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Salivary gland bioengineering - yesterday, today, tomorrow!

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Summary. Salivary glands are specialized structures developed as an extensively compact, arborized design through classical embryogenesis, accompanied by a cascade of events channelized by numerous growth factors and genetic regulatory pathways. Salivary secretions maintain oral homeostasis and, when diminished in certain conditions, present as xerostomia or salivary hypofunction, adversely impacting the patient's quality of life. The current available treatments primarily aim at tackling the immediate symptoms providing temporary relief to the patient. Despite scientific efforts to develop permanent and effective solutions to restore salivation, a significant permanent treatment is yet to be established. Tissue engineering has proven as a promising remedial tool in several diseases, as well as in xerostomia, and aims to restore partial loss of organ function. Recapitulating the physiological cellular microenvironment to *in vitro* culture conditions is constantly evolving. Replicating the dynamic multicellular interactions, genetic pathways, and cytomorphogenic forces, as displayed during salivary gland development have experienced considerable barriers. Through this review, we endeavour to provide an outlook on the evolution of *in vitro* salivary gland research, highlighting the key bioengineering advances and the challenges faced with the current therapeutic strategies for salivary hypofunction, with an insight into our team's scientific contributions.

Key words: Salivary gland, Xerostomia, Regeneration, Bioengineering, Gene therapy, 3D culture

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Introduction

A typical salivary gland (SG) parenchyma is composed of a compound, tubular-acinar, exocrine epithelium, enveloped in the rich connective tissue stroma, with the function to produce and secrete saliva into the oral cavity. The architectural development of the SG densely-packed secretory endpieces in a well-structured arrangement of luminal and abluminal cells can be assigned to physiological processes of 'branching morphogenesis' and 'epithelial-mesenchymal interactions', ultimately forming an extensive arborized pattern (Denny et al., 1997; Patel et al., 2006; Harunaga et al., 2011; Iyer et al., 2021). Saliva, the complex fluid secreted by the SGs, is vital for lubrication and homeostasis of the oral mucosa. Also, saliva is involved in routine functions such as taste perception, mastication, deglutition, and speech (Almansoori et al., 2020; Iyer et al., 2021). Prolonged depletion or salivary hypofunction has proven detrimental to the quality of life because these patients experience dry mouth, difficulty in swallowing, tooth decay, oral infections and taste loss; this malfunction is attributed to various intrinsic or extrinsic factors, such as aging, Sjogren's syndrome (SS), and post-surgical radiotherapy (Almansoori et al., 2020; Rocchi and Emmerson, 2020).

Advances in biophysics, biochemical, and bioengineering spheres are constantly striving towards the fabrication of the specialized salivary epithelium for insights into developmental modelling, disease progression, and restorative approaches (Ozdemir et al., 2016b; Almansoori et al., 2020; Rocchi and Emmerson, 2020). However, considerable barriers have been faced trying to replicate the dynamic cellular interactions, genetic regulatory pathways, as well as cyto-morphogenic and differentiation forces involved in innervation and vascularization, as displayed in native tissues. Through this review, we endeavour to provide an outlook on the evolution of *in vitro* SGs research, highlighting the key bioengineering advances and the challenges faced in current therapeutic strategies for salivary hypofunction.

Salivary glands

The human body comprises three major salivary glands, producing over 90% of whole saliva (~1.5 liters/day); parotid gland, submandibular gland, and sublingual gland, with an additional 800-1000 minor salivary glands in the oral cavity (Holmberg and Hoffman, 2014; Chibly et al., 2022). Although the parotid gland is of oral ectodermal origin, and the submandibular and sublingual glands are derivatives of the embryonic endoderm, the glandular development is initiated by a simple epithelial bud, altogether with cellular proliferation and sequential reciprocal tissue interactions (Nanci, 2012; Kumar, 2014; Holmberg and Hoffman, 2014; Iyer et al., 2021). This cumulative process is responsible for the development of a network of secretory endpieces, with secretory acinar (serous/ mucous) cells, ductal excretory system (intercalated, striated, excretory), and contractile basket cells (myoepithelial cells) (Nanci, 2012; Kumar, 2014; Holmberg and Hoffman, 2014; Iyer et al., 2021), as demonstrated in Fig. 1A,B.

The pyramidal serous cells, with central circular nuclei, produce protein-rich secretions that are reserved as numerous, discrete, secretory zymogen-rich granules, towards the apical cytoplasm. Mucous acini demonstrate a tubular configuration, with peripheral, compressed, basal nuclei and cytoplasm with carbohydrate-rich mucous granules occupying the apical two-thirds of each cell (Nanci, 2012). These acinar cells connect to adjacent cells via tight junctions (TJs). Functionally, the acini differ in secretion, with aqueous secretions high in amylase and ions, antimicrobial in nature, from serous acini to mucous cells secreting viscous mucin as an oral lubricant (Kumar, 2014).

The lumen of secretory endpieces continues into the ductal system, which delivers the acinar-derived secretions into the oral cavity, subsequent to ionic modifications in their composition as they pass through the ductal system. The intercalated ducts lined by a simple cuboidal epithelium with a central nuclei and scanty cytoplasm (Nanci, 2012; Kumar, 2014). The basal surface of the duct cells is bounded by myoepithelial cells, while the apical surface show minute microvilli projections, responsible for the movement of salivary secretions within the lumen, leading to the striated duct (Holmberg and Hoffman, 2014). The striated ducts are lined by columnar epithelium with a central nucleus and acidophilic cytoplasm, with deep infoldings/striations on the basal layer (Nanci, 2012; Kumar, 2014). These striations aid in the active transport of ions across the membrane, thereby modifying the composition of saliva as it enters the excretory duct (Nanci, 2012; Kumar, 2014). The excretory ducts are lined by pseudostratified columnar epithelium, with goblet cells and long microvilli towards the main excretory duct, to facilitate delivery of saliva into the oral cavity (Nanci, 2012; Kumar, 2014).

The acinar and intercalated ductal cells are enveloped by contractile, basket/myoepithelial cells via desmosomal attachments (Holmberg and Hoffman, 2014). The myoepithelial cells, although structurally resemble smooth muscle cells, are of epithelial origin. Those adjacent to the acini are stellate-shaped cells with numerous cytoplasmic processes and flattened nuclei, while the myoepithelial cells surrounding the

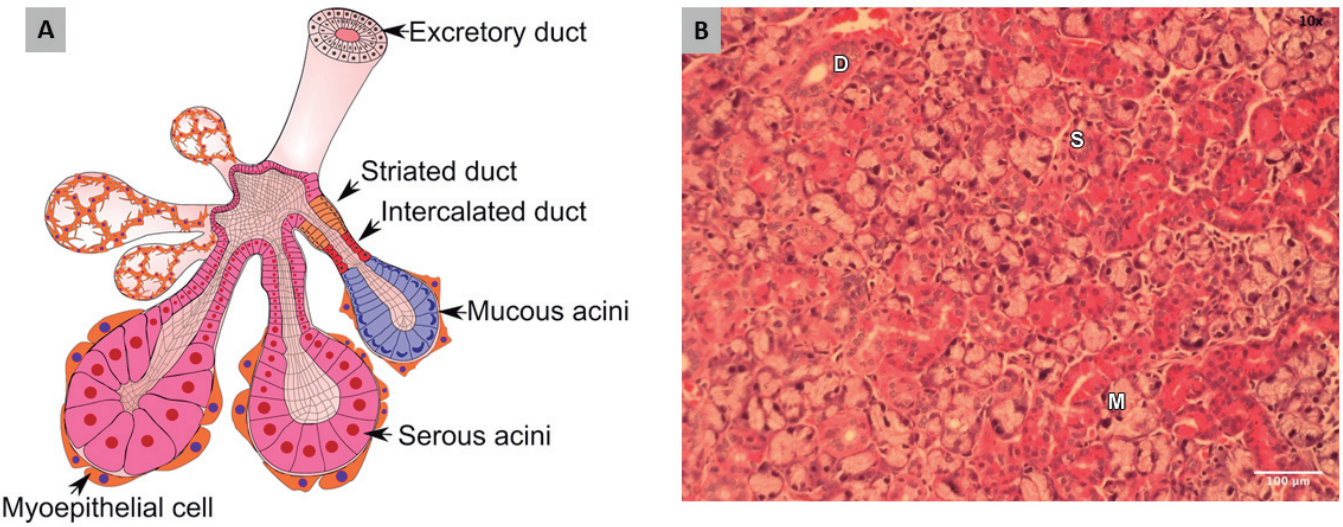

Fig. 1. A. Schematic representation of the histology of salivary glands. Compound, tubulo-acinar, exocrine epithelium, enveloped in a rich connective tissue stroma. The epithelial cells form a network of secretory endpieces, with secretory acinar (serous/ mucous) cells, ductal system (intercalated, striated, excretory), and contractile basket cells (myoepithelial cells) Figure adapted from (Barrows et al., 2020). **B.** Histopathological section of mixed salivary glands stained by routine hematoxylin and eosin, depicting serous acini (S), mucous acini (M), and duct (D). x 100.

intercalated ducts are elongated, fusiform in shape, with fewer processes, running parallel to the ductal system (Nanci, 2012; Kumar, 2014). The contractile action of the myoepithelial cells controls the salivary secretion by the discharge of the acinar secretory granules, which reduces the luminal volume, increases the salivary flow rate, and regulates luminal patency (Nanci, 2012; Kumar, 2014).

This complex parenchymal network is enveloped within a rich connective tissue stroma of collagen and reticular fibers, adipocytes, inflammatory cells, and neurovascular bundle providing sympathetic and parasympathetic nervous control of the salivary secretions in the glandular tissue. This well-established system of epithelium and mesenchyme produce, modify, and secrete saliva into the oral cavity in an orchestrated manner (Holmberg and Hoffman, 2014).

Need of the hour

The rate of salivary flow is considered adequate if unstimulated saliva is about 0.3-0.4 ml/min, with variation from 0.1 ml/min at sleep, to 4.0-5.0 ml/min when stimulated (Iorgulescu, 2009). The secretion of saliva is stimulated upon sight, smell, and taste of food; and is mediated by two integral steps. Initially, the acinar cells form and secrete isotonic primary saliva, chiefly composed of sodium chloride. Thereafter, the sodium and chloride ions get actively and passively reabsorbed, respectively. Further modifications through the ductal network give rise to a hypotonic salivary secretion in the oral cavity (Alhajj, 2020). Dysfunctional glandular architecture or innervation leads to SG pathologies (Talha, 2022).

Xerostomia, or dry mouth, is a commonly reported symptom caused due to reduced or absent salivary flow, usually under 0.1 ml/min of unstimulated saliva or 0.7 ml/min under stimulation (Kapourani et al., 2022). Xerostomia and its associated repercussions, such as dental caries, periodontitis, hypogeusia, dysphagia, oral ulcerations, and infections, lead to poor quality of life (Vivino et al., 2019). It is usually caused by inadequate/altered function of the SGs, by various local or systemic conditions. Local causes may include certain medications, radiation therapy for head and neck malignancies, and consumption of addictive agents such as tobacco and alcohol (Kapourani et al., 2022). Certain autoimmune conditions such as SS, rheumatoid arthritis, endocrine disorders like diabetes mellitus, and infectious diseases (tuberculosis and hepatitis, for instance) are also recognized factors for xerostomia (Kapourani et al., 2022). Despite various scientific advancements, a significant and satisfactory treatment for xerostomia is yet to be established (Jensen et al., 2010; Lim et al., 2013). Recent literature suggests the clinical application of regenerative medicine as a promising and powerful tool in the management of xerostomia associated with oropharyngeal cancer, irradiation injury, and autoimmune disorders.

Regeneration of salivary glands in the laboratory

Cell culture as a tool in studying SG pathophysiology

As previously described, the human SGs are made of three major cell types, the secretory acinar cells, the ductal cells lining the SG ducts, and the myoepithelial cells that contract the acinar cells in response to parasympathetic and sympathetic stimulation to secrete saliva (Brazen and Dyer, 2019; Harrison, 2021). Human SG cell culture techniques play a crucial role in our understanding of the fundamental biological characteristics of SG cells under *in vitro* conditions. In the 1980s, scientists began studying SG pathophysiology by applying standard cell culture techniques using the then-available SG cancer cell lines (Shirasuna et al., 1981; Kurth et al., 1989; O'Connell et al., 1998). Since then, over the past few decades, we have come a long way in developing a deeper understanding of the essential characteristics of SG cells in *in vitro* conditions. In this section, we will discuss the three major components necessary for a successful SG cell culture. Figure 2 illustrated the 3 major components for SG culture (Fig. 2A) and the SG cell organization within the different types of platforms (Fig. 2B-D).

At this juncture, a few vital definitions should be explained. Cell culture is a crucial and indispensable process in basic and applied sciences, including cancer research, drug discovery, and stem cell study to elucidate biological behavior, under controlled conditions while mimicking native environments (Jensen and Teng, 2020). Two-dimensional (2D) cultures are the oldest and most commonly encountered technique in biological research to propagate and assay cells (Ferreira et al., 2018), but with limitations due to the inaccurate representation of cell-cell and cell-extracellular matrix (ECM) interactions that are present in the native three-dimensional (3D) architecture of tissues (Jensen and Teng, 2020).

The 2D cultures allow the proliferation of the cells as a monolayer on rigid surfaces (plastic or glass) (Fig. 2B). Some modifications to the plate surface (low attachment), the addition of chemicals, or physical confinement on 2D culture systems can promote cell aggregations (Song et al., 2014; Jiang et al., 2017; Saglam-Metiner et al., 2019). The 2.5D cultures exploit the advantage of a bioactive molecules-coated surface (ECM or structural proteins such as collagen) to seed the cells, where they can be stimulated to form both monolayer and spheroid growth on the surface, exhibiting partial characteristic of the biological entities growing in 3D environments (Fig. 2C) (Smithmyer et al., 2019; Abugomaa et al., 2020; Zhang et al., 2020).

The 3D cultures can be classified as a scaffold-free and scaffold-based systems. Scaffold-free techniques rely on using a non-bioactive surface coating (agarose or agar) where cells reorganize as clumps or aggregates (Liu and Chen, 2018; Chaicharoenaudomrung et al., 2019). On the other hand, scaffolds can be created and

incorporated into the culture prior to cell seeding that enables the cells to attach to the 3D structure, or cells are embedded into soft materials (e.g., hydrogels), and 3D cell-laden structures can be designed to allow cells to proliferate, migrate, and reorganize as organoids/spheroids (Fig. 2D). The latest system is considered more accurate in mimicking a native 3D cellular environment (Jiang et al., 2017, 2019b; Catoira et al., 2019; Munguia-Lopez et al., 2022).

Components of SG culture

Cells. Isolation and identification of the right cell type are primordial for establishing a successful SG cell culture platform. Each SG cell type hosts innate characteristics which define its role in a fully functioning SG structure. The acinar cells form secretory units and serve as the principal component of the SGs and produce saliva that is transported through an arborised ductal tree finally opening into the oral cavity. Fully differentiated acinar cells maintain homeostasis within the SGs via self-duplication (Aure et al., 2015). These epithelial cells are characterized *in vitro* by the presence of water porin channels like aquaporin-5, TJ proteins like zona-occludens-1 and the baso-lateral membrane co-transporter, the NKCC-1 (sodium, potassium, and chlorine pump), and can be serous (marked by CD44) or mucous (marked by CD166) in nature (Tran et al., 2005;

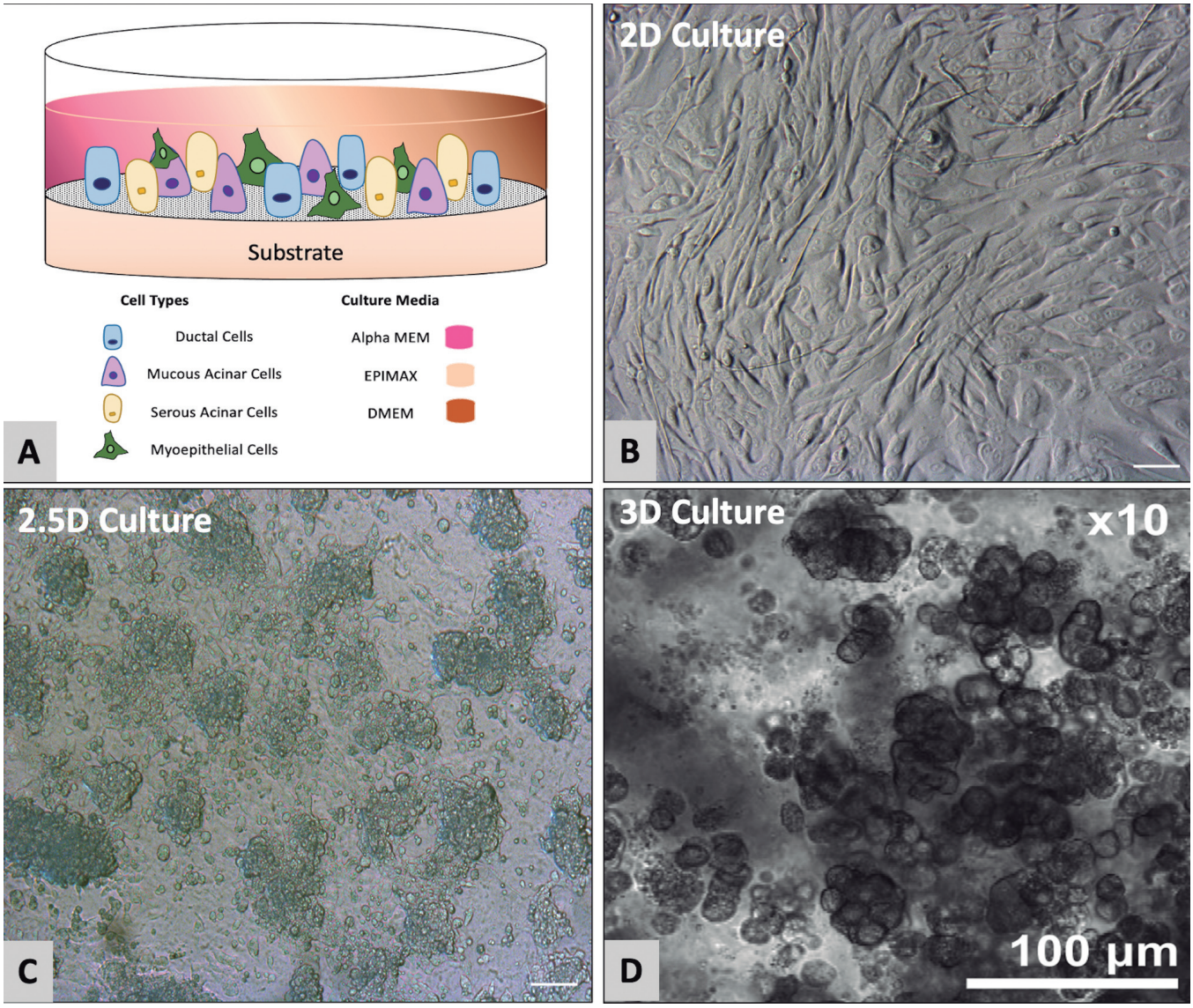

Fig. 2. *In vitro* salivary gland cell culture systems. **A.** Schematic illustration of different types of SG cells cultured on top of a substrate with the commonly used culture mediums. **B.** 2D culture of primary SG single cells cultured on tissue culture plastic and growing as a monolayer. **C.** 2.5D culture of SG cells on top of alginate-egg white (3% alginate) hydrogel-forming spheroids. **D.** 3D culture of primary SG cells in alginate-gelatin-hyaluronic acid hydrogels displaying organoid formation. B, x 100; C, x 5; D, x 10.

Maria et al., 2012).

It is well known that maintaining acinar cell characteristics *in vitro* is challenging, but several studies have provided clues on culture conditions that promote the maintenance of the acinar phenotype (Hiraki et al., 2002; Jang et al., 2015). The ductal cells are the major cell type in the epithelial compartment forming the branched ductal system of SGs, and they play a vital role in maintaining the composition and transportation of saliva. They are characterized *in vitro* by the presence of epithelial surface markers like E-cadherins and TJ proteins like occludens, claudins, and progenitor markers like Keratin 14 (K14), Keratin 5(K5) (Rocchi et al., 2021). The myoepithelial cells are the contractile cells that surround mostly the acinar cells and some ductal cells and cause the saliva secretion from acinar cells and expulsion via ducts in response to neural stimulation. Naturally, owing to their function, the myoepithelial cells are present in low numbers as compared to the acinar and ductal cells and are characterized chiefly by the presence of alpha-smooth muscle actin (α-SMA) protein.

Human primary SG cells can be obtained by either dissociating the major SG tissues using digestive enzymes or by culturing them as explant tissues. We recently published a detailed protocol for the isolation, culture, and characterization of primary SG cells using these methods from human and mice tissues (Su et al., 2022a). In addition to the three major cell types, adult human SGs also comprise of progenitor/stem cells that help maintain cell turnover and homeostasis in response to any injury. For the acinar proliferative unit, these lineage-restricted progenitor/stem cells are characterized by the presence of SOX-2, Mist-1, and PIP markers (Aure et al., 2015; Maruyama et al., 2016; Emmerson et al., 2018) and by c-kit, K14 and K5 for the ductal unit (May et al., 2018).

The epithelial compartment along with the stem cell niche constitute the most representative cell types required to adequately define the native SG *in vitro*. Again, individual SG cells, when cultured *in vitro*, show only limited features in terms of key markers with limited to no secretion. To aide this challenge, several research groups, including ours, have tried to utilize cell clusters to obtain part of acinar and ductal units along with myoepithelial cells to replicate SG function *in vitro* (Shubin et al., 2015; Seo et al., 2019; Song et al., 2021). While these units, when cultured in bioactive scaffolds, are currently the advanced strategies used for SG *in vitro* studies, they still lack several components of the SG niche, such as the progenitor/stem cells along with neurovascular signaling.

While cell lines do not accurately represent the SG cell functions, the lack of stable human cell lines has impeded the pace at which novel *in vitro* models are developed to study SG pathophysiology. Our lab has been working on developing SG cell lines from patient-derived tissues that will serve as essential tools in SG research. In a nutshell, a combination of both novel cell lines representing different adult cells and primary cells either as purified populations or clusters, along with the progenitor components, will act as key cellular components for developing SG models *in vitro*.

Culture medium and soluble factors. A vital component of any cell culture model is an appropriate culture medium specific to the cell type. For SG culture, several commercial and defined culture media have been discussed by different research groups based on their lab-developed protocols. Usually, commercially available media such as Dulbecco's Modified Eagle Medium (DMEM) with Ham's F12 nutrient mixture, HepatoSTIM, Keratinocyte Growth Medium 2 (KGM-2) with added growth factors, and antibiotics are used commonly to culture SG cells (Piraino et al., 2021). The use of serum, such as fetal bovine serum (FBS), along with other growth supporting factors, has also been controversial in growing SG cells, owing to their effect on cell phenotype and the batch-to-batch FBS variability.

Our lab routinely uses the EPIMAX serum-free culture medium to culture primary human and mice SG epithelial cells (Su et al., 2022a). We have noted that supplementing the EPIMAX with 10% FBS significantly increases the proliferation potential in SG cell lines. While primary cells maintain a characteristic cobblestone phenotype with this medium, due to their sensitive nature, it is seen to be beneficial to supplement the medium with 5-10% FBS when passaging them. Especially, in SG single cell culture, this approach promotes the initial attachment of the cell to tissue culture plastic, and the medium can then be replaced with serum-free conditions to allow their proliferation (Su et al., 2022a).

Several growth factors and inhibitors have been seen to profoundly affect SG cells in culture. For examples, fibroblast growth factor (FGF) (Lombaert et al., 2013) and epidermal growth factor (EGF) (Knox et al., 2010) have been frequently used in culturing SG cells, especially primary cells and tissues (Piraino et al., 2021). Specifically, FGF 10 has been shown to improve acinar and pro-acinar cell markers such as AQP-5, α-amylase, and Mist -1 (Sui et al., 2020). Some other factors have also demonstrated the ability to maintain SG cell characteristics in rodent cells, such as the insulin growth factor (IGF-1), which is seen to promote epithelial cell barrier-like functions in rat submandibular cells; however, this has not been well studied in human SG cultures.

Inhibitors like Rho-Kinases (ROCK), EGF receptor (EGFR), and transforming growth factorβ receptors (TGFβR) have been studied for their role in SG cell culture and maintenance. While several studies validate the effect of ROCK inhibitors in embryonic and rodent SG cell maintenance, a recent study by Koslow et al., 2019 demonstrated that ROCK inhibitors promote acinar and ductal progenitor markers like c-kit and Mist-1 and K5 (Koslow et al., 2019). Many EGFR and TGFR have been tested to understand their role in SG culture,

especially in rodent cells (Janebodin et al., 2013) and organoids (Hosseini et al., 2018) but less defined in adult human SG cells. So far, the factors that have been identified to play an essential role in SG culture via promoting epithelial and progenitor cell markers are mainly through studies on rodent and embryonic cells. The synergistic use of these growth factors and inhibitors can create optimal growth conditions for different human SG cell types while maintaining the progenitor populations.

Biomaterials, substrates, and matrices. It is well established now that SG culture *in vitro* is influenced mainly by the presence of appropriate ECM components. Native SGs, during development, require ECM components such as collagens I and IV, laminin, heparan sulfate proteoglycans, nidogens, and fibronectin at different stages of morphogenesis (Porcheri and Mitsiadis, 2019). In the early days of *in vitro* SG culture, researchers primarily used polystyrene tissue culture plates to grow SG cells, especially cell lines, to study cell characteristics. This was succeeded by the use of matrix protein-coated substrates to evaluate the growth and morphological characteristic of SG epithelial cells.

Aframian et al. (2000) examined Poly-L-lactic acid (PLLA), Polyglycolic acid (PGA), or their combination, Poly lactic-co-glycolic acid (PLGA) either as substrates or coated with ECM proteins like fibronectin, laminin, collagen I and IV and gelatin to study cell characteristics (Aframian et al., 2000). These substrate coated experiments provided important guidelines on the capability of a particular polymer and the corresponding ECM combination to allow the successful generation of SG monolayers of adult and embryonic SG tissue culture.

Another approach was the use of inserts with semi-permeable membranes to culture SG cells. Tran et al., 2005 first evidenced the use of transwell inserts to culture primary human SG epithelial cells as monolayers with the ability to polarize and maintain the epithelial barrier, express TJ proteins, and allow fluid movement between the two compartments (Tran et al., 2005). Given the success in culturing SG cells in 2D, researchers further introspecting the role of ECM in SG cell phenotype and function and began using natural matrices to culture SG cells in 2.5D and 3D systems (Joraku et al., 2007).

Pradhan et al. (2009) exhibited that perlecan domain IV peptide and Matrigel trigger the differentiation of SG cells into 3D acini-like structures with the expression of the acinar markers AQP5 and amylase (Pradhan et al., 2009). They further demonstrated that the long-term culture of SG cells in these 3D matrices allowed the development of a necrotic core in the center of acini, demonstrating a lumen formation (Pradhan et al., 2010). Our laboratory evaluated the growth of primary human cells and cell lines in Matrigel demonstrating that SG cells under low Matrigel concentrations organize into polarized 3D acinar units expressing TJs proteins like claudins, occludins, and acinar-specific markers like AQP5 and amylase (Maria et al., 2011a,b).

Currently, other natural matrices such as chitosan, laminin, and collagen have been used to culture SG cells as organoids in combination with soluble cues like fibroblast growth factor (FGFs) (Hosseini et al., 2018; Lee et al., 2018). Hyaluronic acid (HA) based 3D hydrogels have been shown to produce implantable SG spheroids with the ability to regulate fluid secretion in response to neuroreceptor stimulations (Pradhan-Bhatt et al., 2013). Recently, our laboratory has also displayed the potential of egg yolk, egg white, and egg white in combination with alginate as suitable candidates that promote the formation of SG spheroid-like structures in 2.5D cultures (Charbonneau et al., 2019; Zhang et al., 2020; Pham et al., 2021). However, owing to the limited mechanical tunability of natural hydrogels, and batch-to-batch variability in their production, synthetic polymers with tunable mechanical properties such as PLGA, Polyethylene Glycol (PEG), either used alone or in combination with natural substances such as elastin have been well assessed for SG 3D cell culture (Sequeira et al., 2012; Shubin et al., 2015). Electrospun elastin-PLGA nanofiber scaffolds were shown to promote the self-organization of SG cells with apicobasal polarization (Foraida et al., 2017). Matrix metallo-proteases (MMP) degradable- PEGs have revealed the ability to express SG TJ associated proteins like zona-occludens-1 and baso-lateral membrane ion co-transporter NKCC-1 (Shubin, 2017). More recently, Song et al., 2021, used the same hydrogel in combination with a microbubble arrays platform to maintain the SG acinar niche and allow their long-term culture for high content screening (Song et al., 2021). Nevertheless, despite all the success and improvements in SG *in vitro* culture techniques spanning over two decades, there is still a need to develop a tailored biomaterial that can support all the different components of the SG niche, to attain a fully functional artificial SG.

SG organoids as a tool in studying SG pathophysiology

As previously categorized, 3D cultures have evolved to be either scaffold-free or scaffold-based systems, both enabling the proliferation and reorganization of cellular aggregates and spheroids/organoids, with an attempt at simulating the native cellular milieu.

Knowing the different available alternatives to produce cell spheroids/organoids deems it necessary to define the cell organization in 3D culture. Based on the complexity of the 3D cellular structure formed, they can be classified as tumoroid, spheroid, multicellular spheroid (MSC or MCTS, the last one is associated with cancer cell lines), and organoids. Tumoroids are referred to as the use of tumor cells derived from patients assembled as spherical 3D structures (Tatullo et al., 2020). Spheroids are cell aggregates mainly created in a

scaffold-free environment, made from a single cell type. MCS or MSCT are spheroids generated from multiple cell linages, including healthy and cancer cell lines, or a combination of both (Kang et al., 2021). Typically, an organoid is a small tissue fragment, generally epithelial in nature, separated mechanically or enzymatically from the stroma and cultured *in vitro* to mirror its corresponding *in vivo* organ (Simian and Bissell, 2017; de Souza, 2018) in a 3D multicellular organization. Nowadays, the term "organoid" is most commonly used to describe such a 3D construct that is derived from either pluripotent stem cells (embryonic or induced) or adult stem cells obtained from various organs (de Souza, 2018).

The inception of organoid culture dates to the early 1900s, when researchers aimed at recapitulating organogenesis by culturing tissue fragments using the hanging drop tissue technique. The outcome of this research led to the develop of the current methods used to produce organoids (Simian and Bissell, 2017). To our knowledge, the first attempts to isolate and culture SG cells *in vitro* date back to 1975, when the submandibular gland of 4-week-old rats was dissociated by enzymatic and mechanical procedures and cultured over 36 hours before the viability dropped up to 40%. However, the ultrastructural characteristics of acinar, intercalated, and striated duct cells were still well maintained (Kanamura and Barka, 1975). Subsequently, in 1987, rat acinar cells from exorbital lacrimal glands, parotid gland, and pancreas were isolated and cultured on a reconstituted basement membrane (BM) gel matrix. Cells mixed within hydrogel showed morphological changes compared with BM-coated plates, allowing the cells to reorganize as aggregates or tubular appearance as they attached to the surface of the gel. This technique of 3D culture allowed the maintenance of the differentiated acinar state *in vitro* (Oliver et al., 1987).

The organoid culture of SG started in the early 1990s, using rats and mice as biopsy donors. Using the transfilter technique and Matrigel® as the coating material, Takahashi and Nogawa, 1991, were able to recapitulate the SG branching morphogenesis of mice *in vitro*. First, they separated the epithelium and mesenchymal tissues; then, the epithelium was incubated with Matrigel, separated from the mesenchyme by the filter. After three days of culture, the epithelium showed extensive growth and typical branching morphogenesis, with a remarkable differentiation of lobular and stalk regions (Takahashi and Nogawa, 1991). Laminin-1 (LN1-nidogen) also has been used in the formation of 3D environments to promote SG morphogenesis. When mice submandibular epithelial cells were seeded on LN1-nidogen gel using a transwell in mesenchyme-free conditions, the epithelium demonstrated branching comparable to that observed with Matrigel, suggesting that LN1-nidogen supported the branching morphogenesis of submandibular epithelium (Hosokawa et al., 1999).

Organoids in modified 2D surface and suspension cultures

Two-dimensional, 2.5D, and suspension culture systems have been routinely used as a tool to produce 3D SG structures *in vitro*, but with heterogenous spheroid size and morphology (Almansoori et al., 2020). In low-attachment surfaces, coated surfaces, or suspension culture, cells tend to aggregate and proliferate, creating a cell-cell cross-talking while maintaining some of its biological functions, such as the expression of key proteins (Chen et al., 2009). However, the cell-ECM interactions do not resemble the native tissue, which is essential to perform accurate biological functions (Białkowska et al., 2020). Besides, most of the spheroids produced are formed from one cell type, limiting the complex architecture of a native tissue that typically encompasses more than two cell types. Alternatively, partial digestion of the SG tissue has been successfully applied to produce functional SG organoid in a suspension culture system, where the salivary spheroids expressed cell polarization, acinar cell markers, and tight junction proteins, increasing its size and proliferation (Seo et al., 2019; Almansoori et al., 2020).

Organoids in 3D platforms: embedding cells in hydrogels

Since the domain of 3D SG organoid culture is extensive, depending on the platform, we restrict this subsection to the cell-laden biomaterial (hydrogel-based embedded cells) as 3D platform for organoid/spheroid formation. The inception of SG organoids could be considered since the end of early 1980 when basement membrane proteins (i.e., Matrigel®) or ECM-derivatives (such as collagen) became more popular in recapitulating the native tissue microenvironment. Matrigel® has been globally considered as the "gold standard" material for producing organoids; however, the batch-wise variability in mechanical and biological composition, and poor mechanical properties of the formed gel, drove scientific communities to explore better alternatives (Oliver et al., 1987; Fujita et al., 1999; Aisenbrey and Murphy, 2020; Zhang et al., 2020).

Hydrogels are attractive biomaterials due to the tunability on mimicking the mechanical, physico-chemical, and biological properties of human tissue (Jiang et al., 2019a,b; Mantha et al., 2019). As described, hydrogels are defined as polymers with the capability of holding a large amount of water inside of their 3D structure, which can be formed by physical or chemical stimuli (Buwalda et al., 2014; Bashir et al., 2020). Several synthetic and natural polymers are used as precursors to form 3D environments suitable for SG organoid formation and morphogenesis. Alginate, fibronectin, laminin, collagen, and HA, among others, are currently used to produce functional SG organoids *in*

vitro. Primary salivary human stem/progenitor cells (hS/PCs) were encapsulated in a HA hydrogel containing hydrolytically degradable moieties and adaptable disulfide linkages. After three days of culture, cells reorganized as a well-formed, viable multicellular spheroids, increasing their size through time. These 3D SG structures expressed basement membrane components (Collagen IV and laminin), cell-cell junction proteins (occludins), and stem/progenitor markers (K5, and K14) (Ozdemir et al., 2016a).

Therapeutics in SG research

Gene therapy

Gene therapy refers to the insertion of a normal gene to replace an altered or mutated gene using a vector to restore and allow the proper functioning of the gene. Therapeutic use of gene therapy began in the 1980s with a focus on clinical disorders, cancer, and congenital genetic disorders (Anderson, 1984; Rosenberg et al., 1990; Whitsett et al., 1992). The first evidence of gene therapy in SG research was by Mastrangeli et al., in 1994, where they successfully expressed recombinant adenovirus vectors mediated gene in the SG cell lines, which was delivered into rat SGs via retrograde injection, and further into isolated human minor SGs transplanted into mice (Mastrangeli et al., 1994). Since then, several researchers have studied the advantages of using gene therapy to alleviate SG disorders mainly the SG hypofunction due to radiotherapy and SS (Samuni and Baum, 2011). However, most of these proof-of-concept studies were performed on rodent models, which were slowly translated to larger animal models like rhesus monkeys and minipigs (Delporte et al., 1997; He et al., 1998; Shan et al., 2005; Voutetakis et al., 2008). The results from these studies established that SGs are well-encapsulated organs and provide easy access to epithelial cells that are slow dividing and stable targets and thus allow better integration of the vectors in situ (Baum et al., 2015). It was noted that there is a slow translation of these findings to human studies due to a lack of basic understanding in SG physiology with respect the gland secretions and the limited number of investigators working on SG gene therapy (Baum et al., 2015).

The first ever gene therapy performed for human SG regeneration was done by Prof. Baum nearly a decade ago (Baum et al., 2012). This pioneering phase 1 clinical study was based on the positive results on efficacy, distribution, and toxicity of the serotype 5, adenoviral (Ad5) vector (Adh) mediated transfer of human Aquaporin-1 (hAQP1) cDNA in patients undergoing radiotherapy for head and neck cancers (Delporte et al., 1997; Zheng et al., 2006). The study showed three major findings, i) the safety of Ad5 vector in delivering genes to human parotid gland with minimal adverse effects ii) the efficacy of hAQP1gene in restoring parotid gland salivary flow in more than 50% of the subjects (6/11) treated, and iii) positive salivary flow rate observed in the subjects for extended periods which was different from the preclinical *in vivo* experiments (Baum et al., 2012).

Although the study was isolated and had limited participants, it was crucial in establishing the significance of using gene therapy for SG disorders. After the trial, to improve the clinical success of gene therapy in humans and reduce possible immunogenic responses to Ad5-like vectors were studied. Momot et al. in 2014 tested the use of a serotype 2 adeno-associated viral vector (AAV2) in mice to deliver the hAQP1 gene and study the biodistribution and toxicity of the approach. This *in vivo* study showed that the AAV2 vector had limited immune response and toxicity and was well tolerated (Momot et al., 2014).

More recently, in 2017, Baum and colleagues evaluated the late responses of Ad5 mediated hAPQ1 cDNA in the same patients who were treated in 2012 for radiation-induced SG hypofunction (Alevizos et al., 2017). Over the span of 3-4.7 years after the first gene delivery, they examined the salivary flow rates, composition, presence of vectors in the treated glands, clinical tests, and adverse effects. The results showed a marked increase compared to baseline for all parameters with limited adverse effects. The Ad5 vector generally leads to only transient expression of gene with peak values during the first 72 hours and sustaining for ~ 2 weeks. However, this pattern was previously established only in animal models and not in humans (Wang et al., 2000; Li et al., 2004; Voutetakis et al., 2008).

In the only human SG gene therapy using Ad5 vector, the results were quite the opposite. In the first clinical trial, while the peak elevation seen in SG functional parameters occurred for 7-42 days, the effect of the gene therapy surprisingly lasted for ~3-5 years after the initial administration. Moreover, in addition to clinical improvement, results showed that the major cell type expressing the hAQP1 protein was the acinar cells, which do not express AQP1, followed by the myoepithelial and vascular endothelial cells, which do.

In relation, Zheng et al., in 2015 tested the expression of hAQP1 in human and mice SG cells to study the transgene expression. They found that, unlike humans, in rodent SG cell lines, the CMV (cytomegalovirus) promoter gets methylated over time and leads to decreasing expression of AQP1 gene (Zheng et al., 2015). These results point towards and confirm the innate nature of SGs to be an ideal recipient for gene therapy to treat SG hypofunction due to irradiation. The current opinion and recent advances in SG gene delivery has been discussed in detail elsewhere (Baum et al., 2015).

Bioengineering

Baum and colleagues proposed three major research directions for SG regeneration in the year 1999: i) repairing the hypofunctional SG; ii) redesigning the

secretory function; and iii) developing an artificial SG (Baum et al., 1999; Hajiabbas et al., 2022). Among all the efforts that have been made since then, developing an artificial SG via SG tissue engineering aims to provide a permanent solution to SG hypofunction and has achieved substantial progress over the years.

Tissue engineering is a biomedical engineering discipline aiming to replace lost or severely damaged tissues or organs, requiring the amalgamated knowledge and techniques of cell biology, material science, chemistry, molecular biology, engineering, and medicine (Sharma et al., 2019). SG tissue engineering requires three essential components: (1) cell-cell contacts; (2) cell contacts with ECM proteins, and (3) a biocompatible and biodegradable 3D scaffold that can hold these components together (Aframian and Palmon, 2008; Abdulghani and Mitchell, 2019).

Many scaffolds have been proposed, which are porous, and either biologic (e.g., collagen, fibrin, silk, chitosan, alginate, HA) in origin or synthetic biocompatible biomaterials (e.g., poly-glycolic acid, poly-lactic acid, poly lactic-co-glycolic acid (PLGA), and polyethylene glycol), and/or a mixture of both. Depending on their biodegradability, porosity, stiffness, and strength, scaffolds promote cell adhesion, migration, and/or differentiation (Peters et al., 2014). Ideally, engineered scaffolds should structurally and functionally resemble the native SG ECM architecture.

It is essential to first understand the *in vivo* SG extracellular microenvironment before deciding the appropriate scaffold for 3D *in vitro* cell culture. This would help us to provide cues and signals that mimic the morphogenesis in a native SG. There exists a basement membrane (~100 nm thick) beneath the epithelial layer in the acinus or in the duct, mainly composed of collagen IV, laminin, nidogen, and the proteoglycan perlecan/HSPG2 (Ozdemir et al., 2016b). Due to the presence of integrin heterodimers on their basal membrane, epithelial cells build junctions with the basement membrane. In a polarized epithelial cell, the basal membrane contains neurotransmitter receptors and ion channels, while the apical membrane contains aquaporins and mucins. The junctional complexes, including E-cadherin and zonula occludens are located on the cell membrane and near the apex of the lateral membrane respectively (Holmberg and Hoffman, 2014; Saito, 2021). Myoepithelial cells are found lying between the basement membrane and the acini that isolating them from the surrounding stroma (Chitturi et al., 2015). A polarized structure with an orientational secretory function is attribute to the tight regulation of ECM composition and cell-cell interactions (Ozdemir et al., 2016b).

Thus far, various cell types and techniques have been used to effectively generate 3D SG organoids. The study presented by Shin. et al., 2018, introduced a novel bioengineering technique for effectively driving 3D organoids organization using adult tissue stem cells via niche-independent 3D microwell culture (Shin et al., 2018). The nanoscaffold microwell platform was fabricated by photopatterning PEG hydrogel in the presence of an electrospun polycaprolactone nanofibrous scaffold. Human single clonal salivary stem cells (SGSCs) were preconditioned to aggregate and form 3D spheroids in different matrices (Matrigel®, floating dish, or microwells) prior to the induction of 3D organization. The authors found that salivary stem cell markers (LGR5, THY1, ITGB1, HAS, and KRT5) and pluripotency markers (POU5F1, SOX2, and NANOG) expression was more remarkable in 3D spheroid cultures than in 2D plastic culture. The microwell system showed higher levels of acinar, ductal, and tight junction markers levels compared with other 3D cultures, while a decrement on stem-cell marker levels was observed (Shin et al., 2018).

To reach the goal of regenerating a functional SG *in vitro*, we need more than just epithelial acini. Several works have been trying to rebuild a ductal network or reconstruct myoepithelial cells surrounding artificial acini by manipulating the cellular microenvironments. Pradhan and colleagues isolated primary human salivary myoepithelial cells and stem/progenitor cells from normal SG tissues and used these cells to develop a bottom-up approach for generating SG microtissues (Ozdemir et al., 2017). Isolated human stem/progenitor cells growing in 3D modular hyaluronate-based hydrogel system successfully formed spheroids. The myo-epithelial cells "wrapped" the spheroids and were responsive to neuronal signals (Srinivasan et al., 2017). Therefore, a functional organoid model including myoepithelial and secretory acinar cells could be established, which is necessary for understanding the coordinated action of these two cell types in unidirectional fluid secretion (Ozdemir et al., 2017; Srinivasan et al., 2017).

Though there is limited research working on the vascularisation of SG regeneration, progress has been made in other tissue regeneration, which might be able to spark SG *in vitro* vascularisation research. For example, it is reported that the vasculature of human umbilical venous endothelial cells and human dermal lymphatic endothelial cells have been successfully produced by a 3D multi-layered culture of normal human dermal fibroblasts. The 3D vasculature ultrastructure fabricated by ECM-nanofilm-based scaffolds and the cultured fibroblasts and enriched ECM support the vasculatures by acting as connective tissues (Asano et al., 2014). It brought us an idea for further SG regeneration research that the vasculature could be induced by co-culturing SG cells, venous endothelial cells, and dermal fibroblasts in a 3D manner.

Another study reported that FGF7 and FGF10 are needed for branching epithelial morphogenesis (Steinberg et al., 2005; Shubin et al., 2015). Furthermore, progress has been made on acinar polarity and the reconstruction of acini lumen structure *in vitro*. The acinar polarity could be triggered by Matrigel®, which contains basement membrane proteins such as

laminin, collagen type IV, perlecan, and nidogen (Maria, et al., 2011b). While some other hydrogels contain some components of the basement membrane ECM, which could trigger the expression of one or more tight junction proteins, and thus have a similar ability to support acinar polarity (Pradhan et al., 2010; Nam et al., 2019). In addition, one group also found acinar lumens with the expression of AQP5 formed in HA-based hydrogels containing a peptide from perlecan domain IV (Pradhan et al., 2009, 2010).

Transplantation

Progenitor and stem cells exhibit prominent characteristics of proliferation and differentiation, with an added attribute of stem cell self-renewal. Autologous transplantation of SG progenitor cells organized as organoids/salispheres displays significant positivity for c-kit, Sca-1, and CD117 markers, suggesting effective salivary restoration in mice models (Kwak et al., 2018; Urkasemsin and Ferreira, 2019). Advances in 3D organoid and bioprinting methods have enabled Ferreira et al., to development a novel scaffold-free system such as magnetic 3D levitation (Ferreira et al., 2019). This system levitated magnetized SG organoids, thus mimicking the physiological production of 3D ECM in spheres. These functional mini-SGs replicated primary cellular characteristics of a functional SG, with significant expression of NKCC1, M3 receptors, E-Cadherin, K-14, α-SMA, and β3-tubulin, including α-amylase and intracellular calcium activity. Although research in humans is limited, mice studies have demonstrated efficient uptake of isolated submandibular cells when transplanted in irradiated mice (Ferreira et al., 2019).

Transplantation of non-epithelial, multipotent, mesenchymal stem cells from bone marrow, adipose tissues, peripheral blood, placental tissue, dental pulp, and labial mucosa has shown promising results as cell therapy candidates for both *in vivo* and *ex vivo* studies (Kosinski et al., 2020; Chansaenroj et al., 2021; Hajiabbas et al., 2022). Few research groups, including our group, have successfully tested the therapeutic effects of bone marrow-derived stem cells and peripheral blood-derived stem cells in non-obese diabetic (NOD) mice presenting with SS-like disease (Khalili et al., 2010; Tran et al., 2011a; Elghanam et al., 2017). Khalili et al. (2010), concluded that injectable Complete Freund's Adjuvant and MHC Class 1-matched bone marrow cells exhibited salivary regeneration in NOD mice. (Khalili et al., 2010). Tran et al. delved deeper into the pathophysiology of irradiation- and SS-induced xerostomia and explored the possible combination of transdifferentiation, vasculogenesis and paracrine effects of bone marrow-derived cell transplantation, as an adjunct to alleviate dry mouth (Tran et al., 2003, 2007, 2011b; Sumita et al., 2011).

Transplantation of adipose tissue-derived mesenchymal stem cells has demonstrated tissue regeneration, anti-inflammatory and immune modulatory action similar to that of bone marrow-derived stem cells (Grønhøj et al., 2018). Grønhøj and co-workers confirmed promising efficacy of adipose tissue-derived mesenchymal stem cells in alleviating xerostomia, post radiation in patients with oropharyngeal squamous cell carcinoma, through a randomized, placebo-controlled trial (Grønhøj et al., 2018). They measured a significant increase (33% at one month; $p=0.04$, and 50% at four months; $p=0.003$) in the unstimulated whole salivary flow rate, in the experimental group, when compared to the control group ($p=0.6$ at one month, and $p=0.8$ at four months). As secondary outcomes of their trial, the researchers also observed changes in the patient-reported outcome measures, flow-rate induced changes in the composition of inorganic saliva components, changes in the stimulated whole salivary flow rate, changes in unstimulated and stimulated submandibular salivary flow rates, and changes in submandibular gland morphology, based on contrast-induced magnetic resonance imaging and core-needle tissue samples, at one and four months after the administration of adipose cells (Grønhøj et al., 2018).

Cell-free therapies have recently overcome challenges associated with cell-based therapies, such as invasive surgery required for substantial tissue and problems associated with cell delivery, immunogenicity, and storage (Su et al., 2020). Our laboratory has produced considerable results to suggest the efficacy of cell-free therapies in treating salivary hypofunction in irradiated mice SGs (Fang et al., 2015; Abughanam et al., 2019; Su et al., 2020). We recently developed a minimally invasive method to isolate and expand labial stem cells that proved significant results in mitigating xerostomia in as a therapy for irradiated mice (Su et al., 2020). Bone marrow-derived stem cell extract or soup, when tested in NOD mice, upregulated the mRNA expressions of AQP5, EGF, FGF, BMP-7, and Interleukin-10, with improved salivary as well as tear flow in mice with SS-like disease (Abughanam et al., 2019). The proteins in bone marrow-derived extracts (CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4, SDF-1) have also proved beneficial in promoting angiogenesis and restoration of salivary function in irradiated mice SGs (Fang et al., 2015; Su et al., 2022b). The emerging use of conditioned media, secretome components, exosomes, and extracellular vesicles in SG regeneration, seems more quantifiable and stable for long-term storage (Chansaenroj et al., 2021).

Induced pluripotent stem cells (iPSCs) mimic embryonic stem cells reprogramed and differentiated using a cocktail of transcription factors and signaling molecules and have been recently used to form cells from nerves, skin, liver, and pancreas. More recently, iPSCs have piqued the interest of SG researchers, to form functional rudimentary SGs, with positive -amylase, parotid secretion protein, E-cadherin, Sox2, AQP5, M3, -SMA, 3-tubulin and CD31 markers (Ono et al., 2015; Tanaka et al., 2018).

The advances in bioengineering and bioprinting can be exploited for the reorganization and construction of

3D functional innervated SG organoids, with potential applications in organ transplantation research currently restricted to animal studies (Berishvili et al., 2021). Ogawa and team bioengineered a SG germ, through orthotropic transplantation, in a mouse model, that replicated acinar formations with innervation, that produced saliva in response to stimulants (Ogawa et al., 2013).

Despite the constant dynamics in the fields of stem cell-based therapies and tissue engineering, the development of an artificial SG, with secretory acinar, alongside the ductal and myoepithelial cells with supporting non-parenchymal components, seem slightly remote. Although the initial footwork has been accomplished, the lack of scientific contributions solely dedicated to some aspects of SG research may create bottlenecks (Aframian and Palmon, 2008). Further, an amalgamation of collaborative multi-disciplinary approaches for SG tissue engineering and regeneration needs emphasis.

Conclusion

Significant research and advancement towards a deeper understanding of the various exogenous and endogenous factors contributing to xerostomia or salivary disorders has pivoted remarkable progress towards therapeutics and SG regeneration. Besides the evolving knowledge of the physiology and pathology of these conditions, considerable development has been achieved in simulating the microenvironment *in vitro*, but some challenges still remain. Research has demonstrated that though we share similarities with study animal models (rodents), substantial differences may arise during practice, resulting in barriers at recapitulating clinical results. Moreover, it may be noteworthy to mention the possibility of variations among the different major SGs themselves. Regeneration of SG tissue should also be complemented by vital individual patient considerations such as age changes, local damage, and systemic illness, thus warranting individualized precision regenerative strategies. The current well-established research on various components of SG tissue (cells, culture media, biomaterials, matrices, organoid systems) has proved dynamic in bioengineering SG organoids *in vitro*. Future trials employing structural and functional SG transplantations, aimed at bridging the gap between the researcher and clinician, are essential to creating a sizable impact on the treatment of salivary hypofunction in patients.

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