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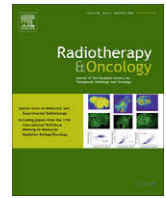
Experimental radiobiology

Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation

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A B S T R A C T

Background: Recently, we showed that transplantation of 100–300 c-Kit^{+} stem cells isolated from cultured salispheres ameliorates radiation-damage in murine salivary glands. The aim of this study is to optimize and translate these findings from mice to man.
Methods: Mouse and human non-malignant parotid and submandibular salivary gland tissue was collected and enzymatically digested. The remaining cell suspension was cultured according to our salisphere culture method optimized for murine salispheres. Salisphere cells were tested using 3D matrix culturing for their *in vitro* stem cell characteristics such as the potential to differentiate into tissue specific cell types. Several potential mouse and human salivary gland stem cells were selected using FACS.
Results: In human salivary gland, c-Kit^{+} cells were only detected in excretory ducts as shown previously in mice. From both human parotid and submandibular gland cell suspensions salispheres could be grown, which when placed in 3D culture developed ductal structures and mucin-expressing acinar-like cells. Moreover, cells dispersed from primary salispheres were able to form secondary spheres in matrigel, a procedure that could be repeated for at least seven passages. Approximately 3000 c-Kit^{+} cells could be isolated from primary human salispheres per biopsy.
Conclusion: Human salivary glands contain a similar 'putative' stem cell population as rodents, expressing c-kit and capable of *in vitro* differentiation and self-renewal. In the future, these cells may have the potential to reduce radiotherapy-induced salivary gland dysfunction in patients.

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Radiotherapy of head and neck tumors commonly induces hyposalivation which in most cases is an irreversible side effect resulting in complaints such as xerostomia (the subjective sensation of a dry mouth). Secondary to this, most patients develop problems such as an increased susceptibility to oral infections and dental caries, impeded swallowing and speech, and nocturnal oral discomfort which have a major detrimental impact on health-related quality of life of these patients [18,19]. Even with the most advanced radiation techniques, such as Intensity-Modulated Radiation Therapy (IMRT), still approximately 40% of these patients develop hyposalivation and consequential life-long complaints [17]. To date, still no satisfactory clinical management of xerostomia exists.

Radiation-induced sterilization of the primitive glandular stem cells which prevents the replenishment of saliva-producing cells seems to be the main cause for irreversible late hyposalivation

[4]. Transplantation of salivary gland stem cells potentially offers a therapy to rescue irradiated salivary glands. For many tissues experimental forms of stem cell transplantation have been shown to result in improvement of tissue function, such as that for bone marrow, skin [2,12], mammary gland [13,15], eye [14], kidney [4], brain [10], and heart [1]. However, only hematopoietic stem cell transplantation and epithelial stem cell-based treatments for skin burns and corneal disorders are routinely applied clinically.

The first attempt to isolate and transplant rat salivary gland stem/progenitor cells was described in 2004, but failed to show formation of saliva-producing acinar cells or functional improvement [16]. Later, stem cells that were selected using the stem cell markers Sca-1, c-Kit or $\alpha6^+\beta1^+$integrin cells from, respectively, mouse [5,11] and rat [6] glands were shown to contribute to tissue regeneration when transplanted via the portal vein into partially hepatectomized animals. The first success in developing an *in vitro* stem cell assay based on colony forming units was performed in 2006 when Kishi and colleagues developed a clonal colony assay for salivary gland cells [6]. However, due to the lack of *in vivo* functional assessment, these as such determined colony forming cells could not be defined as stem cells [6]. Recently, how-

ever, in a series of studies [3,7,9], we showed that salivary glands do contain cells that when stimulated appropriately have the capacity to regenerate damaged tissue after irradiation. Moreover, we developed a culture and isolation procedure to obtain a c-Kit$^+$ cell population from mice submandibular glands that contain stem cells which when transplanted in irradiated salivary gland, prominently restored function and morphology [8].

The aim of this study was to expand our findings to human salivary glands for future development of clinical application. Since this therapy might be applicable to older patients, we also investigated the relationship between age and the number of salivary gland stem cells.

Materials and methods

Animals

Female C57BL/6 mice, 2–3 months of age, were purchased from Harlan (Horst, Netherlands). Salivary glands of 24–26 months old mice were kindly provided by Dr. Brad Dykstra (Department of Cell Biology, section Stem Cell Biology, University Medical Center Groningen, the Netherlands). The mice were kept under clean conventional conditions, and fed *ad libitum* with food pellets (RMH-B, Hope Farms B.V., Woerden, The Netherlands) and acidified tap water (pH 2.8). All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

Salivary gland irradiation

Salivary glands were locally irradiated with a single dose of 15 Gy of X-rays (Philips CMG 41 X, 200 kV, 10 mA, 5 Gy/min). Mice were protected from off-target radiation by a 3 mm lead shield. 15 Gy is a dose known to induce sufficient damage without compromising the general health of the animals. Four days post-irradiation, mice were sacrificed and glands were collected for further investigation.

Irradiated human salivary gland tissue was obtained from a patient (after informed consent) with a squamous cell carcinoma of the oral cavity in whom a neck dissection procedure was performed. This patient was operated and irradiated 15 years ago for an oropharyngeal tumor T4N1M0.

Isolation of salivary gland cells

Salispheres were obtained as previously published [8]. In short, mice were euthanized immediately after blood removal (via cardiac puncture) under full anesthesia (N$_2$O/O$_2$/isoflurane). Submandibular glands were dissected carefully, avoiding contamination from other tissues. A cell suspension was prepared by mincing and enzymatical dissociation with collagenase type II (0.025%), hyaluronidase (0.04%) and CaCl$_2$ (6.25 mM) at 37 °C for 40 min with gentle mechanical movement. After an additional 40 min of fresh enzyme digestion, the tissue cell suspension was filtered with a 100 μm and a 50 μm mesh using a 25G needle, and plated in non-coated 12-well plates at 400,000 cells per well. Per gland, between 2 and 4.6 × 10^6 cells were isolated. The culture medium consisted of DMEM/F12 (Invitrogen, Carlsbad, CA; 11320–074), penicillin, streptomycin, glutamax, EGF (20 ng/mL), FGF-2 (20 ng/mL), N-2 (serum-free supplement for culturing neural cells) (1/100), insulin (10 μg/mL) and dexamethasone (1 μM) further depicted as salivary gland medium (SGM). Fresh medium was added every three days. All growth factors were purchased from Sigma–Aldrich (St. Louis, MO), except for N-2 (Invitrogen, Carlsbad, CA).

Human parotid and submandibular salivary glands were obtained from patients (after informed consent) with a squamous cell carcinoma of the oral cavity in whom a neck dissection procedure was performed. None of the patients had received any other cancer treatment before surgical procedure. Culture conditions for the formation of human salispheres were similar to those for the mouse.

For differentiation experiments three day-old spheres were mixed with Collagen type I (BD 3.68 mg/mL) and cultured for twenty additional days until branches and acinar-like outgrowth could be visualized. Mucin and mucopolysaccharide containing acinar cells were detected by Periodic Acid Schiff's base (PAS).

Immunohistochemical processing

After extirpation, the submandibular glands or cultured cells were incubated for 30 h at 4 °C in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin. Floating spheres were incubated in 4% buffered formaldehyde at 4 °C, washed and resuspended in Histogel (Richard-Allan Scientific, Kalamazoo, MI;HG-4000–012), after dehydration the cells were embedded in paraffin. Five-μm sections were dewaxed and labeled with a polyclonal rabbit anti-human c-Kit antibody (Cat. No. A4502; Dako, Glostrup, Denmark). Visualization was accomplished by adding specific secondary fluorescent antibodies (Jackson Immunoresearch Europe, Suffolk, UK). Nuclei were visualized using 4,6-diamino-2-phenylindole (DAPI). Control sections without primary antibodies were all negative. Cell morphology was visualized by routine histological techniques using hematoxylin-eosin staining.
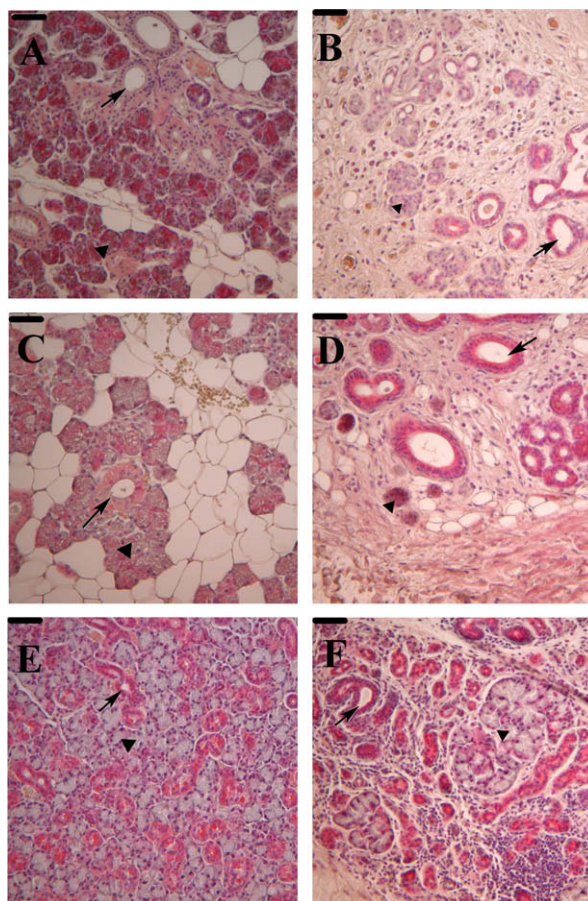
Fig. 1. Human submandibular (A and B) and parotid glands (C and D), before (A and C) and 15 years after radiotherapy for an oropharyngeal tumor T4N1M0 both glands (B and D) showed severe reduction in acinar cells (▶), with some remaining ducts (→), very similar to mouse submandibular glands (E and F). Bars indicate 50 μm.
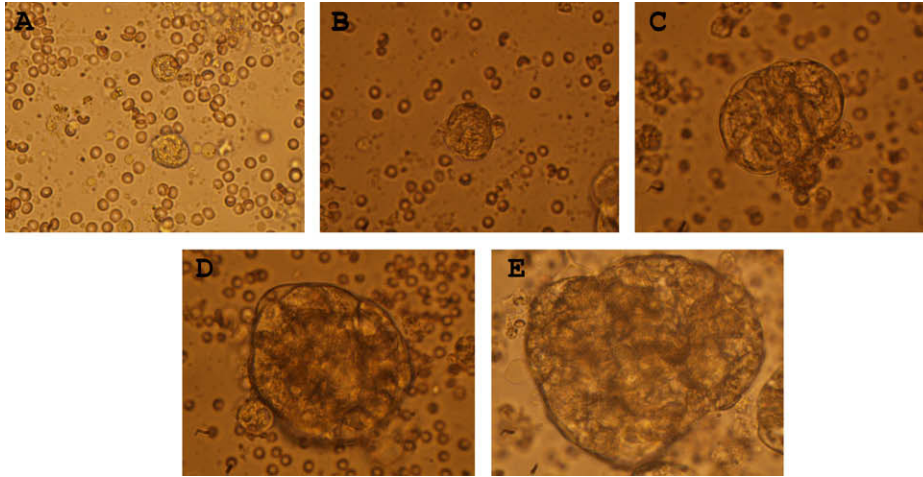
Fig. 2. Salisphere formation. Human submandibular (A–E) gland dispersed cells form salispheres much alike the ones from mice [8]. Magnification 400×.

Flow cytometric analysis and cell purification

Cultured cells were dissociated by 0.05% trypsin–EDTA (Gibco, Invitrogen, Carlsbad, CA;25300) with mechanical use of 26G needles. Incubation with APC-conjugated anti-human C-Kit antibody (BD Biosciences Pharmingen-550412) was performed at 4 °C for 20 min, followed by a washing step in PBS containing 0.2% BSA. Finally, Propidium Iodide (PI, 1 μg/ml) was added to the cells before analysis using a FACS Calibur Flow Cytometer (BD) with a least 100,000 events for each measurement. Data were analyzed by Flow-Jo software (Tree Star, Ashland, OR). Gates for viable (PI-negative) C-Kit+ cells were set by using iso-type controls (BD Biosciences Pharmingen) for APC. From 7 patients c-Kit cells were selected.

Self-renewal assay

After 3 days of culture, cells were dissociated with 0.05% trypsin–EDTA and counted using a hemocytometer and suspended in growth factor-reduced BD Matrigel Matrix (BD Biosciences) with

SGM in a 3:2 ratio (a total volume of 125 μl) with a total number of 10,000 cells. Samples were plated in a 12-well plate and allowed to solidify at 37 °C for 20 min, before 1 ml SGM was added. Medium was replenished every 3 days. Ten days after plating, spheres were counted.

To passage spheres, medium was aspirated off, and Matrigel GFR was digested by incubation with 1 ml dispase solution (Gibco, Invitrogen 1 mg/mL, dissolved in SGM) for 1 h at 37 °C. Digested cultures were collected, pelleted, resuspended, and incubated in 1 ml type 1 collagenase (Gibco, Invitrogen 190 units/mL) for 45 min at 37 °C. Cells were then pelleted, resuspended, and incubated in 0.05% trypsin–EDTA for 10 min at 37 °C, passed through a 26G needle and a 50 μm mesh-filter. Finally, cells were counted by hemocytometer and replated.

Statistical analysis

Statistical analysis was performed with SPSS (version 15) software computer program using a Mann–Whitney test. Statistical
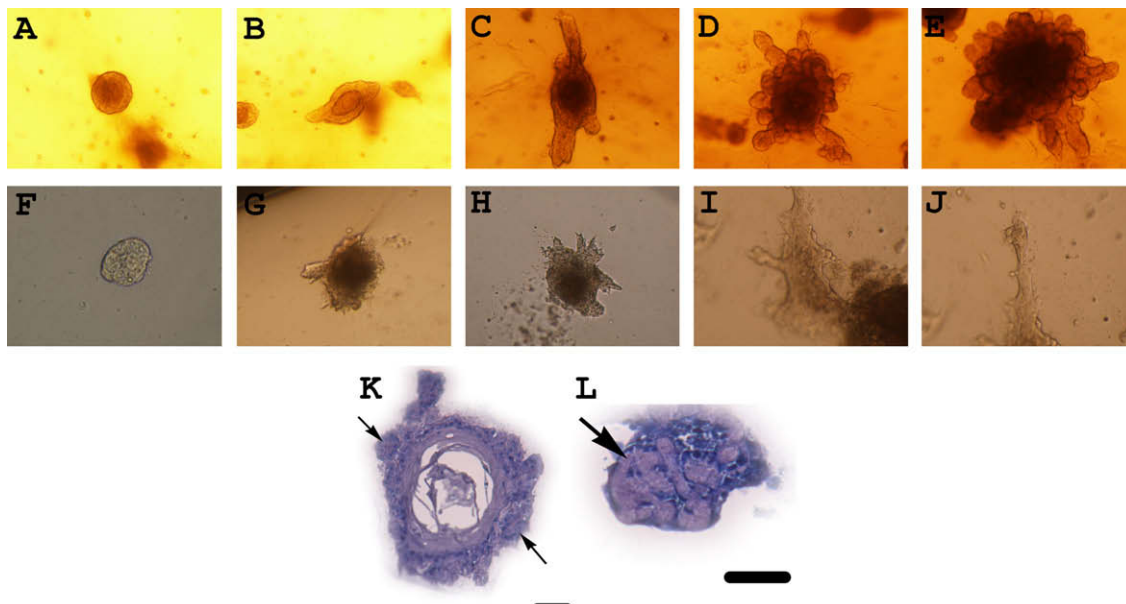
Fig. 3. Differentiation of salisphere cells. Dispersed cells from salispheres form new salispheres in Matrigel which gradually form outgrowth resembling duct cells (A–E) in a very similar way as mouse salispheres (F–J). In time, differentiation into mucin-producing acinar-like cells(human, K and mice L) is present. Bars indicate 50 μm. Magnification (A–E) 50× and mice (F–J) 100×.
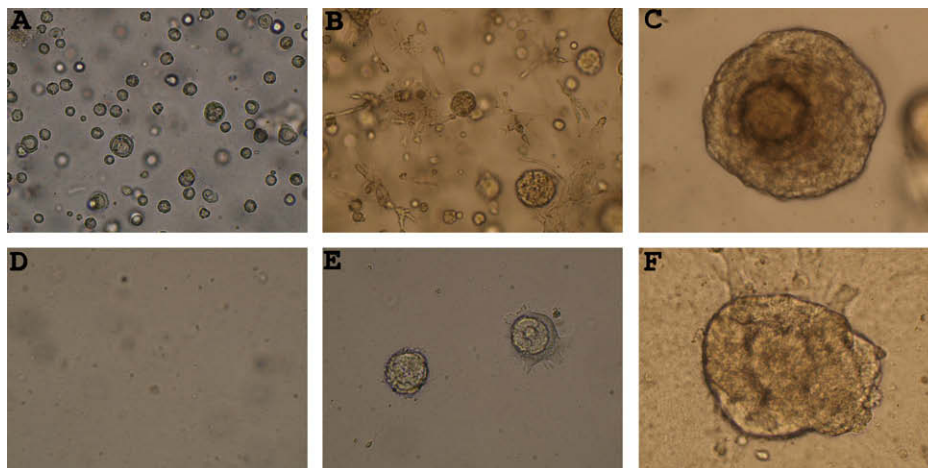
Fig. 4. Self-renewal of human salivary gland cells. Dispersed cells obtained from salispheres gradually form secondary salispheres in matrigel. Human salispheres A = day 0, B = day 3, and C = day 10 and mouse salispheres D = day 0, E = day 3, and F = day 10. Magnification 200×.

significance was defined as $P < 0.05$. Numbers represent average ± SEM, unless otherwise specified.

Results

The successful transplantation of a murine c-kit$^+$ salivary gland stem cell containing cell population that restored the function of irradiated glands [8], warrants translation of the results to humans. The present study was performed to compare some basics in radiation response and stem cell characteristics between human and mouse salivary glands. The success of the mouse salivary gland stem cell transplantation is partly due to the typical response of the tissue to radiation with disappearing acinar cells but with a ductal compartment remaining largely intact. Since, the stem cells of the salivary gland reside in the ducts [8], they provide the environment for engraftment of the transplanted cells. Therefore, we first studied the histopathology of irradiated human salivary gland. Normal human salivary parenchyma comprises of acini, which similar to mice are composed of triangle-shaped saliva-producing acinar cells, and a branchy duct system (Fig. 1A, C, and E). After radiotherapy, acinar cells are replaced by fibrotic tissue but similar to mice still some ductal tissue remains (Fig. 1B, D, and F), which may allow for the engraftment of stem cells in patients.

Next, we investigated the presence of salivary gland stem cells in human salivary gland, using the same method of culturing salispheres as in our mice studies [8]. Indeed, using this method, we were able to grow human salispheres, which usually appeared 2 days after isolation and culturing. In general, the appearance of the salispheres was very similar to that of the spheres derived from mouse tissue (Fig. 2). To show that human salispheres contain cells that are able to differentiate in saliva-producing cells, the dispersed salispheres were placed in collagen type I after 3 days of floating culture. Human salispheres were able to form spheres in 3D collagen type I (Fig. 3), which gradually transformed into duct- and acinar-like structures. Fig. 3A–E shows the gradual differentiation process, indicated by branching of duct-like cells and formation of acinar-like structures. The appearance of PAS (to detect mucins) and amylase-positive cells (Fig. 3K and L) in the salispheres indicates functional differentiation of cells in the spheres. The similarities with mouse salisphere differentiation are striking (Fig. 3 F–J, M, and N).

To show the self-renewal potential of salisphere cells, they were dissociated after 2 days of floating culture and a dispersed cell suspension was plated into matrigel. New salispheres were formed in 10 days (Fig. 4A–F). These spheres were collected and dispersed into a cell suspension which was replated in matrigel to form secondary (tertiary etc.) spheres. This procedure could be repeated at least seven times, without apparent loss of replating efficiency. This assay reveals long-term self-renewal ability *in vitro* to suggest the presence of a stem cell population in human salispheres.

Recently, we showed that especially c-Kit$^+$ cell isolated from 3-day-old spheres cultured from mouse salivary glands contained cells that were able to transdifferentiate in all salivary gland cell types, to regenerate irradiated glands, and to self-renew *in vivo* [8]. Therefore, we investigated human salivary glands for the presence of c-Kit$^+$ cells. Using immuno-fluorescent staining, normal human salivary gland clearly showed c-Kit$^+$ cells in excretory ducts of both submandibular and parotid glands (Fig. 5A and B, respectively). This is in agreement with our findings in mice [8], where c-Kit$^+$ was found to be confined to these type of ducts. Next, we tested if the cultured human salispheres contained c-Kit$^+$ cells. Hereto, we dispersed 3-day-old human salispheres into single cells and analyzed them using flow cytometry. Indeed, a clear
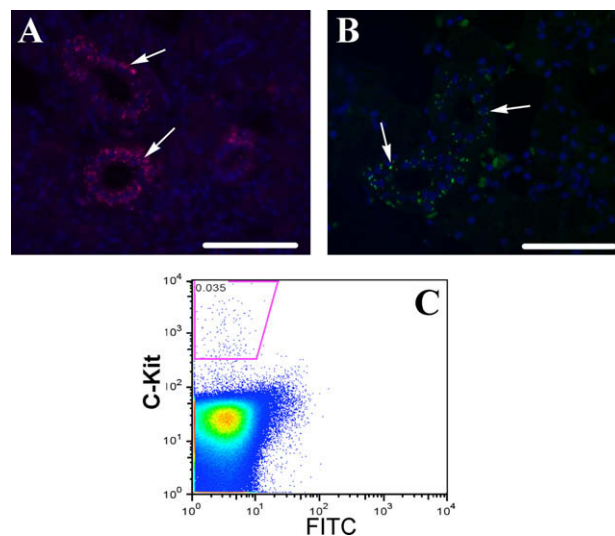
Fig. 5. Human submandibular (A) and parotid (B) gland excretory ducts contain c-Kit$^+$ cells, as indicated by the positive fluorescence label. From the salispheres grown from these glands a clear population of c-Kit cells could be observed (C). Nuclei are blue (Dapi) and c-Kit$^+$ cells are purple (Texas Red; submandibular gland) or green (Cy-2; parotid gland). Bars indicate 50 μm.
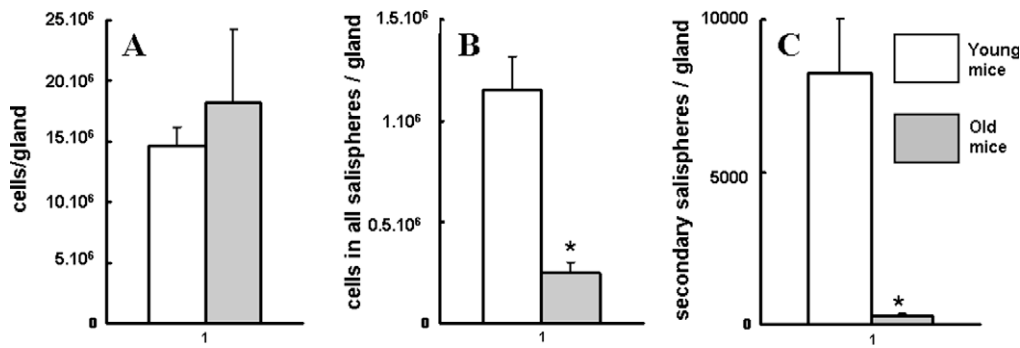
Fig. 6. Reduced number of stem/progenitor cells in older murine submandibular glands. One- to two-year-old mice submandibular glands contain a similar amount of cells as young mice glands (A), but all salispheres that could be grown from old mice together contained fewer cells (B). Furthermore, these cells were diminished in their capability to form secondary spheres in matrigel (C). $^*P < .05$, Error bars depict SEM of $n = 6$.

population of c-Kit$^+$ cells (less than 1%) could be observed (Fig. 5C), albeit in slightly lower frequencies than in mouse salispheres [8].

The salispheres cultured from mouse glands were obtained from rather young (2–3 months) animals. However, patients that are treated for head and neck cancer are often relatively much older and potentially may have different numbers of stem cells. Since we showed that the number of salispheres that can be grown from mouse salivary glands represent the number of stem cells present [9], we tested for a possible relationship between age and the number of salivary gland stem cells. To this end, we quantitatively compared the salisphere culture of old mice and young mice. Salispheres were cultured from 10 old mice and the number of salispheres that could be grown was compared to young mice. After 3 days of culturing, salispheres were counted and subsequently dispersed into a cell suspension, put into matrigel and cultured for another 10 days. As such we were able to measure three different variables: (1) the number of cells after isolation, (2) the number of cells derived from salispheres after 3 days of culturing and (3) the number of secondary spheres formed after passaging in matrigel. The result showed that there was no significant difference ($P > 0.05$) between the number of cells that could be isolated from young or old mouse (Fig. 6A). However, primary salispheres from older mice contain significantly less cells than primary salispheres from young mice ($P < 0.01$, Fig. 6B). Moreover, these cells were less capable to grow spheres in matrigel in the secondary salisphere culture (Fig. 6C).

Discussion

Recently, we discovered a population of c-Kit-expressing cells in mice with remarkable capability to restore salivary glands damaged by radiation [8]. Salispheres cultured from rodent submandibular glands contained cells that expressed many stem cell markers (e.g., Sca-1, c-Kit, Musashi-1) and were able to differentiate into all salivary gland lineages. These cells were also able to self-renew *in vivo* [8]. After stem cell enrichment by flow cytometric selection using c-Kit as a single marker, as few as 100 c-Kit$^+$ cells were able to completely restore salivary gland function and morphology 3 months after transplantation. This study was the first evidence for the potential use of stem cell transplantation to functionally rescue salivary gland damaged by irradiation.

In this study, we investigated the presence and *in vitro* potential of human salivary gland stem cells. We determined the characteristics of human salivary gland for the following aspects: morphology and pathology, salisphere formation, *in vitro* differentiation, self-renewal ability and the presence of c-Kit stem cell marker. Although human and mouse salivary glands are not exactly the same, the tissue architecture after irradiation looks remarkably

similar. In both species, the ductal compartment necessary for stem cell engraftment largely remains intact. Moreover, salisphere formation of human salivary gland cells was very similar to mice. In addition, human salispheres could form duct and acinar-like cells and were able to self-renew for at least seven passages in culture. These results indicate that human salispheres do contain cells with stem cell-like properties. The potential existence of stem cells that may be used for transplantation purposes is further substantiated by the location of c-Kit$^+$ cells in the excretory ducts of both human submandibular and parotid glands. Furthermore, these cells could be isolated from human salispheres in substantial numbers, albeit in lower percentages than from rodent salispheres. This may be due to a lower stem cell number in older people such as the patients with head and neck cancer used in this study. Our finding that older mice may have reduced numbers of cells capable to form salispheres in floating culture is in agreement with this.

Therefore, we conclude that it may very well be possible to develop human adult stem cell therapy to restore the function of irradiated salivary glands in patients. However, before we can proceed a number of problems still have to be addressed. For instance, does the human c-Kit+ cell population contain cells that have *in vivo* regeneration ability? And, can we get sufficient and potent enough stem cells to establish long term regeneration of salivary glands or do we have to expand human salivary gland stem cells *in vitro* before transplantation? Recently, we have shown that *in vivo* expansion of salivary gland stem cells is attempted [9] and it may thus also be possible *in vitro*.

Human and mouse salivary glands contain a similar 'putative' stem cell population, which might have potential to reduce radiotherapy-induced salivary gland dysfunction in the future.

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