1 Implications of Salivary Gland Developmental Mechanisms for the Regeneration of Adult Damaged Tissues

Isabelle M.A. Lombaert

Abstract

The convergence of the fields of tissue engineering and regenerative medicine provides a potential blueprint to repair damaged tissues. Accordingly, a range of therapeutic applications have emerged that hold great potential to regenerate branching organs, such as salivary glands. This unique saliva-secreting organ is required for proper oral health, lubrication, immunity, and food digestion but is susceptible to damage either by co-irradiation as a side effect of radiotherapy cancer treatment, autoimmune-related Sjögren syndrome, disease-related medications, or surgical resection. This chapter focuses on fundamental cellular and molecular processes occurring during organ ontogenesis and in developing branching glands. We cover the growth of the epithelial compartment, which is the major functional component of the gland, but also how surrounding niches such as mesenchymal, endothelial, and neuronal cells communicate, intertwine, and influence the formation of glands and other branching organs. Finally, we highlight how this key information has created new regenerative-related approaches and how these impact future clinical translation.

1.1 Introduction

Increasing our knowledge of how organs develop has profound implications for the design of therapies to regrow and/or repair injured tissues. Understanding the mechanisms regulating cell survival, expansion, specification, movement, communication with neighboring cells, as well as how they respond to damage is critical to navigating the landscape of future therapy designs.

I.M.A. Lombaert, PhD
University of Michigan, School of Dentistry,
2800 Plymouth Road, Ann Arbor, MI 48109, USA

Biointerfaces Institute, 2800 Plymouth Road,
Ann Arbor, MI 48109, USA
e-mail: lombaert@umich.edu

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In order to appropriately translate information gathered from studies on organ development, we need to compare molecular and cellular processes during embryonic development with adult homeostasis and when repair initiates and/or fails after each damaging event. Each of these stages correlates with specific cellular responses, activation of specific signaling pathways, and accumulation of environmental cues. Thus, developmental-related information is instrumental to stimulate regrowth within an existing damaged in vivo organ or to initiate de novo growth.

The majority of our current knowledge on salivary gland organogenesis derives from experimental animal models, primarily mice and rats. While rodent biology is not identical to that of humans, many processes and pathways are very similar. As such, developmental biologists have been and continue to be a valuable resource to other disciplines such as engineering, oral surgery, and oncology to translate conceptual ideas into therapeutic designs.

The advantages of specific biomaterials, gene therapy, and surgical in vivo approaches are outlined in depth in the following chapters. In this review, we focus on different salivary gland cell types and their supportive environment that is needed to form the fully functional secretory branching organ. Subsequently, we outline how this knowledge can render future therapeutic implications and/or what potential complications might arise.

1.2 Epithelial Growth Driven by Stem Cells

Branching organs such as salivary, lacrimal, and mammary glands are comprised of different cell types, including epithelial and the surrounding mesenchymal, endothelial, and neuronal cells (Fig. 1.1). Intertwined within these tissues are circulating hematopoietic-related blood and immune cells. The major component of developing and adult salivary glands (SGs) is the epithelia, which is responsible for saliva secretion and transportation to the oral cavity. Here, we describe how the epithelial compartment of three

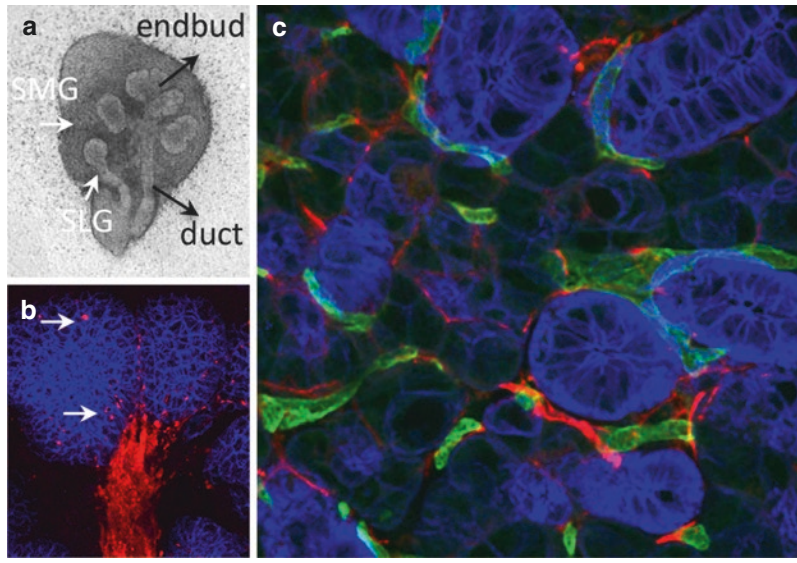

Fig. 1.1 Developing salivary glands in mice. (**a**) Bright field picture represents E13 submandibular (SMG) and sublingual gland (SLG). The epithelial compartment is comprised of a distal endbud and proximal duct area. (**b**) Epithelia (*blue*) innervated by the parasympathetic nerves (PSG, *red*) during embryonic SMG development. The PSG releases neurotransmitters via varicosities (*arrow*). Confocal image of E-cadherin stained epithelia and Tubbulin-3 stained PSG. (**c**) Different niches surrounding the epithelium in adult mouse submandibular gland. Confocal 30 μm projected image of stained SMG with epithelial marker E-cadherin (*blue*), neuronal marker Tubbulin-3 (*red*), and endothelial protein CD31 (*green*)

major glands, which provide 90 % of total saliva, becomes established by tightly controlled mechanisms of cellular interactions.

1.2.1 Morphological Development of Salivary Glands

Salivary glands originate as an invagination of the oral epithelium from a placode at embryonic day (E) 11.5 in mice or Carnegie stage 18 (~44 days, weeks 6–7) in humans. This thickening epithelium arises on the side of the tongue outside of the lamina dentalis at the anlage of the dental arch. Each major gland initiates at slightly different locations: the serous parotid gland (PAR) in the labiogingival sulcus, the mucous sublingual (SLG) in the paralingual sulcus, and seromucous submandibular gland (SMG) in the linguogingival sulcus. Even though glands arise in the tongue area, they grow out during development toward the back of the mouth below the ears, floor of the mouth near the mandibular bone, and the anterior floor of the mouth. While in mice, SMG, SLG, and PAR initiate around E11.5, E12, and E13, respectively, human SLGs initiate later than SMG and PAR at the ninth embryonic month. More detailed descriptions on anatomic locations of human glands have been recently reviewed [41]. The developmental origin of each gland has not been clear, with some classifying the PAR as ectodermal derived and SMG and SLG as endodermal, while genetic experiments in mice suggest they are all ectodermal [87].

Once the epithelial thickening arises, a cell population, termed endbud or tip, forms distally from an elongating stalk (Fig. 1.1a), which developmentally progresses to form major ducts termed Wharton's (SMG), Bharton's (SLG), and Stensen's (PAR) ducts. The unique SG branching pattern is created by repetitive clefting of the initial and subsequently formed endbuds. Ductal structures gradually mature by elongation, lumen formation, and expansion. The clefting endbuds mature by E16 in mice and 19–24 weeks (7th month) in humans to form polarized pro-acinar and pro-myoepithelial cells. While pro-acinar cells do express some secretory-related proteins, they are not yet fully functional and must still undergo specific acinar-lineage maturation so that by birth, in both humans and mice, the organ is comprised of functional secretory compartments.

1.2.2 Contribution of Stem Cells and Their Differentiating Progeny

The stem/progenitor cell theory asserts that all salivary gland cells are initiated from and maintained by stem and progenitor cells. By definition, these cells are characterized by their ability to expand themselves, i.e., self-renew, as well as to propagate multiple more defined cell types, such as acinar cells. Stem cells are further classified as being more potent than progenitor cells in their self-renewal and differentiation potential. When both these cellular processes are tightly controlled, stem/progenitor cells not only give rise to tissues but also maintain and repair organ structures during adulthood. Any deregulation in this regulatory network during development can lead to malformation/absence of the organ and in the adult may cause cancer formation. Over the past years, remarkable progress has been made wherein multiple stem/progenitors have been classified based on their ability to (1) form multiple cell types (mouse genetic lineage tracing, ex vivo culturing), (2) alternate quiescence with proliferation (BrdU incorporation or genetically labeled DNA tracing), and (3) restore radiation-induced damaged SGs (in vivo transplantation assay). One new consensus gathered from this data is that different stem/progenitor cells contribute to the growing SG and that these cells may originate at different time points during development. Importantly, this permits the organ to compensate for any losses in specific stem/progenitor cells and still allows proper development [88]. Known stem/progenitors contributing to SG organogenesis include cells marked by their expression of intracellular cytokeratin 5 (CK5, K5) and CK14 [50, 57], receptors KIT (c-Kit, CD117) and FGFR2b [57], and transcription factors SOX2 [3] and ASCL3 [10]. Remarkably, stem/progenitors contributing to development

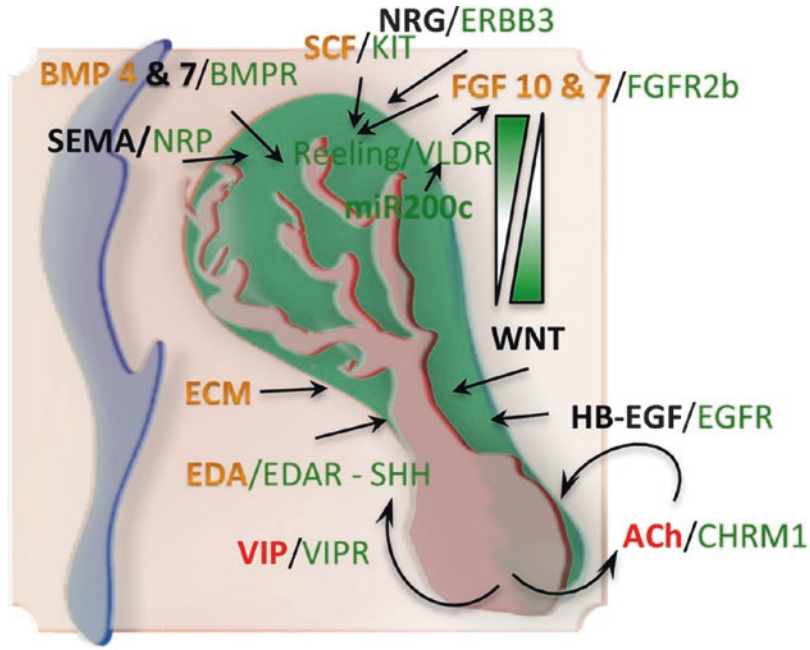

Fig. 1.2 Signaling pathways influencing epithelial growth. Cartoon represents known signaling pathways that influence SG epithelial cell survival, proliferation, expansion, and differentiation. *Green*: expressed by epithelial cells; *orange*: expressed by mesenchyme; *red*: expressed by neuronal cells; *black*: expression by multiple compartments

might not serve a similar role during adult homeostasis. Recent studies observed active proliferation of cells within specific compartments, such as acini and intercalated, striated, and excretory ducts, wherein these cells self-duplicate to replenish their own entity, as reviewed in [4]. To what extent these adult compartmental "reservoir" cells contribute to recovery after injury is a focus of ongoing research. At least after severe radiation-induced damage, which leads to irreversible hyposalivation, there is no active repair initiated by remaining SG cells. This is often a combinatorial result of (a) drastic loss of acinar and duct "reservoir" cells or stem/progenitor cells, (b) decrease in signaling pathways required to activate surviving "reservoir" or stem/progenitor cells, and/or (c) severely damaged cells that can no longer contribute to self-duplication or differentiation. In such cases, multiple strategies ranging from constructing a new gland to gene therapy and stem/progenitor cell transplantations may aid in restoring the functional and morphological components of the gland. Thus far, transplantations of cells selected for their expression of receptor KIT, EPCAM, CD24, and/or CD29 (Integrin β1, ITGβ1) were shown to restore acinar and ductal compartments, leading to significantly increased saliva levels [58, 62, 72, 103]. This does not, however, exclude the potential of other SG-specific epithelial cells, non-SG specific cells, and/or their bioactive cell lysate to contribute to the repair of damaged SGs. These options will be surveyed in following chapters, and their impact on when to use them in different damaging situations has been recently reviewed [61]. In this chapter, we will further outline our current understanding of how SGs are structurally built by various cell types (Fig. 1.1b, c) and how their continuous interactions are informing the design of current and future therapies.

1.2.3 Lessons from Developmental Regulatory Mechanisms Guiding the Epithelium

Often disorders in humans and genetic rodent model systems can provide critical information on what signaling pathways are essential for epithelial cell survival, proliferation, differentiation, and movement (Fig. 1.2). Major examples are *Fgf10*$^{-/-}$ and *Fgfr2b*$^{-/-}$ mice, which are related to human loss-of-function mutations in FGF10 and FGFR2 that result in hereditary diseases including

lacrimal and SG-related aplasia of lacrimal and salivary glands (ALSG), lacrimo-auriculo-dento-digital (LADD) syndrome, and lung-related chronic obstructive pulmonary disease (COPD). In these conditions, SG development is stalled, as FGFR2b+ epithelium no longer receives survival and proliferative cues from the surrounding FGF10-producing mesenchyme [80]. Thus, when invading oral epithelial cells at gland ontogenesis receive FGF10, they initiate an endbud and duct formation. From then on, FGFR2b signaling expands KIT+ progenitors in the continuously clefting endbuds in combination with stem cell factor (SCF)/KIT signaling [57]. As FGF10 has a heparan-binding (HB) core, it evokes and expands more rapid responses once it is bound to specific 3-*O*-sulfated heparin sulfate (3-*O*-HS). This HS belongs to a group of heparan sulfate proteoglycans (HSPGs) located in the basement membrane or at cell surfaces. Interestingly, KIT+ endbud progenitors highly express HS3ST3, the 3-*O*-HS-specific modifying enzyme 3-*O*-sulfotransferase, to rapidly increase their expansion during development [80]. A similar function remains during adult homeostasis. Regulating this FGFR2b signaling pathway is of crucial importance so that every epithelial cell does not undergo extensive proliferation. Ductal cells therefore express FGF antagonists, Sprouty 1 and 2, to lower FGFR2b signaling and upregulate WNT [48]. Both canonical WNT/β-catenin and noncanonical WNT5b pathways drive ductal formation via upregulation of *Tfcp2l1* while inhibiting endbud development. In turn, endbuds repress duct development by FGF-mediated Wnt5b repression and secretion of WNT ligand-sequestering protein SFRP1 [78]. Evidently, a tight FGF-WNT gradient allows for KIT+ progenitor expansion in endbuds, while ductal cells prepare for upcoming lumenization and maturation. In this process, ERBB1 (EGFR)+ ductal K5+ progenitors proliferate in response to HB-EGF to give rise to maturing K19+ cells [50]. One mechanism of action is via induction of membrane-type-2 matrix metalloproteinase (MT2-MMP) and FGFR expression in epithelial cells. MT2-MMP is crucial to release bioactive NC1 domains from extracellular matrix (ECM) protein collagen IV, which in turn promotes branching via epithelial ITGβ1 [84]. MMPs also cleave pro-HB-EGF into an N-terminal and C-terminal fragment at the membrane so that the latter fragment can move to the nucleus to activate cell proliferation via cyclin A [95]. Conversely, another member of the EGF family, neuregulin (NRG), binds ERBB3 on endbuds to aid in their local expansion [70]. NRG1 is further essential for innervation as *Nrg1*$^{-/-}$ mice are devoid of nerves and show aberrant duct formation and lumenization [73].

Similar to FGF10, *Fgf8* hypomorphic and *Fgfr2c* heterozygous mice exhibit hypoplastic glands due to reduced communication between FGF8-producing epithelia and FGFR2c-receiving mesenchyme. In both FGF-deficient mice, initial epithelial invagination occurs, but subsequent SG growth does not occur. To date, FGF8 has been described as a potential target of the EDA pathway. Human mutations in ectodysplasin-A (EDA) or its receptor EDAR result in hypohidrotic ectodermal dysplasia (HED). Defects in teeth, hair, sweat, and salivary glands are noticeable due to reduced cell proliferation and differentiation [45, 74]. In SGs, EDA and downstream target NF-kB aid in ductal lumenization and endbud branching, presumably by inducing ductal maturation within the center of endbuds. Early on, mesenchyme-produced EDA is downstream of mesenchymal WNT and upstream of epithelial SHH (sonic hedgehog) signaling. As such, SHH treatment can rescue SGs deprived of EDA [100]. After E13, EDA does not seem to correlate with WNT anymore, based on their different expression pattern located in the epithelial or mesenchymal compartment [31, 78]. SHH's important role in SG development has been confirmed, as SHH-deficient SGs are hypoplastic with unpolarized epithelial cells and underdeveloped lumen formation [36, 43]. SHH is also linked to FGF8 as both can upregulate each other [43]. Therefore, FGF8 is able to rescue Hedgehog inhibition but surprisingly not EDA deficiency [43]. As such, EDA-FGF8's precise signaling interaction still needs to be determined.

FGF signaling, in particular via FGFR1 (variant b in the epithelium and c in the mesenchyme), can also upregulate bone morphogenetic protein

(BMP) ligands. BMPs are part of the TGFβ signaling family and signal via BMP receptors. FGFR1 signaling regulates BMP7 directly and BMP4 indirectly to regulate epithelial growth. BMP4, which is mesenchyme specific, inhibits epithelial branching, while BMP7, released by both epithelium and mesenchyme, increases it [90]. The role of another member of the TGF family, TGFβ1, is still inconclusive. While TGFβ1-deficient mice have normal SGs, overstimulation of TGFβ1 results in acinar loss, elongated ducts, and/or fibrosis [35, 42].

Additionally, ECM and epithelial integrin cell interactions are just as essential for branching morphogenesis. These ECM molecules line up the basement membrane (BM) separating the epithelium from mesenchyme. Interestingly, isolated epithelial cells can easily grow without the physical presence of mesenchymal cells but not without ECM component(s), such as laminin, fibronectin, perlecan, collagen, or mouse sarcoma-derived reconstituted BM "Matrigel." Deposition of unique ECM components along the clefting endbuds and elongating ducts plays a role in correct branching. Impairing these connections will lead to reduced clefting, endbud number, cell movement, and/or growth. Detailed descriptions of disruptive ECM cell outcomes were recently reviewed in [79].

Finally, an underexplored area contributing to SG formation are microRNAs (miRNAs), which are small, noncoding RNAs that specifically target mRNAs to globally regulate gene expression. Epithelial endbud progenitors highly express miR200c to reduce FGFR-dependent proliferation. miR200c downregulates the autocrine reelin/very low-density lipoprotein receptor (VLDLR) pathway, which positively regulates FGFR signaling [83]. Additionally, it was found that EGF can specifically induce mesenchymal production of miR-21, which decreases multiple target mRNA candidates. One of these, RECK, inhibits MMPs, which subsequently influences ECM degradation to enhance SG branching [37].

In conclusion, various signaling pathways instruct different cell types within the epithelial compartment and not surprisingly interact with and regulate each other to safeguard temporal-spatial proliferation, differentiation, and clefting. Initiation of some of those embryonic signaling pathways has been observed in active repair situations, such as ductal ligation settings where acinar atrophy and hyposalivation is temporarily induced by restricting salivary flow from the major duct [17]. We can thereby try to manipulate the activation and/or repression of specific developmental pathways to stimulate in vivo repair of damaged SGs, as is outlined further in this chapter.

1.3 Environmental Cues Patterning Epithelial Branching and Maturation

SGs are highly vascularized and innervated, all of which integrate within a condensed mesenchyme. Developmentally, SG epithelia invade a condensed mesenchymal placode already containing a complex endothelial network and parasympathetic neuronal bodies awaiting cues for innervation [48]. Both signals for epithelial invasion into the mesenchyme and subsequent branching are transmitted via direct cell-cell contact and/or indirect paracrine signaling pathways, which are discussed below.

1.3.1 Guiding Neurons

Different cranial nerves innervate the pre- and postnatal SG where they exert different functions. While the autonomic nervous system regulates the SG at an unconscious level and in stress conditions, sensory neurons respond to mechanical, thermic, and light signals.

For decades, both the parasympathetic and sympathetic nervous system have been acknowledged as the driving stimulant to release saliva from acinar cells into ducts. While parasympathetic stimulation results in serous secretion and ion release, sympathetic activation stimulates mucous or protein-containing saliva and can also play role in local inflammation and blood flow [23, 67]. Both parasympathetic and sympathetic nerves are part of the autonomic nervous system,

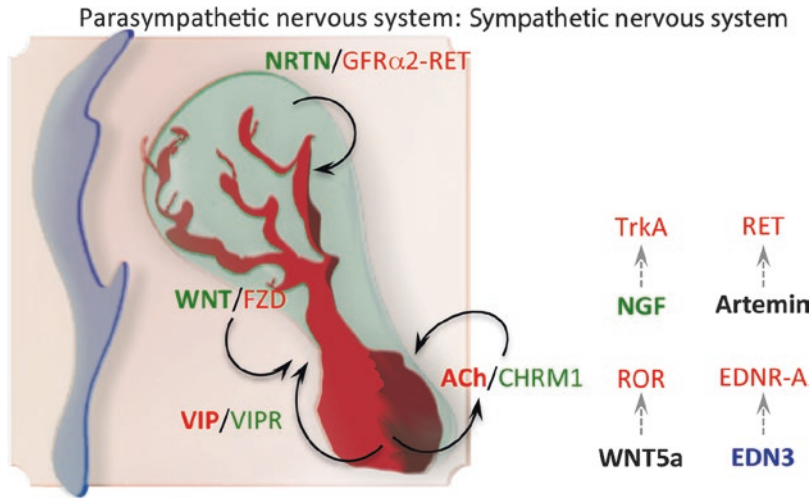

Fig. 1.3 Signaling pathways driving neuronal survival and innervations. Illustrated signaling pathways for parasympathetic nervous system were demonstrated in prenatal glands, sympathetic nervous system in postnatal SGs. *Green*: expressed by epithelial cells; *red*: expressed by neuronal cells; *blue*: expressed by endothelial cells; *black*: undefined which compartment takes part in it

and their neuronal guidance is ensured by axons that sprout along unique paths within the tissue. While the former innervates along the epithelia, the latter follows the vasculature. This directional guidance is driven by neurotrophic factors, secreted by cells in the periphery, as well as the presence of specific receptors on the axons that allow or block their adhesion to the adjacent ECM. The most notable trophic molecules include neurotrophins (e.g., NGF, BDNF, NT-3), netrins, semaphorins, ephrins, and myelin inhibitors. Similarly, axons secrete neurotransmitters in their proximity, exerting a variety of effects through specific receptors on their target cells (Fig. 1.3). Parasympathetic nerves signal via the cholinergic acetylcholine (ACh) pathway, targeting muscarinic receptors on neighboring cells, as well as water channels such as aquaporin 5 (AQP5). In contrast, sympathetic nerves release epinephrine and norepinephrine (i.e., noradrenaline, NA) that bind to β-adrenergic receptors (adrenoceptors) on acini. Other non-ACh, non-NA neurotransmitters can be produced by both parasympathetic and sympathetic nerves and may include vasoactive intestinal peptide (VIP), substance P (SP), neuropeptide Y (NPY), neurokinin A, pituitary adenylate cyclase-activating peptide (PACAP), and calcitonin gene-related peptide (CGRP).

Developmentally, parasympathetic ganglia (PSG) neuron cell bodies migrate along the branches of mandibular arteries [91] to cues from initiating SG epithelia to localize into ganglia around the primary duct and send out axons toward the endbuds [48]. Sympathetic nerves innervate SGs along the blood vessels during later stages of development when final epithelial maturation is needed. As such, developmental experiments can clearly dissect the role of neurotransmitters and neurotrophic factors affecting the PSG. It is now well appreciated that the PSG establishes a communication loop with specific epithelial cells to allow outgrowth of both compartments. When the PSG is absent, the pool of K5-expressing epithelial progenitors is significantly reduced [50], which influences downstream K19+ ductal luminal differentiation and subsequent epithelial outgrowth. This is mediated via a loss in ACh-CHRM1 (muscarinic receptor 1) signaling from the PSG to K5+ cells and resulting in a subsequent reduction of HB-EGF/EGFR pathway signaling that initiates maintenance and differentiation of K5+ progenitors. Lumenization, which marks further ductal maturation, is also coordinated by the PSG but not via the ACh pathway. The neurotransmitter VIP activates a cAMP/protein kinase A (PKA) pathway to induce epithelial duct cell proliferation and formation of a single lumen by the fusion of multiple microlumens. After initial lumen formation, VIP remains essential to expand the lumen size via the cystic fibrosis transmembrane (CFTR) pathway [73].

Organ development also requires proper bidirectional communication. Feedback signaling from epithelial cells toward the PSG stimulates cell survival, migration, and innervation. At SG ontogenesis, WNT-producing epithelia, particularly K5+ progenitors, maintain PSG neuron survival and proliferation [48]. At later stages of branching morphogenesis, the neurotrophic factor neurturin (NRTN), which is mainly secreted by endbud progenitors, not only promotes neuronal survival via GFRα2/RET but also maintains axon outgrowth along ducts toward the endbuds [49]. In the developing lung, there also appears to be a link between nerves and blood vessels. Denervation, in this case via physical cell ablation, resulted in reduced endothelial proliferation, leading to hypo-vascularized lungs [9]. It is unclear whether this is a direct or indirect neuronal-endothelial effect and whether similarities exist within the developing SG.

Detailed anatomical descriptions of nerves in adult SGs are outlined in a recent review [40]. It is assumed that similar communication between nerves and epithelium persists into adulthood as denervation of SGs, via ductal ligation or neurectomy, reduces epithelial content that regenerates after ligation removal if the nerve is intact or reconnected [46, 55, 65]. A morphological difference of early development with later stages and adulthood is that smaller ganglia are found dispersed within adult tissue [40], presumably to reach their target cells more easily as distances are much larger compared to embryonic development.

Even though tyrosine hydroxylase (TH)-expressing sympathetic ganglia are presumed not to be present at SG ontogenesis, some mRNA expression levels of its unique receptor neuropeptide Y receptor 2 (NPY2R) were detected early during development at low levels that increase before birth [23]. Since NPY2R is also present on endothelial cells, some, if not all, of the mRNA expression could be related to blood vessel formation within the SG. However, TH-expressing neuronal cells were detectable by E16.5, which might indicate there is a prenatal presence of sympathetic ganglia [89]. Nevertheless, postnatal sympathetic denervation does lead to hypoplasia of the gland [82] and thus must involve a direct or indirect role for sympathetic nerves in either epithelial cell maintenance or maturation. In the adult gland, RET signaling is also known to be essential for sympathetic neuron survival, but likely via the ligand artemin instead of NRTN. SGs also produce high amounts of NGF and genetic ablation of NGF or its TrkA receptor leads to defective sympathetic innervation, indicating its crucial role in sympathetic neuron survival [22, 27]. Depletion of non-canonical WNT5a in WNT1-derived neural crest cells further leads to incomplete sympathetic innervation and branching in prenatal SGs. While the authors suggest this is due to an autocrine WNT5a/retinoid-related orphan receptor (ROR) pathway in sympathetic neurons, it doesn't rule out that epithelial WNT5a-producing cells might be stimulating sympathetic neurons as well [89]. Similarly, endothelial-released endothelin 3 (EDN3) is also suggested to be a cue for a subset of EDN receptor A+ sympathetic neurons to innervate the prenatal SG along the nascent external carotid arteries [63]. The specific role of other neurotransmitters from the GDNF and NPY family as well as other neurotrophic factors are still being explored. While semaphorins are involved in axon pruning and neuronal migration in the central nervous system, they also appear to have a role in developing SGs. Semaphorin (SEMA) 3A and 3C bind co-receptors neuropilin and plexin. Neuropilin is expressed by epithelial endbuds and by activation with SEMA3A and 3C cleft formation is induced without changing proliferation and, most likely, by affecting cell movement [15]. However, additional FGF7/10 growth-promoting signals from surrounding mesenchyme were required to mediate this cleft formation. Whether additional participation of SEMAs on receptive nerves is required for cleft formation or SG development still remains unclear.

At adulthood, it remains to be determined how sensitive sympathetic nerves are to injuries such as radiation. In rodents, sympathetic nerve function was retained after radiation [52], and increased levels of *TH* as well as *NGF/NGFR* and adrenergic receptor 2 (*ADRA2B*) were detected in radiated human SMGs [49]. Whether a

reestablishment of the balance between parasympathetic and sympathetic nervous system is necessary for regeneration is not known.

Furthermore, it is assumed that sensory neuronal cells are present along the sympathetic and parasympathetic nerve tracks in adult SGs [51], although they have not yet been studied in detail. While sensory neurons can be defined into multiple subtypes based on different criteria such as their origin and molecular expression patterns, they often are loosely classified as unmyelinated capsaicin-sensitive TRPV1+ receptor expressing neurons or myelinated glutamate receptor-expressing neurons. Upon activation, sensory nerves can secrete various neuropeptides, such as Substance P and CGRP. At least in the lung and pancreas, literature indicates that sensory neurons release neurotransmitters in the periphery to serve as direct mediators for recruiting and activating inflammatory cells [68, 86]. Whether a similar mechanism occurs in salivary glands is not known.

In conclusion, innervation plays an essential role for organ development, homeostasis, and repair after injury. Studies on SG biogenesis have been highly informative for defining the involvement of the PSG in branching morphogenesis, not only of SGs but also other organs such as prostate and lungs. The existence and/or loss of bidirectional communication with epithelial stem/progenitor cells have been reported to occur in rodent and human SG homeostasis and postradiation. The inhibition of parasympathetic neuronal function influences adult epithelial K5+ progenitors [49] but it is not known yet whether postradiation regeneration due to epithelial stem/progenitor transplantation repairs neuronal function, even though morphological repair has been suggested [71].

1.3.2 The Role of Blood Vessels

The vasculature in branching organs develops in close proximity to the epithelia, although its spatial pattern differs from parasympathetic nerves. Not only are endothelia important for mediating gas exchange but also as a source of endothelial secretory factors, termed angiocrines, which impact organ development (Fig. 1.4). While research on blood vessels in SGs remains limited, much can be learned from other branching organs. Overall, complex cross-communication between epithelial and endothelial cells appears to regulate both epithelial differentiation and angiogenesis. The initial cues to form an endothelial cell plexus around a condensed mesenchyme do not require epithelia. This is observed in *Fgf10*$^{-/-}$ mice that don't form initial SMG epithelia but where the placode of mesenchyme, blood vessels, and neuronal bodies are present [48]. After SG ontogenesis, however, epithelial-derived angiogenic factors, such as VEGF-A, do play a role as null mutations in *Vegf-A* or its endothelial-expressed receptor (*Vegfr*) show vascular defects in tissues, reduced epithelial budding, and ultimately embryonic lethality [13, 107]. Another epithelial-induced angiogenic mechanism may include the vitamin D pathway. The enzymes CYP27B1/24A1 that activate and catabolize vitamin D are highly upregulated just before birth and in postnatal lung. Exogenous vitamin

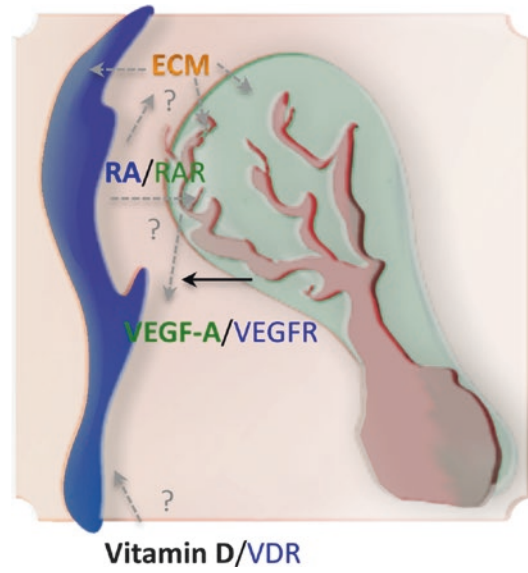

Fig. 1.4 Potential communications initiated by and to endothelial cells. Described pathways were majorly found in other branching organs. Whether they exist in SGs needs to be determined. *Green*: expressed by epithelial cells; *orange*: expressed by mesenchyme; *blue*: expressed by endothelial cells

D positively influences lung growth by inducing maturation in vitamin D receptor-expressing epithelial cells (VDR) [64]. As VDR is also present on endothelial cells, this enhanced growth might be due to direct effects of vitamin D on endothelial cells and/or indirect effects from epithelia to endothelia. Nevertheless, it is clear that epithelial-endothelial communication requires a tight balance as any hyper-vascularization inhibits epithelial growth [14].

Apart from endothelial-epithelial cross-communication, there is also endothelial-mesenchymal communication, as recently reviewed [94]. The early endothelial cells promote survival of pancreatic mesenchymal cells, which in turn have a pivotal role in organ development. A similar complex paracrine signaling network was also found in the lung. Retinoic acid (RA), which is produced by endothelial cells, induces VEGF-A expression in lung epithelia. Evidently, endothelial cells are recruited via VEGF-A, and thus angiogenesis is stimulated via this endothelial-epithelial communication loop. Furthermore, endothelial-released RA also stimulated mesenchymal cells to produce more FGF18 and ECM component elastin, thus increasing epithelial alveolar formation [108]. Other organ-specific angiocrine factors that may follow this paracrine loop include HGF, WNT, NOTCH, and BMP ligands. Mesenchymal cells also signal back to endothelial cells to stimulate survival, proliferation, migration, and autophagy via production of ECM components, such as the perlecan/heparan sulfate proteoglycan (HSPG2) fragment endorepellin, decorin, and endostatin [18, 75].

While blood vessels and nerves can independently respond to their own set of signaling factors, there also seems to be a paracrine connection via epithelial-released VEGF. Even though VEGFR is absent on nerves and not required for innervation, VEGF overexpression in pancreas not only led to hyper-vascularization but also to hyper-innervation [85]. Interestingly, endothelial cells did not produce any known neurotrophic factors, but the effect appeared to be related to their upregulated expression of basement membrane components, such as collagens and laminins. These components in turn served as scaffolds for increased axon outgrowth.

In addition to blood vessels, we must not forget the circulating cells within them. White blood cell monocyte-derived macrophages and dendritic cells arise from the bone marrow and colonize tissues via blood vessels to phagocytose cellular debris and help in the innate non-specific and specific adaptive immune defense. While macrophages normally develop in the bone marrow via granulocyte-macrophage colony-stimulating factor (GM-CSF), mesenchymal cells in tissues can also release GM-CSF to induce a similar differentiation effect on circulating monocytes. While it is clear that both macrophages and dendritic cells may be involved in organ morphogenesis, their exact functions are not always fully understood. Also in adult tissues, for example, in the lung, there is conflicting data on their specific role: antigen-sensing dendritic cells might induce different immune responses depending on their physical location in the tissue while surrounding different epithelial cell types [54]. Similarly, various macrophages invade the mesenchyme where they can interact with dendritic cells, lymphocytes, and epithelia to regulate immunity. Macrophages suppress immune responses by inhibiting both dendritic-mediated T-cell activation and inactive TGFβ production. Subsequent activation of this inactive TGFβ into bioactive TGFβ by lung epithelia is essential in order to prevent spontaneous inflammation after acute injury. Lung alveolar cells in turn secrete various ligands to receptive macrophages to ensure this prevention of inflammatory responses. Whether a similar action or disruption in this communication is occurring in adult SGs after radiation remains to be determined.

Apart from immune regulators, macrophages further shape the branching patterning of organs by remodeling the ECM around the ducts to allow outgrowth as well as survival of endbud stem/progenitor cells [11, 102]. They also regulate angiogenesis by instructing endothelial cells to undergo apoptosis via WNT signaling, counterbalancing a pro-survival factor produced by pericytes, which wrap around endothelial cells to influence functions such as blood flow [2].

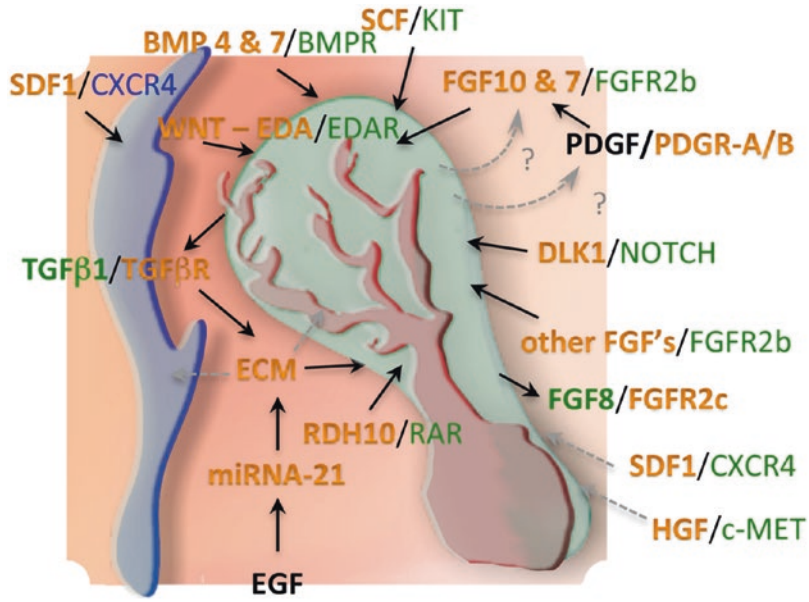

Fig. 1.5 Described signaling interactions with the SG mesenchyme. Most known communications are between the SG mesenchyme and epithelium. *Green*: expressed by epithelial cells; *orange*: expressed by mesenchyme; *blue*: expressed by endothelial cells; *black*: undefined which compartment takes part in it

In sum, blood vessels play important roles during development in other branching tissues not only for oxygen supply but also to maintain essential communication signaling pathways with epithelia and surrounding mesenchyme. The bone marrow-derived cells circulating in the blood vessels also aid in tissue branching morphogenesis and evoke or suppress immune responses after injury. Whether similar mechanisms exist in the developing and adult SGs remains to be determined.

1.3.3 Supporting Mesenchymal Cells

Embryonic SG mesenchymal cells are WNT1+ neural crest-derived cells [44, 105] and provide supportive cues such as growth factors, proteases, and ECM proteins to guide and activate epithelial, neuronal, and endothelial cells (Fig. 1.5). In vitro recombination experiments show that the SG mesenchyme induces an SG-like branching pattern in various epithelia such as a pancreatic, mammary, and pituitary gland [96]. This property, however, is not found in mesenchyme from non-SG tissues, as E11.5–13 SG epithelium does not properly branch unless it is recombined with SG mesenchyme [28, 29, 99]. This indicated that SG mesenchyme has strong and unique multicomponent instructional properties. One of them is the high production of FGF10, which is also essential for lung, lacrimal, and mammary gland initiation. Notably, non-SG epithelia only adapted SG-like branching patterns when they were placed in close vicinity to high FGF10-expressing mesenchymal tissue, confirming the importance of FGF10's spatiotemporal dosage [99]. With this in mind, it is important to understand that the SG mesenchymal component in these experimental conditions contains mesenchymal cells as well as ganglia and blood vessels albeit disconnected from the rest of the body. It can therefore not be excluded that SG-specific neuronal cells and/or blood vessels may have additional contributions to this specific SG patterning.

Interestingly, early-stage E11.5–12.5 SG epithelia, but not later stages, are able to instruct E10.5 mesenchyme from different sources to produce FGF10. However, not every mesenchyme is as competent to receive this signal as only SG and pharyngeal second arch mesenchyme responded and limb mesenchyme, for example, did not. This indicates that this initial signal is exclusively located within specific regions of the embryo, likely to restrict specific organ outgrowth to the correct location in the body. What this initial epithelial signal is

remains a subject of debate. Whereas early limb and lung epithelia secrete FGF8 or FGF9 to initiate this process, it is unlikely that FGF8 serves a similar function in SGs [99]. Neither is the signal FGF4, BMP2, SHH, TGFβ1, or WNT6 [47]. One unexplored candidate is platelet-derived growth factor (PDGF). During development, PDGF-A and PDGF-B ligands are mainly produced by epithelia and mesenchyme, respectively [105], while PDGFR-A and PDGFR-B receptors are expressed in the mesenchyme. By adding exogenous PDGF, epithelial proliferation can accelerate via upregulation of mesenchymal *Fgf7*, *Fgf10*, *Fgf1*, and *Fgf3* and downregulation of growth inhibitory factor *Fgf2*. While this induction was observed during SG morphogenesis (E14), it has not been confirmed that epithelial PDGF-A is a potential FGF10 inducer at SG ontogenesis. Nevertheless, once FGF10 expression is initiated, it persists and becomes independent from epithelial cues. It is also interesting to note that mesenchymal condensation at placode initiation is independent of this FGF10 activation. As such, the mesenchyme can condense around a network of blood vessels and resting PSG cells before SG initiation and in the absence of FGF10 as seen in *Fgf10*$^{-/-}$ mice. This mesenchyme presumably awaits signal(s) from the invading oral epithelia to initiate FGF10, which in turn promotes SG-specific epithelial growth.

Even during branching morphogenesis, mesenchymal cells continue to play a part in multiple bidirectional signaling pathways. Early on, WNT/β-catenin signaling is exclusively induced in the mesenchyme before it is expressed in lumenizing ductal cells [78]. This mesenchymal WNT can activate EDA and, at least in part, influence SG morphogenesis via epithelial EDAR [31]. Branching epithelia also regulate local FGF10 expression to reduce aberrant cell proliferation. Lung epithelia release BMP ligands as well as SHH to spatially downregulate FGF10 and specifically induce secondary bud formation [12]. A recent study further points to an important role for mesenchymal retinoic acid (RA) to enhance branching. RA is a small diffusible hormone-like molecule generated by a two-step enzymatic oxidation of dietary vitamin A via RDH10 and ALDH1A. RDH10, which metabolizes vitamin A in the first step, is exclusively expressed in early SG mesenchyme, while RA activity is mainly observed in RA receptor+ epithelia. Disruption in *Rdh10* results in early embryonic lethality, often before SG epithelial invagination. However, when E13 SGs were treated with an RAR inhibitor reduced branching was observed [101]. In contrast, mesenchymal cells can also secrete signaling inhibitors to slow down branching. DLK1, a non-canonical NOTCH1 ligand produced by the mesenchyme, inhibits branching and subsequent innervation, presumably to modulate cleft formation [25]. Furthermore, DLK1 appears to regulate the epithelial balance of K14+ progenitors, although the precise mechanism is unclear [26]. The role of TGFβ1 is also unclear; there is some evidence that epithelial-secreted TGFβ1 enhances collagen production from Coll1α1+ mesenchymal cells to inhibit SG acinar formation [42].

Other mesenchymal signaling pathways that may influence epithelial branching are hepatocyte growth factor (HGF)/c-MET and SDF1/CXCR4 signaling [38]. Additional cellular instruction mechanisms also include microRNAs (miRNAs). miRNAs are small noncoding RNA molecules that function to silence other mRNAs.

While most research has focused on mesenchymal-epithelial interactions, there may also be mesenchymal-endothelial interactions. When the mesenchymal factor SDF-1 was specifically inhibited from binding CXCR7+ endothelial cells, SG epithelial branching was decreased [38], thus suggesting a tri-directional loop between mesenchymal, endothelial, and epithelial cells. It is also possible that mesenchymal cells may play a role in axonal guidance. As outlined earlier, multiple studies have verified that mesenchymal cells aid in cellular migration, clefting, and differentiation via regulation of ECM production.

1.4 Translation into Future Therapies to Repair Damaged Salivary Glands

There is a tremendous need for long-term therapies to restore salivary flow. Clinical impacts of dry mouth, or xerostomia, not only include difficulty

with food mastication, swallowing, taste, and speech but also increased risks for dental caries, pain, and oral infections [19]. Depending on the type of damage, different therapies could be considered. Moderate damage could be addressed by protein and/or gene activation, while cell therapies and/or bioengineered tissues to restore the entire organ may be more suitable for cases of severe radiation-induced damage. Current bioengineering, gene and mesenchymal stem cell therapies, as well as SG transfers are emphasized in the following chapters. Here, we review how different therapies could contribute to SG repair by correlating them with the developmental concepts described above.

1.4.1 Epithelial Protection and Repair

Since epithelial cells are the major component of SGs, they are the main target for any type of damage, especially radiation. Therefore, the prevention of SG cell apoptosis and/or membrane damage-induced dysfunction of acinar cells is clinically attempted by using intensity modulated radiation therapy (IMRT) rather than conventional radiotherapy. The precise delivery of radiation by IMRT reduces the amount of the SGs being targeted, still, 40 % of head and neck cancer patients experience moderate to severe oral dryness [8]. Recently, it was revealed that exclusion of a subregion of the cranial SG is essential to reduce severe loss of organ function [97]. Not surprisingly, this region appears to harbor the highest number of epithelial SG stem/progenitor cells. Superior dose distribution as delivered by proton therapy is further expected to improve dose sparing of SGs, specifically in this cranial subregion [56].

In the meantime, free radical scavengers (amifostine and tempol) and saliva-stimulating sialogogues (pilocarpine) provide relief to some patients. However, major health-related side effects induced by radical scavengers, such as vomiting and fever, need to be taken in consideration. Also the effectiveness of pilocarpine is related to the severity of tissue damage as this muscarinic agonist relies on some functional SG epithelial cells still being present in the gland. Suppressing cell apoptosis is another treatment that has had some success in animal models. Approaches shown to be effective in mouse models include pretreatment with insulin growth factor 1 (IGF1) [69], FGF2 [30, 53], Tousled kinase (TLK1B) [77], Pkcδ [1], or roscovitine. The latter is a cyclin-dependent kinase inhibitor that transiently inhibits G2/M cell cycle arrest, allowing suppression of apoptosis and DNA repair [66].

Alternatively, epithelial cell proliferation can be stimulated via posttreatment with aldehyde activator ALDH3 [5] or pre- and posttreatment with keratinocyte growth factor (KGF) or FGF7 [60, 109] to ameliorate radiation-induced hyposalivation in mice. Other signaling pathways shown to enhance adult SG regeneration postradiation or post-ductal ligation in mice are concurrent or transient activation of WNT/β-catenin [33, 34], SHH [32], and EDA [39]. Similar to developmental processes, all of these pathways affect epithelial stem/progenitors and aid in their expansion and differentiation. Notably, radiation itself does not alter endogenous WNT, EDA, or SHH pathway components, but evidently overstimulation reinforces epithelial growth mechanisms required to regenerate the damaged tissues. Another approach is to use a cocktail of chemokines, cytokines, and growth factors obtained by mobilizing or injecting bone marrow-derived cells, adipocytes, and/or mesenchymal stem cells into damaged SGs, as reviewed in [61]. These bioactive lysates are proposed to drive SG repair by reactivating signaling pathways in the epithelial and endothelial cells that remain. Whether they also stimulate post-damage neuronal repair is currently unclear.

Restoration of saliva secretion is further feasible by epithelial water channel aquaporin 1 (AQP1) protein gene therapy. After extensive animal research in mice, rats, pigs, and monkeys, the safety of an adenovirus-containing human AQP1 vector (AdhuAQP1) was studied in human Phase I clinical trials [6]. While there was some efficacy reported in some patients, follow-up studies to test the effectiveness of this therapy for long-term maintenance of increased salivary flow are

ongoing. The reader is guided to a following chapter for more in-depth information.

SG cell transplantations have proven to be an effective treatment in rodent models. Several SG epithelial specific and non-SG cell types are able to integrate within the remaining epithelial compartment and contribute to its repair and subsequent homeostasis by differentiating into pools of various ductal and acinar cell types. Their potential use in the clinic has been reviewed in detail [61]. Briefly, autologous SG cell transplantation could occur in patients who still need to undergo radiation and where a SG biopsy could be taken before radiation therapy starts. Subsequent post-therapy transplantation of biopsy-isolated cells could initiate regeneration of the radiated SG. Alternatively, other cell types such as mesenchymal or adipose cells could be transplanted or mobilized post-radiotherapy to positively influence epithelial, mesenchymal, and endothelial repair.

In conclusion, the number of remaining stem/progenitor cells and functional epithelial cells will determine the probability of spontaneous regeneration of the damaged SG, as well as efficiency of sialogogues, apoptosis protectors, and signaling pathway stimulators. Restoring the epithelial compartment could elicit repair of surrounding blood vessels and nerves as well. As indicated from animal models [32, 59, 72], this can occur via paracrine communications from epithelial angiocrine and neurotrophic factor (e.g., BDNF, NRTN) release. One important clinical issue to be determined is whether these proposed treatments lead to undesirable side effects such as radioresistance and/or the acceleration of the patient's tumor cells.

1.4.2 Inducing Neuronal Survival and Reinnervation

Nerves have a limited capacity to regenerate, and irradiated human SGs have been shown to have reduced parasympathetic innervation [49]. The role of nerves and neurotransmitters during organ development and homeostasis is being elucidated, but less is known about their role during gland repair. Apart from neurotrophic release, innervation can also be influenced via cell plasma membrane-derived vesicles or exosomes, which not only mediate transmission of proteins but also mRNA and microRNAs [76]. One study proposes a participating role for mRNA and microRNAs in neuronal myelination and survival. Oligodendrocytes secrete exosomes with specific proteins and RNA toward surrounding neurons in the brain to improve their viability under stress conditions [24]. While exosome-influenced neuronal repair has yet not been investigated in SGs, neurotrophic protein deliveries via injection or gene therapy are currently being explored. In fetal SG experimental settings, radiation-induced neuronal apoptosis and denervation were reduced by postradiation delivery of exogenous neurturin (NRTN), which binds GFRα2/RET receptors on parasympathetic nerves [49]. Adult radiated SGs were shown to benefit from exogenous GDNF, which binds GFRα1 and aids in epithelial stem/progenitor cell expansion [103]. This likely occurs via neuronal communication, although its reinnervation pattern has not yet been studied.

Future efforts will also need to be directed toward determining the impact of radiation on sympathetic nerves and possible positive influences by WNT5a, NGF, or END3, which are known to be important during development of the gland.

In sum, functional neuronal repair and reinnervation may help in the release of neurotransmitters to maintain epithelial stem/progenitor cells and induce epithelial regeneration and ductal maturation. Moreover, their reactivation is necessary for proper saliva release from acinar cells and may also reestablish the communication pathways necessary for repair and subsequent homeostasis. Importantly, the major human SMG and SLG parasympathetic ganglions are located outside the gland in contrast with many rodent models [21]. The prevention or reduction of radiation to these ganglions, as well as the PAR ganglion that is located outside the tissue, could also improve the efficiency of these therapies.

1.4.3 Restoration of Blood Vessel Supply

Radiation severely impacts blood vessels to the point that capillary endothelial cells detach from

the basal lamina, their density reduces, and large blood vessels dilate. Reducing damage to blood vessels will contribute to repair of damaged SGs. As iterated above, endothelial cells communicate not only with epithelial and mesenchymal but also neuronal cells. Furthermore, circulating bone marrow-derived cells migrate via blood vessels into damaged tissues to phagocytose the damaged environment. Several proposed strategies actively contribute to repair of the endothelial compartment. Pre-radiation gene delivery of angiocrine VEGF or FGF2 could ameliorate endothelial damage, resulting in reduced hyposalivation [16]. In vivo mobilization of bone marrow-derived cells is also a feasible approach to increase saliva flow as both their secretory bioactive lysate as well as the presence of endothelial progenitors can contribute to stimulation of endothelial survival and proliferation [59, 92].

1.4.4 Inflammation and Stromal Cell-Induced Fibrosis

Inflammation following radiation is an acute damage response but can persist and become chronic. While recruitment of macrophages is a necessity to phagocytose apoptotic cells [102], too much secretion of inflammatory cytokines and chemokines like IL, CCL2, and TNF can augment epithelial dysfunction. If not immediately controlled, the inflammatory response becomes pathogenic, as seen in autoimmune diseases and tissue fibrosis. In such cases, it is imperative to switch the pro-inflammatory response to an anti-inflammatory state in order to allow repair. Various potential mediators for reducing inflammation include IL-4, IL-13, T-reg, and B1 B-cells, and new evidence has also shown the ameliorating effects of stromal (mesenchymal) stem cells (MSCs) [81].

In adult tissues, the stromal cell pool harbors interstitial fibroblasts and adipocytes that continue to deposit and remodel the ECM. With aging patients, more adipose and fibrotic tissue is apparent that together with acinar cell loss leads to a 30–40 % decrease in parenchymal SG volume [7, 20]. Radiated stromal cells can enhance stress fiber formation and mature their cell matrix focal adhesions to turn into myofibroblasts, thereby producing excessive ECM. Injecting MSC/adipose-derived stem cells reduces muscular fibrosis as a consequence of radiation [93]. In the SG, these cells could presumably reduce inflammation as well as degrade the ECM and induce phagocytosis of apoptotic myofibroblasts. The timing of cell delivery, however, will be crucial as delayed therapy after radiation resulted in increased lung fibrosis due to the differentiation of mesenchymal stem cells into myofibroblasts [106]. Although TGFb has beneficial roles in wound healing and inflammatory responses, excessive levels of TGFβ1 can result in a number of serious conditions that are characterized by fibrosis, including chronic hepatitis, glomerulosclerosis, and postradiation tissue remodeling. As such, treatment with TGFβ inhibitors or genetically engineered TGFβR or HGF-expressing MSCs may be beneficial to attenuate fibrosis, as has been observed in radiated lungs [98, 104].

1.5 Future Prospects

From both this chapter and following chapters, it is clear that there are a number of different strategies to repair damaged SGs. Intravenous protein delivery, particularly of growth factors or agents that increase stem/progenitor cell activity, is often clinically disputed due to concerns regarding activation of any remaining tumor tissue. Local retrograde duct delivery of agents, such as viral vectors as part of gene therapy, provides a localized delivery and safer strategy as SGs are encapsulated and epithelial cells are easily transfected. While there is a possibility of vector diffusion into the bloodstream, this has not been a major issue in preclinical analysis. Similarly, direct cell delivery or mobilization of resident cells will always require evaluation of possible improper growth patterns, and bioengineered tissues will have to integrate with existing tissue and/or connecting ducts, nerves, and blood vessels.

Research on gland morphogenesis and repair will continue to identify targets from which new ways to approach regenerative therapies are being

developed. Clearly, reestablishing crosstalk between different cell types endogenously enhances the regeneration process. Thus, by improving one compartment, one may also indirectly improve repair in another compartment. Recreating an optimal environment wherein cells can multiply and reconstruct the organ will be key. Currently, multiple approaches may be required to improve specific tissue structures within the organ and/or simultaneously influence other compartments to regenerate a damaged organ.

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