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Salivary gland stem cells: a review of development, regeneration and cancer

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Abstract

Salivary glands are responsible for maintaining the health of the oral cavity and are routinely damaged by therapeutic radiation for head and neck cancer as well as by autoimmune diseases such as Sjögren's syndrome. Regenerative approaches based on the reactivation of endogenous stem cells or the transplant of exogenous stem cells hold substantial promise in restoring the structure and function of these organs to improve patient quality of life. However, these approaches have been hampered by a lack of knowledge on the identity of salivary stem cell populations and their regulators. In this review we discuss our current knowledge on salivary stem cells and their regulators during organ development, homeostasis and regeneration. As increasing evidence in other systems suggests that progenitor cells may be a source of cancer, we also review whether these same salivary stem cells may also be cancer initiating cells.

Introduction

Salivary glands (SGs) are one of numerous exocrine organs that have evolved to allow terrestrial living. Although their gross anatomy can vary dramatically across species, the complex serous-mucous liquid produced (saliva) plays an important and often essential role in survival through its impact on diet, for example, mice die within days after major gland removal. Although functional salivary glands are not required for human survival, SG dysfunction that arises from genetic anomalies (e.g., LADD or ASLG syndromes), or damage from surgery, therapeutic radiation for head and neck cancer (Frank *et al.*, 1965; Valdez *et al.*, 1993), or autoimmune diseases such as Sjögren's syndrome (Azuma *et al.*, 1997; Patel and Shahane, 2014; Stewart *et al.*, 2008) impairs oral health, resulting in a myriad of symptoms including mastication and swallowing difficulties (Dusek *et al.*, 1996; Hamlet *et al.*, 1997; Tolentino Ede *et al.*, 2011), speech impairment (Rhodus *et al.*, 1995), mucosal alterations, oral infection (Azizi and Rezaei, 2009; Brown *et al.*, 1975; Davies *et al.*, 2006) and accelerated tooth decay (Lu *et al.*, 2014). Despite these detrimental and life-long effects, current therapies are limited to secretagogues and artificial saliva, with no long-term

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solutions to restore salivary gland function. Consistent with the need to develop regenerative strategies, there has been increasing focus on the identification of stem cell populations and their regulators for the repair or regeneration of injured salivary tissue. Here we aim to provide a perspective on what is currently known about the identity and regulation of salivary stem cells during organ development and adult regeneration. Due to the lack of knowledge on salivary cancers, and the increasing evidence in other systems that progenitor cells may be a source of such neoplasms, we also discuss whether these same salivary stem cells may be the initiators of salivary cancers.

Salivary Gland Structure and Function

Mammalian saliva is primarily secreted by 3 pairs of major salivary glands (parotid (PG), submandibular (SMG) and sublingual (SLG)) and over 1000 minor glands. In humans and mice the SMG, SLG and PG produce >90% of the total saliva secreted into the oral cavity (Miletich, 2010). In humans, the PG is the largest of the three glands and is located inferior and anterior to the ear; the SMGs are located alongside the mandible posterior to the tongue, and the SLGs lie beneath the oral mucosa anterior to the tongue (Figure 1A). The saliva producing secretory units of the gland, acinar cells, are located at the end of the ductal network and are either of a serous or mucous cell type. In humans, acinar cells of the PG are serous, the SMG has both serous and mucous and the SLG is predominantly composed of mucous acini (Martinez-Madrigal and Micheau, 1989). The different acinar cell types can be easily distinguished from each other at the histological level: serous cells are pyramidal with a large round central nucleus, and mucous cells are columnar and contain granules, displacing the flattened nuclei to the basal membrane. Surrounding the acini are myoepithelial cells that are thought to aid in saliva secretion by constricting the acini in response to neuronal cues (Segawa *et al.*, 1995). Once secreted from the acinar cell, saliva travels through a ductal network consisting of intercalated ducts (the smallest ducts composed of simple cuboidal epithelium), striated (simple columnar epithelium), and excretory ducts (pseudo-stratified columnar epithelium) into the oral cavity (Miletich, 2010). Mice have the same three major pairs of salivary glands, located in a similar location (Figure 1B). Mice differ from humans in that the SMG has an additional ductal network that connects the intercalated ducts to the striated ducts and is the main source of growth stimulatory molecules such as nerve growth factor and epidermal growth factor (Gresik *et al.*, 1980; Schenck *et al.*, 2017).

To produce the large quantities of saliva required each day ((0.5–1L per day in humans (Melvin *et al.*, 2005)) the SGs need extensive vascularization (water is derived from the plasma) and innervation. For the PGs, capillaries derived from the external carotid artery wrap around the serous acini while the SMGs and SLGs are vascularised by the submental and sublingual arteries, branches of the lingual and facial arteries. Saliva secretion is primarily controlled by parasympathetic, and to a much lesser extent, sympathetic nerves of the autonomic nervous system (Proctor and Carpenter, 2007). The PGs receive parasympathetic innervation from the glossopharyngeal nerve IX via the otic ganglion, while the SMGs and SLGs are innervated by the chorda tympani via the parasympathetic submandibular ganglion (Holsinger and Bui, 2007). Parasympathetic nerves activate both cholinergic and non-cholinergic receptors to drive salivary flow by increasing water transfer.

Sympathetic nerves, which travel from the superior cervical nuclei in the brain stem to innervate all three major SG, regulate protein secretion and thereby the viscosity of saliva. It is important to note that parasympathetic innervation is not only required for organ function, but is also necessary for tissue maintenance. Removal of the parasympathetic nerves (parasympathectomy) results in glandular atrophy in humans and rodents (Peronace *et al.*, 1964; Snell and Garrett, 1958; Wells and Peronace, 1967), which can be reversed if the tissue is reinnervated (Carpenter *et al.*, 2009). Although sympathetic nerves are not essential for tissue maintenance (Proctor and Asking, 1989), activation of beta-adrenergic receptors promotes cell proliferation and hypertrophy (Hand and Ho, 1985; Johnson, 1984) and has been demonstrated to promote regeneration of salivary tissue (Boshell and Pennington, 1980), suggesting that these nerves play may yet play a role in tissue homeostasis.

Salivary gland development

As for most mammalian glandular organs, the acinar-ductal network of the salivary glands is formed through the fundamental process of epithelial branching morphogenesis. This process, which was first described in the salivary gland by Elio Borghese in 1950 (Borghese, 1950), involves extensive rounds of epithelial tissue expansion, cleft formation, cell differentiation and lumenization that, unlike the lung, occurs in a non-stereotypical fashion (Figure 2). As most studies to date describe the murine SMG, this review will focus on this gland, however, we remind the reader that the PG and SLG differ greatly in position, size and acinar composition, suggesting potential differences in progenitor cell types and mechanisms regulating cell maturation.

The SMG initiates as an invagination of the oral epithelium into a condensing neural crest-derived mesenchyme after embryonic day (E) 11.5. Subsequent SMG development can be divided into 3 major phases: establishment of a relatively undifferentiated branched structure consisting of acinar and ductal precursors (E12–E14; Figure 2) followed by terminal differentiation into secretory cell types (E15–E17.5) and ending in functional maturation (post-natal day (P) 0 – 4 weeks). The first phase consists of an early round of branching, beginning at E12, in which a single epithelial end bud (pre-acini) undergoes multiple rounds of bud and duct formation to establish a tubular network by E15 that consists of KRT19+ duct cells and AQP5+ pre-acinar cells (myoepithelial cells have not emerged from the epithelium). By E16, alpha smooth muscle actin (αSMA)+ myoepithelial cells have emerged and acinar cells have begun to express a master regulator of secretion, MIST1 (BHLHA15), as well as early secretory proteins (e.g., parotid secretory protein (PSP)). After birth the tissue continues to undergo extensive epithelial branching and continued maturation (e.g., production of amylase) to form functional mucous and serous acinar cells, ducts and myoepithelial cells capable of producing the levels of saliva required for life (reviewed in Patel *et al.*, 2006); Figure 3).

Not surprisingly, these complex morphogenic and differentiation events are controlled by multiple signalling networks, including those derived from epithelial-mesenchyme, nerve-epithelial and more recently endothelial-epithelial interactions. These interactions are formed at the beginnings of organogenesis: the E11.5 epithelium invaginates into a condensing neural crest-derived mesenchyme containing a discontinuous CD31+ endothelial

plexus and recruits SOX10+ neural precursors to form a post-ganglionic parasympathetic ganglion (Figure 2). During the next 36 h the endothelium becomes continuous and axons extend from the ganglion and travel along the developing ductal system to envelope newly forming end buds, thus forming an integrated organ system (Coughlin, 1975; Knosp *et al.*, 2015; Knox *et al.*, 2010; Kwon *et al.*, 2017). As would be expected from such a heterogeneous structure, many pathways are involved in regulating epithelial branching, including those mediated by growth factors such as FGF, EGF, WNT, Hedgehog, and EDA (reviewed in Mattingly *et al.*, 2015) and neurotransmitters acetylcholine (ACh) (Knox *et al.*, 2010) and vasoactive intestinal protein (VIP) (Nedvetsky *et al.*, 2014). Although we know a great deal about the impact of these signalling pathways in controlling morphogenesis, we are only beginning to understand their impact on progenitor/stem cell behavior. Here we will review those pathways known to regulate progenitor cells and point the reader to excellent reviews on pathways regulating tissue morphogenesis (Kwon and Larsen, 2015; Mattingly *et al.*, 2015; Patel and Hoffman, 2014).

1. Progenitor cell markers in developing and adult SG

Genetic lineage tracing has become the gold standard technique for identifying stem and progenitor cells in a plethora of developing and adult organs. By permanently labeling a specific cell and all its subsequent progeny (differentiated or not), we are able to identify initiating cells that contribute to the tissue during development, homeostasis and after injury. Here we define these initiating cells as progenitors, as unlike multipotent stem cells in the early embryo, current evidence indicates that their differentiation in the SG is limited. As only a few genetic lineage-tracing studies have been conducted thus far, we also describe potential progenitor populations based on markers identified to be present on progenitor cells in other glandular/epithelial organs via this technique. See Table 1 for the list of genes discussed below.

A) Progenitor markers in Developing SG

Intermediate filaments: Keratin-5, 14, 15 and 19: Basal epithelial cells marked by the acidic cytokeratins KRT5 and 14 have been shown to mark progenitor cells of numerous epithelial tissues including skin, cornea, developing trachea, lung airway epithelia, bladder and salivary glands (Colopy *et al.*, 2014; Cotsarelis *et al.*, 1989; Di Girolamo *et al.*, 2015; Knox *et al.*, 2010; Lombaert *et al.*, 2013; Moll *et al.*, 1982; Peters *et al.*, 2001; Rock *et al.*, 2009). In the SG, genetic lineage tracing using inducible and non- inducible *Krt14* or *Krt5* promoters, have demonstrated that the KRT14+/KRT5+ cells of the invaginating oral epithelium contribute extensively to acinar, ductal and myoepithelial cells (Knox *et al.*, 2010; Lombaert *et al.*, 2013). These cells - and consequently SG morphogenesis - have been shown to be regulated by a number of signaling pathways including those of the HIPPO and retinoic acid (RA) family. YAP, a negative regulator of the HIPPO pathway involved in organ size and cell proliferation (Wu *et al.*, 2003), is enriched in the nucleus of KRT14+ cells in the ducts during early stages of SG development (E13.5). Ablation of *Yap*, and thus activation of the HIPPO pathway, in KRT5+/KRT14+ cells before SG initiation reduces the production of Epiregulin, an ErbB receptor ligand which is involved in cell fate control (Gregorieff *et al.*, 2015) and is required for KRT5+/KRT14+ cell expansion, thereby perturbing epithelial branching and duct formation (Szymaniak *et al.*, 2017). In addition,

restriction of nuclear *Yap* localization, via deletion of *Lats1* and *Lats2*, results in the expansion of KRT5+/KRT14+ cells, aberrant enlargement of ducts and reduced end bud formation, further illustrating the requirement of controlled YAP signaling during lineage formation and epithelial morphogenesis in the developing SG (Szymaniak *et al.*, 2017). Similar to the HIPPO pathway, the RA pathway also regulates proliferation of KRT14+ progenitors. RA signaling occurs in early SMG development (from E10.5), where it plays a role in maintaining SG progenitor cells as well as epithelial morphogenesis. RA-deficient mice exhibit SMG developmental delay (Wright *et al.*, 2015) and similarly, blocking RA signaling in isolated epithelia with the pan-RAR antagonist BMS 493 results in reduced branching morphogenesis (Wright *et al.*, 2015) and repressed cell proliferation (Abashev *et al.*, 2017). Using RAR isoform-specific agonists and inhibitors DeSantis, *et al.* demonstrated isoform-specific roles for retinoic acid receptor (RAR) signaling in maintenance of KRT14+ cells, where RARγ is necessary, but not sufficient, to maintain KRT5+ cells, whereas RARα agonism reduces the number of KRT5+ cells and promotes differentiation (DeSantis *et al.*, 2017).

As expression of KRT14 and KRT5 segregates during development, with the majority of KRT14+ cells in E13 end buds being deficient in KRT5 (Lombaert *et al.*, 2013) (Figure 3), it remains unclear if cells that co-express KRT5 and 14 or those that solely express one but not the other keratin continue to contribute to the different epithelial lineages. Given recent studies showing cells solely expressing KRT5+ or KRT14+ cells but not both are regulated by different mechanisms, these cells likely differ in their progenitor cell properties. Maintenance of KRT5+ but not KRT14+ basal cells is dependent on parasympathetic nerves: acetylcholine secreted by the nerves activates muscarinic/EGFR signaling to promote KRT5+ cell self-renewal (Knox *et al.*, 2010). Intriguingly, KRT5+ cells themselves initiate and maintain their own innervation by producing WNTs that act on neural precursors to promote ganglion formation at the primary duct, a location rich in these cells (Knosp *et al.*, 2015). In contrast, Lombaert and co-workers demonstrated that KRT14+ cells in the end buds expand in response to FGFR2b signaling but are not depleted by the absence of innervation (Lombaert *et al.*, 2013). Thus, multiple mechanisms including nerves, FGF, retinoic acid and Hippo signaling regulate KRT5+ and KRT14+ cells.

In addition to KRT5 and KRT14, two other keratins, KRT15 and KRT19, have also been postulated to mark progenitors in the SG due to their known status as progenitors in other epithelial/glandular organs. KRT15 marks a progenitor cell of the hair follicle (Ito *et al.*, 2005; Lyle *et al.*, 1998; Morris *et al.*, 2004; Wang *et al.*, 2011) and is expressed in the ductal region of the developing SG, similar to KRT5 (Knox *et al.*, 2010; Lombaert *et al.*, 2011). Although genetic lineage tracing of KRT19+ cells has not been performed in the SG, studies in the developing exocrine pancreas and liver reveal that KRT19+ cells contribute exclusively to the ductal compartment (Means *et al.*, 2008), suggesting that these cells are specified to produce ductal cells in epithelial organs. Consistent with this outcome, KRT19 expression in the SG is also limited to the presumptive ducts of the developing SG and the luminal ductal cells of adult SG. In developing SG, inhibition or ablation of EGFR or inhibition of the vasoactive intestinal peptide (VIP)/PKA pathway depletes KRT19+ cell numbers and results in abnormal duct morphogenesis (Jaskoll and Melnick, 1999; Knox *et*

al., 2010; Nedvetsky *et al.*, 2014), supporting the idea that KRT19+ cells are crucial for efficient duct development.

Growth factor receptors: KIT and LGR4/5/6: A number of growth factor receptors and co-receptors have been shown to mark progenitors in developing organs. One of the most well-characterized is KIT, a receptor tyrosine kinase encoded by the oncogene c-kit that functions in multiple cellular processes including cell proliferation, differentiation, cell survival and migration through interaction with its ligand Stem Cell Factor (SCF) (Lennartsson and Ronnstrand, 2012). KIT was originally identified as a marker of hematopoietic stem cells (Shiohara *et al.*, 1993) and was subsequently used as a marker of progenitor cells in other developing organ systems including the kidney (Schmidt-Ott *et al.*, 2006) and salivary gland (Lombaert *et al.*, 2013). However, despite an allele for genetically tracing KIT+ cells being readily available (van Berlo *et al.*, 2014), to date, only the developing lymphatics (Stanczuk *et al.*, 2015) and olfactory epithelium (Goss *et al.*, 2015) have been reported to be derived, at least in part, from KIT+ cells. Support for KIT as a marker of progenitors in the SG is derived from its expression by a subset of KRT14+ progenitors, as well as studies showing reduced epithelial branching of E14 SG from mice deficient in *Kit* (Kitw/w) (Lombaert *et al.*, 2013). Indeed, the expression pattern of KIT is similar to KRT14 in that it is expressed in both pre-acini and presumptive ductal structures and becomes restricted to the end buds by E14 (Lombaert *et al.*, 2013; Wang *et al.*, 2014). Given the requirement for FGF10/FGFR2b in the invagination and expansion of the primordial epithelium, it comes as no surprise that KIT+ and KRT14+ cells are both regulated by FGF10/FGFR2b signaling (Lombaert *et al.*, 2013; Patel *et al.*, 2014). A recent study has implicated mesenchymal-epithelial communication and epigenetic control via the miRNA miR-133b-3p and DIP2B in expansion of this KIT+K14+ population during organogenesis. Exosome transport of microRNA between the mesenchyme and epithelium is essential for SMG development and knockdown of the mature mesenchymal microRNA miR-133b-3p resulted in decreased end-bud morphogenesis and reduced proliferation of KIT + progenitor cells. miR-133b-3p downregulates the target gene *Dip2b* in KIT+ progenitor cells, subsequently influencing cell cycle, and thus acts as a epigenetic regulator of KIT +K14/K5- progenitor cell expansion during SG morphogenesis (Hayashi *et al.*, 2017). However, despite this overlap, a population of KIT+ cells remains KRT14-negative in the pre-acinar cells of the SMG (Lombaert *et al.*, 2013), suggesting that KIT and KRT14 diverge to mark distinct progenitor populations in the SG. In support of this segregation, an inverse expression profile to *Krt14* was apparent for *Kit* expression following RA inhibition, where BMS 493 reduces expression of *Kit* in isolated epithelia explants (Abashev *et al.*, 2017). In addition, KRT14 but not KIT is expressed by emerging SMA+ myoepithelial cells, which in the lacrimal gland give rise only to themselves (Farmer *et al.*, 2017).

The developing SG also expresses a number of other putative progenitor markers that are components of pathways important to salivary gland development. This includes the Leucine-rich repeat containing G protein-coupled receptors (LGR4, 5 and 6), components of the WNT signaling pathway, where WNT functions in SG duct development and gangliogenesis (Knosp *et al.*, 2015; Patel *et al.*, 2011). LGR5 marks progenitor cells in the developing kidney, where they contribute to nephron formation (Barker *et al.*, 2012) and the

embryonic ovaries where they give rise to the ovary surface epithelium (Ng *et al.*, 2014). Genetic lineage tracing experiments have demonstrated that LGR5+ cells can maintain all cell lineages of the hair follicle (HF) and generate an entire new follicle (Jaks *et al.*, 2008) and contribute to the intestinal epithelium (Barker and Clevers, 2010) and the stomach (Barker *et al.*, 2010). In the SG, LGR5 expression is enriched in the primary duct of the developing SMG (similar to KRT5 and SOX2), as well as in the mesenchyme (Salivary Gland Atlas, NIDCR). Although it is not known whether LGR5 marks a progenitor population in the SG, SOX2 and LGR5 co-localize in the mouse pylorus (Arnold *et al.*, 2011) and human minor salivary gland mesenchymal stem cells (HMSGMSCs) that possess self-renewal and multipotent ability express LGR5 (Lu *et al.*, 2015). Although WNT signaling is a regulator of SG development and is crucial to maintain SG stem cell-containing organoids in culture (Maimets *et al.*, 2016), whether LGR5+ cells or LGR5 function are required for SG development has not been reported. However, the mild to normal phenotypes observed in other organs of the *Lgr5*-deficient mouse model suggest that LGR5 itself is not essential to organism development (Kinzel *et al.*, 2014; Morita *et al.*, 2004). The closely related receptor LGR4 is essential for renal development (Kato *et al.*, 2006) and is expressed throughout the developing and adult salivary gland epithelium and mesenchyme (Salivary Gland Atlas, NIDCR; Van Schoore *et al.*, 2005). Ablation of *Lgr4* results in a loss of the crypt cells of the intestine (de Lau *et al.*, 2011), impaired intestinal, kidney and skin development (Kato *et al.*, 2006; Kinzel *et al.*, 2014; Mohri *et al.*, 2008; Mohri *et al.*, 2011) and a failure to generate intestinal organoids in culture, a phenotype exacerbated when in combination with *Lgr5* knockout (de Lau *et al.*, 2011). LGR6+ cells mark stem cells in the hair follicle that contribute to the skin lineages (Snippert *et al.*, 2010), the nail and are essential for digit tip regeneration (Lehoczky and Tabin, 2015). However, deletion of *Lgr6* demonstrates that LGR6+ cells are dispensable for epidermal repair (Jiang *et al.*, 2017).

Transcription factors: ASCL3, SOX2, SOX10, SOX9, P63, PAX6: The basic helix-loop-helix transcription factor ASCL3 (achaete-scute family bHLH transcription factor 3) and its family members ASCL1 and 2 are essential determinants of cell fate and differentiation in multiple tissues (Battiste *et al.*, 2007; van der Flier *et al.*, 2009). ASCL3 was originally named Salivary Glands 1 (Sgn1) due to its prominent expression in a subset of striated and excretory duct cells in the adult mouse SMG (Yoshida et al., 2001). Similarly, in the developing SG expression of *Ascl3* (begins at E14) is also localized to cells in the ductal regions. This location in the ducts correlates with the long-believed notion that the SG progenitors resided in the ductal compartment. Consistent with this, using a non-inducible recombinase under the control of the *Ascl3* promoter (*Ascl3*$^{EGFP\text{-}Cre/+}$) crossed to a *Rosa26R* reporter, Bullard and colleagues determined that ASCL3+ cells give rise to ductal and acinar cells during development (Bullard *et al.*, 2008). However, as not all acinar and duct cells were labeled by *Ascl3* induction, the authors suggested the presence of other progenitor cells that likely contribute to salivary gland development. This was shown to be the case when basal epithelial cells expressing KRT5 or KRT14 were also shown to contribute to all acinar, ductal and myoepithelial cells (Knox *et al.*, 2010; Lombaert *et al.*, 2013). However, whether KRT5 and KRT14 cells contribute to the synthesis of ASCL3+ cells or if this is a population of cells that arises from another progenitor cell type remains to

be investigated. Furthermore, ASLC3+ cells exclusively gives rise to microvillar cells and Bowman's glands of the olfactory epithelium (Weng *et al.*, 2016).

Genetic lineage tracing has also identified the transcription factor SOX2, an essential regulator of pluripotency of embryonic stem cells (ESCs), as a marker of progenitor cells for the duct and acinar lineages in the developing SMG and SLG (PG not investigated (Emmerson *et al.*, 2017)), as well as many other epithelial tissues (Arnold *et al.*, 2011). We recently reported that despite SOX2 being expressed throughout the oral epithelium, SOX2 has an essential role in the generation of acini: genetic ablation of epithelial *Sox2* prior to gland ontogenesis impairs the production of SOX10+ acini but not ducts, in part, through cell death (Emmerson *et al.*, 2017). This lineage specificity was postulated to be mediated by direct regulation of *Sox10*, a known regulator of acinar cell differentiation in the lacrimal glands (Chen *et al.*, 2014). Furthermore, we showed that SOX2 expression and SOX2+ cell proliferation is regulated by neuronal acetylcholine-muscarinic signaling, demonstrating a novel role for parasympathetic nerves and SOX2 in directing a specific lineage during SG development (Emmerson *et al.*, 2017).

Another member of the SOX family, SOX9, marks multipotent progenitor cells in the developing pancreas, mammary gland, lung, liver, duodenum and tendons (Chang *et al.*, 2013; Furuyama *et al.*, 2011; Jo *et al.*, 2014; Malhotra *et al.*, 2014; Rockich *et al.*, 2013; Seymour *et al.*, 2007; Soeda *et al.*, 2010). Similarly, SOX9+ cells have recently been shown to be a bona fide progenitor cell population that gives rise to cells of the acinar and ductal lineages during SG development (Chatzeli *et al.*, 2017). Furthermore, conditional ablation of *Sox9* using the *Krt14* promoter arrested acinar and ductal morphogenesis and impaired specification of distal putative progenitors (marked by Myb and SOX10), indicating an essential role for this transcription factor in morphogenic processes and cell fate. This role is consistent with other studies showing *Sox9* is required for epithelial branching in the developing lung (Chang *et al.*, 2013; Rockich *et al.*, 2013), kidney and ocular glands (Reginensi *et al.*, 2011), as well as the development of the secretory acinar and myoepithelial cells of the lacrimal and harderian glands (Chen *et al.*, 2014). Whether SOX9 continues to mark acinar progenitors and control cell fate in the SG remains to be investigated. In the pancreas SOX9+ cells become lineage restricted over time, contributing solely to the ductal lineage shortly after birth (Kopp *et al.*, 2011), SOX9+ cells in the postnatal mammary gland give rise to estrogen receptor (ER)-negative luminal and basal cells (Malhotra *et al.*, 2014; Wang *et al.*, 2017) and in the hair follicle become restricted to the early bulge progeny of the outer root sheath (ORS) during the tissue growth phase known as anagen (Kadaja *et al.*, 2014).

Although definitive lineage tracing studies have not been performed for SOX10, due to its restricted expression to the pre-acinar cells of the developing lacrimal gland (Chen *et al.*, 2014) and salivary gland (Lombaert *et al.*, 2013; Lombaert and Hoffman, 2010) and its requirement the production of acini and myoepithelial cells during lacrimal gland morphogenesis (Chen *et al.*, 2014), it has been proposed as a marker of acinar progenitor cells in the developing SG. Moreover, SOX9 and SOX10 are co-expressed in these cells, where SOX9 regulates lineage outcomes in the lacrimal glands through SOX10. As SOX10 is also expressed in acinar cells of the adult SG (Ohtomo *et al.*, 2013) (Figure 3) it is likely

to be a marker of acinar progenitors throughout development and homeostasis (Lombaert *et al.*, 2013; Lombaert and Hoffman, 2010). Consistent with the enrichment of SOX9 and SOX10 in the acini, and the essential role for FGF10/FGFR2b in acinar cell expansion, both SOX9 and SOX10 are regulated by FGF signaling (Chatzeli *et al.*, 2017; Chen *et al.*, 2014; Lombaert *et al.*, 2013). Ablation of *Fgf10*, in the developing lacrimal gland results in a depletion of both Sox9 and Sox10 (Chen *et al.*, 2014). In addition, *Sox9* expression is severely reduced in the developmental placodes of the premature SGs of *Fgf10* knockout mice (Chatzeli *et al.*, 2017). Additional studies in the SG have also shown that SOX10 is reduced in the absence of FGF10 or KIT (Lombaert *et al.*, 2013), indicating a common mechanism for maintenance of progenitors marked by SOX9 and SOX10 in glandular tissues.

Transformation-related protein 63 (*Trp63*/P63) and Paired box protein-6 (PAX6) which mark progenitors in multiple epithelial tissues, including lacrimal glands, thymus and skin (Finley *et al.*, 2014; Senoo *et al.*, 2007; Yang *et al.*, 1999), are also postulated as progenitors for the SG. P63 and more specifically the NH2- terminal truncated (ΔN) p63 isoform marks basal epithelial cells and myoepithelial cells in the salivary gland (Bilal *et al.*, 2003), as well as basal epithelial cells in the bladder, prostate (Cheng *et al.*, 2006; Pignon *et al.*, 2013; Signoretti *et al.*, 2000), cornea, skin trachea and lung (Mills *et al.*, 1999; Rock *et al.*, 2009; Yang *et al.*, 1998). Although not reported for all epithelial organs, genetic lineage tracing using a non-inducible Cre under the control of the ΔNp63 promoter has established p63 as a progenitor in the developing prostate, bladder and colorectal epithelium (Pignon *et al.*, 2013). Global ablation of p63 or ΔNp63 results in an absence of all squamous epithelia and their derivatives, including the SGs, lacrimal glands and the stratified epidermis of the skin (Yang *et al.*, 1999). These phenotypes result, at least in part, from apparent defects in stem and progenitor cells' capacity to proliferate or survive (Pellegrini *et al.*, 2001; Senoo *et al.*, 2007; Yang *et al.*, 1999).

PAX6, a protein initially found to regulate neural stem cell self-renewal and differentiation, marks progenitor cells in the developing lens, cornea and lacrimal glands (Li *et al.*, 2015; Lin *et al.*, 2016). PAX6 is essential for ocular organ formation as shown by the absence of eyes and lacrimal gland in *Pax6*$^{-/-}$ embryos (Hill *et al.*, 1991) and impaired eye and lacrimal gland morphogenesis in embryos heterozygous for *Pax6* (Makarenkova *et al.*, 2000; Remez *et al.*, 2017). Although genetic lineage tracing has not been reported in the salivary glands, PAX6+ cells have been identified in developing SG and global ablation of *Pax6* results in a reduction in epithelial branching compared to wild type controls (Jaskoll *et al.*, 2002). Whether ablation of *Pax6* (or other genes) reflects their role as SG progenitors themselves or as regulators of differentiated epithelial cells requires further investigation.

B) Progenitor cell markers in the adult SG

Epithelial progenitors

KRT14, SOX2 and KIT mark lineage restricted epithelial progenitor cells: The intercalated ducts of the adult salivary glands were originally predicted to harbor a stem cell population capable of giving rise to both acini and ducts (Denny *et al.*, 1993; Ihrler *et al.*, 2004; Redman, 1995). However, a number of recent studies based on genetic lineage tracing have

disputed this hypothesis. Kwak *et al.* utilized an inducible Cre under the control of the *Krt14* promotor to demonstrate that cells marked by KRT14 i.e., myoepithelial cells and basal cells located in the intercalated ducts, give rise to cells of the granular convoluted tubules but not intercalated ducts, myoepithelial cells or acinar cells (Kwak *et al.*, 2016). We also recently showed through long term genetic lineage tracing of KIT+ cells that these cells, even after 6 months, contribute solely to the intercalated duct cells and not acinar cells of the homeostatic SG (Emmerson *et al.*, 2018). In concordance with these results, Aure *et al.* demonstrated that acini give rise exclusively to acini and not to the ductal system. Using a tamoxifen inducible Cre under the control of a *Mist1* promoter, where MIST1 labels the acinar cell lineage in mice (Lemercier *et al.*, 1997; Yoshida *et al.*, 2001), they demonstrated that acinar cells are replaced during homeostasis and after injury by labeled acinar cells and not by unlabeled cells arising from the ducts. Although from this study it was postulated that acinar cell replacement is mediated by self-duplication, whether a bona fide progenitor contributes to the acinar lineage was not known. We recently reported the presence of an acinar progenitor population, marked by SOX2 (Arnold et al., 2011; Figure 3), that gives rise to differentiated MUC19+ acinar cells of the SLG during homeostasis and after injury (Emmerson *et al.*, 2018). Furthermore, ablation of SOX2+ cells results in a striking loss of acinar cells and deletion of *Sox2* impairs acinar cell replenishment after radiation-induced damage, suggesting that SOX2+ cells are the sole progenitors of the murine SLG. A recent study using the single cell colony method suggested SOX2+ cells may also be progenitors for human SG (all three major SG have a subpopulation of acini that are SOX2+ (Emmerson et al., 2018)) as these colonies expressed *SOX2* and engrafted into SCID mice (Lu *et al.*, 2015). Similar to the developing SG, SOX2+ cells and SOX2-mediated acinar cell replacement are dependent on functional parasympathetic innervation, with administration of acetylcholine muscarinic mimetics being sufficient to drive acinar cell regeneration in mice and promote SOX2 expression and the acinar lineage in human SG (Emmerson *et al.*, 2018). As parasympathetic nerves and SOX2+ cells are diminished in irradiated human SG (Emmerson *et al.*, 2018), tissue degeneration may be due to a loss of progenitor cells and the cues that regulate them. However, many more studies are needed to determine if these are the sole progenitors or if other subsets of progenitors exist.

Mesenchymal progenitor cells

Hematopoietic stem cell markers: SCA1, KIT, THY1 and CD49f: The mesenchyme surrounding the salivary epithelium is derived from the neural crest and likely contains progenitor cells capable of contributing to the mesenchyme and/or other cell types, however, to date no lineage tracing has been performed to confirm this. In support of this theory, a number of putative progenitor cell surface receptors have been identified in murine salivary mesenchyme, including the hematopoietic stem cell markers SCA1 (stem cell antigen 1, expressed in mouse but not human HSCs), KIT, CD49f (integrin alpha 6; (Hisatomi *et al.*, 2004; Okumura *et al.*, 2003) and THY1 (thymocyte antigen 1; (Matsumoto *et al.*, 2007; Sato *et al.*, 2007) that possess stem/progenitor cell properties. For example, murine SCA1+KIT +CD49f+ cells have the ability to clonally expand under stress conditions, suggesting they have replenishing capacity (David *et al.*, 2008), and are able to differentiate into hepatic, pancreatic or salivary-like cells (Hisatomi *et al.*, 2004; Okumura *et al.*, 2003). When cultured SCA1+KIT1+ cells form salispheres that have the ability to branch (Lombaert *et al.*, 2008).

More recently, CD49f+THY1+ cells were shown to have proliferation potential, form organoids in culture and like SCA1+KIT+CD49f+ cells could also differentiate into pancreatic-like and amylase-expressing cells (Sato *et al.*, 2007). Histatomi *et al.* report that SCA1+ and KIT+ cells are rare in the healthy SG but are found in clusters in the ducts following ligation injury, suggesting that they proliferate and expand under stress or injury conditions in order to replenish the injured tissue (Hisatomi *et al.*, 2004). Indeed, when SCA1+KIT+ cells are transplanted into irradiated SGs they are able to successfully regenerate the gland, forming both acinar and ductal structures (Lombaert *et al.*, 2008). The fact that KIT+ cells derived from SG can transdifferentiate into hepatic and pancreatic lineages (Hisatomi *et al.*, 2004; Okumura *et al.*, 2003) may indicate that they are truly multipotent and are, in fact, regulated by their microenvironment or niche to transdifferentiate into different lineages besides SG epithelial tissue. In addition, far fewer KIT+ cells are required to be transplanted to rescue radiation-induced damage than CD133+, CD49f+ or CD29+ cells, demonstrating their potency as a true progenitor (Nanduri *et al.*, 2011). Furthermore, when KIT+ cells are serially transplanted they are able to expand even after several rounds of regeneration, demonstrating their ability to self-renew as a progenitor cell (Lombaert *et al.*, 2008).

Classical mesenchymal stem cell (MSC) markers: There are a number of markers that are internationally recognized as classical MSCs, including CD24, CD29 and CD44 (Coulombel *et al.*, 1997). A number of studies have reported the successful isolation of putative progenitor cells expressing these markers from human SG via clonal assay (Rotter *et al.*, 2008; Schwarz and Rotter, 2012; Tatsuishi *et al.*, 2009). Following culture the cells resemble mesenchymal stem cells (MSCs) and express classical MSC markers, including CD29, CD44 and CD90. Furthermore, these cells are able to differentiate into osteogenic, adipogenic and chondrogenic lineages (Rotter *et al.*, 2008; Schwarz and Rotter, 2012). CD24+CD29+ cells can proliferate and expand ex vivo and, crucially, can rescue radiation-induced SG dysfunction in vivo (Nanduri *et al.*, 2014). Interestingly, when this CD24+CD29+ population also expresses KIT, and is injected into irradiated SMG they not only recovered saliva flow but also improved tissue architecture (Nanduri *et al.*, 2013). However, in this study the authors do not demonstrate that the isolated cells are directly incorporated into the regenerated epithelia. Indeed, since endothelial cells are sensitive to radiation and vasculature is often adversely affected by radiation therapy (Ying *et al.*, 2007) and vascularization of the tissue was also notably improved in this study it cannot be ruled out that this CD24+CD29+KIT+ population may be, at least in part, also influencing the vasculature of the injured gland via release of VEGF, for example (Beckermann *et al.*, 2008), and thus indirectly improving tissue regeneration.

The report that the highly proliferative SCA1+KIT+CD24+ population can rescue SMG function (saliva flow) and architecture (functional acini) following radiation-induced damage with the addition of glial cell line-derived neurotrophic factor (GDNF) (Xiao *et al.*, 2014) suggests that the niche and external cues are essential for these so-called SG MSCs to elicit their positive effects. Since cell tracing has never been performed in any such experiments it cannot be ruled out that these populations may not be contributing directly to restoration of the SG epithelium, but merely altering the niche or microenvironment or providing trophic

cues to positively enhance epithelial regeneration. Since GDNF is neuroattractive and improved innervation of the SG following radiation-induced damage has been shown to improve epithelial regeneration (Knox *et al.*, 2013) such outcomes could be the result of improvements to the niche, such as occurs during development (Ferreira and Hoffman, 2013). Indeed, a recent study demonstrated that GDNF itself does not protect SG stem cells from radiation-induced damage directly (Peng *et al.*, 2017), suggesting that such outcomes are the result of improvements to the supporting niche. Further tracing experiments are required to unequivocally demonstrate that such isolated cells are directly regenerating damaged SG tissue. Until recently it was thought that CD34 marked hematopoietic stem cells but was a negative marker of MSCs, but recent studies have suggested that this is due to an artefact of cell culture and that a small CD34+ MSC population exists in multiple tissues (Lin *et al.*, 2012; Sidney *et al.*, 2014). A recent study found that CD34 is expressed by MSCs of all three of the major human SGs. Furthermore, these cells express genes involved in ERK, FGF and PDGF signaling, pathways essential to salivary gland development and regeneration, and when transplanted engraft into murine SGs (Togarrati *et al.*, 2017). The co-expression of CD44 in these cells is in agreement with the results of Bahn *et al.* (2013) and both CD34 and CD44 are expressed by murine SG salispheres, the cells of which can rescue SG function following radiation injury (Banh *et al.*, 2011; Lombaert *et al.*, 2008).

Table 2 lists all markers used to enrich for mesenchymal salivary gland progenitor cell populations.

Are long lived cells also progenitors?: Early studies utilized a number of methods to identify stem cells in adult mouse SGs, including a principle property of progenitor cells that they are slow-cycling and replicate infrequently, thus retaining DNA nucleotide labels such as Bromodeoxyuridine and Ethynyldeoxyuridine (BrDU and EdU). Experiments to mark these so-called label retaining cells (LRCs) have led to the discovery of progenitor populations in multiple epithelial tissues, including the skin (reviewed in Terskikh *et al.*, 2012), sweat glands (Leung *et al.*, 2013; Lu *et al.*, 2012), teeth (Seidel *et al.*, 2010), pancreas (Teng *et al.*, 2007) and intestine (Buczacki *et al.*, 2013). Original observations using single injections of radiolabeled thymidine into adult mice or rats (6–7 weeks of age) found LRCs to reside in intercalated ducts and excretory ducts but not acinar cells (Man *et al.*, 2001; Zajicek *et al.*, 1985). Other recent studies in similarly aged rodents using BrdU delivered over 4 or 7 days followed by a chase of 7–8 weeks have found LRCs throughout the gland in both acinar, ductal and myoepithelial cells, as well as the connective tissue, suggesting a larger number of cells are long lived and/or have progenitor cell-like properties than previously thought (Kim *et al.*, 2008; Kimoto *et al.*, 2008). Up until recently the identity of these LRCs was not known, however, a study by Chibly and colleagues determined that LRCs in both acinar and ductal structures co-localize with the embryonic SG progenitor markers KRT5, KRT14, and SOX2 protein and *Kit* mRNA (Chibly *et al.*, 2014). Of these, only KRT5 has not yet been reported to contribute to the adult tissue via lineage tracing experiments. However, intriguingly, these studies contrast to a recent report using a H2BGFP mouse model showing the presence of actively dividing pools of progenitor cells in the intercalated and excretory ducts (Kwak and Ghazizadeh, 2015). As H2B can also acts

in the DNA damage response (Kim *et al.*, 2008), it remains to be determined whether these cells actively contribute to the SG.

Are proliferating salisphere cells the true stem cells of the SG?: In an effort to translate these finding toward a clinical therapeutic a number of studies have attempted to isolate SG progenitor cell populations from adult human SG, based on markers identified in murine studies (Feng *et al.*, 2009) or based on in vitro assays, as described earlier (Jeong *et al.*, 2013; Lu *et al.*, 2015; Okumura *et al.*, 2012; Rotter *et al.*, 2008; Schwarz and Rotter, 2012; Tatsuishi *et al.*, 2009). Feng *et al.* collected tissue from human PG and SMG and by optimizing conditions used for culture of murine cells were able to successfully culture human salispheres that expressed KIT. These human-derived salispheres are able to branch when transferred to a 3D matrix (Okumura *et al.*, 2012), in a manner similar to embryonic mouse epithelial rudiments (Wei *et al.*, 2007), suggesting stem cell-like properties. Conversely, Jeong *et al.* developed a human SG progenitor culture system that negated the requirement for cell sorting and surface markers, and demonstrated that the adherent cells in their culture exhibited MSC-like characteristics and could rescue acinar structure and hyposalivation in irradiated rats. Similarly, Rotter *et al.* used the clonal assay technique to enrich for progenitors that could differentiate into multiple lineages, demonstrating their multipotency. However, until transplantation studies are performed, we remain in the dark regarding the ability of these cells to contribute to the salivary gland in vivo.

2. Is salivary gland cancer derived from salivary progenitors?

Emerging evidence points toward the existence of cancer initiating cells (CICs) that possess multipotency and self-renewal capacity, characteristics attributed to stem cells. CICs can both initiate and maintain a tumour and are often resistant to chemotherapy, thus enabling tumour recurrence, often many years later (reviewed in Adams *et al.*, 2013). Cancers of the SG are the most heterogeneous in humans, consisting of 24 distinct pathological sub-types (Gillespie *et al.*, 2012) and are notoriously difficult to treat given their poor response to chemo- and radio-therapy (Laurie and Licitra, 2006). As such, despite the high survival rate in the first 5–10 years, their ability to evade treatments combined with their high recurrence rate leads to low long-term survival, implicating the possibility of a CIC population capable of being reactivated following therapy. As such, a better understanding of salivary gland malignancies and these possible CICs is essential for the generation of effective therapies.

CICs in human head and neck cancers (HNCs) were first reported in 2007 (Prince *et al.*, 2007). These expressed CD44 and showed high tumorigenicity in NOD/SCID mice. Subsequently, expression of aldehyde dehydrogenase (ALDH), a marker originally used for CICs in the breast (Ginestier *et al.*, 2007) was reported to be a characteristic of HNC CICs (Clay *et al.*, 2010) and salivary CICs with high metastatic potential (Sun and Wang, 2010). More recently a population of ALDH expressing CD24+/CD44+ cells present in human head and neck squamous cell carcinomas (HNSCCs) (Han *et al.*, 2014) and salivary gland malignant neoplasms (SGMNs) (Soave *et al.*, 2013) was reported to exhibit stemness characteristics (Adams *et al.*, 2015), similar to CICs of the pancreas and breast (Al-Hajj *et al.*, 2003; Li *et al.*, 2007). These cells were resistant to chemotherapeutic agents such as Cisplatin and, when injected into nude mice, induced large tumors (Han *et al.*, 2014). WNT/

β-catenin signalling may play a role in regulating these cells as activity correlates with expression of CD44 in SG cancers and treatment of CICs with a WNT/β-catenin active small molecule inhibitor, LF3, blocked their self-renewal capacity (Fang *et al.*, 2016). Indeed, WNT inhibition supresses CIC stemness and induces cellular senescence in SG squamous cell carcinoma (SCC) (Ramachandran *et al.*, 2014), whereas activation of the WNT/β-catenin pathway, via a β-catenin gain of function approach, induces a rapidly growing, aggressive phenotype (Wend *et al.*, 2013). While these studies did not specifically investigate the association between WNT/β-catenin and CD24/CD44 expression in SG CICs, this result suggests that dysregulated WNT/β-catenin signalling may be, at least in part, responsible for the expansion of SG CICs and tumor progression. As WNT/β-catenin signalling has been reported to protect SG function during therapeutic radiation to treat head and neck cancer, presumably by preserving adult SG stem cells (Hai *et al.*, 2012), this pathway may also protect CICs from elimination by radiotherapy. Thus, signalling pathways that control SG stem cells in the healthy gland must be carefully regulated if they are to act as a therapeutic approach and not lead to secondary tumors.

Other markers of SG progenitors have also been reported in tumour tissue; however, whether the expression of these markers specifically correlates with cellular attributes of CICs is yet to be determined. In spite of this, high expression of many of these markers is linked to metastasis and poor patient survival. An example of this is SOX2, which is often aberrantly expressed in HNCs (Dong *et al.*, 2014; Ge *et al.*, 2010; Lee *et al.*, 2014; Li *et al.*, 2014; Schrock *et al.*, 2014) with high SOX2 expression being linked to distant metastasis in adenoid cystic carcinoma (ACC) of the salivary gland (Dai *et al.*, 2014) and high tumour grade in salivary cancer (Sedassari *et al.*, 2017) as well as head and neck cancers in general (Dong *et al.*, 2014). Elevated SOX2 expression is also associated with increased resistance to chemotherapy agents, although whether this is a direct effect is uncertain (Schrock *et al.*, 2014). However, silencing SOX2 in breast cancer cells in culture leads to increased sensitivity to the chemotherapeutic agent paclitaxel and reduction in mammosphere formation (Mukherjee *et al.*, 2017), suggesting a direct effect of SOX2 expression on chemoresistance in cancer cells. In addition, elevated or ectopic SOX2 expression has also been associated with the progression of other cancers, including skin squamous cell carcinoma (SCC) (Boumahdi *et al.*, 2014), glioblastoma (Annovazzi *et al.*, 2011), laryngeal squamous cell carcinoma (LSCC) (Yang *et al.*, 2014), bladder cancer (Zhu *et al.*, 2017) and small-cell lung cancer (SCLC) (Hussenet *et al.*, 2010). Interestingly, other members of the SOX family, which are expressed in salivary glands, may also be linked to cancer progression. SOX9 is overexpressed in a number of cancers, where it promotes cell proliferation and inhibits senescence (Matheu *et al.*, 2012), while SOX10 expression appears to be involved in transcriptional programming and progression of salivary ACC (Ivanov *et al.*, 2013; Ohtomo *et al.*, 2013). *c-Myc,* alongside *Sox2, Klf4 and Oct3/4* is expressed by ESCs and as such is intrinsically involved with pluripotency (Takahashi and Yamanaka, 2006). c-Myc is also highly expressed in salivary gland carcinoma, compared to healthy SG tissue (Schoenhals *et al.*, 2009) and in conjunction with Transforming Growth Factor alpha (TGFα) promotes adenocarcinoma (Amundadottir *et al.*, 1995). Thus, cells expressing high levels of both SOX2 and c-Myc may act as an undifferentiated cancer cell, with the ability to rapidly proliferate and metastasize.

Since SOX2 is regulated by acetylcholine derived from autonomic nerves innervating the developing and adult salivary gland (Emmerson *et al.*, 2018; Emmerson *et al.*, 2017), it would be of therapeutic interest to know if innervation and neuronal signalling also regulates SOX2 expression in salivary cancer. To date no studies have investigated the correlation between SOX2, innervation of salivary gland tumors and cancer progression. A number of recent studies have demonstrated the interaction between nerves and cancer cells known to overexpress SOX2 in other cancers such as those of the stomach and prostate (Magnon *et al.*, 2013; Zhao *et al.*, 2014) (Zhao *et al.*, 2014). As denervation suppresses tumor progression in gastric cancer (Zhao *et al.*, 2014), pancreatic cancer (Saloman *et al.*, 2016) and fibrosarcoma (Lackovicova *et al.*, 2011), nerves may provide trophic cues required for cancer development and for maintenance of SOX2, although expression of SOX2 in these denervated conditions has not been examined. Given the potential success of anti-neurogenic therapeutics in breast and gastric cancer (Hondermarck, 2012; Miknyoczki *et al.*, 2002), modulating innervation and neuronal signalling in salivary tumors may be a therapeutic approach to reduce SOX2 expression and improve patient survival.

Since p63 is considered both a tumour protein and a progenitor cell marker one would expect a high association between p63 and SG cancer. p63 is strongly expressed in basal cell adenoma, adenoid cystic carcinoma (ACC) and polymorphous low-grade adenocarcinoma (PLGA) and these cells may represent a neoplastic population of basal cancer stem cells (Edwards *et al.*, 2004; Emanuel *et al.*, 2005; Sams and Gnepp, 2013). Of importance, expression of p63 may also act as a marker of prognosis: patients who survive for more than 10 years following SG cancer diagnosis exhibit a lower extent of p63 expression than those who died within 10 years (Ramer *et al.*, 2010). In support of this, tumors expressing high levels of p63 are commonly associated with chemoresistance (Rocca *et al.*, 2008; Zangen *et al.*, 2005) via interference with apoptotic pathways (Mundt *et al.*, 2010). Specifically, the NH2- terminal truncated (ΔN) p63 isoform, which lacks the TA-domain, promotes cell proliferation and tumorigenesis in head and neck SCC and downregulation of ΔNp63 via RACK1 determines the cellular response of a tumor to chemotherapy agents, such as Cisplatin (Fomenkov *et al.*, 2004). Thus, degradation of ΔNp63 in cancer cells may provide a therapeutic approach to treat high p63 expressing cells of salivary tumors. Whether p63 marks a true CIC in the adult salivary gland remains to be determined.

High expression of KRT5 is also strongly associated with poor survival rates for SG cancers such as high-grade mucoepidermoid carcinoma, a common SG cancer (Lueck and Robinson, 2008), similar to what has been reported in breast cancer (van de Rijn *et al.*, 2002). In addition, a recent study has demonstrated that pleomorphic adenomas are, at least in part, derived from KRT14+ SG progenitors (Ogawa *et al.*, 2000) and multiple human SG neoplasms are positive for KRT14 (Bansal *et al.*, 2012) implicating the presence a KRT14+ CIC. While KRT5+ cells are regulated by autonomic nerves in the developing salivary gland (Knox *et al.*, 2013; Knox *et al.*, 2010) there has been no correlation demonstrated between KRT5 expression and nerves or neuronal signalling in adult salivary glands or salivary cancers. However, as discussed above, the association between nerve infiltration and tumor progression has been demonstrated in other cancers including those of the head and neck (Dobrenis *et al.*, 2015; Magnon *et al.*, 2013; Pundavela *et al.*, 2014), and as such modulating

neuronal signalling in salivary cancer may provide therapeutic benefit by modulating multiple CICs.

KIT is expressed in multiple SG tumour types, including ACCs and monomorphic adenomas (Andreadis *et al.*, 2006; Edwards *et al.*, 2003; Mino *et al.*, 2003), although there remains controversy about whether polymorphous low-grade adenocarcinomas (PLGAs) also express KIT, with some studies claiming they detect expression (Edwards *et al.*, 2003), while others claim no expression (Meer *et al.*, 2011; Penner *et al.*, 2002). Perineural invasion is a reliable indicator of poor survival in numerous cancers (Beard *et al.*, 2004; Ozaki *et al.*, 1999) and a number of studies have demonstrated a relationship between perineural invasion and KIT expression i.e., tumor cells invading facial nerves exhibit high expression levels of KIT (Phuchareon *et al.*, 2014; Tang *et al.*, 2010; Youssef and Said, 2014). This phenotype is associated with poor patient prognosis (Huyett *et al.*, 2018; Ko *et al.*, 2007) but whether nerves regulate KIT in these tumors, and if KIT is required for perineural invasion, is not known. However, while two case reports suggested that KIT-targeted therapy could inhibit ACC progression (Alcedo *et al.*, 2004; Faivre *et al.*, 2005), larger phase II therapies targeting KIT activity show no efficacious impact on cancers of the head and neck, suggesting that KIT itself is not driving tumor behaviour (Bruce *et al.*, 2005; Hotte *et al.*, 2005; Laurie and Licitra, 2006).

Conclusions and Future Directions

The characterization of multiple distinct SG progenitor populations has now introduced the need to understand the balance and relative contribution of these different populations to SG homeostasis and regeneration as well as the impact of disease on their function. For example, different progenitors may differ in their self-renewal and regenerative capacity and understanding this will be crucial to the generation and application of regenerative strategies. Moreover, it is unclear whether a single progenitor cell type gives rise to a single cell type or whether these cells are derived from more than one progenitor. In the prostate, for example, luminal cells give rise to luminal cells whereas basal cells produce themselves as well as luminal cells (Ousset *et al.*, 2012), thus providing a level of redundancy that benefits damage responses in the case that the luminal cell is compromised. Similarly, in the homeostatic intestine, fast cycling LGR5+ cells produce epithelial cells of the villi but should this cell be ablated a quiescent BMI1+ cell is able to compensate, feeding the villi with new daughter cells (Yan *et al.*, 2012). Another option for tissue repair is the de-differentiation of terminally differentiated cells into stem cell-like cells, as occurs in the pancreas and in the regenerating digit tip of rodents. In the inflamed pancreas, terminally differentiated acinar cells de-differentiate towards the ductal lineage, resulting in cells that express both acinar and ductal markers that are thought to regenerate the tissue (Liu *et al.*, 2016). In the case of the regenerating digit, the epithelial cells are derived from a non-epithelial progenitor cell, that is mature Schwann cells at the damage site are transformed into a more primitive lineage that can repopulate the epithelium (Johnston *et al.*, 2016). Whether such methods contribute to the homeostasis and regeneration of the salivary gland (murine or human) or if such mechanisms are utilized by CICs remains to be discovered. However, given the diversity of salivary cancers that have been identified to date, we speculate that a number of these cell programs may be employed both during cancer initiation as well as progression. In

summary, we are beginning to unfurl the identity and regulation of progenitors in the salivary gland, which will without doubt lead to a better understanding of tissue homeostasis, repair and disease.

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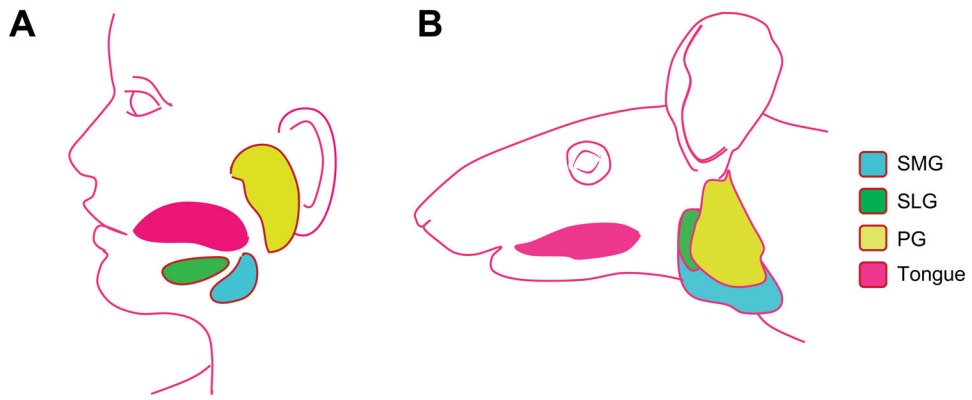

Figure 1.
Schematic to show the localisation of the three major salivary glands in humans (A) and mice B).

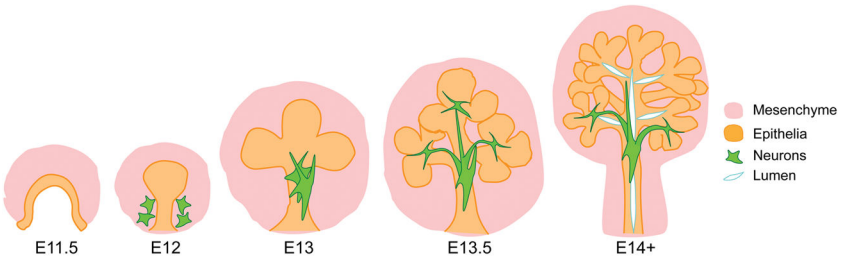

Figure 2.
Schematic of salivary gland development in the fetal mouse.

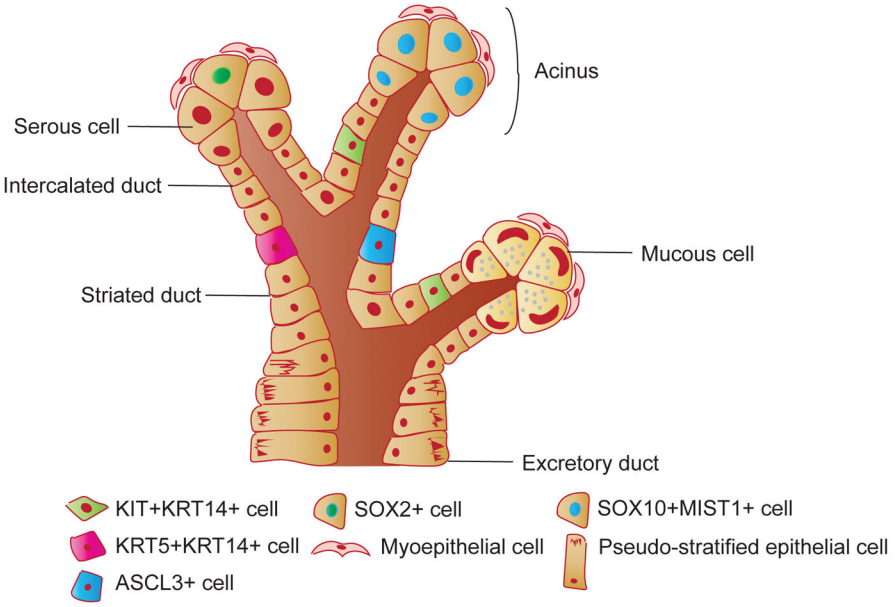

Figure 3.

Schematic of the structure of the salivary gland and the localization of known progenitor cells.

Table 1

Bona fide and putative salivary gland progenitors

Progenitor cell marker	Lineages traced in developing/adult organs	Phenotype from gene ablation/mutation
ASCL3	**SG -** Constitutively active Cre labels duct and acinar cells (Arany, *et al.*, 2011; Bullard, *et al.*, 2008; Rugel-Stahl, *et al.* 2012)	**SG** - No phenotype in *Ascl3*-deficient SG (Arany, *et al.*, 2011)
	Olfactory epithelium – gives rise to microvillar cells and Bowman's gland (Yoshida, *et al.*, 2001; Weng, *et al.* 2016)	**Olfactory epithelium** - *Ascl3*-deficient mouse lacks the non-neuronal microvillar and Bowman's gland support cells (Weng, *et al.* 2016)
KIT	**SG** – duct cells (adult reported; Emmerson *et al.,* 2018).	**SG** - *Kit*-deficient E14 SG ($Kit^{w/w}$) has reduced epithelial branching (Lombaert, *et al.*, 2013)
	Olfactory epithelium (Goss, *et al.* 2015) **Lymphatics** (Stanczuk, *et al.* 2015)	**Kidney** - reductions in ureteric bud branching and nephrons ($Kit^{w/w}$ and via inhibition of c-kit tyrosine phosphorylation; Schmidt-Ott, *et al.*, 2006)
KRT5	**SG** - acini, ducts, myoepithelial cells of fetal gland (adult not reported; Knox, *et al.*, 2010)	**SG** - No *Krt5*-deficient SG studies reported
	Lacrimal gland – duct and myoepithelial cells in adult gland (fetal not reported; Farmer *et al.* 2017) **Developing trachea and lung airway epithelia** (Rock, *et al.* 2009)	**Skin** – *Krt5*(−/−) die shortly after birth and exhibit skin blistering arising from basal cell cytolysis (Peters *et al.* 2001) Human mutations in *KRT5* and *KRT14* cause Epidermolysis bullosa simplex (Peters *et al.* 2001)
KRT14	**SG** – acini (fetal only), ducts, myoepithelial cells (fetal and adult) (Lombaert, *et al.* 2013; Patel, *et al.*, 2014; Kwak, *et al.* 2016)	**SG** - No *Krt14*-deficient SG studies reported
	Skin (Mascre, *et al.* 2012) **Cornea** – epithelium (Di Girolamo, *et al.* 2015) **Developing trachea and lung** (Rock, *et al.* 2009)	**Skin** – *Krt14*(−/−) does not lead to the ablation of a basal cell cytoskeleton (*Krt15* compensation mechanism; Peters *et al.* 2001) Human mutations in *KRT5* and *KRT14* cause Epidermolysis bullosa simplex (Peters *et al.* 2001)
KRT15	**SG** – not reported	**SG** - No *Krt15*-deficient SG studies reported
	Hair follicle – bulge and secondary hair germ (Ito, *et al.* 2005; Wang, *et al.,* 2011; Morris, *et al.* 2004)	No *Krt15*-deficient studies reported
KRT19	**SG** – not reported	**SG** - No *Krt19*-deficient SG studies reported
	Exocrine pancreas, liver – duct cells (Means, *et al.,* 2008) **Stomach, intestine** (Means, *et al.,* 2008)	No *Krt19*-deficient studies reported
LGR4/LGR5/LGR6	**SG** – not reported	**SG** – *Lgr5* KO – fusion of tongue to floor of oral cavity, SG phenotype not reported (Morita, *et al.* 2004)
	Ovary (Ng, *et al.* 2014) **Kidney nephron** (Barker, *et al.* 2012) **Skin and hair follicle** (Jaks, *et al.* 2008) **Intestine** (Barker and Cleavers, 2010) **Stomach** (Barker, *et al.* 2010)	**Kidney** - dilated kidney tubules and ectatic Bowman's spaces in *Lgr4* KO (Kinzel, *et al.* 2014) **Skin** – reduced basal cell proliferation and hair follicles in *Lgr4* KO (Kinzel, *et al.* 2014). No effect on epidermal repair in *Lgr6* KO (Jiang, *et al.* 2017) **Intestine** – loss of stem cells in *Lgr4* KO (Kinzel, *et al.* 2014) and gastrointestinal tract dilation (Morita, *et al.* 2004) *Lgr4* and *Lgr5* KO are perinatal lethal (Kinzel, *et al.* 2014; Morita, *et al.* 2004)
P63	**SG** – not reported	**SG** - aplasia in KO (Yang A, *et al.,* 1999)
	Prostate (fetal) (Pignon, *et al.* 2013) **Bladder (fetal) (**Pignon, *et al.* 2013) **Colorectal epithelium (fetal) (**Pignon, *et al.* 2013)	**Skin** – absence of squamous epithelia and derivatives in KO (Yang A, *et al.,* 1999; Senoo, *et al.* 2007) **Limb** – truncations in KO (Yang A, *et al.,* 1999) **Craniofacial** - defects in KO (Yang A, *et al.,* 1999) **Mammary and lacrimal glands** – absent in KO (Yang A, *et al.,* 1999)
PAX6	**SG** – not reported	**SG** - abnormal development in the KO (Jaskoll, T. *et al.,* 2002)

Progenitor cell marker	Lineages traced in developing/adult organs	Phenotype from gene ablation/mutation
	Cornea and lens (Lin, *et al.* 2016) **Limbs (fetal)** (Li, *et al.* 2015)	**Eye** – impaired retina, lacrimal gland and eye development in the KO (Remez, *et al.* 2017; Marenkova, *et al.* 2000)
SOX2	**SG** – fetal; acini, ducts (Arnold, *et al.*, 2011; Emmerson *et al.* 2017), adult; acini only (Arnold, *et al.*, 2011; Emmerson *et al.* 2018)	**SG** – (fetal) reduced epithelial branching in conditional KO (*K14^{CreERT2}; Sox2$^{fl/fl}$*; Emmerson *et al.* 2017) **SG** – (adult) loss of acini in conditional KO (*Sox2^{CreERT2}; Sox2$^{fl/fl}$* and *Sox2^{CreERT2}; R26^{DTA}*; Emmerson *et al.* 2018)
	Stomach (Arnold, *et al.*, 2011) **Cervix (**Arnold, *et al.*, 2011) **Anus (**Arnold, *et al.*, 2011) **Testes (**Arnold, *et al.*, 2011) **Lens (**Arnold, *et al.*, 2011) **Eosophagus** (Arnold, *et al.*, 2011)	**Dermal papilla of hair follicle** – no phenotype in conditional KO (*K14^{Cre}; Sox2$^{fl/fl}$*; Lesko, *et al.* 2013) **Merkel cells** – decreased number in conditional KO (*K14^{Cre}; Sox2$^{fl/fl}$*; Lesko, *et al.* 2013)
SOX9	**SG** – fetal; acini, ducts (Chatzeli, *et al.* 2017)	**SG** - reduced branching the KO (*Krt14^{CreERT2}; Sox9$^{fl/fl}$*)
	Liver (Furuyama, *et al.* 2011) **Exocrine pancreas** (Furuyama, *et al.* 2011; Seymour, *et al.* 2007) **Intestine** (duodenum) (Furuyama, *et al.* 2011) **Mammary gland (**Malhotra, *et al.* 2014) **Lung** (Rockich, et al. 2013; Chang, et al. 2013) **Tendon (**Soeda, *et al.* 2010)	**Lacrimal gland –** branching defect in conditional KO (*Sox9$^{fl/fl}$; Le-Cre+;* Chen, *et al.* 2014) **Harderian and meibomian glands** - reduced acini and loss of epithelia in conditional KO (*Sox9$^{fl/fl}$; Le-Cre+;* Chen, *et al.* 2014) **Skin** - missing hair in eyelids and facial skin in conditional KO (*Sox9$^{fl/fl}$; Le-Cre+;* Chen, *et al.* 2014)
SOX10	**SG** – not reported	**SG** - No *Sox10*-deficient SG studies reported
	No lineage tracing studies reported	**Lacrimal gland –** reduced acini and branching defect in conditional KO (*Sox10$^{fl/fl}$; Le-Cre+;* Chen, *et al.* 2014) **Harderian glands** - reduced acini and branching defect in conditional KO (*Sox10$^{fl/fl}$; Le-Cre+;* Chen, *et al.* 2014)

Table 2

Cell surface markers used to enrich for mesenchymal salivary gland progenitor cells

Marker	Alternate names	Reference(s)
CD49f	ITGA6, Integrin α6	Okumura, *et al.*, 2003; David, *et al.,* 2008; Matsumoto, *et al.,* 2007; Nanduri, *et al.*, 2011; Nanduri, *et al.*, 2013; Sato, *et al.*, 2007
CD29	ITGB1, Integrin β1	Okumura, *et al.*, 2003; David, *et al.,* 2008; Matsumoto, *et al.* 2007; Nanduri, *et al.*, 2013, Nanduri, *et al.,* 2014
Ly-6A/E	Sca-1	Hisatomi, *et al.* 2004
CD90	Thy-1	Matsumoto, *et al.,* 2007; Sato, *et al.*, 2007
CD117	KIT, c-Kit	Lombaert, *et al.*, 2008; Hisatomi, *et al.*, 2004; Nanduri, *et al.*, 2013; Nanduri, *et al.*, 2011
CD133	Prom1, Prominin 1	Nanduri, *et al.*, 2011
Laminin	---	Okumura, *et al.*, 2003; Matsumoto, *et al.,* 2007
AFP	Alpha fetoprotein	Matsumoto, *et al.,* 2007
CK18	KRT19, Keratin 19	Matsumoto, *et al.,* 2007
CK19	KRT18, Keratin 18	Matsumoto, *et al.,* 2007
CD24	HAS, Heat stable antigen	Nanduri, *et al.*, 2011; Nanduri, *et al.*, 2013, Xiao, *et al.,* 2014
MSI	Musashi 1	Lombaert, *et al.*, 2008
CD34	---	Bahn, *et al.*, 2011, Togarrati, *et al.* 2017
CD44	HCAM, Homing cell adhesion molecule	Bahn, *et al.*, 2011
Nestin	---	Bahn, *et al.*, 2011