Human Salivary Gland Stem Cells Functionally Restore Radiation Damaged Salivary Glands

Sarah Pringle,a,b Martti Maimets,a,b Marianne van der Zwaag,a Monique A. Stokman,a,b,c Diroe van Gosliga,a,b Erik Zwart,d Max J.H. Wijte,s Gerald de Haan,d Ronald van Os,d Rob P. Coppes,a,b

Key Words. Human salivary gland stem cells • Radiation induced xerostomia • Adult stem cells • Differentiation • Organoids

ABSTRACT

Adult stem cells are often touted as therapeutic agents in the regenerative medicine field, however data detailing both the engraftment and functional capabilities of solid tissue derived human adult epithelial stem cells is scarce. Here we show the isolation of adult human salivary gland (SG) stem/progenitor cells and demonstrate at the single cell level in vitro self-renewal and differentiation into multilineage organoids. We also show in vivo functionality, long-term engraftment, and functional restoration in a xenotransplantation model. Indeed, transplanted human salisphere-derived cells restored saliva production and greatly improved the regenerative potential of irradiated SGs. Further selection for c-Kit expression enriched for cells with enhanced regenerative potencies. Interestingly, interaction of transplanted cells with the recipient SG may also be involved in functional recovery. Thus, we show for the first time that salispheres cultured from human SGs contain stem/progenitor cells capable of self-renewal and differentiation and rescue of saliva production. Our study underpins the therapeutic promise of salisphere cell therapy for the treatment of xerostomia. STEM CELLS 2016;34:640–652

SIGNIFICANCE STATEMENT

This study describes the isolation, cultivation, and clinical potential of human salivary gland stem cells to treat radiation-induced xerostomia. We show the ability of human salivary gland derived cells to self-renew and differentiate in vitro and functionally restore irradiated salivary glands after xenotransplantation. The study is the final step toward clinical application.

INTRODUCTION

The salivary glands (SGs) are exocrine organs whose parenchymal tissue manufactures and secretes saliva. Rat submandibular SG duct ligation induces dysfunction/atrophy of saliva-producing acinar cells, and rapid diminishment of saliva production, while ductal cells remained unharmed. Upon deligation, proliferation and differentiation of these ductal cells into acinar cells was observed, and saliva flow rather rapidly returned to preligation levels, indicating the pronounced regenerative potential of SGs [1–5]. Label retaining cell studies hinted at the presence of putative stem/progenitor cell populations residing in the ducts of rodent SGs [6] and indicated that SGs are “slow-turnover” tissues such as intestines. These data imply that SGs harbor a resident stem or progenitor cell population that is capable of regenerating the parenchym of the SG. Stud-
other tissues [19]. These CD24hiCD29hi salisphere cells showed pronounced self-renewal abilities indicated by high potential to form secondary salispheres and to differentiate into organoids containing both ductal and acinar cell lineages [16]. Similarly, CD24+ c-Kit+Sca-1+ murine salisphere cells also demonstrated greater salisphere-formation potential than their nonmarker expressing counterparts in a separate study [20]. In vivo functionality of murine SG stem/progenitor cells has been repeatedly demonstrated, whereby as few as 100 CD117/c-kit expressing counterparts in a separate study [20], 300 CD24+ c-Kit+Sca-1+ cells [20], or 10,000 cultured-enriched CD24+CD29hi murine SG stem progenitor cells [16] rescued radiation-induced hyposalivation in a mouse model. All three stem/progenitor cell phenotypes showed integration into the recipient SG and displayed ductal and acinar cell-type morphologies [12, 16, 20].

These data demonstrate the potential clinical utility of SG stem/progenitor cells as a novel therapeutic strategy to treat SG dysfunction. Hyposalivation, and its collection of associated ailments, resulting in xerostomia, is observed in 40% of patients receiving unavoidable radiation of the SGs during head and neck cancer therapy. Reduction in saliva production is immediate, irreversible, and impacts greatly on patient quality of life. Hyposalivation leaves in its wake life-long oral, dental, speaking, eating and sleeping problems, which have no current cure [21–23]. New therapies for xerostomia therefore represent an unmet clinical need. Although stem cells have been isolated from several adult human tissues such as bone marrow, brain, eye, dental pulp, intestine, adipose tissue, lung, skin and muscle [24–32], very little is known about human SG stem or progenitor cells. Preliminary studies of cultures of various formats from human SGs have indicated expression of surface proteins CD44 [33], CD24 [34], CD29 [34], CD49f [35], CD117 [13, 34, 36], CD133 [34], CD90 [13], CD34 [13], CD166 [33] and aldehyde dehydrogenase [13], similar but not the same to what has been shown in the rodent SG, and with limited examination of in vitro differentiation potential of human salispheres [34, 36]. To date there has been no exploration of the engraftment capabilities or functional attributes of human SG stem/progenitor cells. Therefore, we present in this study the first data exploring the potential of human SG stem/progenitor cells, including their self-renewal and differentiation properties and in vivo engraftment and functionality. Our results pave the way for the development of a cell therapy for xerostomia.

## Materials and Methods

### Source of SG Tissue

Human nonmalignant submandibular SG tissue was obtained from donors (after informed consent and IRB approval) with a squamous cell carcinoma of the oral cavity, in whom an elective head and neck dissection procedure is performed. During this procedure submandibular SG is exposed and removed as part of the dissection procedure. This cohort represents the patient group most eligible for stem cell transplantation, following clinical translation.

### Salisphere Cultures

Submandibular SG biopsies were collected after surgery into Hank’s Balanced Salt Solution (HBSS) containing 1% bovine serum albumin (BSA; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Biopsies were mechanically digested using the gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyi-biotec.com) and simultaneously subjected to digestion in HBSS/1% BSA buffer containing 0.63 mg/mL collagenase II (Invitrogen) and 0.5 mg/mL hyaluronidase (Sigma Aldrich, St. Louis, MO, https://www.sigmaaldrich.com), and calcium chloride at a final concentration of 6.25 mM, for two periods of 30 minutes at 37°C. Twenty mg of tissue was processed per 1 mL buffer volume, total volume was adjusted according to biopsy weight. Digested cells were collected by centrifugation, washed twice in HBSS/1% BSA solution, and passed through 100 μm cell strainers (BD Biosciences, San Diego, http://www.bdbiosciences.com). Resultant cell suspensions were collected again by centrifugation and resuspended in Dulbecco’s modified Eagle’s medium:F12 medium containing Pen/Strep antibiotics (Invitrogen), Glutamax (Invitrogen), 20 ng/mL epidermal growth factor (EGF) (Sigma Aldrich, https://www.sigmaaldrich.com/), 20 ng mL fibroblast growth factor-2 (Sigma), N2 (Invitrogen), 10 μg/mL insulin (Sigma), and 1 μM dexamethasone (Sigma), and plated at a density of 400,000 cells per well of a 12-well plate. For salisphere culture from mouse SG, both submandibular glands from a single mouse were digested in a 2 mL volume of the same hyaluronidase/collagenase buffer solution and processed otherwise in the same manner as human tissue.

### Self-Renewal Assay

Salisphere cultures of 3–5 day (d) were dispersed to form single cell suspensions using 0.05% trypsin-EDTA (Invitrogen), enumerated, and concentration adjusted to 0.4 × 106 cells per mL. 25 μL of this cell solution was combined on ice with 50 μL volumes of Basement Membrane Matrigel (BD Biosciences, Franklin Lakes, NJ, https://wwwbdbiosciences.com) and deposited in the center of 12-well tissue culture plates. After solidifying the Matrigel for 20 minutes at 37°C, gels were covered in salisphere medium as defined above. New spheres appeared 2–3 days postseeding of single cells in Matrigel. One week after seeding, Matrigel was dissolved by incubation with Dispase enzyme (1 mg/mL for 30 minutes to 1 hour at 37°C; Sigma). Spheres released from the gels were processed to a single cell suspension using 0.05% trypsin-EDTA, cell number and sphere number noted, and encapsulation in Matrigel repeated. This cycle was repeated up to five times (5 passages). Cell numbers seeded at the start of each passage and harvested at the end were used to calculate the number of population doublings that had occurred, using the following formula, where \( PD = \frac{\ln(\text{harvested cells/seeded cells})}{\ln{2}} \).

For time-lapse microscopy, single cells were seeded as described, and imaged every hour for 96 hours, using the Zeiss 780 confocal inverted microscope.

### Organoid Differentiation

Single cell-derived salispheres were encapsulated in a three-dimensional matrix consisting of a 60:40 ratio of Type I rat tail collagen to growth factor reduced Matrigel (BD Biosciences). After solidifying the gel at 37°C for 20 minutes,
salisphere medium containing 10% fetal calf serum (FCS, Invitrogen) was added and cultures maintained for up to 3 weeks.

**Immunostaining**

Organoids were removed from plate and fixed in 4% formaldehyde (FA; 20 minutes, 4°C), before washing, embedding in paraffin wax, and processing to 5 μm sections. Transplanted murine SGs were either snap-frozen in liquid nitrogen, stored at −80°C and processed to 8 μm cryostat sections or FA fixed (24 hours, room temperature [RT]), and processed for paraffin sections. After air drying, frozen sections were fixed in 4% FA (10 minutes, RT), and washed with phosphate buffered saline (PBS). Hematoxylin and Eosin staining was performed according to standard protocols. For PKH26 cell-tracing, nuclear counterstaining with 0.2 μg/mL 4’,6-diamidino-2-phenylindole (DAPI; 10 minutes, RT) was performed, and sections visualized in the phycoerythrin-fluorescence channel. An average of 100 slides per SG were cut, and PKH26 screening performed every 10 slides. For immunohistological analysis of frozen tissues, relevant primary antibodies were added to fixed tissue in PBS (2 hrs, RT), washed thrice with PBS, incubated with secondary antibodies (1 hr, RT) and counterstained with DAPI as above. Dilutions of primary antibodies used for immunostaining of frozen sections were: mouse anti-human nuclei (1:50, Chemicon, Temecula, CA, http://www.chemicon.com; clone 235-1); mouse anti-cytokeratin (1:00, Abcam, Cambridge, U.K., http://www.abcam.com; clone AE1/AE3); rabbit anti-human α-amylase (1:100, Sigma, polyclonal); rabbit anti-aquaporin-5 (1:100, Abcam polyclonal). Secondary antibodies were goat anti-mouse-Alexaflour-488 or goat anti-rabbit-Alexaflour-594 conjugates, used at 1:300 dilution. Fluorescent stainings were performed according to standard protocols. For PKH26 cell-tracing, nuclear counterstaining with 0.2 μg/mL 4’,6-diamidino-2-phenylindole (DAPI; 10 minutes, RT) was performed, and sections visualized in the phycoerythrin-fluorescence channel. An average of 100 slides per SG were cut, and PKH26 screening performed every 10 slides. For immunohistological analysis of frozen tissues, relevant primary antibodies were added to fixed tissue in PBS (2 hrs, RT), washed thrice with PBS, incubated with secondary antibodies (1 hr, RT) and counterstained with DAPI as above. Dilutions of primary antibodies used for immunostaining of frozen sections were: mouse anti-human nuclei (1:50, Chemicon, Temecula, CA, http://www.chemicon.com; clone 235-1); mouse anti-cytokeratin (1:00, Abcam, Cambridge, U.K., http://www.abcam.com; clone AE1/AE3); rabbit anti-human α-amylase (1:100, Sigma, polyclonal); rabbit anti-aquaporin-5 (1:100, Abcam polyclonal). Secondary antibodies were goat anti-mouse-Alexaflour-488 or goat anti-rabbit-Alexaflour-594 conjugates, used at 1:300 dilution. Fluorescent stainings were performed using the Leica 6000 series microscope or the Leica TCS SP8 confocal laser scanning microscope and Leica Application Suite software. For immunostaining on paraffin wax-embedded human submandibular gland sections and transplanted glands, sections were boiled for 10 minutes in preheated 10 mM sodium citrate buffer (pH 6.0) containing 0.05% Tween 20 and washed prior to primary antibody exposure. No antigen retrieval was necessary for organoid sections. Dilutions of primary antibodies for paraffin-section immunostainings were: rabbit anti-mouse Ki67 (1:100, Cell Marque); rabbit-anti mouse fibronectin (1:500, Millipore, Billerica, MA, http://www.millipore.com), mouse anti-mouse β-catenin (1:100, BD Laboratories). Secondary conjugates as above, nuclear counterstaining was performed with DRAQ5 (1:1,000, BD Laboratories).

**Masson’s Trichrome Staining**

Tissue sections of 5 μm were incubated in Erhlich’s Hematoxylin for 5 minutes and rinsed in tap water. Sections were then incubated for 5 minutes in 1% (w/v) acid fuchsien (Sigma Aldrich) in 1% (v/v) glacial acetic acid (aq) with 1% Ponceau Xylidine (Sigma Aldrich) in 3% glacial acetic acid (aq). After washing in deionized water, sections were incubated for 1 minute in 1 % Aniline Blue (Klinipath, Duiven, Netherlands, http://www.klinipath.nl) in 3% glacial acetic acid (aq), washed in deionized water, and finally incubated for 5 minutes in 1% (w/v) molybdenum phosphoric acid (aq) (Alfa Aesar, Halerville, MA, https://www.alfa.com) and washed again. Sections were dehydrated and mounted as standard.

**Quantitative Polymerase Chain Reaction**

Genomic DNA was extracted from human salisphere-transplanted SGs at relevant time points, using the Qiagen DNeasy Blood and Tissue kit and adjusted to a concentration of 5 μg/mL (Qiagen, Valencia, CA, https://www.qiagen.com). Total RNA was extracted using the Qiagen RNeasy MiniKit. For cDNA generation, 1 μg of total RNA was reverse transcribed using 0.5 μg oligo(dT) 15-18 primers, 0.5 mM dNTPs, 1 × First-strand Buffer, 0.01 M dithiothreitol, 400 U RnaseOut, and 200 U of M-MLV Reverse Transcriptase (all Invitrogen), in a total volume of 20 μL per reaction. Quantitative polymerase chain reaction (Bio-Rad, Hercules, CA, http://www.bio-rad.com) (qPCR) was performed using Bio-Rad iQ SYBR Green Supermix according to manufacturer’s instruction, primers at a final concentration of 1.67 μM and 25 ng load of genomic DNA or cDNA per reaction. Primer sequences are listed in Supplementary Information Table S1. For detection of human mitochondrial DNA, a standard curve was generated using a dilution series of human genomic DNA in mouse genomic DNA, with a constant load of 25 ng DNA per qPCR reaction. A two-step qPCR reaction using the Bio-Rad iCycler was used to amplify human mitochondrial DNA, and approximate proportion of human cells in transplanted glands inferred from standard curve.

**Irradiation, Cell Transplantation, and Saliva Collection of/into/from Mouse SG**

All mice were housed in individually ventilated cages and in accordance with the Wet op de dierproeven (1977). Mice were fed ad libitum. SGs of NOD.Cg-Pkdcre+/−Il2rg−/−Mm1S129 (NSG) mice were locally irradiated with a single X-ray dose of 5 Gy under isofluorane anesthesia. This dose ablated function of SGs without compromising general health of the animals. Cell transplantation was performed 1 month following irradiation. Cultures of human salispheres between 3 and 5 days postirradiation were trypsinized to single cell suspensions using 0.05% trypsin-EDTA and labeled with PKH26 Red Fluorescent Cell Linker Kit (Sigma). Upon cell proliferation, the intensity of the PKH26 labeling is halved, hence PKH26-mediated fluorescence indicates proliferative abilities of the cells. Following labeling, cells were suspended in a 5 μL volume of alpha-modified eagle’s medium with 2% FCS. For transplantation, a 5 mm incision was made in the neck of NSG mice under isofluorane anesthesia and the submandibular SG exposed. 500, 5,000, or 50,000 human salisphere cells per gland were injected into the submandibular SG, whereafter the wounds were closed by suturing. At 1, 2, and 3 months postirradiation, whole stimulated saliva was collected from transplanted and control animals. Two mg/kg pilocarpine was administered subcutaneously to the animals, and saliva collected by suction pump for 15 minutes. The quantity of saliva was determined gravimetrically, assuming a density of 1 g/mL saliva and normalized to the weight of the animal and preirradiation saliva flow rate.

**Genome-Wide Expression Analysis**

Genome-wide gene expression was profiled in NSG submandibular SGs transplanted with 100,000 human or autologous NSG salisphere cells per animal, at 1 week posttransplantation. Time-matched irradiated controls were used.
to eliminate effects of radiation on the transcriptome. Samples were analyzed in triplicate. Total RNA was isolated using the RNeasy Mini Kit. Highly pure total RNA (300 ng/sample) was used for expression profiling on Illumina WG6 v2.0 expression bead chip kit. RNA was amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, http://www.ambion.com/Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) and hybridized to Sentrix MouseWG-6 Version 2.0 expression beadchips (Illumina, San Diego, CA, www.illumina.com) according to the manufacturer’s instructions. Hybridization and washing were performed by in-house Genome Analysis Facility (University Medical Centre Groningen). Scanning was carried out on the iScan System (Illumina). Data were extracted using GenomeStudio software (Illumina). The data were normalized using the R version 3.0.1 norm function of the BioConductor version 2.12 library limma 3.16.5 (Smyth, 2005) [37] by control background correction, quantile normalization, and log2 transformation and batch effects between arrays. Differential expression analysis was performed using the eBayes function of the BioConductor version 2.12 library limma and an adjustment method BH (Benjamini Hochberg) to exclude false positives, with a p value of .05. Assignment of probes upregulated at 1 week post-transplantation to biological pathways was performed using ENrichR online resources (http://amp.pharm.mssm.edu/Enrichr/).

**Statistical Analysis**

A two-way ANOVA and Bonferroni post hoc test with x values of 0.05 were applied to the time course analysis of saliva flow. n numbers for tested groups are stated in figure legend. A non-parametric one-way ANOVA (Kruskal Wallis test) and Dunn’s post hoc testing with x values of 0.05 was applied to qPCR data in Figure 4. Additional methods for supplementary figures can be found in Supplementary Information file.

**RESULTS**

**Single Cell-Derived In Vitro Self-Renewal and Organoid Formation from Human SG Stem Cells**

Human salispheres were cultured from healthy human SG biopsies according to an optimized previously published protocol [36]. Primary human salispheres cultured from such mechanically and enzymatically dissociated human submandibular (SG) biopsies grew in size over time (Fig. 1A), in a similar manner to those from the mouse [12, 16]. These cells were actively dividing as indicated by abundant expression of proliferating cell nuclear antigen (Supplementary Information Fig. S1A, S1B). Culturing primary human salispheres did not induce karyotypic abnormalities, as demonstrated by karyotype spread analysis (Supplementary Information Fig. S2).

To determine whether human salispheres contain stem/progenitors, in vitro self-renewal and differentiation potential was assessed. When primary human salispheres were enzymatically dispersed into single cells, they were able to form secondary human salispheres in a 3D matrix (Fig. 1A; Supplementary Information Video 1). Moreover, this procedure could be repeated for at least 5 passages and up to 10 passages in some cases, indicating extensive self-renewal potential. The maximum salisphere cloning frequency was 4.37% ± 1.09% SEM at passage 3 (Fig. 1A, 1B). When self-renewal potential declines, we observed an increase in apoptotic cells (Supplementary Information Fig. S3), indicating that our culture conditions are not optimal yet for long-term in vitro self-renewal. Thus, human salispheres derived from clinical biopsies contain cells that are able to self-renew in vitro at the single cell level. To evaluate the potential of human salispheres cells to generate functionally mature SG cell lineages, we performed in vitro differentiation studies. Some differentiation into mucin producing acinar cells was observed in salispheres themselves (Supplementary Information Fig. S1C), but transferring single cell-derived human salispheres to a Matrigel/collagen matrix induced formation of organoids with SG structures, with branching occurring as early as 2 days after transfer (Fig. 1C). After 12 days, complex structures developed which contained both branching and lobular structures (Fig. 1C, 1D). Moreover, branches expressed the ductal cell marker Cytokeratin (Fig. 1E; Supplementary Information Fig. S4) while lobular structures expressed α-amylase (Fig. 1F; Supplementary Information Fig. S4), and aquaporin-5 (AQP-5; Fig. 1E), a water channel protein expressed in the apical membrane of acinar cells, indicative of differentiation into multiple SG lineages. Collectively, the in vitro data demonstrate that human salispheres contain stem/progenitor cells capable of both self-renewal and multilineage differentiation.

**In Vivo Proliferation and Differentiation of Human Salisphere Cells**

Next we investigated the regenerative potential of human salispheres in vivo. Immunodeficient NOD/SCIDIL2Rg−/− (NSG) mice were locally irradiated with 5 Gy in the neck region. After 1 month, mice received intra-submandibular SG transplantsations of 500, 5,000, or 50,000 enzymatically dispersed human salisphere cells per gland, isolated from 3–5 day cultured primary human salisphere cells. Both glands in each mouse received equal cell numbers, so that a total cell number of 1,000, 10,000, or 100,000 cells were transplanted per recipient mouse. At least seven animals per group were transplanted with human salisphere cells obtained from at least three donors, which were transplanted separately (see scheme in Fig. 2A). Prior to transplantation human salisphere cells were labeled with the PKH26 fluorescent cell membrane dye allowing visualization of donor cells after transplantation (Supplementary Information Fig. S5A). PKHfluorescent cells were found scattered throughout the gland 1 day after injection (Fig. 2B). Injection of PKH26 alone did not result in labeling of the SG (Supplementary Information Fig. S5B). At 2–3 months post-irradiation, scattered cells and foci of PKH26-labelled cells could be observed, with a peripheral dilution of PKH26-labelling intensity (Fig. 2C, 2D), indicating proliferation of the transplanted cells. Within PKH26 foci, duct-like arrangements of PKH26 cells were present, suggesting cellular organization into functional units (Fig. 2E). Immunostaining with an antibody specific to human nuclei revealed colocalization with PKH26 foci, confirming that PKH26 foci are transplanted human salisphere cells (Fig. 2F, for specificity see Supplementary Information Fig. S5C). Human cells were also detected beyond PKH26 foci, indicating that the label was diluted below the detection threshold (Fig. 2G). Quantification of PKH26 foci revealed
that 12% ± 7% and 36% ± 8% of foci cells displayed recognizable ductal (rectangular narrow shape, arrangement around lumen) or acinar (triangular shape with basally located nucleus) cell morphologies, respectively (Fig. 3A–3C; Supplementary Information Fig. S5D). Quantification of MHC Class I labeled cells mirrored this distribution (4% ± 5% ductal and 10% ± 8% acinar cells, Supplementary Information Fig. S5E). Having established that PKH26<sup>+</sup> cells are transplanted human cells with an ability to form organized structures, we then examined expression of marker proteins associated with the functional human SG. Differentiation of the transplanted cells into functionally mature tissue in the recipient gland was indicated by the colocalization of α-amylase, AQP-5, and cytokeratins with, or in close proximity to, PKH26<sup>+</sup> cells (Fig. 3, for specificity of antibodies see Supplementary Information Fig. S4). These in vivo data demonstrate that human salisphere derived cells are capable of proliferation, differentiation, and long-term engraftment after xenotransplantation into an irradiated environment.

**Rescue of Hyposalivation by Human Salisphere Cells**

To assess the functionality of the transplanted human salisphere cells, we determined pilocarpine stimulated whole saliva flow rate in the transplanted animals (Fig. 2A).
Figure 2. Transplanted human salisphere (hS) cells proliferate in the irradiated mouse SG. (A): Scheme of workflow. (B): hS cells visualized using the PKH26 label at 1 day post-SG transplantation (1 month + 1 day post-irradiation). At 2 months post-irradiation, PKH26<sup>+</sup> cells were either not observed, detected as scattered cells, or detected as organized foci (C, summarized in D). (E): When PKH26<sup>+</sup> foci were observed, dilution of the PKH26 label could be seen, indicative of proliferation of human salisphere cells. High resolution microscopy of white-boxed inset areas are shown. *Marks PKH26<sup>+</sup> putative duct structure. (F): Colocalization of anti-human nuclei (AHN) immunostaining with PKH26 label 2 months post-irradiation. (G): AHN immunostaining and correlation with PKH26 foci at different sites within a human salisphere transplanted gland. Numbers 1–3 correspond to boxed numbered regions in first panel. Scale bars = 100 μM (B, C, and E) and 25 μM (high resolution E). Scale bars = 50 μM, 500 μM, and 50 μM (in F and G, respectively). Nuclei counterstained with DAPI where applicable. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; SG, salivary gland.
irradiated nontransplanted animals saliva production dropped to 46% ± 4% of pre-irradiation values and was further maintained at 49% ± 4% at 3 months post-irradiation. Mice receiving sham transplantations of PBS displayed a similar reduction in saliva production, to 51% ± 5% at 3 months post-irradiation (Supplementary Information Fig. S7A). Primary human salispheres were isolated and transplanted 2–5 days following isolation. Saliva flow in mice transplanted with 100,000 human salisphere cells increased significantly to 79% ± 8% (p < .001) and 70% ± 7% (p < .05) of pre-irradiation saliva flow at 2 and 3 month human salisphere post-irradiation, respectively (Fig. 4A). Area under curve analysis demonstrated a significant (p < .01) increase in saliva production in human salisphere transplanted mice, compared to irradiated controls (Fig. 4B). Increasing the number of transplanted cells led to an increase in gland functionality, emphasizing the cell dose-dependent nature of the hyposalivation rescue (Fig. 4C). The range of functional responses to human salisphere transplantation can be partly explained by the heterogeneous nature of human biopsy material, as transplants from some donors were more efficacious than others (Supplementary Information Fig. S7B). Transplantation of human salisphere cells also

Figure 3. Transplanted human salisphere cells differentiate and express proteins associated with the human salivary gland in the irradiated mouse salivary gland. (A–C): Representative images of acinar and ductal-like cells quantification in PKH26 foci. (A) PKH26 foci and (B) same PKH26 as analyzed for acinar (yellow line) and ductal (green line) cells presence. Boundaries of analyzed foci are denoted with a white dashed line. (C): Quantification of proportion of acinar- and ductal-like cells in PKH26 foci. PKH26 foci at three depths within each transplanted glands were analyzed, from a total of three separate transplanted glands. Scale bars = SEM. (D–F): Immunostaining for the acinar cell marker proteins (D) α-amylase, (E) AQP-5, and (F) ductal cell-associated proteins cytokeratins colocalizes with or is in close proximity to PKH26 foci in cryostat sections of recipient gland, 2 months post-irradiation. White boxed insets are shown in high resolution. Scale bars = 50 μm. Abbreviations: AQP-5, aquaporin 5; DAPI, 4',6-diamidino-2-phenylindole.
increased the wet weight (p < .05) of transplanted glands in a time-dependent manner when compared to time-matched irradiated controls and sham transplanted animals (Fig. 4D).

To examine the effect of human salisphere cell therapy on the regenerative potential of SGs, we assessed the possibility of irradiated and transplanted glands to generate new primary salispheres in culture. Irradiation alone reduced salisphere formation to 33% ± 7% SEM of age-matched non-irradiated controls (Fig. 4E, "0" group), reflecting a deficit in resident SG stem/progenitor cells. Two months after irradiation, animals receiving 100,000 cells demonstrated enhanced salisphere formation compared to cultures generated from the irradiated control group (Fig. 4E). This effect was further dependent on transplanted cell dose, as mice receiving 1,000 or 10,000 cells showed less salisphere generation capability (Fig. 4E). Salisphere cultures from transplanted glands could furthermore be maintained in vitro in 3D cultures for more passages, producing more salispheres than nontransplanted glands, indicating a higher proliferative potential (Fig. 4F). These data provide further evidence that human salisphere transplants replenish SG stem/progenitor cell populations.

Previous reports from our lab and others have suggested that stem and progenitor populations reside within salisphere cultures from rodent SGs, however this remains unresolved in human salisphere cultures [9, 12, 34]. In order to assess the potential of stem/progenitor cell populations within the human salisphere pool, we selected cells expressing the established stem cell marker protein c-Kit, from human salisphere cultures. c-Kit

Figure 4. Transplanted hS cells are capable of rescuing radiation-induced hyposalivation and salisphere count, in a mouse model. (A): Salispheres in time course analysis of relative saliva production, in comparison to control and irradiated control animals Statistical analysis is shown in comparison to irradiated control group (***, p < .001; *, p < .05 at relevant time point. n = ≥18 animals per time point in hS group and ≥9 for control groups. Scale bars = SEM. (B): Area under the curve analysis of saliva flow data in (A), **, p < .01, Student’s t test. Scale bars = SEM. (C): Relative saliva production in animals transplanted with 100, 10,000, or 100,000 hS cells per mouse, compared to nontransplanted, irradiated animals ("0" group). Data are normalized to animal weight. Scale bars = SEM. n ≥ 7 mice per group. *, p < .05, Student’s t test. (D): Wet weights of submandibular salivary glands (SGs from irradiated (irr), irradiated-transplanted (irr+hS), and sham-transplanted (sham) mice at 1, 2, and 8 weeks following irradiation. Transplanted mice received 100,000 human salisphere cells per animal. n ≥ 3 mice per control and sham groups and n ≥ 10 for hS transplanted groups. *, p < .05, Student’s t test. (E): Salisphere cultures from mouse SGs transplanted with 1,000, 10,000, or 100,000 hS cells per gland harvested at three months post-irradiation and compared to age-matched nontransplanted, irradiated control group ("0"). Scale bars = SEM. n ≥ 7 mice per group. *, p < .05, Student’s t test. (F): Self-renewal assay using mouse SGs transplanted with 100,000 hS cells per animal, harvested 3 months post-irradiation. n = 9 animals in transplant group, n = 4 and 5 in control and irradiated control groups, respectively. Error bars represent SD.
age, in agreement with our previous studies showing decrease in overall primary salisphere yield with age [36, 38]. These data suggest that other, more abundant stem cell marker proteins may be more suitable (Fig. 5C).

To investigate to what extent the observed functional recovery was dependent on the continued presence of the human salisphere-derived cells, human chimerism was assessed using qPCR and primers specific for human mitochondrial DNA (for assay design see Supplementary Information Fig. S8). Human cells were detected at up to 9% ± 3% SEM of total cells following transplantation, a frequency which suggests the functional rescue observed might not completely be attributed to human cell engraftment. Regenerative signals emanating from the endogenous murine cells following human salisphere transplantation might also contribute to rescue. Analysis of RNA extracted from human salisphere-transplanted SGs 1 week post-transplantation, when human cells were most easily detected, revealed 471 probes upregulated, in comparison to time-matched irradiated control SGs (GEO accession number: GSE72871). The predominant function of these genes however appeared to be immune rejection/reaction (Fig. 6A; Supplementary Information Fig. S9A). Apparently, NSG mice still exhibit an immune response, which may explain spread in engraftment observed and the reduction in human cells present in the recipient tissue over time (Fig. 6B). Whole genome mRNA expression analysis of syngeneic salisphere transplantation (NSG salispheres into NSG SGs) was performed to circumvent the immune rejection phenotype, and revealed significant (p < .05) upregulation of 510 genes at 1 week following salisphere transplantation.

Figure 5. Human salispheres contain a subpopulation of c-Kit<sup>+</sup> cells capable of organoid formation and functional rescue. (A): Single c-Kit<sup>+</sup> cell derived organoids cultured for 10 days. Scale bar = 50 μM. (B): Relative saliva production in mice receiving 100, 1,000, or 100,000 unselected human salisphere cells total, no cell transplant (“0” group), or 600 c-Kit<sup>+</sup> cells per gland. Scale bars = SEM. n ≥ 7 mice per group. *, p < .05, Student’s t test. Data are normalized to pre-irradiation saliva production value for each animal. (C): Frequency of c-Kit<sup>+</sup> cells in patient biopsies grouped by age.
compared to irradiated controls (Fig. 6C, 6D) (GEO accession number: GSE72871). Using a significance threshold of \( p < .05 \) and a minimal fold increase of 2, upregulated genes could be functionally organized into tissue remodeling (including extracellular matrix (ECM)-, cytoskeletal- and cell junction-associated genes) and proliferation-associated genes, in addition to highly upregulated expression of the canonical Wnt-signaling regulator \( \beta\)-catenin (Fig. 6E). Further analysis demonstrated a moderate upregulation of soluble factors associated with SG branching and development (transforming growth factor (TGF)b1, TGFb3, bone morphogenetic protein1) [38, 39], SG stem cell maintenance (insulin growth factor, EGF) (Supplementary Information Fig. S9B), and SG functionality (\( \alpha\)-amylase, AQP-1, DCPP2, DCPP3, PSP) (Fig. 6F; Supplementary Information Fig. S9C). In order to translate these data to xenotransplants of human salisphere cells, we returned to check the expression of genes identified in Figure 6C–6E in NSG glands transplanted with human salisphere cells. Indeed, murine-specific transcripts upregulated in syngeneic salisphere microarray analysis, including regulators of the Wnt pathway, were also upregulated in the human salisphere xenotransplantation microarray (Fig. 7A, 7B). To assess the expression of some of the translated proteins, we performed immunohistochemical and histological stainings on human salisphere-transplanted NSG SGs. We observed \( \beta\)-catenin expression, the key mediator of the canonical Wnt-signaling pathway, in the

---

Figure 6. Gene expression profiles of SGs 1 week following human and murine salisphere transplantation. (A): Top 20 upregulated genes in human salisphere (hS)-transplanted glands, following whole genome microarray analysis and compared with irradiated control mice. "Upregulated" defined as significantly different expression of a gene \( (p < .05) \) from irradiated control group, with a minimal fold increase in expression of 2. (B): Detection of human mitochondrial DNA in hS-transplanted SGs by quantitative polymerase chain reaction. Each data point represents a separate mouse, all mice received 100,000 hS cells. (C): Heat map showing differential expression of genes in NSG mice transplanted with autologous NSG salispheres (irr+r-mS), compared to irradiated (irr) NSG controls. Numbers represent biological replicates. (D): Top 20 upregulated genes in mS-transplanted glands following whole genome microarray analysis and compared with irradiated control mice. "Upregulated" defined as (B). (E): Functional grouping of upregulated genes from mS-transplanted glands. Grouping was performed using EnrichR software, grouping genes into functional related clusters. Gene acronyms are standard Gene Symbol abbreviations. All data are relative to irradiated control expression (irr) and above a fold increase of 2 (threshold). \( n = 3 \) biological replicates in transplanted and control group, errors bars = SEM. (F): Expression of SG-associated \( \alpha\)-amylase in NSG control (con), SG-irradiated and salisphere-transplanted (irr+r-mS), and time-matched irradiated NSG glands (irr). \( n = 3 \) biological replicates, Student’s t test, * \( p < .05 \). Error bars = SEM. Abbreviations: ECM, extracellular matrix; SG, salivary gland.
ducts of human salisphere transplanted glands, whereas it was lost in irradiated but nontransplanted glands (Fig. 7C). Similarly, immunostainings for collagen (Fig. 7D) and fibronectin (Fig. 7E) validated the microarray data suggesting upregulation of extracellular matrix proteins, and Ki67 labeling further confirmed the presence of more proliferating cells in both ducts and acinar cell compartments of human salisphere-transplanted glands (Fig. 7F; Supplementary Information Fig. S9A, S9B). Interestingly, when c-Kit+ cells were transplanted and mice sacrificed at the same time point (1 week following transplantation), increased expression of some genes in this regeneration-associated cohort, including the Wnt signaling reporter Axin-2 was observed (Supplementary Information Fig. S11A, S11B). This suggests that a small number of selected c-Kit+ stem/progenitor cells have similar potencies as unselected human salisphere cells, with respect to initiating functional recovery of the gland. The enhanced expression of these genes in recipient SGs at 1 week after c-Kit+ salisphere cell transplantation and the greater rescue in SG function at 2 months following transplant, compared to unselected cells (Supplementary Information Fig. S11C) would suggest indeed that subsets of human salisphere cells such as c-Kit+ cells are superior in inducing regeneration than others.

These combined data suggest that transplanted human salisphere-derived cells restore homeostasis of the SGs by a combination of engraftment, proliferation, differentiation, and potential stimulation of recipient cells and that salispheres contain within them subsets of cells with greater regenerative potential.

**DISCUSSION**

Adult stem cells hold great therapeutic promise in regenerative medicine, however, translation to the clinic is hampered by rarity of data showing engraftment and functional capabilities of these cells. Here, we characterize for the first time the
pulmonary, differentiation, and regenerative potential of stem/progenitors from the human SG, at the single cell level in vitro, including their long-term engraftment capabilities in vivo. We additionally demonstrate that a population of more potent stem cells, namely c-Kit+ cells, exists within the human SG stem cell pool. These cells, capable of rescuing hyposalivation after low cell dose transplantation, are the first documentation of in vivo functional properties of a defined stem cell population from the adult human SG. Our results follow on recent studies in the murine SG, where c-Kit+ cells rescued function of the irradiated SG and differentiated and engrafted within the recipient mouse [12, 16, 20]. Of note, 300 transplanted c-Kit+ murine SG stem cells per SG were capable of rescuing hyposalivation in vivo, comparable with 500 human c-Kit+ cells per SG [12]. Indeed, we have previously shown the expression of a panel of stem cell associated marker proteins in human salispheres, suggesting that defining the specific stem cell may permit even greater therapeutic potential for the treatment of hyposalivation [34]. While many stem cell populations have been characterized in mouse tissues and some have been tested in human stem cell populations, few human stem cell populations have shown engraftment and functional rescue potential such as that presented in this study [19, 39–44].

Further to these data, we also suggest that transplantation of human salispheres and consequential regeneration might involve stem cell-associated signals for the recipient SG. Our expression analysis revealed enhanced expression of ECM protein expression and stimulation of endogenous stem cell proliferation as probable mediators of this effect. Several studies suggest that murine SG stem cells are receptive to such signaling from TGF/BMP, FGF, and Wnt signaling pathways [18, 45–50]. The low radiation dose (5 Gy) used to induce SG hypofunction in this study (chosen because of the severity of esophageal tract side effects in NSG mice irradiated with higher doses) permits the survival of endogenous murine SG stem/progenitor cells with regenerative potential, which might be amenable to signaling stimuli from transplanted human salispheres. Indeed, the study by Hai et al. demonstrated recovery of the post-irradiation murine SG following transient Wnt pathway activation (and not following irradiation alone), lending further weight to our hypothesis that Wnt signaling plays a critical role in functional recovery of radiation-damaged SG [47].

We present the first evidence showing therapeutic potential of a new population of clinically relevant adult human stem cells. Syngeneic transplantation studies in the mouse have demonstrated long-term engraftment potential of salispheres, and we predict great engraftment potential of human salispheres cells, and amelioration of xerostomia, when autologous transplantations are performed. The autologous setting may provide better incorporation of the transplanted cells in SGs in patients in which few or no resident stem cells survive [12, 16, 20]. Surviving endogenous patient cells might additionally benefit from stimulation via transplanted salisphere cells and provide a boost to SG function [51].

**Conclusion**

In summary, we showed the presence of SG stem/progenitor cells in cultured human salispheres. These cells are capable of self-renewal and differentiation, which when transplanted into irradiated recipients, restore glandular function. The present data highlight the promising therapeutic potential of human SG stem/progenitor-like cells cultured from biopsy material for treatment of radiation-induced hyposalivation.

**Acknowledgments**

This work was supported by grants from The Netherlands Organisation for Health Research and Development (ZonMW-Grant nr. 11.600.1023), the Netherlands Institute for Regenerative Medicine (NIRM, Grant No. FES0908), and the Dutch Cancer Society (RUG2008-4022). We thank the help from Dr. Viktor Guryev (European Research Institute for the Biology of Ageing, The Netherlands), for design of anti-human mitochondrial primers, Dr. Leonid Bystrykh for microarray study guidance, Hette Faber for irradiation and the maxillofacial surgical team (University Medical Center Groningen and Medical Center Leeuwarden) for donor biopsies. We also thank Arjan Vis-sink and Hans Langendijk (University Medical Center Groningen) for fruitful discussions of data, and the Genetics Department of the University Medical Center Groningen for their support with karyotype analysis.

**Author Contributions**

S.P.: conception and design, collection and/or assembly of data, data analysis and/or interpretation, and manuscript writing; M.M., M.v.d.Z., and D.v.G.: collection and/or assembly of data and data analysis and/or interpretation; M.A.S.: conception and design and provision of study materials or patients; E.Z.: data analysis of interpretation; M.J.H.W.: conception and design and provision of study materials or patients; G.d.H. and R.v.O.: conception and design and manuscript writing. R.P.C. conception and design, data interpretation, financial support, and manuscript writing.

**Disclosure of Potential Conflicts of Interest**

The authors indicate no potential conflicts of interest.