII in endothelial cells reduced fibrotic remodeling and endothelial-mesenchymal transition in kidney tissue.[266] A recent preprint from Altreith et al. reported the results of single-cell transcriptomic analyses that compared submandibular and sublingual glands after 14 days of ductal ligation or after 14 days of ligation and five days of deligation, using homeostatic or mock glands as controls.[243] Though there is much to consider from this robust dataset, the authors reported that after the 5-day deligation, the CellChat data analysis predicted that chemokine (C-X-C motif) and EPH signaling in endothelial cells promote salivary gland regeneration.[243] These findings suggest that endothelial cells are relevant to the regenerative process and strongly support a role in tissue regeneration for TGF-β signaling in endothelial cells and perhaps other cell types that warrant further investigation.

As we interpret the findings described in this review, we should consider the limitations and context of the *in vitro* and *in vivo* models employed. For example, the ductal ligation model is most widely used to study salivary gland fibrosis and regeneration; however, optimum time courses for evaluating fibrosis development and tissue regeneration have not been uniformly employed. For example, data collected at 7 or 14 days after ligation are commonly used to assess fibrosis development, whereas data collected at 5, 7, or 14 days post-deligation have been used to evaluate the extent of tissue regeneration. Thus, we have included details on the time courses used in the studies reviewed. Still, we acknowledge that the optimal times to collect data likely vary widely based on tissue types and the experimental approaches used. When considering irradiation models of tissue injury that mimic collateral tissue damage caused by common radiotherapies in cancer patients, it is important to recognize that most experimental *in vivo* radiation exposure studies evaluate the effects of targeted irradiation of tissues, such as salivary glands, in the absence of a proximal tumor, which does not mimic the clinical setting for cancer patients. During tumor development, the immune and stromal microenvironment adjacent to salivary glands and other tissues is likely to be strikingly different than under healthy conditions, which could limit the significance of tissue irradiation studies done in the absence of tumors. Another consideration, especially as we investigate diseases that affect aging populations such as SjD, is the role TGF-β plays in normal physiologic aging and fibrotic development and distinguishing these roles from pathologic dysfunction and fibrosis. As mentioned earlier, TGF-β can mediate normal physiologic aging-related fibrosis,[170] but the extent to which these contributions translate to salivary gland aging is unknown. One study utilizing mice of 12–18 months of age determined that inhibitory SMAD7 was protective of salivary gland function with age,[267] as loss of SMAD7 resulted in more pronounced loss of function with age. These findings suggest that TGF-β signaling is important in salivary gland aging and diminished function, but more research is needed in this area.

Targeting TGF-β signaling components may be useful for promoting salivary gland regeneration following damage caused by myriad conditions. However, much more work is needed to elucidate the optimum therapeutic strategy for targeting this pathway *in vivo* and to further identify the cellular contributors to these responses and side effects that could limit the acceptance of a promising regenerative therapy.

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