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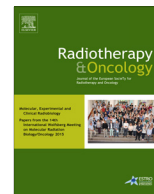
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Experimental radiobiology

Similar *ex vivo* expansion and post-irradiation regenerative potential of juvenile and aged salivary gland stem cellsMartti Maimets^{a,b}, Reinier Bron^a, Gerald de Haan^c, Ronald van Os^c, Robert P. Coppes^{a,b,*}^a Department of Cell Biology; ^b Department of Radiation Oncology; and ^c Laboratory of Ageing Biology and Stem Cells, European Research Institute for the Biology of Aging (ERIBA), University Medical Center Groningen, University of Groningen, The Netherlands

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ABSTRACT

Background and purpose: Salivary gland dysfunction is a major side effect of radiotherapy for head and neck cancer patients, which in the future might be salvaged by autologous adult salivary gland stem cell (SGSC) therapy. Since frail elderly patients may have decreased activity of SGSCs, we aimed to characterize the potential of aged SGSC-population in a murine model.

Materials and methods: Salivary glands and salisphere-derived cells from young and old mice were tested for CD24 and CD29 stem cell marker expression using FACS. Moreover, *in vitro* expansion capability and *in vivo* regeneration potential upon post-irradiation transplantation of young and aged SGSCs were measured.

Results: An increase in CD24^{hi}/CD29^{hi} putative stem cells was detected in aged salivary glands albeit with a decrease in functional ability to form salispheres. However, the salispheres formed from aged mice salivary glands expressed CD24^{hi}/CD29^{hi} to the same extent as the ones from young mice. Moreover, following exposure to adequate growth conditions old and young SGSCs exhibited similar *in vitro* expansion- and *in vivo* regeneration potential.

Conclusions: Aged SGSCs although reduced in number are *in vitro* indistinguishable from young SGSCs and could potentially be used to ameliorate age- or treatment related salivary gland dysfunction.

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Head and neck cancer (HNC) is the sixth most common cancer worldwide, with an estimated 55,070 new cases in the U.S. annually [1]. The majority of these patients will be treated with radiotherapy, alone or in combination with chemotherapy and/or surgery. Although radiotherapy treatment significantly improves the patient's chances of survival, it often coincides with side-effects due to the unavoidable co-irradiation of normal tissues surrounding the tumor, including salivary glands.

A potential option for salvaging radiation-damaged salivary glands and the consequential hyposalivation is adult stem cell therapy. In a preclinical model, we [2,3] and others [4] have previously shown that stem/progenitor cell transplantation not only rescues hyposalivation, but also restores tissue homeostasis of the irradiated gland, necessary for long-term maintenance of adult tissue [5]. Additionally, we demonstrated optimized culturing conditions for long-term *in vitro* expansion of CD24^{hi}/CD29^{hi} salivary gland stem cells (SGSCs) [6]. Moreover, similar results have been found with human salivary gland derived cells [7,8]. However,

these preclinical studies have been performed in young and healthy donor animals. According to the Netherlands Cancer Registry database (Nederlandse Kankerregistratie: <http://www.cijfersoverkanker.nl>), the age of approximately 50% of all patients diagnosed with HNC in the Netherlands between 1989 and 1998 was 65 or above, making HNC essentially a cancer for aged population. In addition, the incidence of newly diagnosed HNC cases among elderly is expected to have increased by more than 60% in the year 2030 [9]. These data imply that studies performed in aged organisms would have increased clinical relevance in order to improve quality of life of the elderly. Aging can be generally characterized with a pronounced decline in functionality of tissue homeostasis and regeneration capacity [10]. In muscle [11], blood [12], liver [13] and brain [14] this decline has been accredited to a reduced responsiveness to proliferation stimuli of tissue-specific stem and progenitor cells. In many elderly individuals salivary gland dysfunction with consequential dry-mouth syndrome (xerostomia) is a major complaint [15]. However, whether the functional reduction of salivary gland activity during aging could be attributed to decreased activity of salivary gland stem cells has until now been unexplored. To date attempts of culturing and expanding salivary gland stem cells from an aged organism have not been successful [7] possibly due to analyzing a

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heterogeneous population of cells and the lack of optimized culturing conditions. Therefore, we set out to study phenotypical and functional properties of purified salivary gland stem cells isolated from young and old mice to determine directly whether these cells change with age.

Methods

Mice

Young (8–12 week old) and old (22–26 month old) female C57BL/6 mice were purchased from Harlan. The mice were maintained under conventional conditions and fed ad libitum with food pellets (RMH-B, Hope Farms B.V.) and water. All experiments were approved by the Ethics Committee on animal testing of the University of Groningen.

Cell sorting and single cell salivary gland sphere culture

Salivary glands were harvested from healthy adult mice, mechanically disrupted by gentle MACS Dissociator (Milteny) followed by enzymatic digestion with collagenase type II (0.63 mg/ml; Gibco), hyaluronidase (0.5 mg/ml; Sigma–Aldrich) and CaCl_2 (6.25 mM; Sigma–Aldrich). After filtering through 100 μm cell strainer the suspension was dissociated using 0.05% trypsin–EDTA (Gibco) following filtering through 35 μm strainer. Cell pellets were incubated with anti-mouse CD31-PE (eBioscience), CD45-PE (Biolegend), TER-119-PE/Cy7 (Biolegend) and EpCAM-APC antibody (eBiosciences) for 15' on room

temperature. After washing thoroughly cells were suspended in a solution containing propidium iodide (PI; 1 mg/ml; Sigma–Aldrich), MgSO_4 (10 mM; Sigma–Aldrich) and DNase I (50 $\mu\text{g}/\text{ml}$; Sigma–Aldrich). Pulse-width gating excluded cell doublets while dead cells were excluded by gating on PI negative cells.

Primary salispheres were cultured as published previously [16]. In short, cell suspensions were prepared first by mechanical disruption with gentleMACS (Milteny) followed by enzymatic digestion with collagenase type II (0.63 mg/ml; Gibco), hyaluronidase (0.5 mg/ml; Sigma–Aldrich) and CaCl_2 (6.25 mM; Sigma–Aldrich). After washing thoroughly cell suspensions were resuspended in DMEM:F12 medium containing $1\times$ Pen/Strep antibiotics (Invitrogen), Glutamax ($1\times$; Invitrogen), EGF (20 ng/ml; Sigma–Aldrich), FGF-2 (20 ng/ml; Sigma–Aldrich), N2 ($1\times$; Gibco), insulin (10 $\mu\text{g}/\text{ml}$; Sigma–Aldrich) and dexamethasone (1 μM ; Sigma–Aldrich), at a density of 400,000 cells per well of a 12-well plate. For estimation of the number of salispheres, 3-day salisphere cultures were collected, suspended in 10 ml of PBS containing 0.2% BSA and enumerated.

For cell sorting, 3-day salisphere cultures were dispersed to single cell suspensions using 0.05% trypsin–EDTA (Invitrogen). Cell pellets were incubated with anti-mouse CD24-PB (Biolegend) and CD29-FITC antibody (BD biosciences) for 15' on room temperature. After washing thoroughly cells were suspended in a solution containing propidium iodide (PI; 1 mg/ml; Sigma–Aldrich) and processed to cell sorting. Sorted cells were embedded in Basement Membrane Matrigel (BD Biosciences) and seeded in 12-well. Cells were cultured in expansion medium (EM) as previously published [6] (DMEM:F12 containing Pen/Strep antibiotics ($1\times$; Invitrogen),

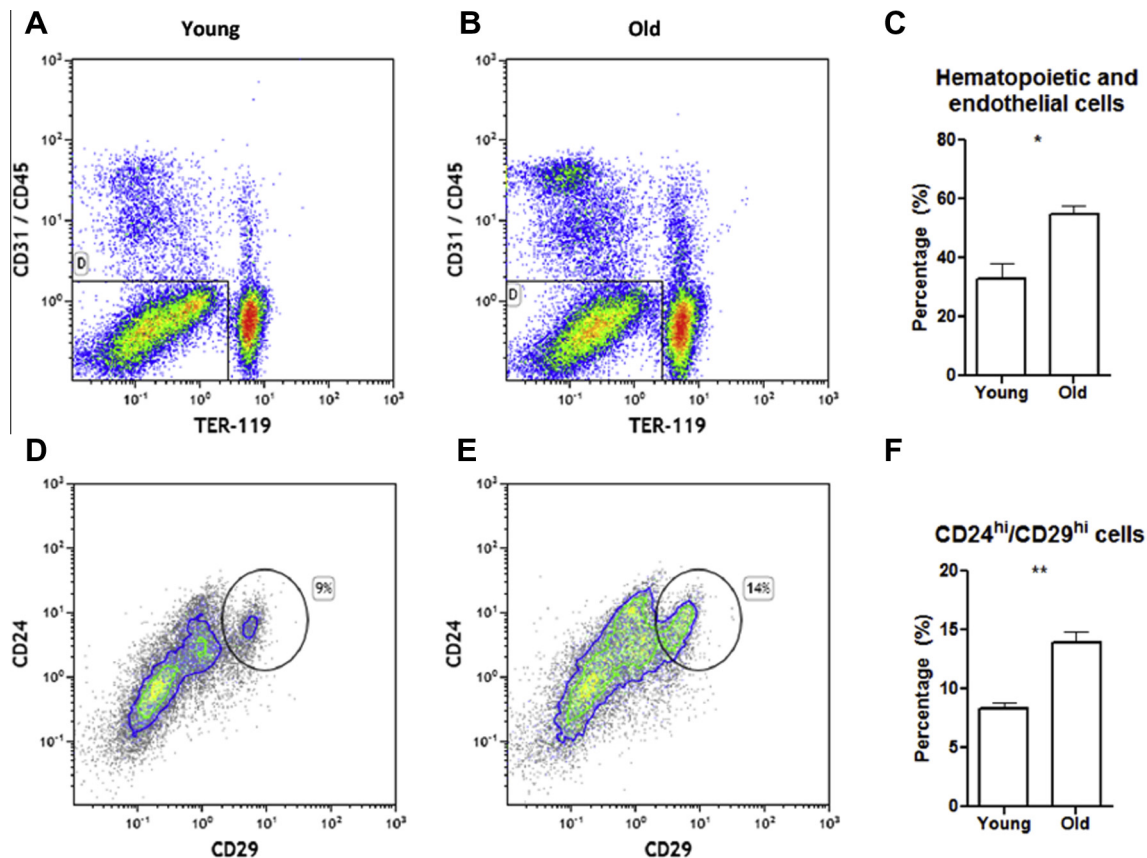


Fig. 1. Quantification of putative stem cells in young and old mice. (A and B) Representative fluorescence-activated cell sorting (FACS) gating strategy for lineage negative cells from (A) young and (B) old mice. (C) Quantification of endothelial and hematopoietic cells in young and old mice. (D and E) Representative FACS plot for CD24 and CD29 putative stem cell marker expression from (D) young and (E) old mice. (F) Quantification of CD24^{hi}/CD29^{hi} putative stem cells in young or old mice. Error bars represent the standard error of the mean, $p \leq 0.05$, $**p \leq 0.01$.

Glutamax (1×; Invitrogen), N2 (1×; Gibco), EGF (20 ng/ml; Sigma–Aldrich), FGF2 (20 ng/ml; Sigma–Aldrich), insulin (10 µg/ml; Sigma–Aldrich), dexamethasone (1 µM; Sigma–Aldrich), Y-27632 (10 µM; Sigma–Aldrich)).

Self-renewal assay

Self-renewal capacity of primary salisphere-derived cells was determined as published previously [6]. Shortly, 3-day salisphere cultures were dispersed to single cell suspensions using 0.05% trypsin–EDTA (Invitrogen), enumerated and concentration adjusted to 0.4×10^6 cells/ml. 25 µl of cell suspension was combined on ice with 50 µl of BM Matrigel and deposited in the center of 12-well tissue culture plates. After solidifying the gels for 20 min at 37 °C, gels were covered in EM [6]. Seven days after seeding, Matrigel was dissolved by incubation with Dispase (1 mg/ml; Sigma) for 1 h at 37 °C. Passaging: Spheres released from the gels were processed to a single cell suspension using 0.05% trypsin EDTA and encapsulation in Matrigel was repeated.

Irradiation and regeneration assay

The irradiation and regeneration assay employed here was described earlier [2]. Shortly, salivary glands of female C57BL/6 mice were locally irradiated with a single dose of 15 Gy (Precision X-ray Inc.). Four weeks after irradiation, mice were anesthetized and SMG was exposed by small incision. As a source of donor cells passage 1 salisphere cultures derived from young or old mice were dissociated into single cell solution, 10,000 cells suspended in equal volumes of α -MEM (Gibco) and injected into both

submandibular glands of irradiated mice intra-glandular. Saliva was collected for 15 min at 30, 60, 90 and 120 days post irradiation.

Data analysis

All values are represented as mean \pm standard error of the mean (SEM) (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Student's *t*-test was used for testing statistical significance in cell sorting and cell culture experiments. A 2-way analysis of variance (ANOVA) and Bonferroni post hoc test with alpha values of 0.05 were applied to the analysis of saliva flow. *n* Numbers for tested groups throughout all experiments equal 3. All calculations were performed using GraphPad Prism (GraphPad software) software.

Results

We sought to study the initial number of putative stem cells in young and old mice using molecular markers that exclusively characterize purified SGSCs. Recently, we have shown that in the mouse, most, if not all, SGSCs reside within CD24^{hi}/CD29^{hi} fraction of salisphere derived cells [6]. Therefore, we quantified the number of CD24^{hi}/CD29^{hi} putative stem cells in young and aged salivary glands of 2–3 or 22–24 month old healthy mice. That in mind, the glands were isolated and digested into single cell suspension. After removing cell clumps, dead cells and debris, we excluded CD45⁺ and Ter119⁺ hematopoietic and CD31⁺ endothelial cells (Fig. 1A and B). We observed an enlargement in hematopoietic and endothelial cell compartment from $33.0 \pm 5.2\%$ in young to 54.8 ± 2.7 ($p = 0.0101$) in old mice (Fig. 1C). Interestingly, we noted

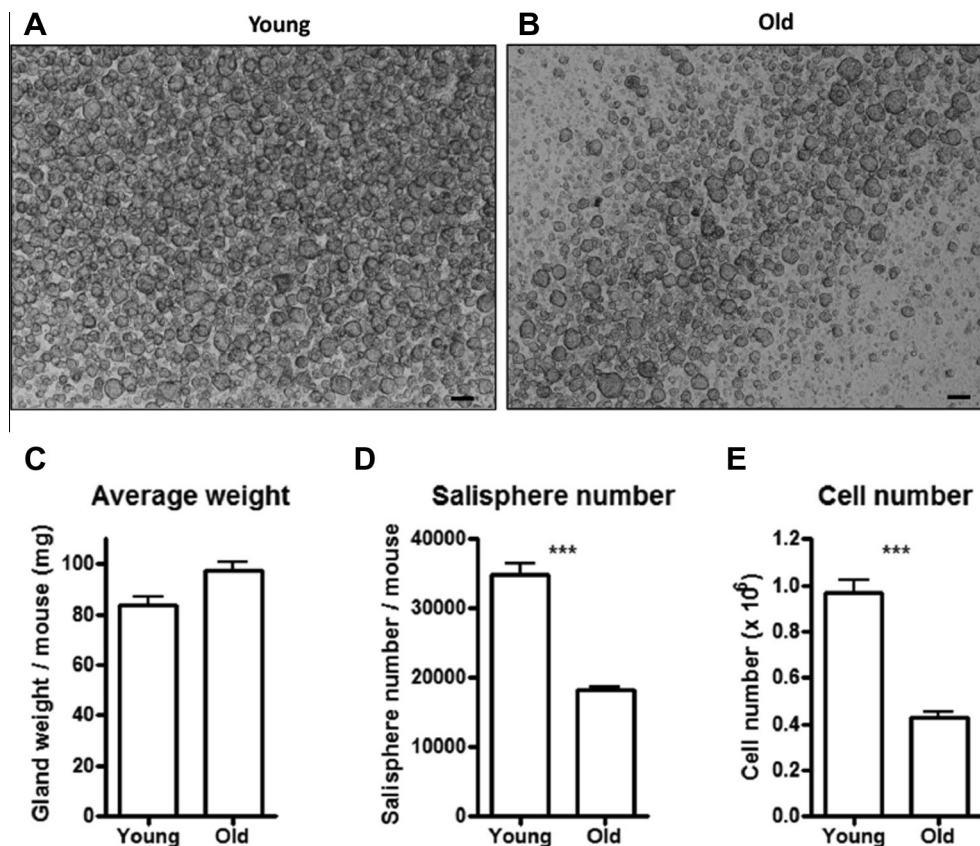


Fig. 2. Reduction in functional activity of SGSCs isolated from aged mice. (A and B) Differential interference contrast (DIC) images of 3-day old salispheres derived from (A) young or (B) old mice. (C–E) Comparison of (C) submandibular gland weight, (D) number of salispheres derived and (E) number of cells enclosed in 3-day salispheres, between young and old mice. Scale bars 100 µm. Error bars represent the standard error of the mean, *** $p \leq 0.001$.

a near 2-fold increase in the relative numbers of CD24^{hi}/CD29^{hi} cells from $8.3 \pm 0.5\%$ in young mice (Fig. 1D and F) to $13.9 \pm 1.0\%$ in old mice (Fig. 1E and F) ($p = 0.0067$). To investigate whether aged stem cells in the salivary gland are functionally inert, we next asked if the regenerative potential of salivary glands changes during aging. Previously, we have shown that the number of spheres grown from salivary glands represents the number of stem cells present [16]. With that goal, the salivary glands from young and old mice were isolated and digested into single cells or small aggregates of cells, which formed primary salispheres within 3 days (Fig. 2A and B). We did not observe any significant change in submandibular gland weight between young and old animals (Fig. 2C). Interestingly, the number of salispheres (Fig. 2D) as well as the number of cells enclosed (Fig. 2E) reduced more than 2-fold from $34,600 \pm 1700$ spheres and $960,000 \pm 50,000$ cells, respectively per mouse in young to $18,300 \pm 400$ spheres and $430,000 \pm 20,000$ cells, respectively per mouse in old animals. These data indicate that although containing a larger CD24^{hi}/CD29^{hi} population, aging salivary glands are associated with a cell intrinsic reduction in the ability of SGSCs to give rise to new salispheres.

We next asked the question whether the cell surface phenotype of SGSCs changes over time. To this end, we tested if subpopulations of CD24 and/or CD29 as shown previously by Nanduri et al. [6] exist in primary salisphere-derived cells isolated from old mice vs young mice using flow cytometry. First, based on population density analysis we separated cells into three subsets (CD24^{hi}/CD29^{hi}, CD24^{hi}/CD29^{med} and CD24^{low}/CD29^{low}) (Fig. 3A and B). Further analysis of these subsets revealed no significant difference between salispheres grown from young and old mice

(Fig. 3C). Next, we sorted these three subsets and plated them into Basement Membrane Matrigel supplemented with our previously published Expansion Medium (EM) [6], known to allow self-renewal and expansion of young stem cells. After 5–7 days, secondary spheres were formed primarily from CD24^{hi}/CD29^{hi}, less from CD24^{hi}/CD29^{med} and not at all from CD24^{low}/CD29^{low} subsets (Fig. 3D), in agreement with what was found previously [6]. Interestingly, quantification of sphere forming capacity showed no significant difference between cells derived from young or old salivary glands (Fig. 3E). These data establish that cell surface phenotype of SGSCs as defined by CD24 and CD29 expression of cells grown in primary salispheres, is not altered with age and show the same *in vitro* sphere forming potential.

As a measure of stemness, self-renewal potential of young and old salisphere derived stem/progenitor cells was assessed. Primary salispheres were enzymatically dissociated after which the enclosed cells were again embedded into Basement Membrane Matrigel. To ensure the long-term survival of SGSCs we used EM to supplement our cultures. At weekly intervals secondary spheres were dissociated and re-plated. Under these conditions we did not observe any significant difference in the self-renewing potential of young and aged SGSCs within 4 passages (Fig. 4A) after the initial reduction in sphere forming capacity of primary salisphere cultures (Fig. 2).

Finally, to gauge the qualitative potential of young and old cells to rescue radiation-induced hyposalivation *in vivo* was compared. For this purpose, sphere cultures from young and old mice were initiated and expanded for one passage in Basement Membrane Matrigel supplemented with EM. Subsequently, cultures were collected, enzymatically dissociated into single-cell suspension and

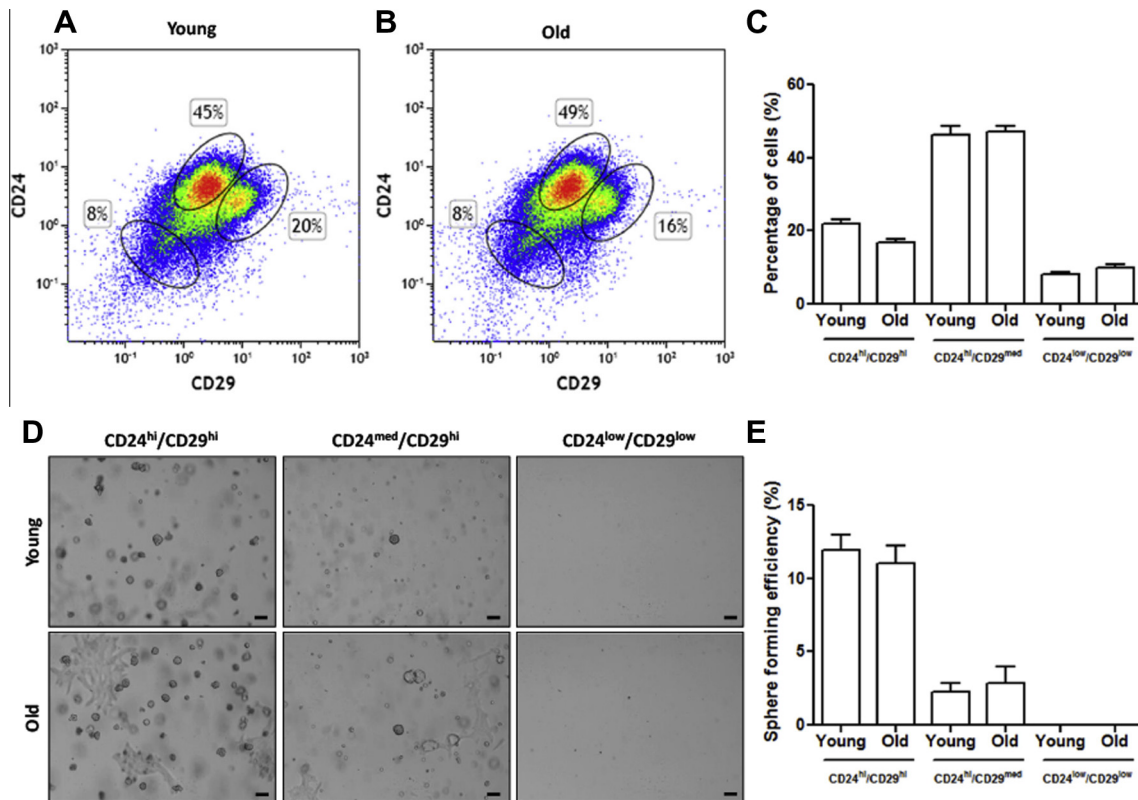


Fig. 3. Cell surface phenotype of SGSCs is unaltered during the course of aging. (A and B) Representative FACS gating strategy used to isolate CD24^{hi}/CD29^{hi}, CD24^{hi}/CD29^{med} and CD24^{low}/CD29^{low} cell populations from 3-day old salispheres derived from (A) young or (B) old mice. (C) Quantification of the three CD24/29 subpopulations in 3-day old salispheres. (D) DIC images of secondary spheres in culture (end of passage 1) formed from CD24/29 subpopulations. (E) Quantification of sphere formation capability of CD24/29 subpopulations in the end of passage 1. Scale bars 100 μ m. Error bars represent the standard error of the mean, * $p \leq 0.01$.

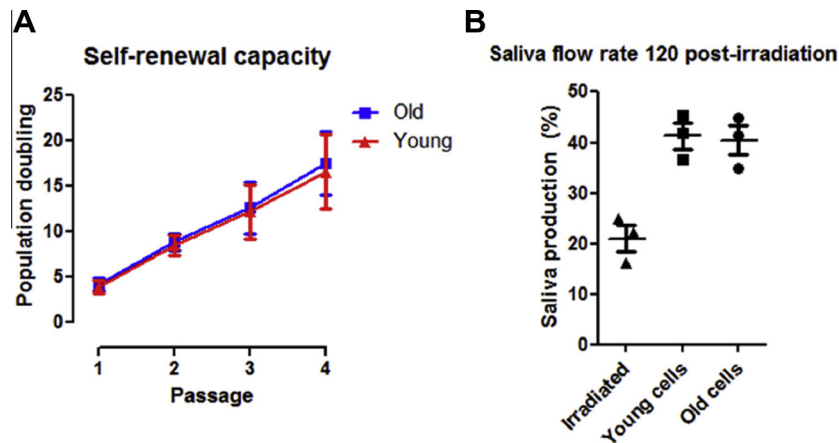


Fig. 4. Similar self-renewal and regeneration potential of young and old SGSCs. (A) Population dynamics plot of expanded SGSCs derived from young or old mice. (B) Relative saliva production at 120 days after irradiation in animals transplanted with one-time passaged SGSCs isolated from young or old mice. Error bars represent the standard error of the mean, $^*p \leq 0.01$.

transplanted intraglandularly into young C57BL/6 recipient mice, which were previously locally irradiated with 15 Gy to the head and neck region [2]. Both glands in each mouse received equal cell numbers, so that a total cell number of 10,000 was transplanted per recipient mouse. The functionality of the transplanted glands was determined by pilocarpine stimulated saliva flow rate as described previously [2]. As expected, 120 days after irradiation, in control animals (irradiated and non-transplanted), saliva production dropped to $21 \pm 3\%$ of pre-irradiation values (Fig. 4B). In contrast, saliva flow of mice transplanted with young or old expanded SGSCs increased significantly to $41 \pm 3\%$ and $40 \pm 3\%$, respectively (Fig. 4B). Collectively these data indicate that apart from an initial drop in plating efficiency, aged SGSCs have normal growth capacity *in vitro* and reconstitution ability *in vivo*.

Discussion

Here, we provide evidence that aged salivary glands contain more cells that express stem cell makers albeit with reduced potential. However, the remaining stem cells hold similar expansion- and *in vivo* regeneration potential as their young counterparts when cultured in a setting supplemented with appropriate extrinsic factors.

Our data suggest that the tissue environment has a significant role in SGSC maintenance *in vivo* leading to reduced proliferation *in vitro*. Upon manipulation of systemic factors which promote proliferative expansion, aged SGSCs do have a similar *in vitro* growth and *in vivo* regeneration potential as their young counterparts. We therefore hypothesize that the aged micro-environment in the salivary gland is suboptimal for resident adult stem cells. A similar observation, albeit with partial restoration of the proliferation and differentiation potential of satellite cells, has been made for muscle stem cells upon exposure to young serum [11,17,18]. However, these studies did not explore the possibility of increasing the number of functionally capable stem cells to the numbers reported here.

Until now, the cause for aging-related decline of salivary gland function has remained unexplored. Recently, Yamakoshi and colleagues [19] reported age-related dysregulation of Bmi-1/p16^{Ink4a} pathway and a subsequent decline of stem or progenitor cell function in submandibular glands. While extending the current understanding of the molecular mechanisms underlying the aging-related decline of SMG function, the study [19] did not focus on refined populations of SGSCs. Due to widespread variation in attempts to characterize aging it is of vital importance to conduct analysis in purified cell populations. Here, with series of *in vitro* and *in vivo* assays we confirmed that the cell surface phenotype

of SGSCs is not altered during aging and that the cells with the highest self-renewal potential remain in the CD24^{hi}/CD29^{hi} fraction of cells. Similar observations have been made in long-term HSCs [20] and skeletal muscle [21]. However, chromosomal rearrangements, stem cell malignant transformation and with age genomic instability may increase and have to be assessed before clinical application.

In conclusion, our studies show that the age-dependent decline in tissue regenerative potential of salivary glands may be caused due to age-related changes in micro-environment which can be reversed through exposure to extrinsic factors. This further suggests that tissue-specific stem cells retain much of their intrinsic proliferative potential during aging and that age-related changes in the salivary gland stem cell niche might prevent adequate activation of these cells for beneficial tissue recovery *in vivo*. Identification of all critical stimuli required for *in vivo* maintenance of SGSCs which decline with aging may increase our understanding of physiological regulation of these cells and may be exploited for clinical purposes. Therefore, it may become feasible to expand old SGSCs *in vitro* which holds clinical promise for regeneration of damaged salivary glands regardless of patient's age.

Conflict of interest

The authors declare no conflicts of interests.

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