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Concise Review: Adult Salivary Gland Stem Cells and a Potential Therapy for Xerostomia

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ABSTRACT

The ability to speak, swallow, masticate, taste food, and maintain a healthy oral cavity is heavily reliant on the presence of saliva, the hugely important effect of which on our everyday lives is often unappreciated. Hyposalivation, frequently experienced by people receiving radiation therapy for head and neck cancers, results in a plethora of symptoms whose combined effect can drastically reduce quality of life. Although artificial lubricants and drugs stimulating residual function are available to ameliorate the consequences of hyposalivation, their effects are at best transient. Such management techniques do not address the source of the problem: a lack of functional saliva-producing acinar cells, resulting from radiation-induced stem cell sterilization. Post-radiotherapy stimulation of cell proliferation only results in improved saliva secretion when part of the tissue has been spared or when the dose to the salivary gland (SG) remains below a certain level. Therefore, stem cell replacement therapy may be a good option to treat radiation-induced hyposalivation. Substantial progress has been made lately in the understanding of cell turnover in the SG, and the recent identification of stem and progenitor cell populations in the SG provides a basis for studies toward development of a stem cell-based therapy for xerostomia. Here, we review the current state of knowledge of SG stem cells and their potential for use in a cell-based therapy that may provide a more durable cure for hyposalivation. STEM CELLS *2013;31:613–619*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

More than 40,000 new patients in the U.S.A. are expected to be diagnosed with head and neck cancer in 2012 [1]. The majority of these patients will be treated with radiotherapy (RT) alone, or in combination with chemotherapy and/or surgery, with a consequent 5-year-survival rate of approximately 50% for non-metastatic locally advanced disease [2]. While significantly improving the patient's chances of survival, RT treatment often results in unavoidable co-irradiation of normal tissues surrounding the tumor, such as the salivary glands (SGs). Although protocols have been developed to minimize early and late loss of gland function following RT, 40% of head and neck cancer patients receiving the most modern intensity modulated RT will still experience moderate or severe xerostomia [3–7].

Induced by radiation, SG dysfunction and consequential hyposalivation causes many post-treatment complications, including hampered speech, dental problems, difficulties with swallowing and food mastication, impaired taste, and nocturnal oral discomfort. Hyposalivation and the resultant symptoms are together termed xerostomia ("dry mouth syndrome"), can lead to a dramatic loss in quality of life for the patient, and remains extremely difficult to manage [3, 4, 8, 9]. This review describes recent progress in our comprehension of radiation-induced hyposalivation, the characterization of rodent and human SG stem cells, and advances in design of an adult stem cell-based therapy for long-term treatment of hyposalivation in post-RT patients.

A CELLULAR BASIS FOR RADIATION-INDUCED LONG-TERM HYPOSALIVATION

The SGs of mice, rats, and humans are composed basically of two saliva-producing cells types, namely mucous and serous acinar cells, myoepithelial cells, which facilitate saliva expulsion and a ductal cell system which modifies saliva composition and through which saliva is secreted into the oral cavity (Fig. 1). Intertwined cholinergic and adrenergic nerve fibers stimulate saliva production and also indirectly affect SG secretion through innervation of the blood vessels that supply the glands. The whole consortium of cells is kept in close physical proximity to each other by supporting stromal tissue [10, 11] (Fig. 1). The impact of RT on function of SGs is bifaceted. Saliva-producing acinar cells are largely postmitotic in nature, and according

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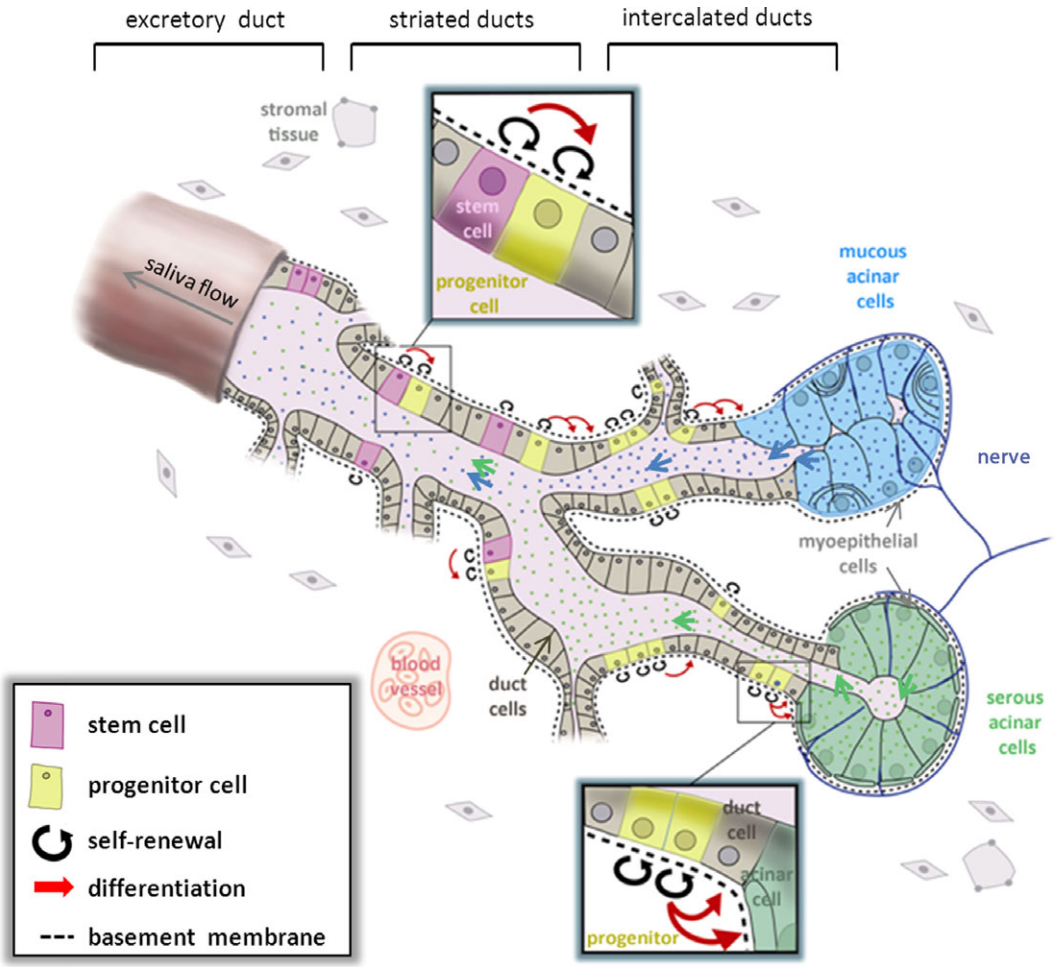

Figure 1. Schematic representation of a generic salivary gland showing component cell types and theorized stem and progenitor cell locations. Visualization of proposed location of primitive stem cells, within larger excretory and striated ducts, and progenitor cells, within the striated and intercalated ducts. The stem cell pool supplies in the progenitor cell pool, which in turn replenishes the population of functionally mature duct and acinar cell types. Both stem and progenitor cells have the capacity to self-renew and differentiate. Schematic diagram generated based on available duct ligation, label-retaining cell, and stem/progenitor cell marker expression data.

classical radiobiology theory not predicted to be radiation-sensitive [12]. RT of the SG however induces severe early (phases 1 and 2, 0–10 days and 10–60 days, respectively) loss in saliva production, suggesting that the SG is more radiosensitive than anticipated [13]. Debate is still ongoing as to whether this observed early RT-induced hyposalivation is attributable to apoptosis or to membrane damage-induced dysfunction of the acinar cells [3, 13–19]. The later phases of RT-induced hyposalivation (phases 3 and 4, from 60 to 120 and 120 to 240 days, respectively), wherein functionally mature acinar cells senesce and are not replenished with new ones, are now suggested to be due to RT-induced sterilization of a SG stem/progenitor cell population (SSPCs) ([13, 14, 20–26]; Fig. 1). Stem or progenitor cells are characterized by their self-renewal and differentiation capabilities, can replenish damaged cells, and have been identified in many tissues within the mouse and human [27–33]. In this hypothesis therefore, the number of remaining undamaged SSPCs will determine the regenerative capacity of the gland after irradiation. Recovery and compensatory responses in nonirradiated regions (presumably containing SSPCs) have been observed after radiation, indicating the potential of surviving SSPCs to regenerate the tissue [23, 34]. We now review the evidence for the existence of such a SSPC population that is both responsible for SG homeostasis, and for long-term hyposalivation when sterilized.

Adult sg Stem/Progenitor Cells

Through label-retaining cell studies using nucleotide analogs such as bromodeoxyuridine and ^{3}H-thymidine, proliferating cells have been localized mainly to the excretory and intercalated ducts in the SG ([20–22, 24, 35]; Fig. 1). Ligating the major excretory duct of the SG, creating a dysfunctional/apoptotic acinar cell environment, results in the proliferation of intercalated and excretory duct cells [21, 36–41]. The initial functional ablation in ligated glands can be rescued after deligation through proliferation and suggested differentiation of these ductal cells, and saliva flow will rather rapidly return to pre-ligation levels. Label-retaining cell studies have also demonstrated that acinar cells themselves display a limited degree of proliferative ability, but the total ablation of acinar cell function in ligation experiments suggests that acinar cell proliferation is unlikely to account for the rescue of function. The above studies imply that cells capable of proliferation and differentiation reside within the ducts of SGs and may represent a potent SSPC population. Further studies have also suggested that these putative SSPCs are responsive to growth factor-mediated stimulation, whereby RT-induced hyposalivation was rescued through administration of keratinocyte

Table 1. Summary of current salivary gland stem cell phenotypic studies presented in chronological order within species

Species	Marker/s of interest	Culture method	Tested for		First author	Ref
			In vitro differentiation?	*In vivo* function?		
Mouse	CD117	Salispheres	Yes	Yes	Lombaert	[44]
	CD49f, CD29, CD24, CD117	Salispheres	No	Yes	Nanduri	[45]
	CD117 and ALDH	Salispheres	Yes	No	Banh	[46]
	SP cells, Sca-1, clusterin	No culture	No	Yes	Mishima	[47]
	Ascl-3	Salispheres	Yes[a]	No	Rugel-Stahl	[48]
Rat	No marker	Monolayer	Yes	No	Kishi	[49]
	CD49f, CD29	Monolayer	No	No	David	[50]
	CD49f, CD29, CD117	Monolayer	Yes	No	Neumann	[51]
Human	CD49f, CD90	Monolayer	Yes[b]	No	Sato	[52]
	CD117	Salispheres	Yes	No	Feng	[53]
	CD34, CD117, ALDH, CD90, CD44	Salispheres	Yes	No	Banh	[46]
	CD44, CD166	No culture	—	—	Maria	[54]
	CD49f, CD29	Monolayer	No	No	Palmon	[55]

—, indicates not applicable in study. [a]Lineage tracing data. [b]Pancreatic-like differentiation shown. CD nomenclature are given where possible. Pseudonyms are: CD49f = integrin $\alpha 6$; CD117 = c-Kit; CD90 = Thy-1; CD166 = activated leukocyte cell adhesion molecule (ALCAM); CD24 = heat stable antigen (HSA); CD29 = integrin $\beta 1$. Abbreviations: ALDH, aldehyde dehydrogenase; CD, cluster of differentiation; SP, side population cells.

growth factor (KGF), or GFs secreted by bone marrow cells (BMCs) mobilized to the SG via granulocyte-colony-stimulating factor (G-CSF) [42, 43]. Tissue recovery in G-CSF- or KGF-treated animals was markedly higher than that in control animals, as assessed by a significantly higher acinar cell content in and saliva production from treated glands [42, 43]. However, reducing the number of surviving putative SSPCs by increasing the radiation dose prevented these growth factors from rescuing SG function [42, 43].

These data serve to emphasize further the importance of a functional residual SSPC population in the SG for hyposalivation recovery. Pre-RT isolation of SSPCs followed by post-RT replacement into the patient could therefore increase the regenerative potential of the SG and potentially completely restore tissue homeostasis following RT. In order to develop such a cell-based therapy for hyposalivation, determine the most potent SSPC population, and further characterize these cells, the ability to manipulate putative SSPCs *in vitro* is paramount.

Rodent Sspcs

Numerous studies have now demonstrated that *in vitro* culture of processed SG tissue is possible, a summary of which can be found in Table 1. Some of these studies have used a monolayer culture technique, where adherent, proliferative colonies of presumed SSPCs were cultured from rat SGs, and after 7 days of culture with added epidermal growth factor and hepatocyte growth factor demonstrated expression of ductal (cytokeratins 18 and 19 and c-Met), acinar (amylase and aquaporin-5), and myoepithelial (vimentin and α-smooth muscle actin) differentiation marker proteins (Table 1). Also CD24/CD49f ($\alpha 6\beta 1$ integrin) and CD117 (c-Kit) stem-cell-associated proteins were found at frequencies of 90% and 6%, respectively, in these cultures [49, 50]. Recent developments in the study of stem cell populations from other glandular tissues such as the prostrate and mammary gland, and also from the neural system, have used nonadherent culture methods to derive functional populations of adult progenitor cells [27–32]. Following these advancements, our lab developed a non-adherent method for culturing potential murine SSPCs ([44, 45, 56]; Table 1). After mechanical and enzymatic digestion, aggregates of cells cultured in suspension, which were named salispheres, increased in size over time in culture and contained proliferating cells [44, 56]. Murine salispheres were found to express the adult stem cell marker proteins CD117, CD24, CD29, CD49f, Sca-1, Musashi-1, CD44, CD90, and CD34, expression of most of which has been localized to ducts in naïve SGs ([44–46]; Fig. 1), with the exception of CD44, whose expression was also suggested to be associated with differentiated serous acinar cells [54]. Interestingly, CD117 expression in 3-day cultured salispheres (>0.6%) was markedly higher than that immediately following salisphere isolation (< 0.01%), suggesting that salisphere culture represents a form of lineage selection and could be used as a tool to enrich for stem cells prior to therapeutic use [45]. Spontaneous differentiation into cells expressing acinar (α-amylase) and ductal cell (cytokeratins 7 and 14) marker proteins during culture was also reported in salisphere cultures [44, 46]. Thus, through ligation, label-retaining cell, growth factor, and culture-based studies, we can surmise that a stem cell-like population is likely to be contained within SG duct cells. For the development of a (stem) cell therapy for hyposalivation, ductal-like cells from salisphere or monolayer cultures may be promising candidates.

The first evidence of ductal-like SSPC functionality *in vivo* was reported from studies in which donor cells isolated from salisphere cultures were transplanted back into irradiated recipient murine glands [44, 45]. Recovery of SG function of 70% of the transplanted animals was achieved with as few as 300 c-Kit$^+$ SSPCs from primary salispheres. In serial transplantation experiments, only 100 c-Kit$^+$ donor-derived cells isolated from salispheres grown from primary recipient glands repopulated glands in a secondary transplant. Non-c-Kit-expressing cells were much less potent leading to 33% recovery following transplantation of 10,000–90,000 cells [44]. Importantly, and in contrast to studies involving transplanted BMCs, the transplanted c-Kit$^+$ SSPCs had functionally integrated within the recipient gland, expressed donor-derived markers, and displayed ductal and acinar cell-type morphologies [44]. Studies of the regenerative capacity of potential SSPCs expressing the CD24, CD49f, and CD133 ductal-associated marker proteins yielded similar exciting functional recovery, with effective cell numbers of approximately 5,000

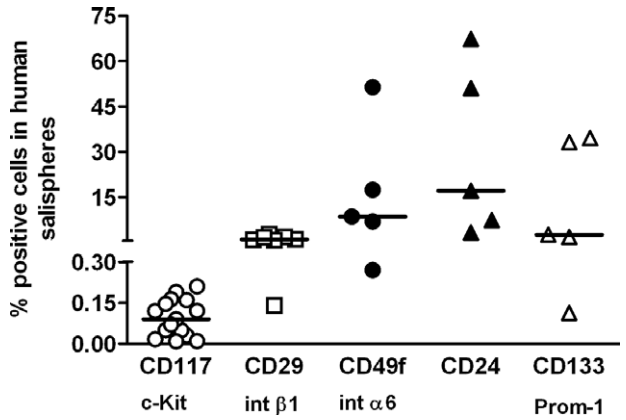

Figure 2. Human salispheres express adult stem cell marker proteins. Adult human salivary gland biopsies were subject to mechanical and enzymatic digestion and cultured as previously reported [45, 56]. Flow cytometry was performed between 3 and 5 days post-isolation, to detect expression of the CD117, CD29, CD49f, CD24, and CD133 stem cell-associated proteins. Data points within each marker protein are from separate patient isolations. Bars represent mean percentage expression. Alternative nomenclatures if applicable are also stated. Abbreviation: int, integrin prom-1, prominin-1.

CD24, CD29, or CD133-expressing cells [45]. When side population (SP) cells, shown to be stem cell-like cells in other tissues, were isolated from SGs and immediately transplanted into irradiated recipient glands without a culture period, functional recovery was indeed observed within 2 months, however integrated acinar and ductal-like donor cells were not detected in the recipient SGs [47, 71–78]. These data suggest that selection for donor ductal-like cells, both by inclusion of an *in vitro* cell culture phase, and/or employment of marker proteins enhances functional recovery *in vivo*.

Human Sspcs

Preliminary data suggests that salisphere-based culture principles and the employment of protein markers can be utilized in the study of human SSPCs (hSSPCs) ([44, 46, 53]; see also Table 1). Data from our own lab demonstrates that CD117, CD24, CD29 and CD49f are expressed by a proportion of cells in 3-5 day old human salisphere cultures (Fig. 2). Preliminary data showing some human salisphere differentiation into three-dimensional organoid structures containing acinar and ductal-like regions is also encouraging in terms of the potential differentiation capabilities of these cells [53]. Alternatively, cells grown in monolayers have also been shown to express a panel of stem-cell-associated marker proteins (CD44, CD49f, CD24/CD49f, CD90, CD104, and p75NGFR). The colocalization of two such markers, CD49f and CD90, in the periductal region of a native gland was further suggested to be evidence for the ductal location of hSSPCs [52, 55].

Studies regarding hSSPCs are few in number as yet and crucial assays for the reliable assessment of hSSPC differentiation and proliferation capabilities are still lacking. Even if hSSPCs mirror the *in vivo* functional ability of murine SSPCs, they still represent by no means the only cell-based option for a xerostomia therapy. Since 1998, a huge effort has been directed toward the investigation of human embryonic stem cell (hESC) potential as a source of cells for therapeutic applications, based on their capability to turn into any cell type in the body and self-renew indefinitely [57, 58]. In 2001, the first clinical trial using hESC-derived cells began, as a therapy for spinal cord injury [59]. An hESC-based approach to xerostomia therapy has not yet been reported, and may be hazardous due to the vulnerable nature of post-RT patients, in combination with the inherent teratogenicity of hESCs and their tendency to acquire karyotypic abnormalities during *in vitro* culture, exclusive of the ethically contentious nature of hESC research [60, 61]. Technically, the expertise required to generate hSSPCs from hESCs is currently still lacking, but it remains possible that hESC-derived hSSPCs may represent an interesting option for xerostomia therapy in the future. Transplantation of BMCs into numerous disease-like mouse models and the progression toward clinical trials using BMCs suggest also that existing adult human stem cells represent a simple source of cells for xerostomia therapy [42–44, 62–70]. Although mobilized BMCs seem to have some ameliorating effect on hyposalivation in studies described above, this effect was most likely due to growth factor secretion. Transdifferentiation of BMCs into acinar cells was not observed, and functional recovery was attributed to stimulation of surviving endogenous SSPCs [42, 43, 64–70]. BMC-mediated hyposalivation rescue is therefore limited first by the requirement for surviving SSPCs and second by the lifespan of the growth factor-secreting BMCs. We hypothesize that hSSPCs are likely to be preferable to hESCs and BMCs as therapeutic agents for hyposalivation, when considering the ability of murine SSPCs to differentiate appropriately into saliva-producing cells, integrate effectively into host tissue, and rescue hyposalivation. We speculate further that a long-term cell therapy for hyposalivation is feasible, through the employment of hSSPCs.

Challenges

The above studies are encouraging in terms of the development of a stem cell therapy for hyposalivation, however the most potent SSPC population within the mouse and rat system remains to be defined, and further translated to the human system. Indeed, the hSSPC hierarchy may not necessarily mirror that observed in the mouse system, and furthermore, the effect of prolonged *in vitro* culture on expression of cell-surface markers that may define this hierarchy is still unclear. Moreover, due to the relatively long turnover time of SG tissue and following the protocol of the hematopoietic system, most likely a cocktail of stem and progenitor cells will need to be given to effectively induce SG recovery. In that scenario, short-term recovery may result from the progenitors within the graft and long-term sustained improvement from the stem cells. A definitive minimal SSPC number required for SG rescue is also unknown and is likely to differ depending on for instance patient age and extent of irradiation. A number of additional challenges clutter the path toward a hSSPC-based therapy, including ensuring the efficacious delivery of the hSSPCs. Putative SSPC populations are currently delivered to recipient mice by means of site non-specific injection directly into the gland. Due to the lobular nature of SGs, the exact localization of injected SSPCs cannot be guaranteed. Unpublished data from our group suggest that retrograde injection of SSPC solutions directly into the opening of the rat submandibular or parotid SGs might be used to control transplantation direction and efficacy, while other studies suggest that echo guidance may also be useful to overcome this problem [71, 72]. Both the above techniques suggest that ductal delivery for hSSPCs is most desirable. Aside from the delivery of the cells, various facets of the culture

systems must be further optimized. In salisphere culture for example, the majority of culture components are now compliant with current good manufacturing practice (cGMP)-regulations, although isolation still relies on an enzyme of bovine origin, hyaluronidase. cGMP guidelines dictate that all complicit reagents must be derived from non-animal sources, thus substitute cGMP-approved reagents must be sourced. cGMP-compliant selection of hSSPCs from monolayer or salisphere cultures should be achievable using magnetic activated cell sorting (MACS) and cGMP-approved antibodies [55].

RT treatment schedules with curative intent generally last between 5 and 7 weeks, not including extra time required for biopsy of the SG, pre-RT. In an ideal situation, transplantation should be performed as soon as possible after RT, before onset of tissue fibrosis which is likely to be detrimental to cell engraftment. Thus, hSSPCs will probably be cultured briefly during this 5–7-week period and then undergo cryopreservation until the desired time point. Both manipulations present their own challenges. Culture of some stem cell populations, albeit mostly hESCs, has been documented to increase the incidence of karyotypic abnormalities in the cells, thus genomic stability must be demonstrated in human salisphere cultures to ensure potentially oncogenic cells are not delivered to a vulnerable patient [60, 61, 73]. Cryopreservation is already possible using cGMP-approved reagents, and the preserved function of $CD24^{+}CD49f^{+}$ putative rat SSPCs frozen for 3 years has been documented [51]. Once thawed, these SSPCs demonstrated equal and in some cases better proliferative ability and expression of differentiation markers compared to their noncryopreserved counterparts [51]. Parallel experiments using hSSPCs remain to be performed, to provide the equivalent functional guarantee for patients awaiting transplantation. In conclusion, further optimization of culture methods and application of additional procedures is required in the near future.

Future Perspectives

Research into the true identity of SG stem or progenitor cells is gathering pace. This is important as an ever increasing deluge of new head and neck cancer patients are admitted every year into hospitals worldwide. Regretfully, most of these patients are of old age and have been suggested to respond even more dramatically to the deleterious effects of radiation on the SGs [74]. Moreover, we observed a reduction in salisphere-forming capability of cells from SGs of mice of old age [53]. This combined with the fact that only a small piece of tissue from the patient may be obtained prior to the RT makes it essential to multiply the number of SSPCs before transplantation, and it is therefore of eminent importance to find protocols that safely permit this. Current *in vitro* culture, self-renewal, and differentiation assays for SSPCs open new possibilities for the screening of novel factors and genes that may be useful tools for SSPC amplification. Administration of KGF and/or manipulation of the Wnt/β-catenin and Notch pathways represent potential approaches for SSPC amplification. The involvement of Notch signaling pathway has been implicated in postnatal SG development and regeneration, and the protective effects of both KGF treatment and of the transiently activated Wnt pathway against radiation-induced damage of the SG have been suggested [43, 75–77]. When successful, novel allogeneic stem cell selection and expansion protocols, pending further investigation into the immune rejection of such transplanted hSSPCs, will greatly expand the reach of the future SSPC therapies, for example, to treat diseases such as Sjögrens syndrome and aging-related xerostomia. Although consensus is that some form of SG cellular therapy is feasible to increase the quality of life of head and neck cancer patients post-RT, the hurdles facing the development of a cellular therapy for hyposalivation are considerable. Perhaps SG researchers should take heart from the complete integration of bone marrow transplantation into our clinical practices, as an example of what is possible using an adult stem cell population in a clinical situation, to dramatically improve the quality of life of patients.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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