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Human Cancer Biology

Clinical Cancer Research

A Novel Aldehyde Dehydrogenase-3 Activator Leads to Adult Salivary Stem Cell Enrichment *In Vivo*

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Abstract

Purpose: To assess aldehyde dehydrogenase (ALDH) expression in adult human and murine submandibular gland (SMG) stem cells and to determine the effect of ALDH3 activation in SMG stem cell enrichment.

Experimental Design: Adult human and murine SMG stem cells were selected by cell surface markers (CD34 for human and c-Kit for mouse) and characterized for various other stem cell surface markers by flow cytometry and ALDH isozymes expression by quantitative reverse transcriptase PCR. Sphere formation and bromodeoxyuridine (BrdUrd) incorporation assays were used on selected cells to confirm their renewal capacity and three-dimensional (3D) collagen matrix culture was applied to observe differentiation. To determine whether ALDH3 activation would increase stem cell yield, adult mice were infused with a novel ALDH3 activator (Alda-89) or with vehicle followed by quantification of c-Kit$^+$/CD90$^+$ SMG stem cells and BrdUrd$^+$ salispheres.

Results: More than 99% of CD34$^+$ huSMG stem cells stained positive for c-Kit, CD90 and 70% colocalized with CD44, Nestin. Similarly, 73.8% c-Kit$^+$ mSMG stem cells colocalized with Sca-1, whereas 80.7% with CD90. Functionally, these cells formed BrdUrd$^+$ salispheres, which differentiated into acinar- and ductal-like structures when cultured in 3D collagen. Both adult human and murine SMG stem cells showed higher expression of ALDH3 than in their non–stem cells and 84% of these cells have measurable ALDH1 activity. Alda-89 infusion in adult mice significantly increased c-Kit$^+$/CD90$^+$ SMG population and BrdUrd$^+$ sphere formation compared with control.

Conclusion: This is the first study to characterize expression of different ALDH isozymes in SMG stem cells. *In vivo* activation of ALDH3 can increase SMG stem cell yield, thus providing a novel means for SMG stem cell enrichment for future stem cell therapy. *Clin Cancer Res; 17(23); 1–8. ©2011 AACR.*

Introduction

Most patients with head and neck cancer (HNC) receive radiotherapy as part of their cancer management (1–3). Radiation exposure results in permanent damage to the salivary glands, causing subsequent radiotherapy-related xerostomia or dry mouth (1, 2). Patients with radiotherapy-related xerostomia experience reduced saliva, which leads to considerable morbidities, including dysphagia, chronic dental caries, recurrent oral infections, and rare mandibular osteoradionecrosis (1–4). It is estimated that more than 80% of patients receiving head and neck radiotherapy suffer from these side effects (5). Current approved medical managements for radiotherapy-related xerostomia include the use of salivary substitutes, lubricants, and cholinergic agonists to stimulate salivary secretion. These treatments remain palliative in nature, require chronic use, and are often ineffective (1, 6, 7). Intensity-modulated radiotherapy (IMRT) can protect the parotid glands from direct radiation injury in selective cases; however, it often cannot spare the submandibular glands (SMG), which are responsible for resting salivary production (8). The vicinity of the SMG to the level II nodes, which are the most commonly involved nodes in HNC, makes it harder to spare it from direct radiotherapy beams. At least one randomized study indicated that although IMRT resulted in improved parotid sparing, it did not result in significant improvement of patient's subjective xerostomia (9). In contrast, SMG transfer and sparing from direct radiotherapy beams was

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Translational Relevance

The most common late morbidity of head and neck irradiation is xerostomia or dry mouth with more than 80% of treated patients experiencing this side effect. The morbidity is because of radiation damage to the surrounding salivary gland tissues, which can be partially mitigated but not completely prevented with the use of intensity-modulated radiotherapy. There is heightened interest in using adult salivary gland stem cells to rescue function in patients with radiation-induced xerostomia; however, this treatment strategy is hampered by the rarity of these stem cells in the adult gland. This manuscript reports on a novel finding that certain ALDH enzymes, specifically ALDH3 is expressed at high level in adult mouse and human salivary stem cells. Most importantly, pharmacologic activator using a novel small molecular activator that is specific for ALDH3 resulted in significantly higher stem cell yield for future therapy.

associated with a significantly better subjective xerostomia function as assessed by quality of life questionnaires (10, 11). Therefore, despite widespread IMRT use in HNC, development of methods to reconstitute salivary gland tissue, specifically SMG, and recovery of physiologic salivary secretion after radiotherapy is needed in patients with HNC.

Recently, there is increased interest in using salivary stem cells to replenish functional cells after radiotherapy (2, 4, 7). Past studies have shown that human adult salivary stem cells can be isolated from both parotid glands and SMGs (3, 7, 12, 13). Lombaert and colleagues showed that intraglandular transplantation of murine c-Kit$^+$ cells into irradiated recipient murine SMG resulted in pronounced improvement of saliva secretion (2). However, these cells are sparse, difficult to culture and inadequately characterized. Methods to obtain sufficient numbers of adult salivary stem cells for therapy have not yet been established.

Aldehyde dehydrogenase (ALDH) isozymes are responsible for oxidizing intracellular aldehydes and protecting stem cells from oxidative insult (14, 15). ALDH1 is the most abundant isoform found in human hematopoietic stem cells and is commonly used as a stem cell marker (14–16). Both ALDH1 and ALDH3 can catalyze the detoxification of certain chemotherapies such as cyclophosphamide and oxazaphosphorines, thus protecting cells from chemotherapeutic damage (17, 18). In addition, ALDH3 has been implicated in protecting ocular tissue from UV radiation (18). Studies have also shown that ALDH1 activity modulates stem cell proliferation and differentiation via the production of retinoic acid (15, 16). The protective role of ALDH from aldehyde accumulation because of oxidative stress in salivary stem cells has not been investigated.

In this study, we isolated and characterized human and murine SMG stem cells and determined the expression of different ALDH members in these cells as compared with their non–stem cell counterparts. We found that adult human SMG stem cells express different stem cell surface markers, including c-Kit, CD90, Nestin, and CD44. They also have higher levels of ALDH3 isozymes relative to non–stem cells. Functionally, SMG stem cells retain the ability to proliferate by forming bromodeoxyuridine (BrdUrd$^+$) salispheres and to differentiate into different SMG lineages in culture. However, this cell population is rare, hence there is a need to improve its yield. Treatment of adult mice with Alda-89, a selective ALDH3 activator, resulted in more than doubling of c-Kit$^+$/CD90$^+$ SMG stem cell and BrdUrd$^+$ salisphere number. Our data are the first to show that ALDH3 plays a role in SMG stem cell survival and activating this enzyme can be used for enrichment of these cells for future therapy.

Materials and Methods

Human salivary tissue collection

Salivary gland tissues were obtained from patients who underwent a neck dissection. All patients signed an informed consent approved by the Stanford Institutional Review Board (IRB).

Salivary gland tissue dissociation

Isolation of human and mouse SMG tissues was carried out as previously described (13). Salivary gland tissues were minced and dissociated in Dulbecco's Modified Eagle's Medium (DMEM)/F12 media (Gibco; Invitrogen) containing 0.1% trypsin-EDTA and 0.2 U/mL of liberase blendzyme 3 (Roche) for 4 hours at 37°C, centrifuged at 300 × *g*, and filtered through a Millipore filter (100 μm pore size, Millipore). Primary cells were resuspended in DMEM/F12 medium supplemented with N2, B27, EGF (20 ng/mL), FGF2 (10 ng/mL), IGF-1 (50 ng/mL), penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin-B (2.5 mg/mL; Gibco; Invitrogen). CD34$^+$ (human) and c-Kit$^+$ (murine) cells were isolated using an EasySep human CD34 and mouse c-Kit–positive selection kit (STEMCELL Technologies Inc.).

Salispheres formation and BrdUrd analysis

After 3 days of culture, cells were stained with BrdUrd according to the manufacturer's instructions (Invitrogen). The total number of salispheres and BrdUrd-positive salispheres per 96 wells was counted under light microscopy. For each group, 20 wells were counted and all experiments were repeated at least 3 times.

The salispheres were also cultured in rat tail collagen three-dimensional (3D) matrix (Roche). After 7 days of culture, the formation of ductal structures was examined. Images were captured with light microscopy under 400× magnification.

Immunohistochemistry

Frozen tissue sections were warmed to room temperature and fixed on ice in acetone for 20 minutes. Paraffin

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sections were deparaffined, rehydrated, and antigen retrieved (Vector laboratories). Slides were washed in PBS solution, incubated overnight at 4°C with different primary antibodies: mouse anti-CD34 (BD Biosciences), anti-c-Kit (R&D Systems), anti-Sca1 (R&D Systems), anti-CK5 (Covance), or anti-CK14 (Abcam), rinsed with PBS and incubated with anti-mouse secondary antibody (1:200 dilution; Invitrogen) at room temperature for 1 hour. The sections were then mounted with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Immunofluorescence images were acquired at 400× magnification using a Leica TCS SP2 confocal microscope (Leica Microsystems Inc.).

Fluorescence-activated cell-sorting analysis for stem cell markers

$CD34^+$-selected huSMG cells were incubated simultaneously with anti-human CD90, c-Kit, CD44, and Nestin antibodies (all simultaneously) for 30 minutes on ice. Isolated mouse SMG cells were incubated with anti-mouse c-Kit and Sca-1, or c-Kit and CD90 antibodies, respectively. The ALDEFLUOR Kit was used to detect ALDH1 activity (STEMCELL Technologies Inc.). Cell viability was detected with a LIVE/DEAD fixable staining kit (Invitrogen) following the manufacturer's protocol. ArC amine-reactive compensation beads (Invitrogen) were used as a positive staining control. Results were analyzed using the LSR II Analyzer (BD Biosciences) and FlowJo software (Tree Star).

A separate experiment was carried out to estimate the percentage of stem cells in human SMGs ($n = 3$). SMG tissues were digested as described; viable cells were identified using LIVE/DEAD fixable stain and then were incubated with anti-human CD34, CD90, c-Kit, CD44, and Nestin. The total cell number was determined by the CountBright absolute counting beads (Invitrogen) according to the manufacturer's protocol. The percentage of huSMG stem cells was calculated by dividing the total viable stem cells (defined as positive for all 5 surface markers) by the total viable cells collected per sample.

RNA purification, reverse transcription, and quantitative PCR

Total RNA was extracted from CD34 (human) and c-Kit (mouse) positive and negative cells, respectively, using TRIzol (Invitrogen). Reverse transcription was carried out on 500 ng of total RNA using random hexamer primers (Applied Biosystems). Quantitative PCR was carried out on the cDNA samples using a 7900HT detection system (Applied Biosystems). The primers for human samples are listed in supplementary information. The primers for mouse ALDH1A3, ALDH2, ALDH3A1, ALDH3A2, and ALDH6A were previously described (19). All PCR reactions were carried out in triplicate. Quantification of the samples was calculated with the threshold cycle by $\Delta\Delta C_t$ method. Experiments were repeated 4 times on different samples.

ALDH enzymatic assay

For *in vitro* assays, 20 μg of purified human recombinant ALDH enzymes, ALDH1, 2, 3, and 5 was used in each assay. The effect of Alda-89 on the enzymatic activity of each ALDH isozyme was determined by standard spectrophotometry, as described previously (20). The catalytic activity of ALDH was measured by the reduction of NAD^+ to NADH at $A_{340\ nm}$ in the presence or absence of Alda-89 (50 μmol/L). All kinetic assays were monitored for 3 minutes at 25°C in 100 mmol/L sodium pyrophosphate buffer, pH 8.8, 2.5 mmol/L NAD^+ using 10 mmol/L acetaldehyde as the substrate. For the *in vivo* assays, after 7 days of treatment with Alda-89 using the Alzet osmotic pump for sustained delivery of drug at 34 mg/kg/d, esophageal tissue (known to express ALDH3) was isolated from the Alda-89–treated or vehicle control animals. Homogenization was carried out in 100 mmol/L Tris HCl buffer, pH 8.0, with 10 mmol/L DTT, 20% glycerol and 1% Triton X-100. Three hundred micrograms of total protein from each supernatant was used in the enzymatic assay. ALDH activity was expressed as millimole per NADH/min/mg protein on the basis of the conversion of 1 OD = 6.22 mmol/L NADH.

In vivo treatment with ALDH3 activator (Alda-89)

All animal procedures were approved by the Institutional Animal Care and Use Committee at Stanford University. Female C57BL/6 mice (4–5 weeks old; Jackson Laboratory) were used. Alda-89 (34 mg/kg/d, $n = 5$) and vehicle control (PEG400/DMSO, 50:50, $n = 5$) were delivered in a sustained fashion using an ALZET osmotic pump (DURECT Corporation). The osmotic pumps, which contained 200 mmol/L of Alda-89 and delivered the drug at a rate of 1.0 μL/h, were implanted subcutaneously into C57BL/6 mice. The mice were euthanized after 7 days of treatment and salivary gland tissues were collected for analysis of salisphere formation and BrdUrd incorporation as described above. In addition, fluorescence-activated cell-sorting (FACS) analysis for the duel c-Kit/$CD90^+$ cell population was also carried out in pooled SMG samples.

The osmotic pumps with drugs lasting for 2 weeks were implanted intraperitoneally in C57BL/6 mice ($n = 8$ for each group) to deliver Alda-89 or vehicle control as above. One week postpump placement, the salivary glands were exposed to a single dose of 15 Gy radiotherapy with the rest of the body protected. Saliva collection was carried out at post radiation 1, 2, 3, 4, and 6 weeks after 2 mg/kg pilocarpine injection (s.c.) as previously described (21). The measured saliva secretion was normalized to the mouse body weight and to the pre-radiotherapy secretion level.

Statistics

Data are expressed as mean ± SEM. Statistical analysis of variance (ANOVA) and *t* tests were use to compare the different number of salispheres, BrdUrd incorporation, and various ALDH isoform expressions in CD34 or c-Kit–positive cells from human and mouse salivary tissues. A value $P \leq 0.05$ is considered to be significant.

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Results

Isolation of human salivary stem cells

Patient samples were dissociated and selected for $CD34^+$ cells. These cells were used for colocalization studies to characterize the presence of other stem cell surface markers by flow cytometry. As shown in Fig. 1A, more than 99% of viable $CD34^+$ cells also harbored c-Kit and CD90 on the cell surface and 70% were also positive for CD44 and Nestin. Functionally, these $CD34^+$ cells were able to form highly proliferative $BrdUrd^+$ salispheres in culture (Fig. 1B). The proliferative salispheres also retained positive staining for CD34 and c-Kit (Fig. 1C). When these spheres were placed in a 3D collagen matrix culture, they formed acinar- and ductal-like structures at day 7. Long-term cultured (10 days) salispheres stained positive for periodic acid—Schiff (PAS), confirming acinar differentiation (Fig. 1D). The overall stem cell yield (defined as cells positive for all 5 surface markers) by FACS analysis was $0.3\% \pm 0.01\%$ of total viable cells. These results show that we have been able to isolate viable adult salivary stem cell from patients' SMG and these cells are quite rare in adult unirradiated glands.

Similarly, we also successfully isolated $c\text{-}Kit^+$ stem cells from mouse SMG. Most of these cells coexpressed Sca-1 (73.8%) or CD90 (80.7%) and formed proliferative $BrdUrd^+$ salisphere in culture. Long-term culture (10 days) salispheres stained positive for ductal markers CK5 and CK14 as well as stem cell markers c-Kit and Sca-1. These salispheres also stained positive for PAS and formed ductal-like structures in 3D collagen matrix culture (Supplementary Fig. S1).

ALDH3 activation increased $c\text{-}Kit^+/CD90^+$ salivary stem cell population

Quantitative PCR analysis of mouse SMG $c\text{-}Kit^+$ cells showed significantly higher levels of ALDH3A2 (1.70 ± 0.44) and ALDH6A (1.76 ± 0.22) expression than $c\text{-}Kit^-$ cells (Fig. 2A). The ALDH1A3 (1.04 ± 0.02) and ALDH3A1 (1.10 ± 0.05) expression levels in $c\text{-}Kit^+$ cells did not differ from the $c\text{-}Kit^-$ cells. In addition, ALDH2 (0.49 ± 0.03) expression was significantly lower in $c\text{-}Kit^+$ cells than in the negative control cells. Similarly, human SMG $CD34^+$ cells have higher expressions of ALDH3A2 (7.94 ± 3.91) when normalized to the respective expressions in $CD34^-$ cells (Fig. 2B). Human SMG $CD34^+$ cells also had higher levels of ALDH1A1 (1.90 ± 0.33) and ALDH1A3 (4.55 ± 1.31) than non–stem cell counterparts (Fig. 2B). The higher expression of ALDH2 (2.00 ± 1.09) in the $CD34^+$ cells was not significant. Analysis using the ALDEFLUOR assay, a dye that measures mainly ALDH1 activity, showed that 84% of the human SMG stem cells have high ALDH activity (Fig. 2B). Fewer mouse SMG stem cells (69.2%) had high ALDH activity than diethylaminobenzaldehyde (DEAB)-treated control (Fig. 2A). Amplification of ALDH3A1 and ALDH6 was incomplete in human cells, whereas amplification of ALDH1A1 was incomplete in mouse cells.

Because the expression of the ALDH3A2 isoform was elevated in both human and murine SMG stem cell relative

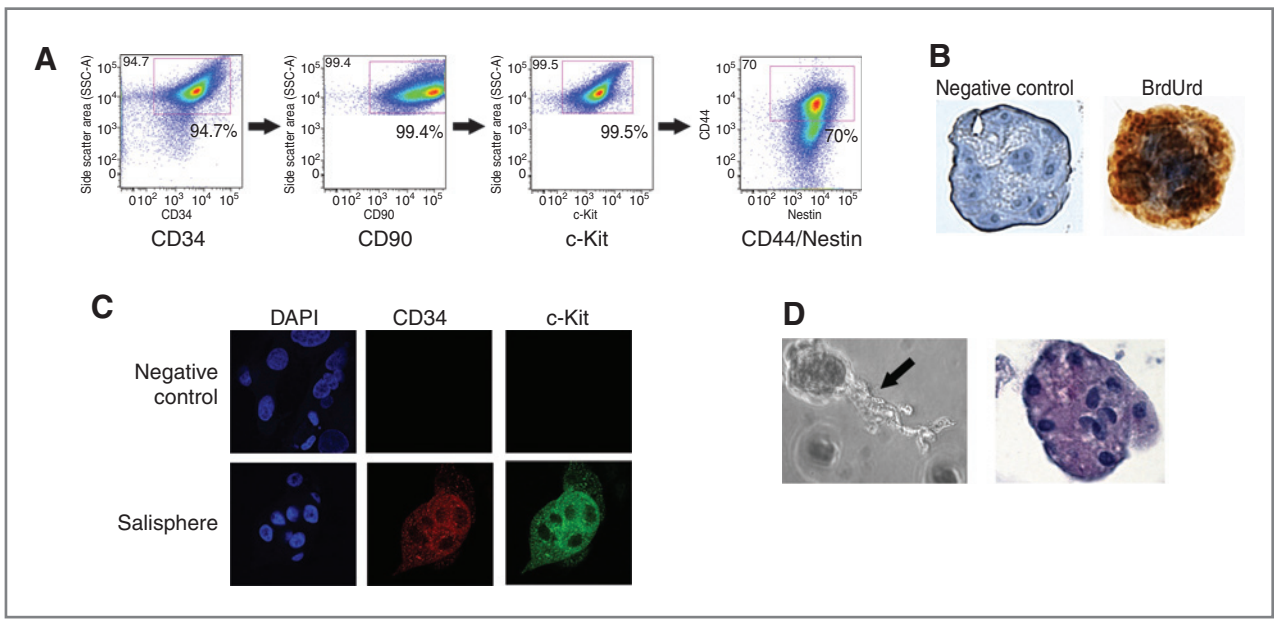

Figure 1. Isolation of human salivary stem cells. A, flow cytometric analysis of CD34-positive cells isolated from human SMGs shows colocalization of CD34 (94.7%), CD90 (99.4%), c-Kit (99.5%), and CD44/Nestin (70%). B, the cultured salispheres show positive staining for BrdUrd when compared with negative staining control. C, the salispheres showed positive staining for CD34 and c-Kit when compared with negative staining control. D, salispheres formed ductal-like structures (indicated by arrow) when cultured in 3D collagen matrix. The acinar structure is shown by periodic acid—Schiff (PAS) staining.

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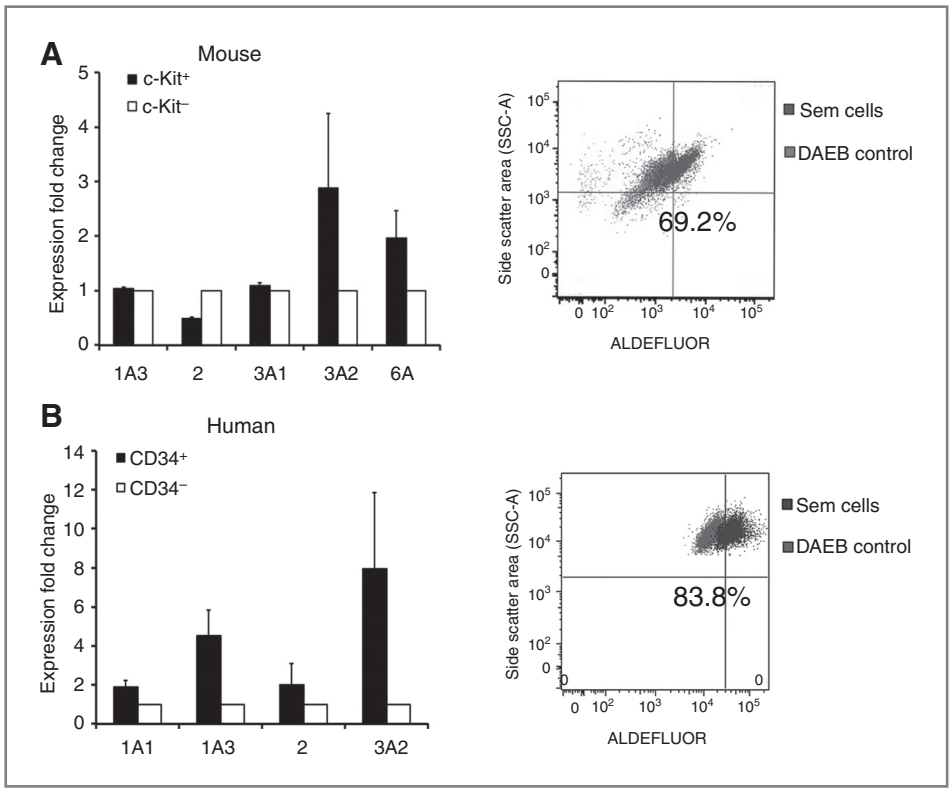

Figure 2. ALDH expression in salivary stem cells. A, quantitative PCR analysis of expression of different ALDHs in mouse SMG c-Kit$^+$ cells showed that there is a significant ($P < 0.05$) increase in the expression of ALDH3A2 (1.70 ± 0.44) and ALDH6A (1.76 ± 0.22) isozymes when compared with the normalized c-Kit$^-$ cells (1.00 ± 0.00). ALDH1A3 (1.04 ± 0.02) and ALDH3A1 (1.10 ± 0.05) expression did not differ from the c-Kit$^-$ cells. In contrast, ALDH2 (0.49 ± 0.03, $P < 0.05$) expression was significantly lower in the c-Kit$^+$ cells. Analysis using the ALDEFLUOR assay showed that 69% of the mouse SMG stem cells have elevated ALDH1 activity. B, quantitative PCR analysis of human SMG CD34$^+$ cells shows a significant ($P < 0.05$) increase in the expression of ALDH3A2 (7.94 ± 3.91), ALDH1A1 (1.90 ± 0.33), and ALDH1A3 (4.55 ± 1.31) when normalized to their respective expressions in CD34$^-$ cells. The increased expression of ALDH2 (2.00 ± 1.09, $P > 0.05$) in the CD34$^+$ cells was not significant. Analysis using the ALDEFLUOR assay showed that 84% of the human SMG stem cells have elevated ALDH1 activity.

to their NCS counterpart, we explored the effect of an activator of this enzyme on salivary stem cell yield in mice. Using a high throughput screen, we identified a novel ALDH3 small molecular activator (Alda-89), which specifically activates ALDH3 enzymatically (Fig. 3A, left). Alda-89 (5-allyl-1,3-benzodioxol; molecular weight = 162) is a related structure to Alda1, an ALDH2 activator (20). At 50 μmol/L, Alda-89 significantly increases the catalytic activity of human ALDH3 recombinant enzyme by 304.4% ± 7.1% of control ($n = 3$, $P < 0.01$), but has no effect on ALDH1, ALDH2, or ALDH5 isozymes (Fig. 3A, left). Infusion of Alda-89 into mice with an osmotic infusion pump successfully enhanced ALDH3 enzyme activity *in vivo* by 29% in esophageal homogenate as compared with vehicle control ($n = 5$, $P < 0.05$, Fig. 3A, right). Mouse SMGs were collected after 7 days of Alda-89 treatment and assayed by flow cytometry. Alda-89 treatment more than doubled the number of c-Kit$^+$/CD90$^+$ cells than the vehicle control (17.8% ± 0.95% vs. 7.37% ± 0.45%, $P < 0.05$), as shown in Fig. 3B (left). In contrast, it resulted in a slight reduction of c-Kit–negative cells but the difference was not statistically significant (Fig. 3B, right). Not surprisingly, ALDEFLUOR activity was not increased by Alda-89 treatment because the drug does not activate ALDH1 (Supplementary Fig. S2A and S2B). In addition, unselected SMG cells were collected and cultured to examine the effects of Alda-89 on BrdUrd$^+$ salisphere formation. Parallel to the increase in c-Kit$^+$/CD90$^+$ cells, the total number of salispheres were significantly greater in the Alda-89–treated group than in vehicle control (121 ± 0.83 spheres/1×10^5 cells vs. 86 ± 0.58; $P < 0.05$; Fig. 3C, left). Similarly, the number of BrdUrd$^+$ spheres was significantly higher in the Alda-89–treated group (91 ± 0.83 spheres/1×10^5 cells vs. 66 ± 1.42, $P < 0.05$, Fig. 3C, right). The sphere morphology was not different between the Alda-89 and the vehicle group (Supplementary Fig. S3). These data indicated that pharmacologic activation of ALDH3 was sufficient to double the yield of adult SMG c-Kit$^+$/CD90$^+$ stem cells.

We also conducted a very preliminary study to evaluate the effect of Alda-89 treatment on saliva production after radiotherapy. Mice ($n = 8$ per group) were pretreated with Alda-89 or vehicle for 1 week, followed by a single radiotherapy of 15 Gy to the SMGs, then by one more week of either Alda-89 or vehicle treatment. As shown in

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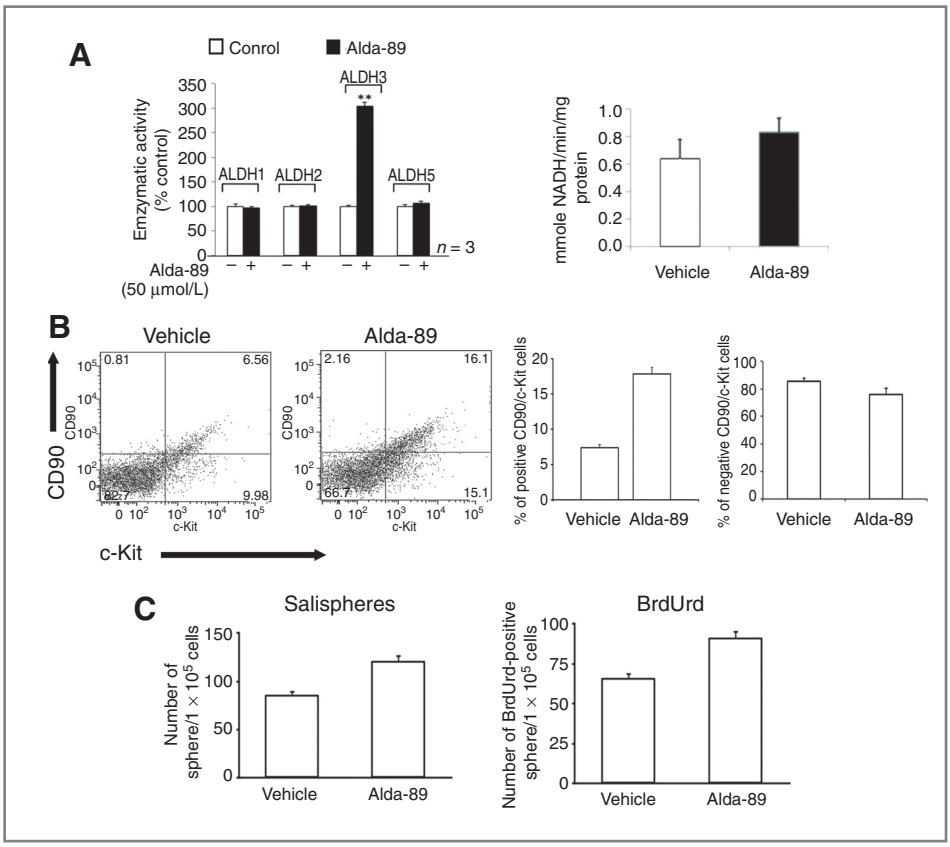

Figure 3. *In vivo* ALDH3 activation enriches salivary stem cells. A, Alda-89 (5-allyl-1,3-benzodioxol; molecular weight = 162) is an ALDH3 isozyme-selective activator. At 50 μmol/L, Alda-89 significantly increases the catalytic activity of human ALDH3 recombinant enzyme by 304.4% ± 7.1% of control ($n = 3$, $P < 0.01$), but has no effect on ALDH1, ALDH2, or ALDH5 isozymes (left). *In vivo*, infusion of Alda-89 into mice by an osmotic pump at 34 mg/kg/d enhances ALDH3 enzymatic activity by 29% in esophageal homogenate as compared with vehicle control ($n = 5$, $P < 0.05$). B, flow cytometric analysis of mouse SMGs collected after 7 days of Alda-89 treatment showed a significant increase of c-Kit$^+$/CD90$^+$ cells when compared with the vehicle control (17.8% ± 0.95% vs. 7.37% ± 0.45%, $P < 0.05$). In contrast, there was a slightly lower increase of c-Kit$^-$ cells (right). C, unselected SMG cells were collected and cultured to examine the effects of Alda-89 on BrdUrd$^+$ salisphere formation. The total number of salispheres were significantly greater in the Alda-89–treated group than in vehicle control (121 ± 0.83 spheres/1 × 10^5 cells vs. 86 ± 0.58; $P < 0.05$, left). The number of BrdUrd$^+$ spheres were also significantly higher in the Alda-89–treated group than in the vehicle control (91 ± 0.83 spheres/1 × 10^5 cells vs. 66 ± 1.42, $P < 0.05$, right).

Supplementary Fig. S4, although there was a trend for more saliva production in the Alda-89–treated group up to 4 weeks after radiotherapy, the difference was not statistically significant.

Discussion

For years, putative adult stem cells were isolated from rodent salivary glands that can form salispheres, which contain cells expressing different stem cell markers including c-Kit, Sca-1, and Musashi (22). However, the definitive functional proof that these are indeed stem cell did not materialize until 2008, when Lombaert and colleagues showed that a population of c-Kit$^+$ murine SMG cells possessed the capacity for self renewal *in vitro* and could differentiate into different salivary gland lineages. Most importantly, as few as 300 to 1,000 male murine c-Kit$^+$ cells were able to rescue salivary gland function and morphology up to 3 months after implantation into an irradiated female recipient gland (2). Secondary spheres cultured from recipient female murine glands were positive for the Y-chromosome, indicating that these spheres were derived from the donor male mouse (2). These results indicate that c-Kit$^+$ SMG cells are able to proliferate and differentiate *in vitro* and *in vivo*, and have established c-Kit as a novel SMG stem cell marker. The same group also reported that using the c-Kit, they were able to similarly isolate adult human salivary stem cells with the ability to self-renew *in vitro* (2). However, *in vivo* function of these cells has not been established. CD34 has long been considered to be a hematopoietic stem cell marker. Endothelial progenitor cells are positive for CD34. It is presently the main surface marker approved by the FDA for hematopoietic stem cell selection. Using CD34 instead of c-Kit as a selection marker, we have isolated a similar adult human SMG stem cell population with *in vitro* self-renewal and differentiation capacity. We believe that this population is similar to those isolated by Feng and colleagues as more than 99% of these

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cells also expressed c-Kit (23). In addition, most of these cells also expressed other stem cell markers including CD90, Nestin, and CD44.

Similar to prior reports, our human SMG stem cell yield was only 0.3%. The rarity of these cells makes it difficult to isolate enough viable cells for culture. Therefore, strategies to improve enrichment of these cells are needed prior to clinical implementation of stem cell therapy. To design enrichment strategy, understanding of the molecular pathways governing SMG stem cell proliferation and survival is necessary.

Although several studies have reported on the isolation of adult salivary stem cells, little is known about the pathways governing their survival. A lineage tracing study has shown that these cells express Ascl3, a transcriptional factor localized primarily to ductal cells (24) and reverse transcriptase PCR studies confirmed that they highly express the pluripotency markers Nanog and Oct3/4 (4, 25). Yet, nothing is known about the expression of the ALDH family, which are highly expressed in other normal tissue stem cells. ALDHs are enzymes involved in aldehyde detoxification and in the metabolism of retinoic acid, biogenic amines, and neurotransmitters (26). They are important for many biologic activities relevant to this study and are critical in reducing injury to cells because of oxidative stress (27). Elevated ALDH1 level is found in murine and human stem cells or progenitor cells (27), including hematopoietic (28, 29) or neural stem cells (27, 30) and muscle (31), hepatic (32), or adipose progenitor cells (33). The exact mechanism of how ALDH activity is involved in stem cell survival, proliferation, and differentiation is largely unknown and the relative level or activity of the different ALDH isozymes in the stem cell and non–stem cell compartments has not been well characterized. To date the most commonly studied ALDH isozyme in normal tissue stem cell is ALDH1. Stem cells with elevated activity of this isozyme are more likely to differentiate into different tissues than those with low activity through retinoic acid pathway (30, 34).

Unlike in other normal tissue stem cells, we found that the expression of ALDH3 rather than ALDH1 is elevated in both human and mouse SMG stem cell. Although ALDH1 expression was elevated in human SMG stem cells, its level was the same in mouse SMG stem cells. These findings were also supported by a higher percentage of ALDEFLUOR-positive cells in human SMG stem cells which primarily reflects ALDH1 activity. On the basis of these findings, we decided to focus on ALDH3 and identified a novel ALDH3 activator (Alda-89) based on structure–function relationship. We showed that Alda-89 specifically activated ALDH3 enzymatically but not other tested ALDH enzymes, including ALDH1. As a proof of concept, we showed that treatment of adult mice with Alda-89 resulted in increased stem cell yield and $BrdUrd^+$ sphere formation. We validated that Alda-89 is on target as it activates ALDH3 *in vivo*. As expected, treatment with Alda-89 did not change ALDEFLUOR activity as it is not expected to affect ALDH1 activity. The mechanism by which activation of ALDH3 augments the stem cell yield *in vivo* is largely unknown. Increased isolation of these putative SMG stem cells can be a result of (i) better survival of these cells during the isolation process, (ii) expansion of the stem cell compartment alone, or (ii) expansion of both stem cell and non–stem cell compartments. Because the total number of c-Kit–negative cells did not increase with Alda-89 infusion, it is unlikely that the drug caused expansion of both compartments. However, our current data cannot distinguish the first 2 options. *In vitro* studies are ongoing to examine the effect of Alda-89 on stem cell survival or expansion. Because of the poor solubility of this compound in water, we are in the process of modifying it chemically for *in vitro* studies.

Our preliminary experiment suggested that short-term Alda-89 infusion might result in more saliva production after radiation; however, the difference was small and did not reach statistical significance. We are in the process of testing different doses and duration of Alda-89 treatment and to determine the best sequence of the drug administration in relation to radiotherapy; however, this is beyond the scope of this study. We hope that we can identify the optimal dose and treatment duration of this drug for future clinical use.

In summary, we have been able to successfully isolate both human and murine SMG stem cells and found that they express higher ALDH3 mRNA than their non–stem cell controls. We are the first group to show that activating ALDH3 function with a specific small molecular activator can enrich SMG stem cell isolation. The mechanism by which ALDH3 activation enhances stem cell yield *in vivo* remains to be elucidated; however, enrichment of SMG stem cells is important for their future clinical application in the management of radiotherapy-related xerostomia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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