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Original Article

Functional regeneration of irradiated salivary glands with human amniotic epithelial cells transplantation

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Abstract: This study aimed to investigate the functional restoration of radiation-damaged salivary gland with human amniotic epithelial cells (hAECs) transplantation by intra-glandular injection. hAECs were isolated from the amnion tissues. After primary culture, the phenotype of hAECs of the second passage was identified by flow cytometry (FCM) and immunocytochemical staining. Then, hAECs were intra-glandularly injected into the irradiated glands of mice. At different time points after transplantation, the glands were collected for hematoxylin-eosin (HE) staining and immunofluorescence staining, and the saliva flow rate was also determined. Results showed these cells were positive for CD29, CD73 and CK19 and negative for CD44, CD34, CD45 and CD71. The transplanted hAECs in the recipient glands could differentiate into acinar-like cells and resulted in morphological and functional restoration of salivary gland.

Keywords: Functional regeneration, salivary gland, irradiated, amniotic epithelial cells

Introduction

Worldwide, there are more than 500,000 patients diagnosed with squamous cell carcinoma of the head and neck (HNSCC) every year [1]. Clinically, the treatment of the head and neck cancer often involves radiation either alone or in combination other modalities including chemotherapy. In the radiotherapy, an important dose-limiting factor is the sensitivity of normal tissues in the radiation field. Radiotherapy for HNSCC may inevitably cause damage to the normal salivary glands [2]. The quality of life in these patients is significantly reduced because of xerostomia (dry mouth syndrome) secondary to the radiation-induced damage to the salivary gland. Moreover, these patients may suffer from mucositis, dental caries, and problems of speech and food mastication, impaired taste and nocturnal oral discomfort [3, 4].

Up to approximately 90% of the salivary gland volume is constituted by acinar cells (ACs), which are responsible for water and protein secretion. The ductal system consists of intercalated duct, granular convoluted tubule, striated duct and excretory duct [5]. Stem cells with regeneration potential reside in the duct compartment where complete recovery is induced within a week after the main duct is blocked [6]. Irreversible damage to the salivary glands is mainly caused by the sterilization of these primitive glandular stem cells, which prevents the replenishment of aged saliva producing cells [7].

Currently, there is no effective therapy for this condition. Amniotic membrane is a translucent film attached to the surface of the placental chorionic plate. It is mainly composed by human amniotic epithelial cells (hAECs) and human amniotic mesenchymal cells (hAMCs). Researchers [8-10] have reported that hAECs have the characteristics of stem cell and can differentiate into hepatocytes, adipocytes, cardiomyocytes, and neuronal cells *in vitro*. hACEs are easily accessible and have the ability to differentiate into some functional cells. In our previous study, results showed hAECs could trans-

differentiate into acinar-like cells *in vitro* using a Double-Chamber System [11]. Transplantation of hAECs may represent a relatively simple and elegant approach to rebuild the functions of injured salivary gland. These characteristics make hAECs a promising source for future clinical application in regeneration medicine for the treatment of damaged salivary gland. In the present study, hACEs were isolated from the amniotic membrane and cultured *in vitro*. These cells were then intra-glandularly injected into the irradiation-damaged submandibular gland of rats, and the transdifferentiation of hAECs was evaluated in the recipient rats.

Materials and methods

Tissue collection and processing

This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of Zun Yi Medical College. Written informed consent was obtained from each participant before study. Fetal membranes were obtained from 3 healthy women with elective cesarean delivery. hAECs were isolated, cultured and passaged according to previously reported [11]. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zun Yi Medical College.

Characterization of hAECs

To detect the cell-surface markers, cell suspensions were incubated for 20 min at 4°C with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) - conjugated isotype control or antibodies against human CD29 (clone MAR4), CD73, CD44, CD34, CD45 or CD71 (clone IM7). Samples were analyzed with a FACSCalibur and the cell Quest software. A fraction of hAECs was seeded on the coverslips for fixation in 4% paraformaldehyde for 30 min, washed with PBS thrice, treated with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS for 20 min, washed with PBS thrice, and blocked with 1% BSA for 20 min. The primary antibody was anti-vimentin or CK19 monoclonal antibody; secondary antibodies (Dako Cytomation) were allowed to react with these cells for 30 min at 37°C. Immunostained hAECs were identified by microscopy. hAECs of the second passage were prepared for intraglandular transplantation.

Irradiation and collection of saliva

The salivary glands of 50 C57BL/6 mice aged 8 weeks were locally irradiated with X-ray at 15 Gy once (Philips CMG 41 X, 200 kV, 10 mA, 5 Gy/min). This dose is known to induce sufficient damage to the salivary glands without compromising the general health of animals. At 30 days post-irradiation, the whole saliva flow rate was determined. Animals were fixed in a restraining device [12] after pilocarpine injection (2 mg/kg, s.c.) and the saliva was collected for 15 min. The amount of saliva was determined gravimetrically, assuming a density of 1 g/mL for saliva.

Intraglandular transplantation of hAECs

For transplantation, hAECs of the second passage were collected and digested by 0.05% trypsin-EDTA. Cells were suspended in DMEM/F12 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), and Indian Ink (1/200) solution was injected to visualize the injected fluid. Cell suspension (5-10 μl, 1×10^6 cells per gland) was injected into the submandibular glands of mice at 30 days after irradiation by using a 33G needle and a Microliter Syringe (NanoFil USA) under anesthesia.

Histological and immuno-fluorescent analysis of submandibular glands

The submandibular glands were collected, fixed in formal sucrose, embedded in paraffin and sectioned (5 μm in thickness). After deparaffinization, these sections were treated with following antibodies: cytokeratin 7 (CK 7) (Bioss China), cytokeratin 14 (CK 14) (Bioss China), Amylase (H-281) (Santa Cruz, USA), MAB1281 (Millipore, USA), goat anti-rat IgG-FITC (Santa Cruz Biotechnology Inc., California, USA), or goat anti rabbit Ig-Texas Red (Santa Cruz Biotechnology Inc., California, USA). DAPI staining Visualization for bright field microscopy was accomplished by adding specific secondary biotin-conjugated antibodies (Dako, Carpinteria, CA), EnVision™Detection SystemsPeroxidase/DAB, Rabbit/Mouse (Gene Tech Shanghai, China), Nuclear counter staining was performed with hematoxylin or methylene green. Sections without treatment with primary antibodies served as negative controls.

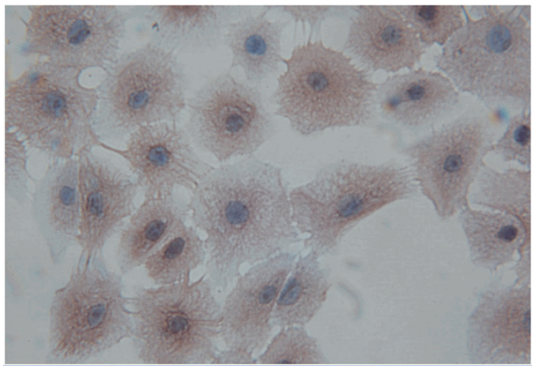

Figure 1. Immunocytochemistry staining for CK19 in hAECs (400 ×).

Submandibular glands were processed with routine histological technique for hematoxylin-eosin (HE) staining. Mucin and mucopolysaccharide containing acinar cells were detected by Periodic Acid Schiff (PAS) staining.

Statistical analysis

Data were compared with one way analysis of variance. A value of $P < 0.05$ was considered statistically significant, and SPSS version 16.0 was used for statistical analysis. Data were presented as mean ± standard error (SEM), unless otherwise specified.

Results

Identification of hAECs

After mechanical separation of amnion and chorion membranes and digestion with trypsin, about 5.0×10^7 of hAECs were obtained from each amnion. Cell viability was > 90%, and surface antigens on hAECs were detected by flow cytometry. Results showed these cells within six passages were positive for CD29 (95.75%) and CD73 (92.48%) but negative for CD44, CD34, CD45 and CD71. hAECs from human term fetal membranes within six passages were characterized after immunocytochemistry staining (**Figure 1**), and results showed they were positive for epithelial CK19. However, the proliferation was slowed down and the cell body changed after passaging six times.

hAECs transplantation

The *in vivo* capacity of hAECs to produce saliva was investigated in the submandibular glands of mice receiving irradiation at 15 Gy. At 30 days post-irradiation, a pronounced loss of acinar cells was found in the irradiation-damaged glands as compared to un-irradiated controls (**Figure 2A**, **2B**). hAECs from amnion fragments of 3 healthy women were intra-glandularly injected into submandibular glands (1×10^6 cells). The morphology of transplanted submandibular glands was detected by HE staining. As shown in **Figure 2**, at 30 days after hAECs transplantation, the transplanted glands appeared similar morphology as non-irradiated submandibular glands (**Figure 2A**, **2E**) and contained a large number of acinar cells. The functions of these cells were detected by PAS staining (**Figure 2F**, **2G**). Cells in control group had high mucin production (pink staining in **Figure 2F**), while, the mucin production was dramatically reduced after irradiation (**Figure 2G**). Irradiated glands almost had no acinar cells as demonstrated by PAS staining.

Intra-glandularly injected hAECs were observed in the irradiated submandibular gland after double immunofluorescent staining of MAB-1281 and CK 7, CK14, amylases, respectively. On the 30th day, the transplanted hAECs survived, and MAB1281 positive cells were observed in the irradiated glands after transplantation. Expressions of CK7, CK14 and amylase were detected in the MAB1281 positive cells as demonstrated by double immunostaining (**Figures 3**-**5**).

Whether these newly formed cells were functional was also investigated. After pilocarpine treatment, total saliva was measured in normal mice and irradiated mice with and without hAECs transplantation. The salivary secretion, measured at 30 days post-irradiation, was significantly increased after hAECs transplantation (125.68 ± 18.64 μL/15 min), as compared to irradiated animals (87.3 ± 8.29 μL/15 min). The un-irradiated mice produced 259.36 ± 9.74 μL/15 min saliva. Of importance, the total saliva was collected from the parotid, sublingual and submandibular glands. However, although all glands were irradiated, hACEs transplantation was performed in only the submandibular gland. Therefore, the comparison of total saliva production is likely to underestimate the gland recovery after transplantation. This result was further confirmed by the morphological observation after hAECs transplantation.

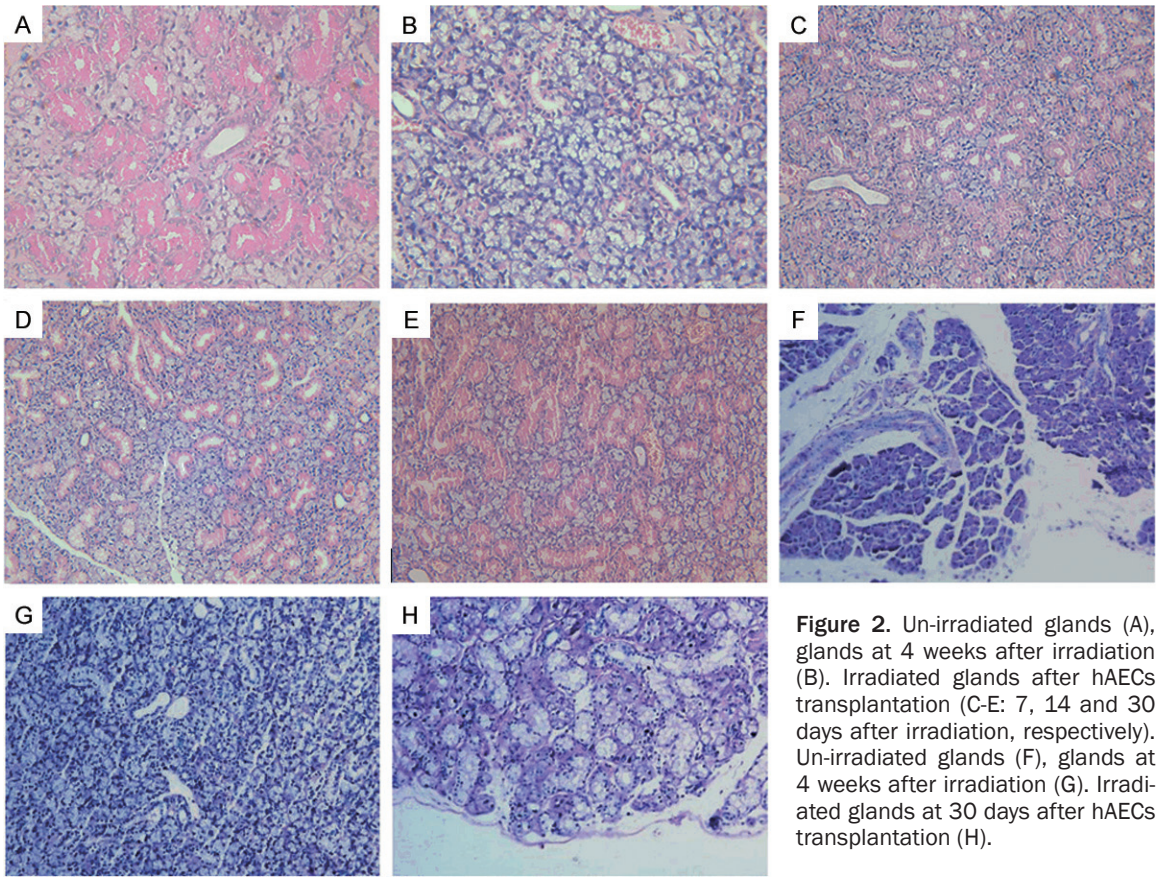

Figure 2. Un-irradiated glands (A), glands at 4 weeks after irradiation (B). Irradiated glands after hAECs transplantation (C-E: 7, 14 and 30 days after irradiation, respectively). Un-irradiated glands (F), glands at 4 weeks after irradiation (G). Irradiated glands at 30 days after hAECs transplantation (H).

Discussion

Radiotherapy is considered an effective local treatment for head and neck cancer, either alone or in combination with other therapeutic modalities such as surgery and/or chemotherapy. However, this treatment usually causes damage to the salivary gland that is accompanied by hypo salivation (xerostomia), a condition that has a negative impact on patients' quality of life. In the present study, a clinically applicable method was introduced for future rebuilding of salivary gland function after irradiation for the head and neck cancer. Our results showed the restoration of functions of irreversibly damaged mouse submandibular glands with *in vitro* intra-glandular injection of hAECs. Our findings suggest that hAECs are transplantation a promising method for the treatment of damaged salivary gland after radiation for head and neck cancer.

Irreversible damage to the salivary gland secondary to radiotherapy for head and neck cancer is mainly caused by sterilization of primitive glandular stem cells, which prevents the replenishment of aged saliva producing cells [13]. Currently, there are still no effective therapies for hypo-salivation caused by irradiation.

Clinical managements of xerostomia usually focus on the sialogogues aiming to improve the functions of remaining gland, and/or stringent oral hygiene and on saliva substitute agents to relieve symptoms. However, these treatment are not sufficient to normalize the complex environment of saliva [14-16].

In some cases, the damage to the salivary glands can be prevented by using intensity modulated radiation therapy (IMRT) [17], cytoprotectants like amifostine [13], prophylactic pilocarpine [18], or surgically transferring glands out of the irradiated field before radiotherapy [19]. However, these modalities are infeasible if little or no acinar cells remain in the glands. Therefore, to develop an effective treatment by using alternative strategies, such as gene therapy, tissue engineering, or cell-based therapy, is imperative [20-22].

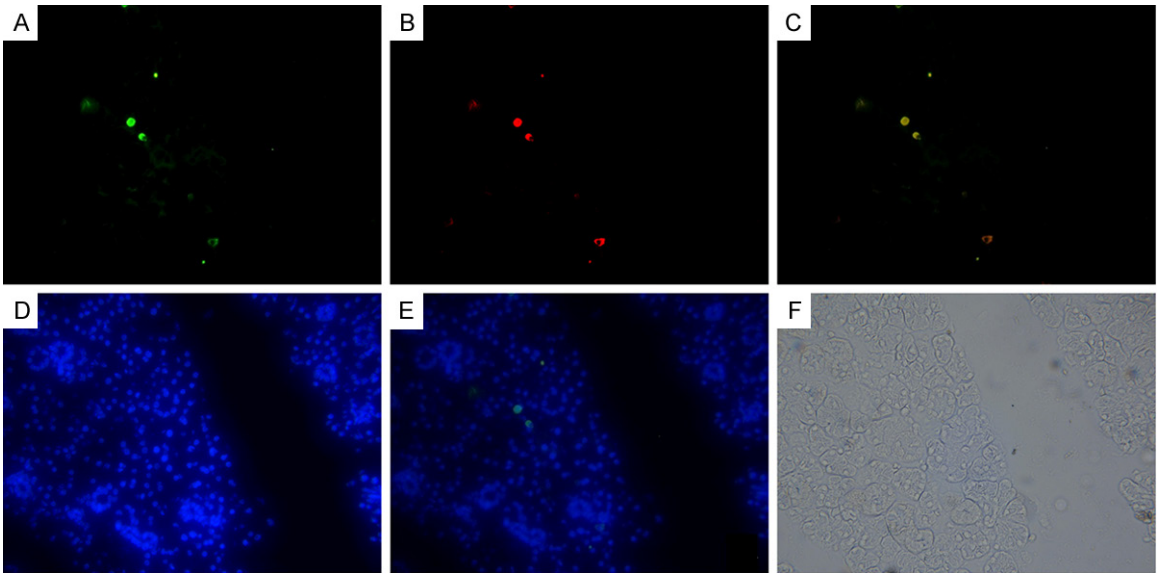

Figure 3. Intra-glandularly injected hAECs were detected in the submandibular gland at 30 days after transplantation. A: MAB1281 positive cells (green fluorescence), B: CK 14 positive (red fluorescence), C: Merge of A and B. D: DAPI staining (blue fluorescence), E: Merge of A and D. F: Bright field image.

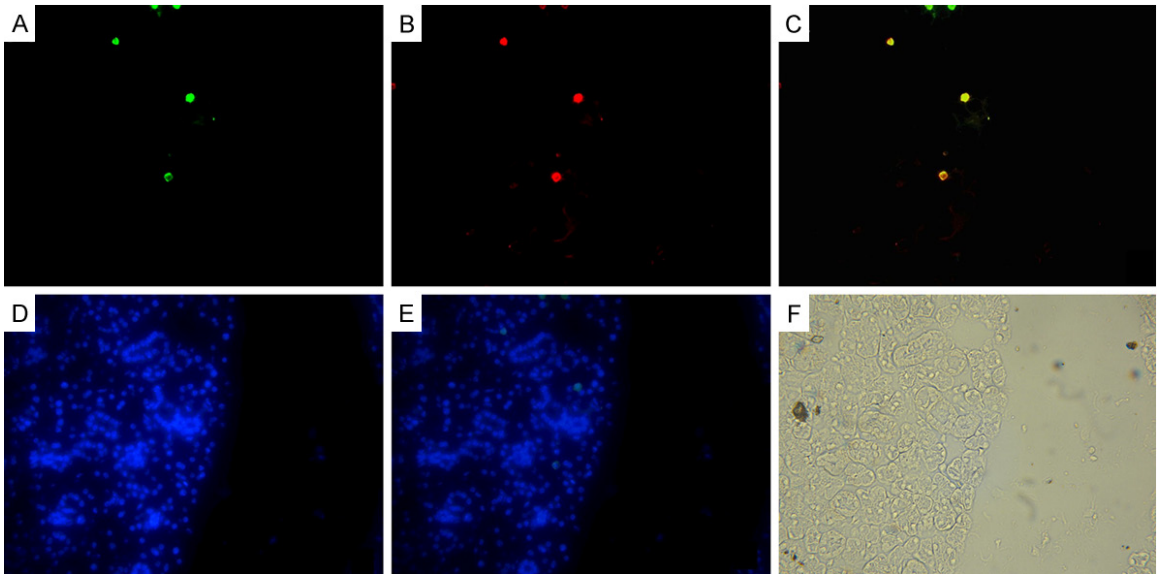

Figure 4. Intra-glandularly injected hAECs were detected in the submandibular gland at 30 days after transplantation. A: MAB1281 positive cells (green fluorescence), B: CK7 positive (red fluorescence), C: Merge of A and B. D: DAPI staining (blue fluorescence), E: Merge of A and D. F: Bright field image.

However, none of these seems to be sufficient and applicable to all patients. Stem cell therapy may be an option to reduce radiation-induced damage to the salivary glands permanently.

Bone marrow-derived cells (BMCs) have been suggested as an easy, accessible source of multipotent stem cells that can potentially transdifferentiate and/or repair non-hematopoietic organs [23-26].

Bone marrow-derived cells (BMCs) have been suggested as an easy accessible source for multipotent stem cells that could potentially transdifferentiate into non-hematopoietic tissues/or organs [7, 18, 19, 23], including the

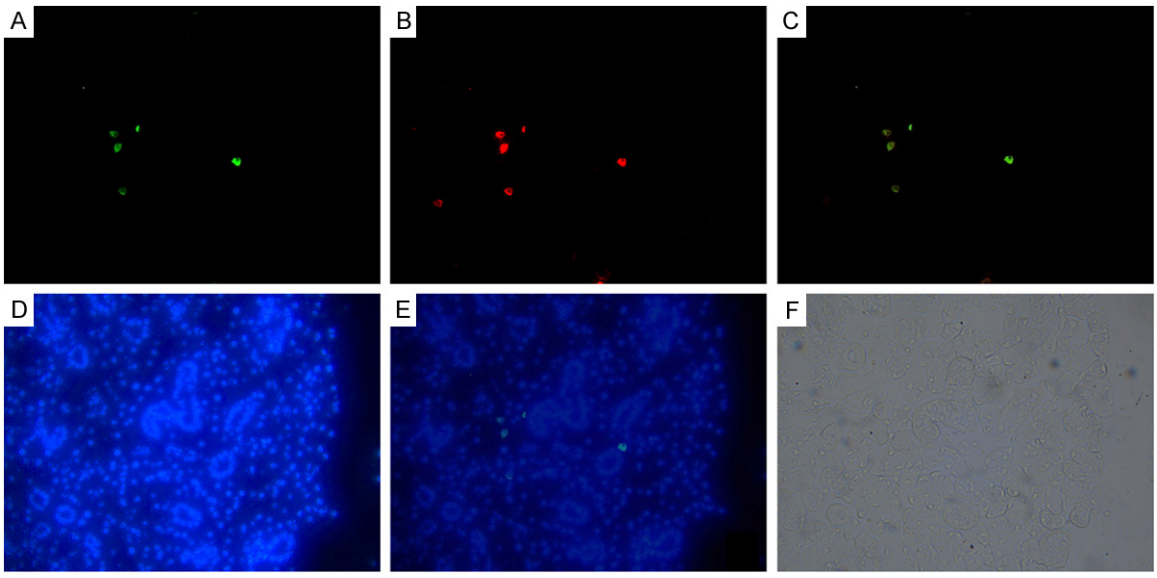

Figure 5. Intra-glandularly injected hAECs were detected in the submandibular gland at 30 days after transplantation. A: MAB1281 positive cells (green fluorescence), B: Amylase positive cells (red fluorescence), C: Merge of A and B. D: DAPI staining (blue fluorescence), E: Merge of A and D. F: Bright field image.

salivary glands [24]. The efficacy of BMCs in solid tissue repair remains controversial. BMCs can mobilize and home to the injured salivary gland after irradiation, and induce limited repair of the function and morphology of both submandibular gland and blood vessels. However, this process has no involvement of BMCs transdifferentiation into salivary gland cells [27, 28].

Transplantation of salivary gland stem cells may be a more adequate and elegant way to repair irradiation-damaged salivary glands. These cells can restore the gland functions after transplantation. However, this strategy may be difficult for clinical application if an insufficient number of stem cells are obtained from the gland of patients with the head and neck cancer. Another question is that the irradiation damaged salivary gland as a scaffold for stem cell transplantation is removed due to collection of stem cells. Researchers have found that the salivary gland stem/progenitor cells with self-renewal, high proliferation and multipotent differentiation activities in the salivary glands, even after irradiation. The survival of these cells depends on the radiation dose and cell aging [29].

Recently, researchers report that hAECs have multipotent differentiation ability and may serve as seed cells for cell transplantation. hAECs possess considerably advantageous characteristics. They can differentiate into all three germ layers, such as neurocytes, hepatocytes, and islet cells [30-33] and even acinar cells [11]; they have low immunogenicity and may exert anti-inflammatory effect [34, 35]; and their isolation has no requirement of sacrifice of human embryos, which avoids the use of human embryonic stem cells. Therefore, hAECs derived from the discarded amnion after parturition is expected to serve as an attractive seed cells in the regenerative medicine [36]. In this study, hAECs were collected and used to restore the functions of submandibular glands after irradiation, and our results highlighted a bright prospect on this approach.

In the present study, hAECs were successfully injected into the irreversibly damaged submandibular gland after irradiation. FCM showed hAECs of the second passage were positive for CD29, CD73 and CK19 but negative for CD44, CD34, CD45 and CD71. At 30 days after hAECs transplantation, the acinus, ductal structures were formed in the recipient gland. The glands receiving transplantation appeared similar morphology as un-irradiated glands and had a large number of acinar cells. The functions of these cells were further confirmed by PAS staining. The glands in control group had a large amount of mucin-producing cells, which were significantly reduced after irradiation. Mucin-

containing acinar cells colonized large areas of gland 30 days after transplantation. In addition, the transplanted hAECs showed remarkable morphological and functional restoring abilities in a long time. They could transdifferentiate *in vivo* into functional cells positive for CK7, CK14 and amylase in the irradiation-damaged recipient submandibular glands at 30 days after transplantation. Moreover, these CK7, CK14 and amylase expressing cells were also positive for MAB1281. These confirmed that these cells were hAECs.

To assess whether these newly formed cells were fully functional, total saliva secretion was measured after pilocarpine stimulation in normal mice and irradiated mice with and without transplantation. Saliva production, measured 30 days post-irradiation, was significantly enhanced after cell transplantation, as compared to irradiated animals without transplantation. The un-irradiated mice produced 259.36 ± 9.74 μL/15 min saliva, and mice with successful transplantation of hAECs restored the saliva production to 48% of that in control group. It should be noted that, in the detection of saliva flow, saliva was collected from the parotid, sublingual and submandibular glands. However, although all glands had been irradiated, only the submandibular gland received cell transplantation. Therefore, comparison of total saliva production is likely to underestimate the gland recovery after transplantation. Strikingly, a clear separation in 3 groups was observed; either very few, an intermediate number or a large number of acinar cells were found after transplantation. Of note, the saliva restoration was not achieved in all animals, which might be due to the technical difficulties in the injection.

In conclusion, our findings suggest that the intra-glandular transplantation of hAECs to the irradiated gland can result in the functional improvement of injured glands. Isolated hAECs from human amniotic membrane (hAM) can provide a sufficient number of cells. Thus, hAECs can be used as seed cells for the cell-based therapy to restore the hypofunction of irradiated salivary glands in mice. However, some questions still remain unanswered. To achieve more efficient transplantation, studies are still needed to overcome the limitations of general application of hAECs and optimize the techniques of stem cell injection into the damaged salivary glands to increase the overall outcome.

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Disclosure of conflict of interest

None declared.

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