Characterization of Primary Epithelial Cells Derived from Human Salivary Gland Contributing to in vivo Formation of Acini-like Structures

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Patients with head and neck cancer are treated with therapeutic irradiation, which can result in irreversible salivary gland dysfunction. Because there is no complete cure for such patients, stem cell therapy is an emerging alternative for functional restoration of salivary glands. In this study, we investigated in vitro characteristics of primarily isolated epithelial cells from human salivary gland (Epi-SGs) and in vivo formation of acini-like structures by Epi-SGs. Primarily isolated Epi-SGs showed typical epithelial cell-like morphology and expressed E-cadherin but not N-cadherin. Epi-SGs expressed epithelial stem cell (EpiSC) and embryonic stem cell (ESC) markers. During long-term culture, the expression of EpiSC and ESC markers was highly detected and maintained within the core population with small size and low cytoplasmic complexity. The core population expressed cytokeratin 7 and cytokeratin 14, known as duct markers indicating that Epi-SGs might be originated from the duct. When Epi-SGs were transplanted in vivo with Matrigel, acini-like structures were readily formed at 4 days after transplantation and they were maintained at 7 days after transplantation. Taken together, our data suggested that Epi-SGs might contain stem cells which were positive for EpiSC and ESC markers, and Epi-SGs might contribute to the regeneration of acini-like structures in vivo. We expect that Epi-SGs will be useful source for the functional restoration of damaged salivary gland.

Keywords: acinar and duct, epithelial cell, head and neck cancer, salivary gland, stem cell

INTRODUCTION

Patients who suffer from head and neck cancer are commonly treated with therapeutic irradiation. However, therapeutic irradiation can lead to salivary gland dysfunction such as reduced saliva production (Vissink et al., 2003a; 2003b). Although saliva substitutes such as artificial lubrication have been developed, there are still no relevant therapeutics to restore functional salivary gland. To overcome these limitations, stem cell therapy is emerging as an alternative therapeutics (Lombaert et al., 2017; Pringle et al., 2016). Salivary gland is composed of multiple cell types, including epithelial, myoepithelial, mesenchymal, neuronal and endothelial cells. Among them, functional components are acinar...
and ductal epithelial cells, which may be therapeutic targets by stem cells. Recently, salivary gland stem cells (SGSCs) have been identified (Feng et al., 2009; Lombaert et al., 2008; Nanduri et al., 2014) and their efficacy has been proved in experimental animal models (Lombaert et al., 2017; Pringle et al., 2016), which suggests potential clinical application of SGSCs.

For the clinically relevant development of stem cell therapy, it is necessary to expand stem cells to acquire enough number of cells for transplantation. Sphere culture method has been used for ex vivo expansion of SGSCs (Nanduri et al., 2014). In this study, we primarily isolated epithelial cells derived from human salivary gland (Epi-SGs) and investigated whether Epi-SGs had stem cell-like characteristics and the stem cell-like characteristics of Epi-SGs could be maintained during long-term culture. Moreover, to answer the origin of Epi-SGs, the expression of cytokeratins was analyzed. Finally, the in vivo functional roles of Epi-SGs were determined via transplantation into immunodeficient mouse.

MATERIALS AND METHODS

Primary isolation and culture
The experimental protocol was approved by the Institutional Review Board (CR106002) of Seoul National University Dental Hospital. Informed consent was obtained from the patients. Human submandibular glands were obtained from patients with squamous cell carcinoma of the oral cavity requiring a neck dissection procedure. None of the patients had received any other cancer treatments prior to the surgical procedure. The submandibular glands were carefully dissected to avoid contamination from other tissues. A cell suspension was prepared by mincing and enzymatic dissociation with 1 mg/mL collagenase type I and 2.4 mg/mL of dispase (Gibco, USA) at 37°C for 30 min with gentle agitation. After an additional 30 min of digestion with fresh enzymes, the suspension containing tissue and cells was filtered through 100-μm mesh (BD, USA). After enzyme inactivation, the cells were suspended in Minimum Essential Medium Alpha (α-MEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and plated in a 6-well plate (SPL Life Sciences, Korea) for 1 day. At next day, the medium was removed and washed with PBS. New serum-free keratinocyte growth medium (KGM: Lonza Rockland, USA) with the provided supplements, was added. To remove mesenchymal cells, 0.01% Trypsin-EDTA (Gibco) was applied for 2 min. The cells were sub-cultured using 0.25% Trypsin-EDTA (Gibco) when they reached 70-80% confluence. The cells were counted and photographed at each passage, and the population doubling level (PDL) was calculated. The primary isolation and culture conditions of dental pulp stem cells (DPSCs), normal human oral keratinocytes (NHOKs), normal human oral fibroblasts (NHOFs), and human embryonic stem cells (hESCs) were written in Supplementary Materials and Methods.

FACS analysis
For FACS analysis, the cells were harvested and washed with PBS supplemented with 2% FBS. The antibodies are listed in Supplementary Table 1. Each primary antibody was incubated with 10,000 cells for 30 min on ice. After washing, the secondary antibody was applied for 30 min on ice. After washing, the cells were fixed with 4% paraformaldehyde at 4°C before analysis. For intracellular staining, the cells were fixed with 0.4% paraformaldehyde for 10 min and permeabilized with ice-cold methanol for 10 min before incubation with the primary antibody. The fluorescence intensity was measured on a FACSCalibur (Becton Dickinson, USA), and the data were analyzed using FLOWJO software (Tree Star, Inc., USA).

RT-PCR
Total RNA was obtained from cells using an RNasy Mini Kit (Qiagen, USA). The total RNA (2 μg) was reverse-transcribed with M-MLV (Invitrogen TM, USA) and oligo dT by incubating at 42°C for 1 h and inaction at 90°C for 15 min. The resulting cDNAs were used as templates for PCR. The PCR was performed with an i-MAXII (Intron, Korea). The conditions used for the PCR and the oligonucleotide sequences of the gene-specific primer pairs used for the amplification of the EpSC-related genes (ABCG2, ΔNp63, and p75) and the ESC-related genes (Oct4 and Sox2) were described previously (Nam and Lee, 2009). The PCR products were separated on 1.5% agarose gels containing ethidium bromide.

Immunocytochemistry
Cells were cultured in 4-well plate (SPL Life Sciences) to be at 70-80% confluence and fixed with ice-cold methanol for 10 min at -20°C. The cells were washed with PBS, and then blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories Inc., USA) for 1 h at room temperature. The following primary antibodies were used: rabbit anti-keratin 7 (1:100; Cell signaling) and mouse anti-keratin 14 (1:100; Millipore). Primary antibodies were applied overnight at 4°C. Secondary antibody incubations were carried out for 1 h at room temperature using Alexa 488-conjugated goat-anti rabbit IgG (1:700; Invitrogen) and Alexa 594-conjugated goat-anti mouse IgG (1:700; Invitrogen) antibodies. All the fluorescent-stained sections were counterstained with DAPI (Sigma-Aldrich). Slides were observed using a confocal laser scanning microscope (Fluoview FV 300, Olympus, Japan).

In vivo transplantation
All animal experiments were approved by the appropriate Institutional Review Boards and conducted in accordance with the ‘National Institute of Health Guide for the Care and Use of Laboratory Animals’ (NIH publication No. 80-23, revised in 1996). A total of 3 × 10⁶ cells was resuspended in 200 μl of ice-cold Phenol Red-free Matrigel (BD Bioscience, USA). Implants of Matrigel alone were served as controls. The mixture was transplanted subcutaneously into the dorsal surface of 6-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague-Dawley, USA) using a 25-gauge needle. At 4 and 7 days after injection, mice were sacrificed in a CO₂ chamber and Matrigel implants were removed for histological analysis.
Immunohistochemistry
Matrigel implants were removed, fixed in 10% buffered formalin overnight, embedded in paraffin, and sectioned at 5 μm-thickness. 5-μm-thick sections were deparaffinized in histoclear (National Diagnostics, USA) and rehydrated through a series of graded alcohols and distilled water. For histological analysis, slides were stained with hematoxylin and eosin (H&E) and examined for the presence of acini-like structure. For immunohistochemistry, slides were incubated in 3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase. After blocking with 10% normal goat serum for 1 h, primary antibodies, anti-p63 (Santa Cruz Biotechnology, USA), anti-Aquaporin 5 (Santa Cruz), and anti-Pan-cytokeratin (Santa Cruz) were treated at 4°C overnight. Secondary antibody incubations were carried out for 1 h at room temperature using Alexa 488-conjugated goat-anti rabbit IgG (1:700; Invitrogen) and Alexa 594-conjugated goat-anti mouse IgG (1:700; Invitrogen) antibodies. All the fluorescent-stained sections were counterstained with DAPI (Sigma-Aldrich). Slides were observed using a confocal laser scanning microscope (Fluoview FV 300, Olympus, Japan).

RESULTS
Primary isolation and characterization of epithelial cells from human salivary gland
Human salivary glands were minced and enzymatically digested into small clumps. Although the use of trypsin could dissociate such clumps into single-cell suspensions, it resulted in reduced viability and even the loss of rare stem cell populations. Therefore, we chose the clump culture method to obtain outgrowing cells from adherent small clumps. Media containing fetal bovine serum (FBS) was used at the initiation of the culture to efficiently attach small clumps. The next day, the culture medium was replaced with serum-free keratinocyte growth medium (KGM) to exclude mesenchymal cells; however, the media switch was not sufficient to completely remove the mesenchymal cells. Within a week, we acquired pure epithelial cells by discarding mesenchymal cells using 0.01% trypsin, which was optimal concentration for the removal of mesenchymal cells.
Primarily isolated epithelial cells from human salivary gland (Epi-SGs) showed typical epithelial cell-like morphology which was maintained during the culture period (Fig. 1A). However, the size of Epi-SGs and the number of vesicles within the cytoplasm increased as the passaging progressed. Although Epi-SGs could be maintained for more than 6 passages, the cells ceased to proliferate during the later passages (Fig. 1B). Although the size and cytoplasmic complexity of Epi-SGs increased, a small population of cells with small size and a low number of vesicles was maintained during the late passages. The immunophenotype of Epi-SGs was determined by FACS analysis. As shown in Fig. 1C, Epi-SGs were positive for some mesenchymal stem cell (MSC) markers, CD29, CD73, and CD90. However, the expression of CD44 and CD105 was low, and the cells were negative for hematopoietic (CD14, CD34, CD45, and CD117) and endothelial (CD31) markers. Epi-SGs showed different expression pattern from dental pulp stem cells (DPSCs) which have typical MSCs-like characteristics (Supplementary Fig. S1). The results by immunofluorescent staining demonstrated that Epi-SGs expressed E-cadherin highly. However, N-cadherin was rarely expressed compared to E-cadherin.

In vitro characterization of stem cell-like characteristics of Epi-SGs

To investigate the stem cell-like characteristics of Epi-SGs, the expression of epithelial stem cell (EpiSC) and embryonic stem cell (ESC) markers was determined by RT-PCR. The PCR conditions for EpiSC- and ESC-related genes were validated in Hertwig’s epithelial root sheath (HERS) cell line, human embryonic stem cell (hESC), and normal human oral fibroblast (NHOF). The RT-PCR data showed that three independent cell lines were positive for EpiSC markers including ABCG2, ΔNp63, and p75 and ESC markers including Oct-4 and Sox2 (Fig. 2A). To confirm the expression of EpiSC and ESC markers, we conducted FACS analysis of Epi-SGs using EpiSC and ESC markers, which were validated using HERS cell line (Supplementary Fig. S3). As shown in the result of forward and side scatter, the cytoplasmic complexity of Epi-SGs increased dependent on the number of passaging (Fig. 2B). Epi-SGs were subdivided into two populations including core population with small size and lower cytoplasmic complexity and other population with higher cytoplasmic complexity. However, the core population with small size and low vesicle complexity was maintained (Fig. 2B). Interestingly, EpiSC and ESC markers were highly expressed within core population, because the expression of EpiSC and ESC markers decreased when the region of analyzed population based on the forward and side scatter profiles was bigger (Supplementary Fig. S2). Most cells with large and irregular surface were viable and their viability was similar to core population (Supplementary Fig. S4). These data suggested that
core population expressing EpiSC and ESC markers was maintained and that there might be a hierarchical organization of Epi-SGs during the culture period.

**The origin of Epi-SGs**

The expression levels of cytokeratin 7 (CK7) and cytokeratin 14 (CK14) were assessed to evaluate the origin of Epi-SGs. The expression of CK7 and CK14 in the core population was determined by FACS analysis (Fig. 3A and Supplementary Fig. S5) and confirmed by immunofluorescent staining (Fig. 3B). During the culture period, expression of CK7 and CK14 was maintained in the Epi-SGs. These data suggested that Epi-SGs might be derived from salivary ducts.

**In vivo transplantation of Epi-SGs**

To investigate in vivo functional roles, Epi-SGs were transplanted into the dorsal region of immunodeficient mouse via subcutaneous injection route with Matrigel. Two different numbers of Epi-SGs, $3 \times 10^6$ cells per injection or $4 \times 10^6$ cells per injection were mixed with Matrigel and injected into the dorsal region of immunodeficient mouse. At 4 and 7 days after transplantation, Matrigel implants were removed and analyzed by hematoxylin and eosin (H&E) staining. In the result of Matrigel implants at 4 days after transplantation, we could observe acini-like structures (Fig. 4A). In the marginal region of Matrigel implants, more numbers of acini-like structures were observed. These acini-like structures were maintained at 7 days after transplantation (Fig. 4A). In the result of Matrigel implants with $3 \times 10^6$ cells at day 4 after transplantation, we could observe acini-like structures with increased size compared to the results of Matrigel implants with $3 \times 10^6$ cells (Fig. 4B). However, at 7 days after transplantation, the number of acini-like structures decreased (Fig. 4B) but the size of acini-like structure increased, which might be derived by fusion of small acini-like structures.

To determine the differentiating status of Epi-SGs after transplantation, the expression of stem cell marker (p63), differentiated cell marker (Aquaporin-5), and epithelial cell marker (Pan-cytokeratin) was determined by immunofluorescent staining. In salivary gland tissue, the expression of p63 was only detected in duct-like structures (Supplementary Fig. S6A). Epi-SGs expressed p63 with different expression levels. After transplantation of Epi-SGs, the expression of p63 was
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Fig. 4. In vivo transplantation of Epi-SGs. For this experiment, $3 \times 10^6$ cells or $4 \times 10^6$ cells of Epi-SGs were transplanted into immunodeficient mice. (A) After transplantation with $3 \times 10^6$ cells, acini-like structures were observed at 4 days after transplantation. They were maintained at 7 days after transplantation. (B) After transplantation with $4 \times 10^6$ cells, acini-like structures were also observed at 4 days after transplantation with more number of total acini-like structures. However, they were not maintained at 7 days after transplantation.

not completely disappeared and localized in the outer layer of acini-like structures. In salivary gland tissue, Aquaporin 5 and Pan-cytokeratin were localized in acini-like and duct-like structures, respectively (Supplementary Fig. S6B). Most of Epi-SGs showed differential expression of Aquaporin 5 and Pan-cytokeratin. After transplantation of Epi-SGs, Aquaporin 5 and Pan-cytokeratin were not co-localized within each acini-like structure. These data suggested that Epi-SGs might be heterogeneous population and after transplantation, Epi-SGs could form acini-like structures, consisting of Aquaporin 5-positive differentiated cells.

**DISCUSSION**

The important functional components of salivary glands are the acinar and ductal epithelial cells. Although previous reports suggested the existence of mesenchymal stem cell-like cells (Gorjup et al., 2009; Rotter et al., 2008) based on the developmental origin of the acini and ducts, other reports proposed that stem cells that could regenerate the acinar and ductal structures, which had epithelial characteristics. Moreover, primitive stem cells such as embryonic stem cells have been reported to possess epithelial characteristics (Huang et al., 2011; Kolle et al., 2009). Salivary glands are composed of multiple cell types, including epithelial, myoepithelial, mesenchymal, neuronal and endothelial cells. Moreover, based on the embryonic development of the salivary gland, it is postulated that the salivary gland may contain different stem cell populations with different differentiation potentials, thereby indicating a heterogeneous and hierarchical organization of the salivary gland. We therefore isolated and enriched stem cells with epithelial-like characteristics from the human salivary gland. The use of serum-free media and differential concentrations of trypsin to exclude mesenchymal cells was sufficient to enrich purified epithelial-like cells. We confirmed that the remaining cells had epithelial
characteristics using FACS analysis and immunofluorescent staining. This simple culture method was very convenient and would be applicable for other epithelial organs to enrich epithelial cells.

There have been reports about the expression of EpISC markers and their functional roles during the development of epithelial organs (Lombaert and Hoffman, 2010). However, the expression of EpISC markers in salivary gland has not been well studied. Among various types of EpISC markers, ABCG2, ΔNp63, p75 have been used to identify EpISCs in human multilayered epithelial tissues (Chen et al., 2004; de Paiva et al., 2005; Zhou et al., 2001). Similar to other epithelial organs, EpISCs expressed EpISC markers including ABCG2, ΔNp63, and p75. The expression of EpISC markers suggested that we could culture stem cells with epithelial characteristics from the human salivary gland. We further characterized the expression of ESC markers in EpISCs. Primitive stem cells such as induced pluripotent stem cells (iPSCs) and ESCs contain core transcriptional regulation by Oct-4, Sox2, Nanog known as pluripotent genes. Recently, cellular reprogramming by overexpressing pluripotent genes demonstrated that their expression could be an indicator of stemness (Okita et al., 2007). In our study, EpISCs expressed not only EpISC markers, but also ESC markers. The expression of pluripotent genes suggested the existence of primitive stem cells within EpISCs. However, we did not identify specific markers of most of the undifferentiated stem cells within EpISCs. Clonal analysis may be a solution for the identification of specific markers.

To acquire enough number of stem cells, proper ex vivo expansion system is necessary. During expansion, stem cells proliferate, differentiate, and finally lose their stemness. Therefore, it is important to maintain the original stem cell characteristics during long-term culture. The hierarchical organization of adult stem cells has been reported for hema-topoietic stem cells and epithelial stem cells (Ema et al., 2014; McQualter et al., 2010). Stem cells have also been reported to be small in size (Izumi et al., 2007), which could be critical criteria to determine stemness of epithelial stem cells. Although the size and complexity of the EpISCs increased, the core population with small size and low cytoplasmic complexity was maintained and expressed EpISC and ESC markers during long-term culture. These data suggested that core population within EpISCs might have primitive stem cell-like characteristics. For ex vivo expansion of the core population within EpISCs, it is important to identify the underlying mechanisms involved in the maintenance of stemness. In the biology of ESCs, a feeder layer such as mouse embryonic fibroblast (MEF) has been used to culture ESCs because MEF has functional roles as a microenvironment for the maintenance of stemness. Feeder systems may also be feasible for retaining the stemness of EpISCs, especially the core population. Moreover, leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) are critical for the stemness of mouse and human ESCs, respectively. FGF signaling plays important roles in the morphogenesis and stemness in mouse submandibular gland development (De Moerlooze et al., 2000; Jaskoll et al., 2005).

Considering the clinical applications of EpISCs, it may be better to develop serum-free and xeno-free culture method. The origin of murine SGSCs has been suggested as the ducts (Feng et al., 2009; Lombaert et al., 2008). Lineage tracing using genetic labeling, label-retaining assays, and duct ligation were applied to identify SGSCs (Okumura et al., 2012). However, these techniques are impossible to apply to human salivary glands. After primary isolation of SGSCs, FACS analysis can be used to identify stem/progenitor cells from adult tissue. Differential expression patterns of keratin have been used to characterize basal, intermediate, and differentiated cell types in many epithelial tissues (Purkis et al., 1990; van Leenders et al., 2000). Murine SGSCs express both CK7 and CK14, also applicable to determine the origin of human SGSCs. In our result, EpISCs expressed CK7 and CK14, which was maintained throughout the culture period. Moreover, the core population of EpISCs expressed CK7 and CK14 during long-term culture, which suggested that in our culture conditions, the original characteristics of core population was not changed.

We could culture EpISCs via adherent culture method with serum-free media. However, in previous reports, SGSCs were isolated and cultured via non-adherent sphere culture method also called salisphere culture (Feng et al., 2009; Nanduri et al., 2014). For salisphere culture, c-kit-positive SGSCs were isolated and expanded. The most difference between EpISCs and SGSCs was also the expression of c-Kit, because the expression of c-Kit was rarely detected in EpISCs. However, through adherent culture method, we could acquire approximately 60 × 10⁶ to 90 × 10⁶ per one biopsy. The functionality of EpISCs was confirmed via transplantation into immunodeficient mouse. EpISCs could form sphere-like structures after transplantation but they did not show typical acini-like structures, which might be similar to the results in previous reports (Feng et al., 2009; Lombaert et al., 2008). The number of injecting EpISCs did not affect the ability of acini-like structure formations, suggestive of preclinical and clinical optimization of dose.

In conclusion, there is an unmet need to identify and characterize SGSCs for functional restoration of salivary gland. Here, we could demonstrate the stem cell-like characteristics of EpISCs and heterogenous organization during long-term culture. Moreover, we proved the possible origin of EpISCs as ducts. We could not conclude that EpISCs would be functional after transplantation, because EpISCs did not form typical acini-like structures and show immunophenotypes, suggesting of further study. We expect that our results will contribute to the better understanding of stem cells in salivary gland and the development of potential stem cell therapy for the patients with an irreversible loss of salivary gland function.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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