Chapter 9
Unveiling Stem Cell Heterogeneity Toward the Development of Salivary Gland Regenerative Strategies

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Abstract Epithelial damage in the salivary gland (SG) resulting in irreversible dry mouth can be commonly induced by gamma radiation therapy. This radiation depletes the SG stem/progenitor cell niche slowing healing and natural gland regeneration. Biologists have been focused in understanding the development and differentiation of epithelial stem and progenitor cell niches during SG organogenesis. These organogenesis studies gave insights into novel cell-based therapies to recreate the three-dimensional (3D) salivary gland (SG) organ, recapitulate the SG native physiology, and restore saliva secretion. Such therapeutical strategies apply techniques that assemble, in a 3D organotypic culture, progenitor and stem cell lines to develop SG organ-like organoids or mini-transplants. Future studies will employ a combination of organoids, decellularized matrices, and smart biomaterials to create viable and functional SG transplants to repair the site of SG injury and reestablish saliva production.

Keywords Exocrine glands · Salivary glands · Radiotherapy · Sjögren's syndrome · Hypofunction · Dry mouth · Xerostomia · Regenerative medicine · Tissue engineering · Epithelial cell · Progenitor cell · Stem cell · Three-dimensional cultures · Bio-printing

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A. Birbrair (ed.), *Stem Cells Heterogeneity - Novel Concepts*,
Advances in Experimental Medicine and Biology 1123,
https://doi.org/10.1007/978-3-030-11096-3_9

Introduction

Salivary gland damage resulting in irreversible dry mouth (or xerostomia) can be commonly induced by radiation therapy for head and neck cancers (HNC). Xerostomia is also usually observed in several systemic diseases, particularly autoimmune, such as graft-versus-host disease, Sjögren's syndrome, granulomatous diseases, and uncontrolled diabetes among others [1].

Xerostomia is a major complication of radiation therapy (RT), which can target more than 500,000 new cases of head and neck cancer that develop every year worldwide. Since saliva is required for food digestion, lubrication, and buffering effects and for protection against environmental hazards, xerostomia can cause various life-disturbing adverse effects, such as progressive caries, unbearable pain, oral fungal infections, speech deficits, taste loss, and swallowing impairment, which greatly impair patients' oral and systemic health [2]. A multicenter randomized controlled trial by Nutting et al. [3] showed that the prevalence of xerostomia (grade 2 and above) can go up to approximately 40% in 12 months even after novel RT modalities are used (such as salivary gland-sparing or intensity-modulated radiation therapy). When the radiation field (during RT) lays on the salivary glands (SG), radiation injury is elicited on secretory epithelial cells, blood vessels, and adjacent nerves [4, 5]. Salivary glands consist of two types of secretory epithelial cells: 80% acinar and 20% ductal. Following RT, patients lose the majority of acinar cells with the surviving secretory cells being primarily ductal; consequently, RT will irreversibly impact salivary secretion and cause inflammatory damage and fibrosis on the long term. This radiation damage further depletes the SG stem/progenitor cell niche deterring healing and natural gland regeneration [4, 6–8]. Yet, no effective therapy has been devised to treat RT-induced xerostomia, and current treatment strategies are confined to the minimization of SG radiation damage or to the administration of artificial salivary substitutes and saliva secretion stimulators [2, 4].

Radiation-induced xerostomia can be an irreversible lifelong condition that will significantly affect the quality of life of cancer patients. Thus, novel and effective therapeutical strategies for SG hypofunction are required. Due to the depletion of the stem cell pool during RT damage, stem/progenitor cell therapies are vital to engender new SG secretory tissues and repair the damaged ones, for the restoration of salivary flow in xerostomia patients [9, 10].

Several biological therapies have been proposed in the last decade for SG regeneration at preclinical stages (the most relevant are summarized in Tables 9.1 and 9.2), which can involve the following biotechnology strategies:

1. Implantation of SG stem/progenitor cells as salispheres into the irradiated gland to replace the functionally damaged cells [9, 24].
2. Transplantation of adult stem cells (e.g., mesenchymal stem cells) with different differentiation cues in mono- or co-culture systems [25, 26].
3. Tissue engineering techniques combining cells with or without environmental cues in 3D biomaterial constructs [21, 27].

Table 9.1 Advantages and limitations of different stem cell culture techniques for salivary gland (SG) repair or regeneration

Culture model	Advantages	Limitations	References
Mouse salisphere cultures with C-KIT-positive cells for transplantation	• Restoration of submandibular gland homeostasis and salivary flow (~55%)	• Lack of clinical prospect due to use of nonhuman cell lines	[9, 11]
Mouse SG salisphere-derived single cells enriched in CD24/CD29	• Fourfold expansion after seven passages	• Long-term cultures (13 passages) can produce karyotypic changes • Lack of clinical prospect	[12]
Co-cultures of mouse fetal epithelium and MSC to generate SG organ germs	• Development of salivary gland-like morphology in 3 days • Uses mesenchymal-epithelial instructive interactions as a template	• Potential differentiation toward divergent lineages after extended in vitro culture due to cellular heterogeneity • Lack of clinical prospect	[13]
Expansion of human SG cells in monolayer culture	• Enhances in vitro expansion of human SG cells • Induces polarization of human SG cells	• Lack of long-term cellular functionality (<9 days) • Lack saliva qualitative studies • Lack of clinical prospect	[14, 15]
SG-derived clonal stem cells expanded by modified sub-fractionation culture	• Genetic and differentiation characteristics similar to bone marrow MSC • Express tight junction markers (i.e., ZO-1)	• Lack of clinical prospect	[16]

MSC mesenchymal stem cells

4. Epithelial cells can potentially be assembled as a 3D organotypic spheroid culture with capabilities to grow and mature into a secretory organ-like appearance (or organoid) [22].

Stem and Progenitor Cells

The first proof of concept study on autologous transplantation of SG cells to functionally rescue salivary hypofunction used in vitro floating spheroid-like cultures of mouse SG progenitor cells, named salispheres [9]. In vitro salisphere cultures have been shown to enrich for SG stem/progenitor cell populations that include KIT (C-KIT, CD117), Sca-1, and Mushashi-1 (Table 9.1) [9]. KIT-expressing (KIT+) progenitors are also found in other epithelial organs beside the SG, such as the prostate gland and lungs, where KIT+ progenitors have remarkable regeneration capabilities [28, 29]. In a salisphere study in mice, 100–300 KIT+ donor-derived cells isolated from the salisphere cultures were sufficient to form both new acini and saliva-transporting ductal structures, restoring the morphology and function of irradiated SG (Table 9.1) [9].

Table 9.2 Advantages and limitations of different biomaterials used in salivary gland (SG) tissue engineering (TE) constructs for SG repair or regeneration

Biomaterial	TE technique	Advantages	Limitations	References
Collagen type I	3D matrix loaded with salispheres of human SG progenitors	• Differentiation of SG progenitors • Long-term self-renewal ability	• Lack of clinical prospect due to presence of xenogeneic substrates	[17]
Matrigel + Collagen	3D matrix with mouse SG salisphere-derived $CD24^{hi}/CD29^{hi}$ single cells	• Differentiated into distinct ductal/lobular organoids with multiple SG cell lineages • Restoration of salivary flow (~46%)	• Require long-term cultures • Lack of clinical prospect	[12]
Matrigel/ Perlecan domain IV peptide	Culture of human SG cells	• Differentiation into self-assembled acini expressing tight junction and water channel proteins	• Lack of proper acinar cell polarity	[18]
PLGA	3D nanofibers construct loaded with SG epithelial cells	• Supports growth, proliferation, and survival of SG cells • Facilitates self-assembly of SG cells to 3D structure	• Lack of 3D branching and proper tight junctions • Lack of saliva flow studies	[19]
PLGA	Lithographically-based patterning with rat SG epithelial cells	• Supports apicobasal polarization • Improves epithelial differentiation	• Long-term in vitro culture • Lack of saliva flow studies	[20]
PLGA coupled with chitosan and laminin-111	Nanofibers for SG epithelial cell proliferation	• Supports apicobasal polarization and maturation of the SG epithelial tight junctions	• Long-term in vitro culture • Lack of saliva flow studies	[21]
HA (2.5D/3D)	3D organotypic culture of human SG cells	• Develop functional 3D spheroids in long-term in vitro cultures with alpha-amylase expression	• Lack of in vivo saliva flow studies	[22]
Laminin-111	3D clusters of mouse SG cells in feeder layers of hair follicle-derived MSC with laminin	• Organization of SG cells in clusters with multilumen formation • Hair follicle-derived MSC feeder layers support SG cell growth	• Lack of saliva flow studies • Lack of clinical prospect	[23]

HA hyaluronic acid, *PLGA* poly(lactic-co-glycolic acid), *MSC* mesenchymal stem cells

Regrettably, human major SG biopsies hold a very limited number of KIT+ progenitor cells [17]. Also, due to the heterogeneity of the KIT+ cell population, further studies have included co-expression of other putative salivary stem cell markers, such as CD24 (HSA) and CD49f (Itga6) [11]. KIT+ cells co-expressing CD24 and CD49f showed an enhanced functional recovery compared to a heterogeneous KIT+ population, which indicates that this subpopulation of KIT+ cells is enriched for SG stem/progenitor cells (Table 9.1) [11]. It is yet to be determined whether human KIT+/CD24+/CD49f+ cells have similar stem−/progenitor-like functions. Nevertheless, recently, SG sphere-derived single cells expressing high CD24 and CD29 markers ($CD24^{hi}/CD29^{hi}$) could be expanded ex vivo by fourfold after seven passages [12], though karyotypic changes (chromosome doubling) were noticed after passage 3. The same research group placed the same spheres in a 3D matrix mixture with Matrigel and collagen, and spheres differentiated in vitro into organoids with ductal/lobular structures. Upon in vivo transplantation of differentiated spheres into an irradiated mouse model, salivary flow was restored to ~46% (of pre-irradiated levels). Interestingly, undifferentiated spheres also partially restored the salivary flow, which denotes these cells may be secreting microenvironment cues that are stimulating the repair of the remaining gland [12]. Despite the abnormal chromosome number, tumor formation was not observed within 4 months (120 days) post-RT. Though, long-term follow-up studies are necessary to confirm tissues are tumor-free. Furthermore, enrichment of C-KIT+ cells within the $CD24^{hi}/CD29^{hi}$ and the CD24+/CD49f+ subpopulations showed similar salivary flow outcomes [12]. The formation of acini and ductal-containing organoids from single cells is an important achievement for the field. However, this salisphere model cannot be translated into humans as it is yet to be demonstrated whether similar salispheres can be obtained from human SG biopsies and in particularly from elderly patients. Salisphere formation is in fact problematic in SG of old age mice [17].

To overcome the above limitations of salisphere-based cultures, our laboratory tested novel 3D spheroid bio-printing cell assembly systems incorporating human dental pulp stem cells expressing KIT+ with high expansion capabilities and binding/tagging them with magnetic nanoparticles [30–36]. Interestingly, KIT is clearly expressed in neural crest progenitors found in the dental pulp of human permanent teeth [37, 38]. These progenitors are termed human dental pulp stem cells, and our research group is enriching them to move SG cell-based therapies from mice to clinically relevant human SG organoids.

Consequently, methodologies for cryopreservation and biobanking of these progenitors have been established. Neumann and others [39] have developed a stem cell biobanking setup where salivary gland integrin α6β1+ cells have been cryopreserved in the long term without affecting their functional and genetic stability, serving as a future therapy in cancer patients. Furthermore, it is crucial to understand how progenitors proliferate and expand particularly during organogenesis. Several researcher groups have demonstrated that KIT and fibroblast growth factor receptor 2b (FGFR2b) signaling are essential for progenitor survival and expansion in the fetal submandibular gland, lung, pancreas, tooth, and skin [40–42].

Moreover, other putative markers that can be used to isolate SG stem/progenitor cells include K5 (Cytokeratin 5), CD29 (Itga1), CD133 (Prom1), Sca1, CD44, CD34, CD90 (Thy1), CD105, CD9, and CD81, but only few populations were proven to actively restore damaged glands [9, 11, 43–45]. Yet, the KIT+ cell population still appears to have the highest stem−/progenitor-like potential in mice. Analysis of regenerated SGs after transplantation of enriched KIT+ progenitor cells shows restoration of tissue homeostasis following irradiation whereby upon an increase in cytokeratin markers of epithelial ductal cells (K7, K8, K14, and K18) and in stem cell markers (KIT, CD133, CD24, and CD49f) induces normalization of vasculature and reduces fibrosis [9, 11]. Other populations of epithelial progenitor/stem cells have been found to be required for glandular branching in the developing mouse model, which are positive for K5+ [43, 46]. These K5+ progenitor cells are from neural crest and may have the potential for SG regeneration by supporting gland innervation [47].

A major obstacle in stem/progenitor cell therapies is the limited lifespan of the cells obtained from in vitro cultivation systems, hence needing to be used within a short time window. Thus, other cell culture systems and cell sources are necessary for the regeneration of salivary glands as well as systems to enrich sufficient numbers of autologous SG progenitor cells. Cell culture systems have been recently established on human minor salivary gland epithelial cells (phmSG) to achieve the maintenance of these cells in an acinar-like phenotype after optimizing growth conditions [48]. These phmSG cells displayed progenitor cell markers (K5 and Nanog) as well as acinar-specific markers such as α-amylase, cystatin C, TMEM16A, and NKCC1. After beta-adrenergic receptor stimulation, phmSG cultures exhibited calcium ion mobilization and formed an epithelial monolayer with transepithelial electrical resistance (TER) and polarization.

This study raises the question on whether the limited available number of human minor SG cells can generate enough saliva to ameliorate the irreversible hyposalivation found in several patients (post-RT, Sjögren's, etc.). Major SG transplants (i.e., from the parotid gland) may be a more feasible option to accomplish higher salivary secretion rates [17], but they are not always available. To generate a reasonable salivary flow, larger in vitro salivary tissues are needed and, consequently, new cell sources capable of generating high cell numbers (in short-term passaging) and a matrix-rich environment [12]. Adult stem cell sources can potentially offer predictable high expansion rates, and due to their heterogeneity, they can be combined into organotypic cultures to generate larger organoids capable of restoring the salivary flow.

Adult Stem Cells

Recently, intraglandular bone marrow-derived (BM) transplants using either mesenchymal stem cells (MSC) or BM bioactive lysates have been shown to induce paracrine pro-survival effects on remaining SG tissues and to potentially induce

site-specific multi-lineage transdifferentiation toward a more functional SG tissue architecture [25, 26]. For example, intraglandular transplantation of BM-MSC improves saliva production, reduces apoptosis, and increases microvessel density in irradiated mice, and transdifferentiation into acinar cells was observed [26]. Highly homogenous bone marrow clonal MSC (BM-cMSC) have recently shown potential to regenerate the SMG, although currently, the regenerative mechanisms are not well understood [25]. Earlier studies in mice have shown that granulocyte colony-stimulating factor-mobilized BM-derived cells can partially regenerate and also functionally restore an irradiated SG [49]. In addition, an in vitro study using BM stem cells (BMSCs) that are co-cultured with neonatal rat parotid acinar cells using a double chamber system showed an increase in the induction of acinar-specific α-amylase expression in BMSCs [50]. This observation indicates that BMSCs can transdifferentiate into acinar-like cells. Yet, transdifferentiation of BMSCs into acinar-like cells was found to occur only in 50% of the cells after co-culturing for 2 weeks. Further studies are still needed to test the secretory function of these acinar-like cells from bone marrow sources. Transdifferentiated BMSCs have not convincingly showed a proper secretory function in vivo.

Interestingly, studies using human adipose-derived mesenchymal stem cells (hAdMSCs) via systemic administration exhibit improved salivary flow rates 4 months after radiation therapy [51]. hAdMSC-transplanted SGs showed lesser tissue fibrosis and epithelial acinar apoptosis and higher secretory mucin and amylase levels. At 4 weeks, a large number of infused hAdMSCs were detected in vivo and were found to have differentiated, whereas in vitro, only low number of co-cultured hAdMSCs (13–18%) were found to transdifferentiate into salivary epithelial-like cells [51]. More recently, soluble signals from feeder layers of hair follicle-derived MSC (that were mitotically inactive) coupled with laminin-111 substrates supported the formation of clusters of mouse submandibular gland cells with multiple lumens [23]. This was a successful attempt to improve the differentiation and organization of SG cells, though its clinical applicability is yet to be demonstrated.

Nonetheless, three-dimensional (3D) tissue/organ constructs are still required to integrate multiple BM-derived tissues and cell lines in biomaterial constructs or extracellular matrices (ECM) under specific growth factor conditions in order to generate whole SG organ-like structures or organoids.

Three-Dimensional Tissue Engineering Strategies

A recent advancement in SG regenerative medicine showed that a bioengineered gland made from embryonic epithelium and mesenchyme can be transplanted into an adult mouse to produce a whole functional SG [13]. This bioengineered SG was composed of a variety of progenitor and stem cells, including cell from epithelial, mesenchymal, endothelial, and neuronal origins. More interestingly, the SG reconnected with the existing ductal system and possessed functional activity. The new

SG was able to secrete saliva, protect the oral cavity from bacteria, and restore swallowing functions.

Thus, future research may translate these bioengineering strategies to animal models with salivary glands that have more structurally and functionally similarities to the human SG. Further studies may also focus on the usage of stem cells or adult salivary progenitors with high expansion capabilities in 3D scaffolds in order to form a bioengineered construct that grows into a functional gland in the adult microenvironment.

Salivary gland tissue engineering requires three essential components: (1) the stem/progenitor cells that retain epithelial progenitor biomarkers typical of the native salivary gland (SG), (2) the extracellular matrix (ECM) proteins that can orchestrate the differentiation of progenitor cells into functional structures, and (3) a biocompatible and biodegradable three-dimensional (3D) scaffold that can hold these components together to recreate the microenvironment found in the native SG [27].

Since dynamic cell-ECM interactions are essential in processes such as epithelial ductal formation/branching, a recent strategy has been to engineer scaffolds that structurally and functionally resemble native ECM architecture. Three-dimensional (3D) collagen matrices have been used for homing salisphere stem/progenitor cells which form epithelial ductal structures with mucin-positive acini, indicating their capability to differentiate in response to the ECM environment [12]. Various biomaterials such as collagen type I, Matrigel, and other animal-derived products have been showing promising results in the differentiation and organization of human SG cells [12, 14, 15]; nevertheless, these biomaterials are not human-compatible. Thus, tissue engineering-based research is gearing toward the creation of xeno-free biomaterials, which can eventually be transplanted into humans.

Recently, researchers have started to utilize the soft hyaluronic acid (HA) hydrogels, which are human-compatible, as biocompatible substrates for SG tissue engineering [52]. When encapsulated in HA hydrogels, human SG cells can grow into organized spheroid structures that merge and proliferate to form larger acini-like structures with a central lumen and are maintained for long term in these gels in vitro [52]. These in vitro 3D acini-like structures also secrete α-amylase, express β-adrenergic and muscarinic receptors that activate protein transport, and induce calcium oscillations upon treatment with cholinergic stimulants. Furthermore, these 3D spheroids continue to secrete α-amylase when hydrogels were implanted in vivo in an athymic rat model [22]. However, these latter 3D structures have reversed polarity suggesting that further environmental cues from the ECM and the myoepithelial cells may be needed to reverse inside-out acini and correct the polarity.

Culture of salivary gland progenitor cells on human perlecan domain IV peptide has been shown to support the formation of 3D acini-like salivary units that express α-amylase [18]. It will be useful to incorporate the perlecan IV domain peptide into biomaterial scaffolds to mediate differentiation and correct polarity and directional secretion of the 3D salivary gland cell cultures in the future.

Other research groups have used poly(lactic)-co-glycolic acid (PLGA), an FDA-approved constituent in implantable dental and orthopedic devices, as a synthetic

material to show that it can support the attachment, proliferation, and survival of salivary gland epithelial cells [19]. The same group further shows that nanofiber PLGA scaffolds can support development and morphogenesis of intact fetal SMG organ cultures and promote natural self-organization of dissociated SMG cells into branched SG-like structures [53]. However, adult SG cells grown on flat polymeric substrates fail to form a complex 3D branching structure and are unable to assemble tight junctions that are needed for unidirectional flow of saliva. To overcome this, recent studies generated lithographically based micropatterning curved "craters" that mimic the physical structure of the basement membrane, which have increased both the surface area and allowed apicobasal polarization and differentiation of salivary gland epithelial cells [20]. An increase in aquaporin-5, a water channel protein marking acinar differentiation, was also detected in SG cells cultured on higher curvature scaffolds. Further studies with PLGA nanofibers coupled with laminin-111 and chitosan showed that laminin-111 promotes the formation of mature epithelial tight junctions and apicobasal polarization, and on the other hand, the chitosan antagonizes this phenomenon [22].

Taken together, current cell-based therapies and tissue engineering studies have provided a promising outlook to regenerate SG and restore the saliva secretory function. However, in order to test these techniques in humans, several hurdles need to be surpassed. To overcome these hurdles, further research steps should include: (1) the elimination of xenogeneic elements from transplants for feasible human use to comply with good manufacturing practices, (2) a thorough assessment of histocompatibility barriers, (3) an evaluation of long-term transplant survival and saliva secretion in larger animal models with a better SG human resemblance (i.e., pigs), and (4) an assessment of tumor sensitivity to bioengineered transplants in SG cancer models. Recent studies indicate that the above research steps are currently being pondered [18, 22, 54, 55].

Novel 3D Bio-printed Magnetic Nanotechnologies for SG Regeneration

Biomedical researchers have been moving toward cell culture technologies in 3D to better recapitulate native cellular environments and ultimately develop organotypic cultures [18, 22]. Novel bio-printing nanotechnologies have been recently developed using magnetic patterning or levitation in which cells bind with a magnetic nanoparticle assembly overnight to render them magnetic (Fig. 9.1) [30–36]. This nanoparticle assembly includes gold, iron oxide, and poly-L-lysine, which can easily tag via electrostatic interaction different cell types at the plasma membrane level. When resuspended in medium, a mild external magnetic field can concentrate and magnetically bio-print cells at the bottom of a cell-repellent plate, where they assemble to form larger 3D spheroids or organoids (Fig. 9.1). The resulting dense cultures can synthesize ECM and can be analyzed similarly to other 2D/3D culture systems, using assays/techniques such as cytotoxicity assays, immunohistochemical

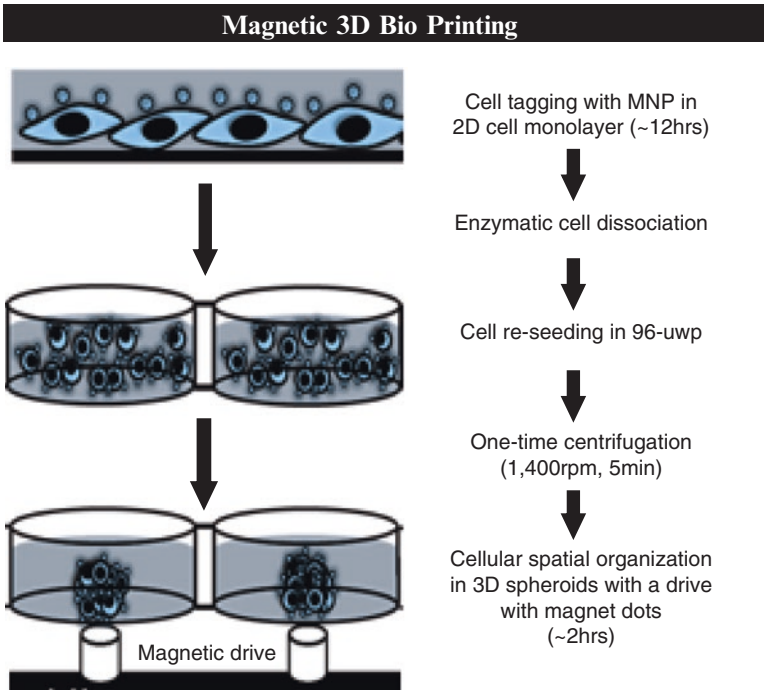

Fig. 9.1 Flowchart with biofabrication steps required for the formation of 3D spheroids by magnetic 3D bioprinting. *MNP* magnetic nanoparticles, *uwp* ultra-low attachment well plate

analysis, Western blotting, and other biochemical assays [56]. These 3D bio-printed systems have been recently found to recapitulate the native ECM from several tissues such as fat, lung, aortic valve, blood vessels, and breast and glioblastoma tumors [30–36]. Further, our research group has showed that these magnetic bioprinting systems can develop SG epithelial organoids with innervation, secretory function upon cholinergic stimulation, and epithelial polarity [57]. The apicobasal polarity in epithelial cells is essential to overcome the challenges related to the directionality of salivary flow. These SG-like organoids will provide a better understanding of human SG physiology in vitro and in vivo, in homeostatic and disease states, although these organoids still lack a robust vascular network [57]. The use of natural ECM can be a plausible alternative that can be accomplished by decellularizing organs with detergents followed by reseeding primary SG cells onto the ECM core [27, 58]. A decellularized SG can be further tested in combination with 3D bio-printed SG epithelial organoids.

The in vitro biofabrication of human SG-like transplants or organoids is crucial to:

1. Generate scaled-up xeno-free biocompatible 3D structures that provide the native architecture with environmental cues to support cell growth [30, 31, 56],

differentiation, and biointegration in the remaining tissues (after damage) to restore homeostasis and functionality [57, 58].
2. Establish methodologies to generate SG-like organoids for scale-up production. These methodologies may need in vitro co-culture systems to integrate in a 3D architecture the cellular complexity of all human SG compartments, such as the acinar and ductal epithelia, myoepithelia, and the networks of parasympathetic nerves and lumenized ducts and vessels [55].
3. Lastly, test new surgical techniques in vivo with ex vivo bio-printed SG transplants/organoids to promptly repair SG damage particularly after RT.

Conclusion

Organotypic 3D bioengineered culture systems are on the rise in regenerative medicine. These novel systems are essential to recapitulate the different cellular components of the SG and create an artificial gland for restoration of secretory function. Researchers have used floating salisphere culture systems combined or not with biomaterial 3D constructs to mimic native environments. Nevertheless, these models provided limited cellular expansion, poor acinar epithelial polarization, and skewed directionality of salivary flow. Bio-printing strategies in 3D using human cells are an avenue that has revealed promising outcomes in several types of tissue including exocrine glands [35, 57].

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