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Characteristics of Labial Gland Mesenchymal Stem Cells of Healthy Individuals and Patients with Sjögren's Syndrome: A Preliminary Study

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Sjögren's syndrome (SS) is a systemic autoimmune disease that is characterized by focal lymphocytic infiltration into exocrine organs such as salivary and lacrimal glands, resulting in dry mouth and eyes, and other systemic injuries. There is no curative clinical therapy for SS, and stem cell therapy has shown great potential in this area. The mesenchymal stem cells (MSCs) in the salivary glands of healthy individuals and in patients with SS have not been extensively studied. The aim of this study was to elucidate the characteristics of MSCs from the labial glands of healthy controls and of those from patients with SS to elucidate the related pathogenesis and to uncover potential avenues for novel clinical interventions. Labial glands from patients with SS and healthy subjects were obtained, and MSCs were isolated and cultured by using the tissue adherent method. The MSC characteristics of the cultured cells were confirmed by using morphology, proliferation, colony forming-unit (CFU) efficiency, and multipotentiality, including osteogenic, adipogenic, and salivary gland differentiation. The MSCs from the healthy controls and SS patients expressed characteristic MSC markers, including CD29, CD44, CD73, CD90, and CD105; they were negative for CD34, CD45, and CD106, and also negative for the salivary gland epithelium markers (CD49f and CD117). Labial gland MSCs from both groups were capable of osteogenic and adipogenic differentiation. The CFU efficiency and adipogenic differentiation potential of MSCs were significantly lower in the SS group compared with the healthy controls. Cells from both groups could also be induced into salivary gland-like cells. Real-time polymerase chain reaction and immunofluorescence staining showed that the gene and protein expression of *AMY1*, *AQP5*, and *ZO-1* in cells from the SS group was lower than that in cells from the healthy group. Thus, MSCs from the labial glands in patients with SS could lack certain characteristics and functions, especially related to salivary secretion. These preliminary data provided insights that could lead to the development of novel therapeutic strategies for the treatment of SS.

Keywords: mesenchymal stem cells, Sjögren's syndrome, salivary glands, labial glands

Introduction

Sjögren's syndrome (SS) is a common chronic systemic autoimmune disease that is characterized by lymphocytic infiltration into exocrine glands, primarily the salivary and lachrymal glands. Besides symptoms such as dry mouth and eyes, SS can cause other systemic injuries that affect quality of life and could increase the risk of lymphoma. SS can be classified as primary or secondary, depending on the presence of other concomitant autoimmune diseases [1,2]. The etiology and pathogenesis of SS remain unknown. The occurrence of SS could be attributed to heredity, environment, innate immune system, or adaptive immune system [3], which is characterized by the presence of rheumatoid factor, hypergammaglobulinemia, and autoantibody to Ro/Sjogren's-syndrome-related antigen A and La/Sjogren's-syndrome-related antigen B [4].

To date, there is no curative clinical therapy for SS, and current treatments for SS primarily include symptomatic and immune suppression therapies [5,6]. Although these treatments have a certain inhibitory effect on inflammation, there are adverse reactions. Moreover, no effective strategies are available to treat salivary gland damage. Muscarinic agonist medications such as pilocarpine and cevimeline have been used to induce salivary secretion from residual functional salivary gland tissue [7]. However, these treatments have a

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minimal effect on the recovery of damaged tissue and would likely not be effective in case of extensive salivary gland cell loss. Alternative therapeutic strategies to treat salivary gland damage are, therefore, required, and tissue engineering and stem cell therapy have shown great potential in this regard.

Mesenchymal stem cells (MSCs) have strong anti-inflammatory properties, and they promote immunosuppression and regeneration of damaged tissue. Recently, MSC transplantation for the treatment of SS and other salivary gland conditions has received much attention in the field of regenerative medicine. In 2011, Lin et al. [8] transplanted MSCs into the salivary gland in an animal model of xerostomia, and they found that salivary secretory volume, gland quality, and body weight of the treated animals increased compared with the control group.

In 2012, Khalili et al. [9] showed that an intravenous injection of bone marrow MSCs could prevent the decrease of salivary secretory volume, reduce lymphocyte infiltration and, therefore, decrease the number of T and B cells in the salivary gland, and decrease the production of Treg cells and inflammatory factors. Further, Chen et al. (2012) [10] treated 24 patients with SS (including 11 patients with xerostomia) with an intravenous injection of allogeneic MSCs, and they concluded that this method was safe and reliable. After treatment, the 11 patients with xerostomia showed an increase in salivary secretion volume. Thus, stem cell therapy has shown some efficacy in the treatment of salivary gland hypofunction. However, all the stem cells used in these studies were derived from tissues other than the salivary gland, and few studies have focused on stem cells derived from salivary glands.

Recently, stem cells have been successfully isolated from normal and damaged adult salivary glands, and demonstrated multilineage differentiation potential [11,12]. In this study, we studied the characteristics of stem cells isolated from the salivary gland, such as morphology, proliferation, surface markers, and multilineage differentiation potential, of healthy individuals and patients with SS, to elucidate the pathogenesis of SS, and to uncover new avenues for the treatment of SS by using labial gland stem cells.

Materials and Methods

This study was approved by the institutional review board of Peking University School of Stomatology (PKUSSIRB-201520016). All patients gave written informed consent before their participation in this study.

Primary culture of labial gland stem cells from patients with SS

SS groups. Five female patients with SS (mean age: 47.5 years), who visited the Department of Oral Medicine or the Department of Maxillofacial Surgery at Peking University School of Stomatology from March 2015 to October 2015 and were clinically and pathologically confirmed to have SS (according to 2002 American–European Consensus Group classification criteria [13]), were recruited in this group. After obtaining informed consent, the labial glands of these patients were resected under sterile conditions.

Healthy control group. Five female non-SS patients (mean age: 45.6 years), who visited the Department of Oral and Maxillofacial Surgery at Peking University School of Stomatology from March 2015 to October 2015 and needed surgery to remove lip mucocele, agreed to participate in this study. After obtaining their informed consent, their labial glands were resected under sterile conditions.

Isolation and culture of labial gland stem cells by using the monolayer method. After the resection of labial gland tissues, they were immediately soaked in mesenchymal stem cell medium (MSCM) and transferred to a biosafety cabinet. Fibrous tissues, capsules, and blood vessels around the labial glands were removed by using ophthalmic scissors. The tissues were minced into 1-mm^3 pieces by using ophthalmic scissors, placed in 10-cm cell culture dishes, and washed three times with PBS and once with MSCM. The medium was aspirated, and the dishes were placed in a cell-hatching box at 37°C.

After the tissue block attached to the wall, 10 mL MSCM was slowly added for primary cell culture at 37°C and 5% CO_2. After the cells grew out of the tissue block, we started observing cellular morphology; we began observation on day 5. When 80% cell confluence was achieved, we performed passage cultivation. The culture medium was refreshed every 3 days, and third-generation cells were used for subsequent experiments after 80% confluence was achieved.

Study of proliferation characteristics of labial gland stem cells

Samples of third-generation labial gland stem cells from SS and control groups were subjected to digestion, centrifugation, and counting after re-suspension. A 100-μL aliquot of the cell suspension solution was added to each well of a 96-well plate, with five replicates for each group and ~3,000 cells per well. From day 1 to 10 after inoculation, a CCK-8 test was conducted each day. Optical density (OD) at wavelength 450 nm was recorded for each well. With cell cultivation time as abscissas and OD values as ordinates, a cell proliferation curve was plotted.

Flow cytometric detection of the surface markers of labial gland stem cells

Third-generation labial gland stem cells from the test and control groups were subjected to digestion, centrifugation, and counting after re-suspension. Cells were resuspended to a concentration of 1×10^7 cells/mL, and 100 μL of the cell suspension was added to round-bottomed wells of microwell plates. Fluorescent-label-conjugated antibodies against CD29, CD34, CD44, CD 45, CD73, CD90, CD105, CD49f, CD106, and CD117 were added and the plates were incubated for 20–45 min on ice, in the dark. The cells were washed twice with 100–200 μL of stain buffer and resuspended in 200 μL of stain buffer. The labeled cell samples were analyzed by flow cytometry.

Colony-forming efficiency assay

Third-generation cells were sampled during an exponential growth period, and they were suspended in MSCM to obtain a single-cell suspension with a single-cell percentage above 95%; the cell concentration was adjusted to 1×10^4 cells/mL. Cells were inoculated into a six-well plate at a density of 100 cells/well, and six replicate wells were used for each group of cells. The plates were placed in an incubator at

37°C under 5% CO_2. When visible clones appeared after 7 days of cultivation, the culture was stopped and the clones were subjected to GIEMSA staining (Sigma-Aldrich), followed by air drying and counting of clones.

Differentiation of labial gland stem cells

Osteogenic differentiation. Third-generation labial gland stem cells from the SS and control groups and human bone marrow MSCs (hBMSCs, No. 12976; Sciencell Research Laboratories) were inoculated at a density of 5×10^4 cells/well in a six-well plate and cell climbing slides in the six-well plate. After 24-h cultivation in MSCM, the medium in the osteogenic induction group was replaced with osteogenic induction medium, which consisted of high-glucose DMEM (StemPro®, gibco), 10% FBS (StemPro, gibco), 10 nmol/L dexamethasone (Sigma-Aldrich), 0.2 mmol/L ascorbic acid (Sigma-Aldrich), 10 mmol/L β-glycol-2-diosodium phosphate salt hydrate (Sigma-Aldrich), and 1% penicillin–streptomycin solution (StemPro, gibco) [14]. The control groups were cultured in MSCM, which was refreshed every 3 days.

Adipogenic differentiation. Third-generation labial gland stem cells from the SS and control groups and human adipose-derived MSCs (hASCs; No. 11537; Sciencell Research Laboratories) were used for adipogenic differentiation experiments. The cells were seeded into 24- and 6-well plates. When compete cell confluence was achieved, adipogenic induction was initiated by using an adipogenesis differentiation kit (StemPro, gibco) and noninduced control groups were cultured in MSCM. After 21 days of cultivation of the adipogenic induction groups, the cells were subjected to Oil Red O staining and real-time polymerase chain reaction (PCR) detection.

Induction of differentiation into salivary gland epithelioid-like cells. Third-generation labial gland stem cells from the SS and control groups and hBMSCs were subjected to induction culture for differentiation into salivary gland epithelioid-like cells. The cells were seeded into six-well plates. After 80% cellular confluence was achieved, the induction was conducted. The rat submandibular gland cell line (SMG-C6; Department of Pathology and Physiology, Peking University Medical Laboratory) culture solutions were collected and filtered, and epithelial cell culture medium (Gibco) was added to the culture for differentiation into salivary gland epithelioid-like cells. The cells of the noninduced control groups were cultured in MSCM. Cells were subjected to immunofluorescence staining and real-time PCR detection after 7 days of induction.

Alkaline phosphatase staining. After 7 days of induced osteogenic differentiation, each group of stem cells was subjected to alkaline phosphatase (ALP) staining by using a commercial ALP reagent kit (CWBIO, Inc., China), according to the manufacturer's instructions. Gray-brown staining was considered a positive result.

Alizarin red staining for mineralized nodules. After 21 days of induced osteogenic differentiation, each group of stem cells was subjected to alizarin red staining; 200 μL 1% alizarin red solution was added to the culture wells, and the cells were incubated at room temperature for 30 min. Deionized water was used to rinse the cells until nonspecific stains were completely removed. Formation of red mineralized nodules was considered a positive result.

Alizarin red staining semi-quantitative method: 1% cetylpyridine solution was added into each stained hole. After being completely dissolved, 100 μL of the solution was added into a 96-well plate, and the absorbance was measured at 490 nm.

Observation of adipogenic differentiation by using Red Oil O staining. After a 21-day adipogenic induction, the cells in each group were subjected to Red Oil O staining to observe cellular morphology and red adipose droplets. After removal of the culture medium from the culture dishes, the cells were rinsed once with PBS and fixed with 10% formaldehyde-calcium solution at room temperature for 30 min. After rinsing once with 70% ethanol, 2% Red Oil O staining solution was added to the cells, and the cells were allowed to stand at room temperature for 30 min, followed by several seconds of differentiation with 70% ethanol. After the cells were rinsed three times with deionized water, the formation of adipose droplets was observed under a microscope.

Immunofluorescence staining for OCN*,* RUNX-2*,* AMY1*,* AQP5*, and* ZO-1. After a 7-day induced osteogenic differentiation in each group of stem cells, we conducted immunofluorescence staining for osteogenic proteins, and observed the distribution and expression of osteocalcin (*OCN*) and Runt-related transcription factor 2 (*RUNX-2*) in the cells. After 7 days of induced differentiation into salivary gland epithelial cells, cells were subjected to immunofluorescence staining for characteristic salivary gland proteins (*AMY1*, *AQP5*, and *ZO-1*). After the cells were rinsed with PBS solution, fixed with 4% paraformaldehyde, and treated with 0.1% Triton™X-100, 3% hydrogen peroxide was added and the cells were allowed to stand for 10 min; then, the cells were sealed with 3% goat serum for 1 h. Next, *OCN*, *RUNX-2*, *AMY1*, *AQP5*, and *ZO-1* primary antibodies were added to cell climbing slides at 4°C, respectively, and allowed to stay overnight; after PBS rinsing, secondary antibodies were added to the slides with a 1:1,000 dilution ratio, and they were stained for 1 h in the dark. Antiquenching mounting medium that contained DAPI was added drop wise to the cells, followed by a 5-min staining in the dark. The protein expression differences among each group were observed with a laser confocal microscope.

Semi-quantitative method for immunofluorescence: According to the method in the Ref. [15], the number of protein expression in the image was counted by using Image pro plus 6.0 software.

Detection of OCN, RUNX-2, CEBPα, PPARγ, AMY1, AQP5, and ZO-1 mRNA expression with real-time PCR. After 7 days of induced osteogenic differentiation in each group of stem cells, osteogenic gene expression was detected by using real-time PCR. After a 21-day adipogenic differentiation of the labial gland stem cells, the expression of adipogenic genes (*CEBPα*, *PPARγ*) was detected by using real-time PCR. After 7 days of induced differentiation into salivary gland epithelioid-like cells, the expression of genes (*AMY1*, *AQP5*, and *ZO-1*) typically expressed in the salivary glands was detected by using real-time PCR. Total RNA was extracted by using a reagent kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR assays were performed according to the manufacturer's protocol (KAPA Biosystems). The primers for *OCN*, *RUNX-2*, *CEBPα*, *PPARγ*, *AMY1*, *AQP5*, and *ZO-1* were synthesized by Invitrogen, and the sequences are listed in Table 1 β-actin was used as an

Table 1. Sequences of Primers Used for Real-Time Polymerase Chain Reaction

Genes	*Forward primer (5′-3′)*	*Reverse primer (5′-3′)*
RUNX-2	CGCATTCCTCATCCCAGTAT	AGGGGTAAGACTGGTCATAGGA
OCN	CTGTATCAATGGCTGGGAGC	GCCTGGAGAGGAGCAGAACT
CEBPα	GTTTTGCTCGGATACTTGCCA	CCCCCAGGATCAAAAGTAATC
PPARγ	GGAATTAGATGACAGCGACTTGG	GGCTTGTAGCAGGTTGTCTTGAA
AMY1	CCATTCACAACCCTTTCAGAC	CACATAAATACGAACCCCAACA
APQ5	CAACCCTTCCTCAAGAGCTGA	CCCCACTCTAAACACCAGCAG
ZO-1	GAACGAGGCATCATCCCTAA	CCAGCTTCTCGAAGAACCAC
β-actin	AGCACAATGAAGATCAAGATCAT	ACTCGTCATACTCCTGCTTGC

internal standard. The cycle threshold values (Ct values) were used to calculate the fold differences by the ΔΔCt method.

Statistical analysis

Data were expressed as the mean ± standard deviation and were analyzed by using SPSS 17.0 software. Two-tailed paired *t* tests were used to investigate the changes in cell proliferation (the OD value, a representative indicator of cell proliferation characteristics), stem-cell colony-forming efficiency, and the flow cytometry data in the SS and normal control groups. After confirmation of homogeneity of variance, one-way analysis of variance plus the least-significant difference test were performed for an overall evaluation of immunofluorescence semi-quantification, and relative quantification of RNA expression (the Ct values) in each group. For all tests, *P* values lower than 0.05 were considered statistically significant.

Results

Primary culture of labial gland stem cells

Stem cell-like cells and a few epithelium-like cells grew as a monolayer from around the tissue block after a 5-day in vitro culture of the labial gland tissues collected from the SS and control groups. Primary cells proliferated rapidly after a 7-day in vitro cultivation. Figure 1A shows the complex cellular array containing spindle-shaped stem cell-like cells and polygonal epithelium-like cells in the primary monolayer culture. The P1 cells, which were obtained by long-time enzymolysis, were all spindle-shaped stem cell-like cells (Fig. 1B). The cellular morphology in the normal control group was not significantly different from that in the

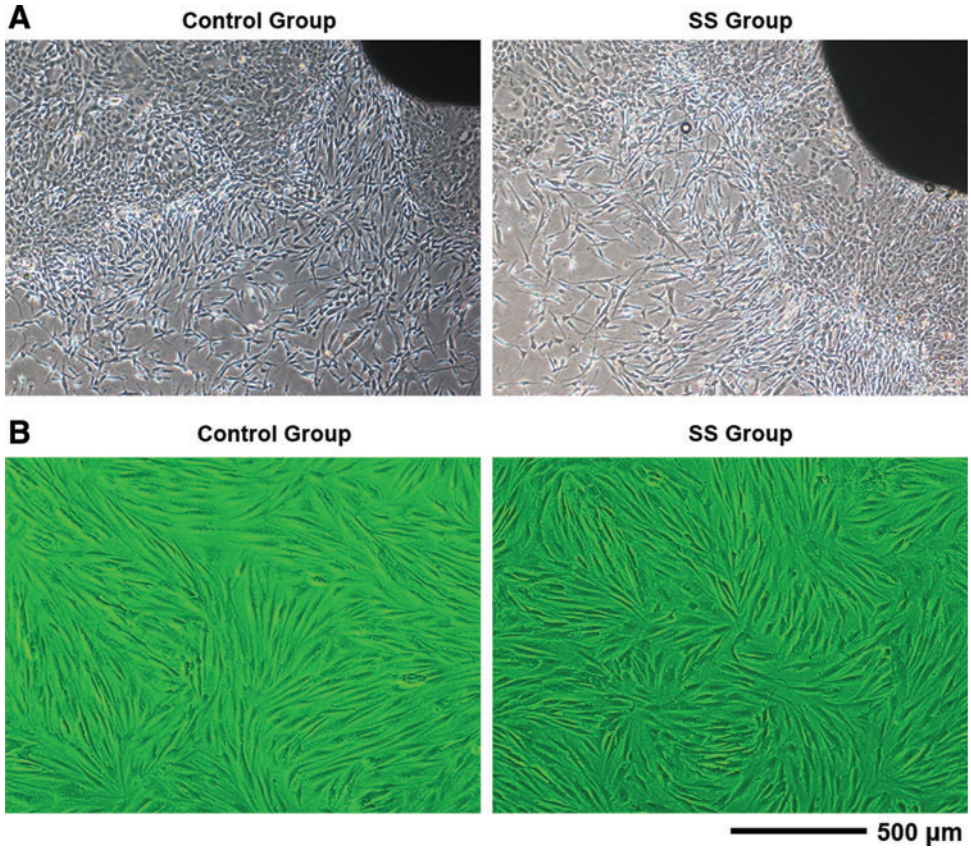


FIG. 1. Light microscope observation of labial gland stem cells after a 7-day in vitro cultivation in both the control group and the SS group. **(A)** Primary cell P0, and the figure show that there is a complex cellular array containing both the *long spindle-shaped* primary cells and *polygon-shaped* epithelial cells around the labial tissues in both the control group and the SS group; **(B)** the first-generation cells P1, and the cells obtained by long-time enzymolysis show a morphology with a *spindle-like shape*, and they are not mixed with epithelial cells (40×). SS, Sjögren's syndrome. Color images available online at www.liebertpub.com/scd

SS group, and P1 cells obtained in both the groups were all stem cell-like cells.

Proliferation characteristics and stem-cell colony-forming efficiency of labial gland stem cells

Proliferation characteristics of the labial gland stem cells were determined by CCK-8 assay in the SS and normal control groups. Cell proliferation was detected each day from day 1 to 10, and the data were used to generate a cell proliferation growth curve (Fig. 2A). The growth curves for the control and SS groups were S shaped. Proliferation was rapid between days 3 and 6, but slowed down between days 6 and 8, and reached a plateau starting from day 8, forming a proliferation curve similar to that observed for stem cells derived from other tissues. The cell proliferation of the SS group was not significantly different from that of the control group at any time-point other than day 5 ($P > 0.05$), indicating no statistically significant difference in the proliferation characteristics of labial gland stem cells between the SS and normal control groups.

The Giemsa staining results of MSCs from the control and SS groups are shown in Fig. 2B. The colony-forming unit efficiency of MSCs in the SS group was $27.65\% \pm 2.31\%$, which was significantly lower than that in the normal group ($34.25\% \pm 3.46\%$); $P < 0.05$.

Flow cytometric detection of stem cell surface markers

The results for flow cytometry detection of the surface markers of labial gland stem cells in the SS and control groups are shown in Fig. 3. The MSCs in both groups expressed characteristic MSC markers, including CD29, CD44, CD73, CD90, and CD105, but were negative for CD34, CD45, and CD106, and also negative for the salivary gland epithelium markers (CD49f and CD117). There were no statistically significant differences in the flow cytometry results between the two groups, and all the detected cell surface markers were markers that were specific to MSCs, indicating that the primary culture cells used in this study were MSC-like cells.

Osteogenic differentiation potential of the labial gland stem cells

ALP staining results. After a 7-day osteogenic induction, the osteogenically induced cultures of labial stem cells of the control and SS groups and hBMSCs showed strong positive ALP staining, whereas noninduced controls did not. As shown in Fig. 4A, ALP staining in the osteogenically induced stem cells was significantly weaker in the SS group than that in the control group, with both groups showing weaker ALP staining than that in the hBMSCs after osteogenic induction.

Alizarin red staining results. After a 7-, 14-, and 21-day osteogenic induction of labial stem cells of the SS and control groups and hBMSCs, the cultures were subjected to alizarin red staining to detect the formation of mineralized nodules. As shown in Fig. 4B, after a 7-day osteogenic induction, only the hBMSCs showed formation of mineralized nodules (Fig. 4Bc). After a 14- and 21-day osteogenic induction, osteogenically induced cells from all the three groups showed red-stained mineralized nodules, whereas noninduced control cultures did not. The highest abundance of mineralized nodules was observed in hBMSCs, and the

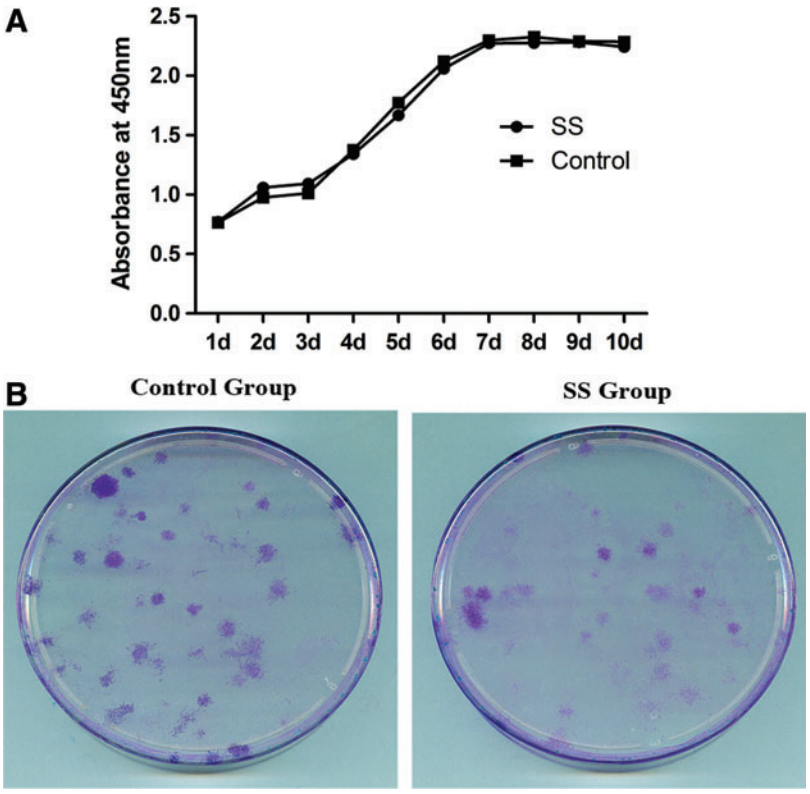


FIG. 2. The growth curves and Giemsa staining results of labial gland stem cells in both the SS group and the control group. **(A)** The stem cell growth curves in both the groups are S shaped, with a rapid proliferation during day 3 and 6 and a proliferation plateau starting from day 8; **(B)** the number of clones in the SS group was significantly higher than that in the control group, and the cloned cell population showed *purple* staining. Color images available online at www.liebertpub.com/scd

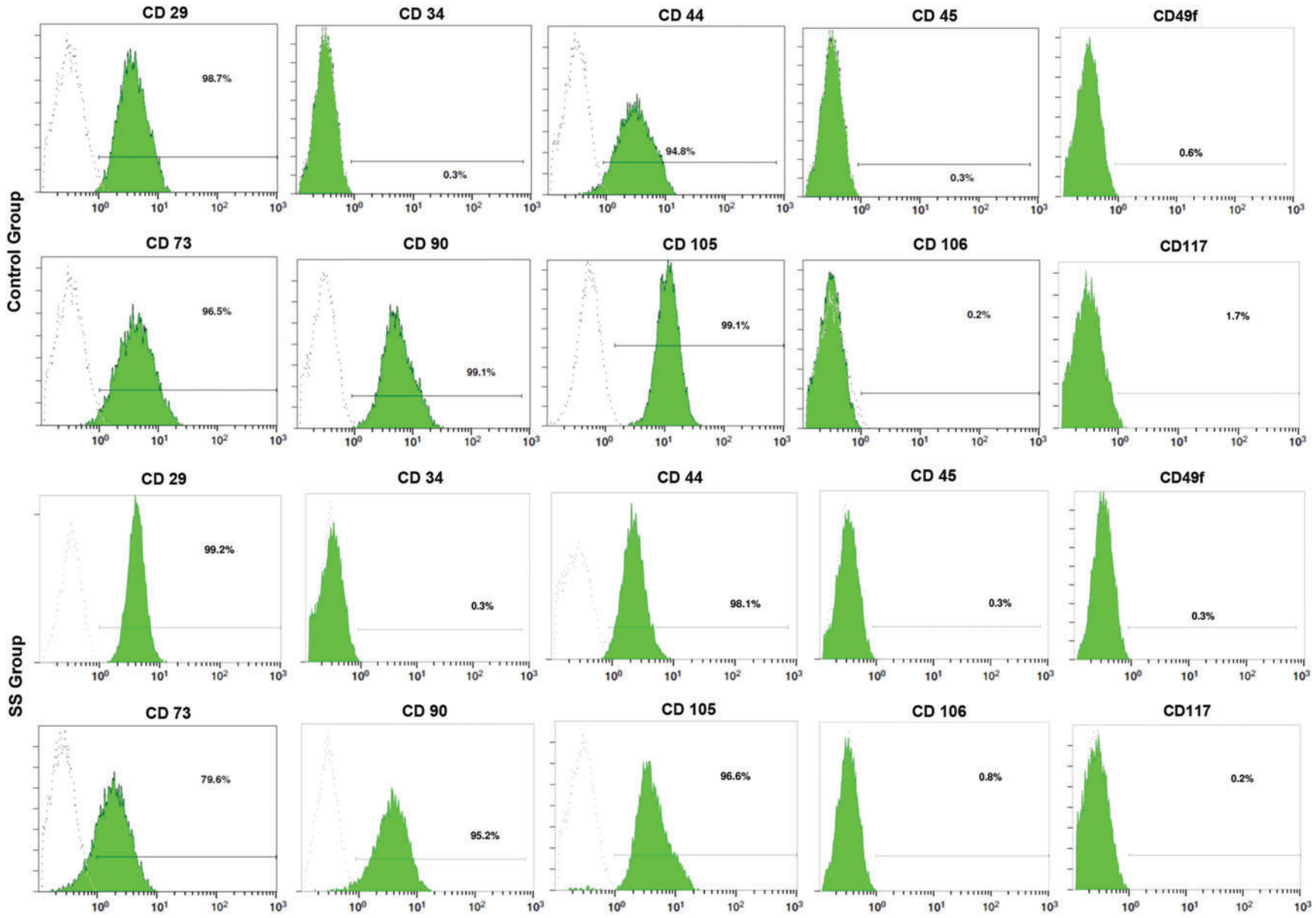


FIG. 3. The detection results of surface markers of labial gland stem cells from the SS patients. The figure showed the MSCs in both groups expressed characteristic MSC markers, including CD29, CD44, CD73, CD90, and CD105, but were negative for CD34, CD45, and CD106, and also negative for the salivary gland epithelium markers (CD49f and CD117). MSC, mesenchymal stem cell. Color images available online at www.liebertpub.com/scd

lowest abundance was observed in the SS group (Fig. 4Ba and Bb). Semi-quantitative detection of alizarin red staining yielded results that were consistent with the results of alizarin red staining (Fig. 4Bd); after the 7-, 14-, and 21-day osteogenic induction, there were statistically significant differences among the three groups ($P < 0.05$).

Immunofluorescence staining of osteogenic proteins. The expression of osteogenic proteins (*OCN*, *RUNX2*) in each group and the differences in expression among the groups after the 7-day osteogenic induction are shown in Fig. 4D and E. As shown in Fig. 4D, *OCN* expression, characterized by scattered red-stained granules in the cytoplasm, was observed in cells of all three osteogenically induced groups but not in the noninduced groups (Fig. 4Da, Db, and Dc). The *OCN* expression level in the hBMSCs was significantly higher than that in the other two groups, and there was a statistically significant difference in *OCN* expression level between the SS and control groups ($P < 0.05$; Fig. 4Dd).

RUNX2 expression in each group is shown in Fig. 4E. *RUNX2* expression was observed as scattered green granules in the cell nucleus and was present in the noninduced and induced groups. The *RUNX2* expression level in the cells of each induction group was significantly higher than that in the cells of the noninduction group (Fig. 4Ea, Eb, and Ec), and it was highest in the induced hBMSCs. There was no statistically significant difference in *RUNX2* expression levels between the SS and control groups ($P < 0.05$, Fig. 4Ed).

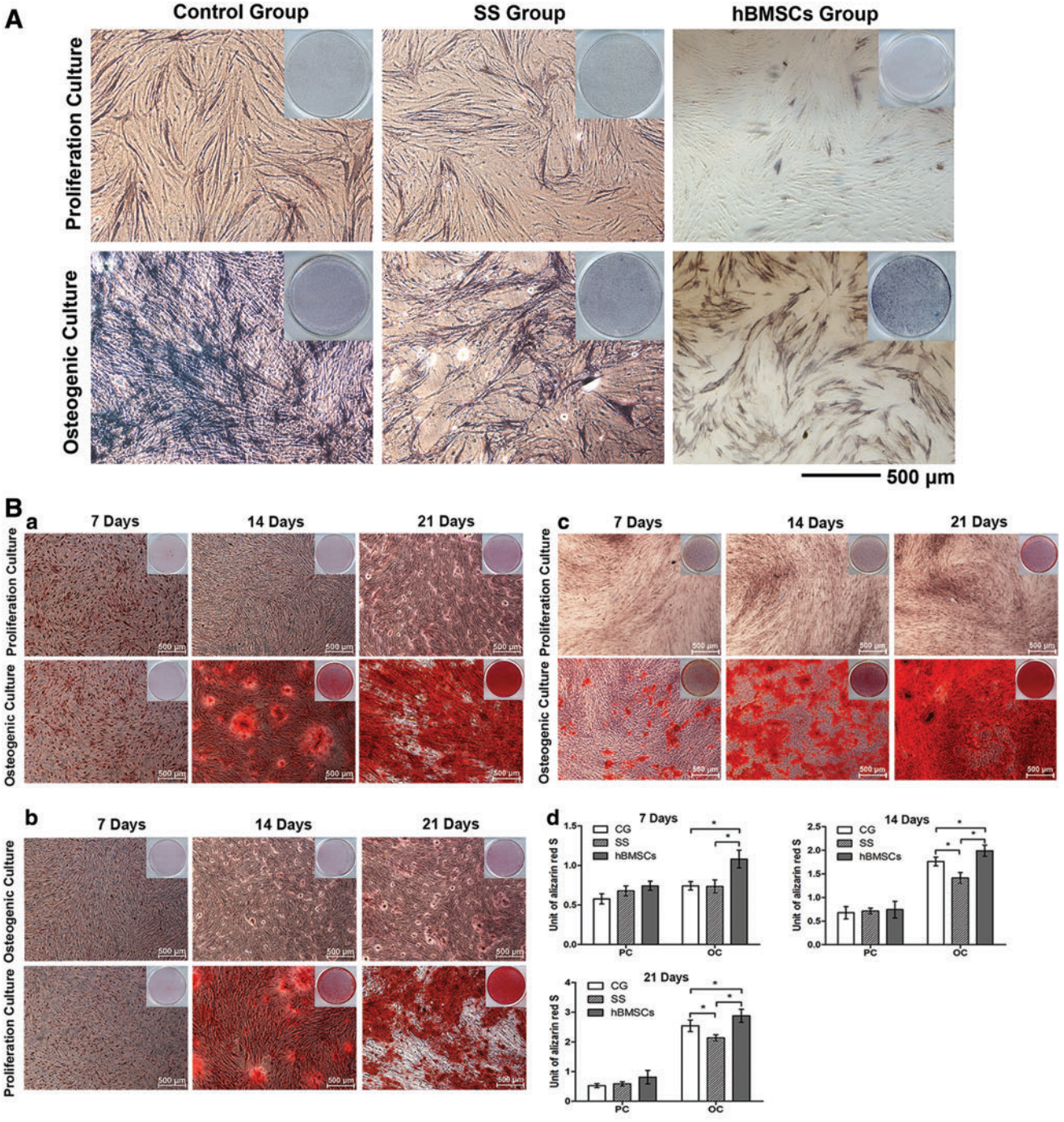
Real-time PCR detection of osteogenic genes. After a 7-day osteogenic induction, the expression of osteogenic genes (*OCN*, *RUNX2*) was analyzed by real-time PCR; the results are shown in Fig. 4C. The results of *OCN* gene detection were consistent with those of the immunofluorescence staining. There was no significant difference in *OCN* gene expression among the three noninduction groups. After osteogenic induction, the *OCN* gene expression level was highest in the hBMSCs and lowest in the SS group, and the level in the SS group was significantly lower than that in the control group ($P < 0.05$). The results of *RUNX2* gene detection were consistent with those of immunofluorescence staining. The *RUNX2* gene expression level was highest in the noninduced and osteogenically induced hBMSCs, and there was no statistically significant difference in *RUNX2* gene expression levels between the SS and control groups ($P > 0.05$).

Adipogenic differentiation potential of the labial gland stem cells

Oil Red O staining results. After 21 days of adipogenic induction, changes in cellular morphology and formation of adipose droplets were monitored (Fig. 5A). No cells in the three noninduction groups differentiated into adipocytes, whereas adipocyte-like cells were observed in the three adipogenically induced groups. The differentiated adipocytes in the hASC group were polygonal and larger than those in the two groups of labial stem cells. Microscopic observation after Oil Red O staining showed adipose droplets in the three adipogenically induced groups, appearing as bright red staining around the cells and in the cytoplasm. Adipose droplets were most abundant in the adipogenically induced hASCs and least abundant in the adipogenically induced SS group, wherein the droplet number was lower than that in the adipogenically induced control group.

Real-time PCR detection of adipogenic genes. After a 21-day adipogenic induction, real-time PCR was used to detect the expression of adipogenic genes (*CEBPα*, *PPARγ*); the results are shown in Fig. 5B. The PCR results of adipogenic gene expression were consistent with those of the Oil Red O staining. There were no significant differences in *CEBPα* gene expression among the three noninduction groups, whereas *PPARγ* gene expression in the noninduction hASCs group was higher than that in the remaining noninduction groups. The differences in *CEBPα* and *PPARγ* gene expression among the three adipogenic induction groups were statistically significant ($P < 0.05$). The expression levels of the two genes were highest in the hASC induction group and lowest in the SS induction group, wherein the level was significantly lower than that in the control induction group.

FIG. 4. Osteogenic differentiation potential of the labial gland stem cells. **(A)** The ALP staining showed that the osteogenically induced hBMSCs had the highest level of ALP expression whereas the lowest level was in the SS group. 40×. **(B)** The detection results of mineralized nodule formation after the 7-, 14- and 21-day osteogenic induction, 40×. **(a)** The control group, **(b)** the SS group, **(c)** the hBMSCs group, and **(d)** the semi-quantitative detection of the mineralized nodule formation indicated that there were statistically significant differences among the three osteogenically induced groups, $*P < 0.05$; **(C)** Real-time PCR detection results of the expression differences of osteogenic genes: The *OCN* and *RUNX2* gene expression levels in the induction groups were higher than those in the noninduction groups, and those in the labial stem cell groups were lower than those in the hBMSCs group. There was a statistically significant difference in the *OCN* gene expression level between the SS group and the control group, whereas no statistically significant difference was observed in the *RUNX2* gene expression level between the two groups. $*P < 0.05$; $**P < 0.01$. **(D)** Immunofluorescence staining results for *OCN* expression after the 7-day osteogenic induction: DAPI is presented by blue staining and *OCN* is presented by red staining, 200×. **(a)** The labial stem cells of the control group, **(b)** the labial stem cells of the SS group, **(c)** the hBMSCs group, and **(d)** the semi-quantitative analysis of the *OCN* expression level indicated that the level was highest in the hBMSCs group and lowest in the SS group. $*P < 0.05$; **(E)** Immunofluorescence staining results for the *RUNX2* expression after the 7-day osteogenic induction: DAPI is presented by blue staining and *OCN* is presented by green staining, 200×. **(a)** The labial stem cells of the control group, **(b)** the labial stem cells of the SS group, **(c)** the hBMSCs group, and **(d)** the semi-quantitative analysis of the *RUNX2* expression level indicated that the level was highest in the hBMSCs group, and there was no statistically significant difference in the level between the SS group and the control group. $*P < 0.05$. ALP, alkaline phosphatase; hBMSC, human bone marrow MSC; PCR, polymerase chain reaction. Color images available online at www.liebertpub.com/scd



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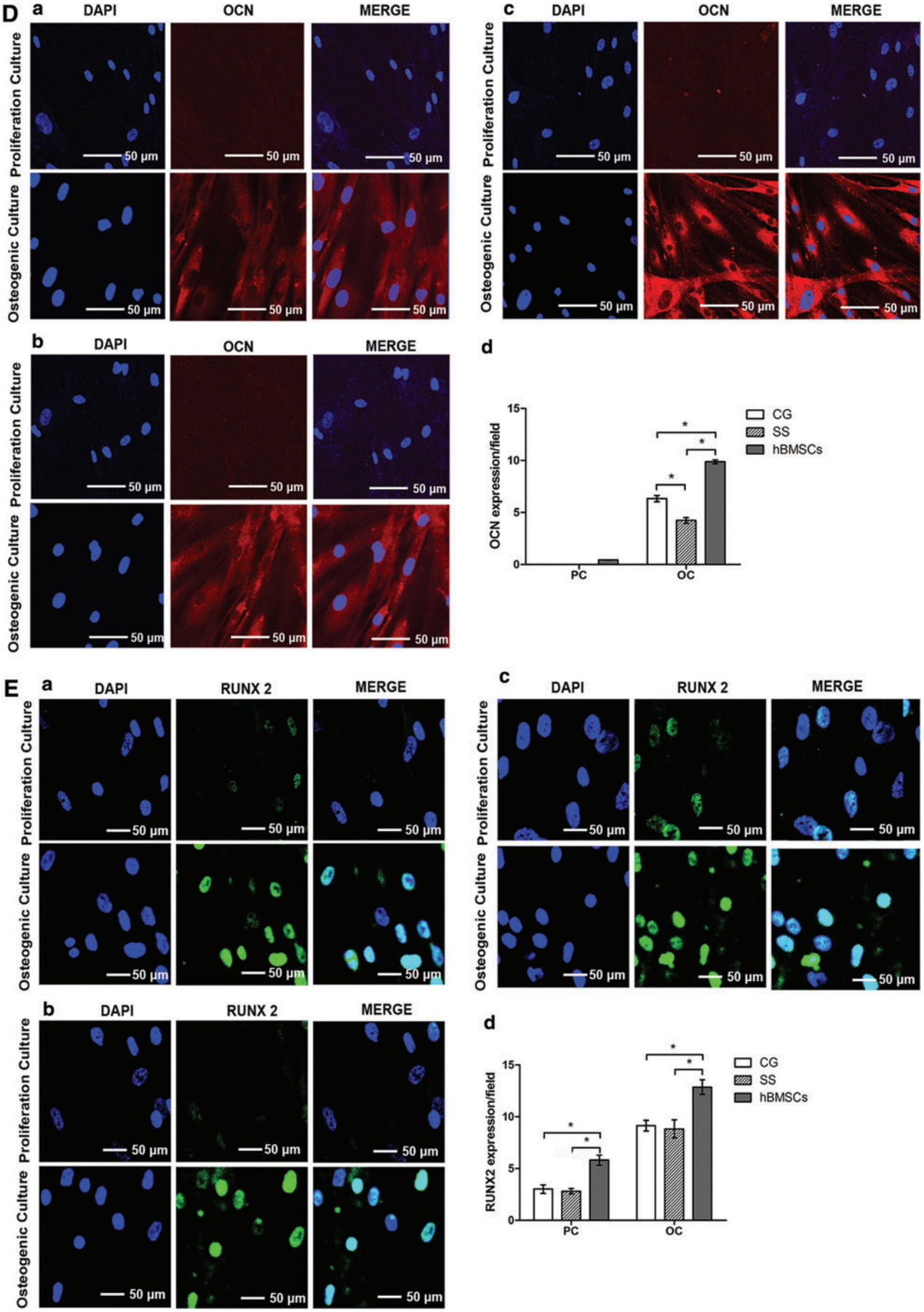


FIG. 4. (Continued).

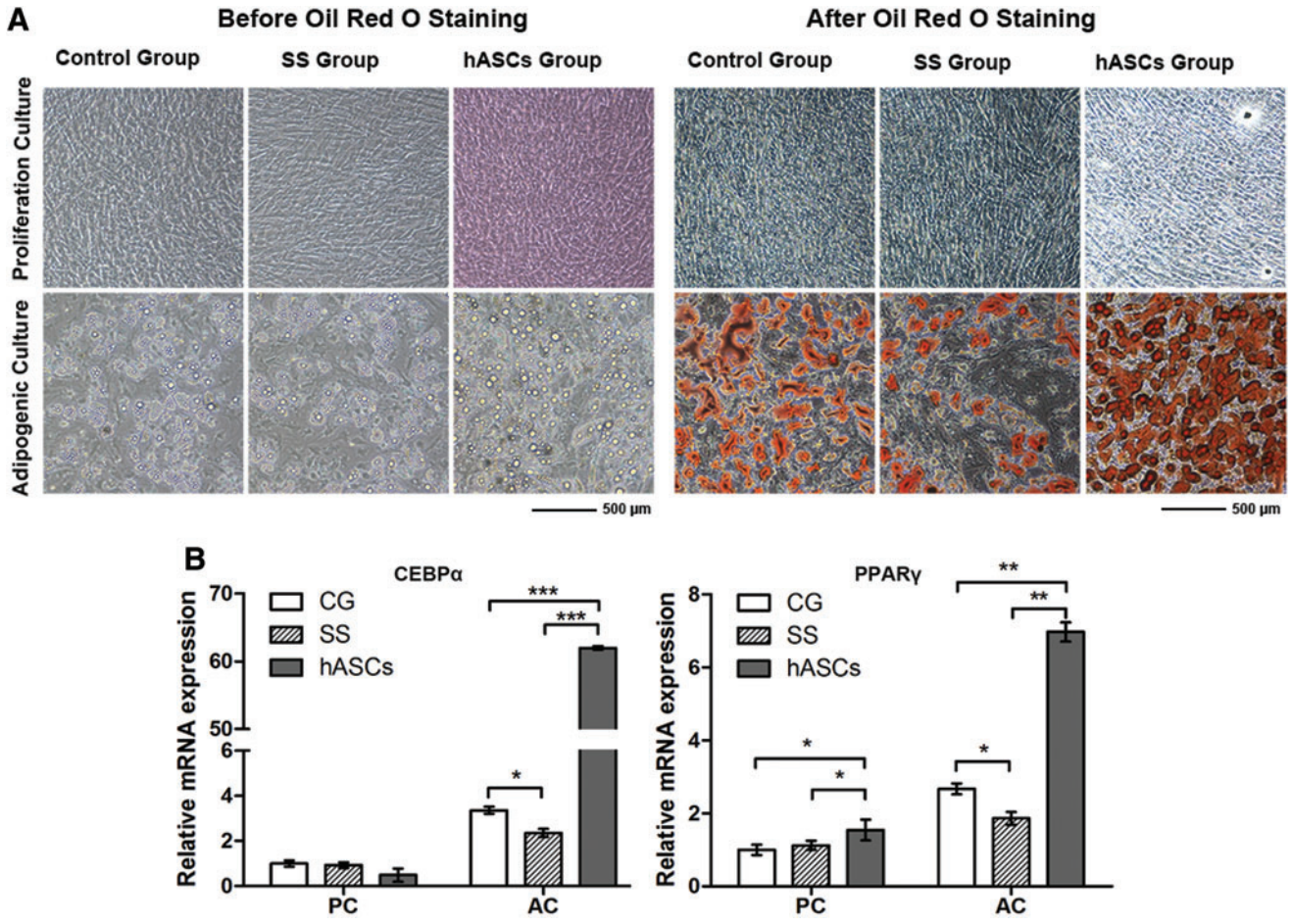
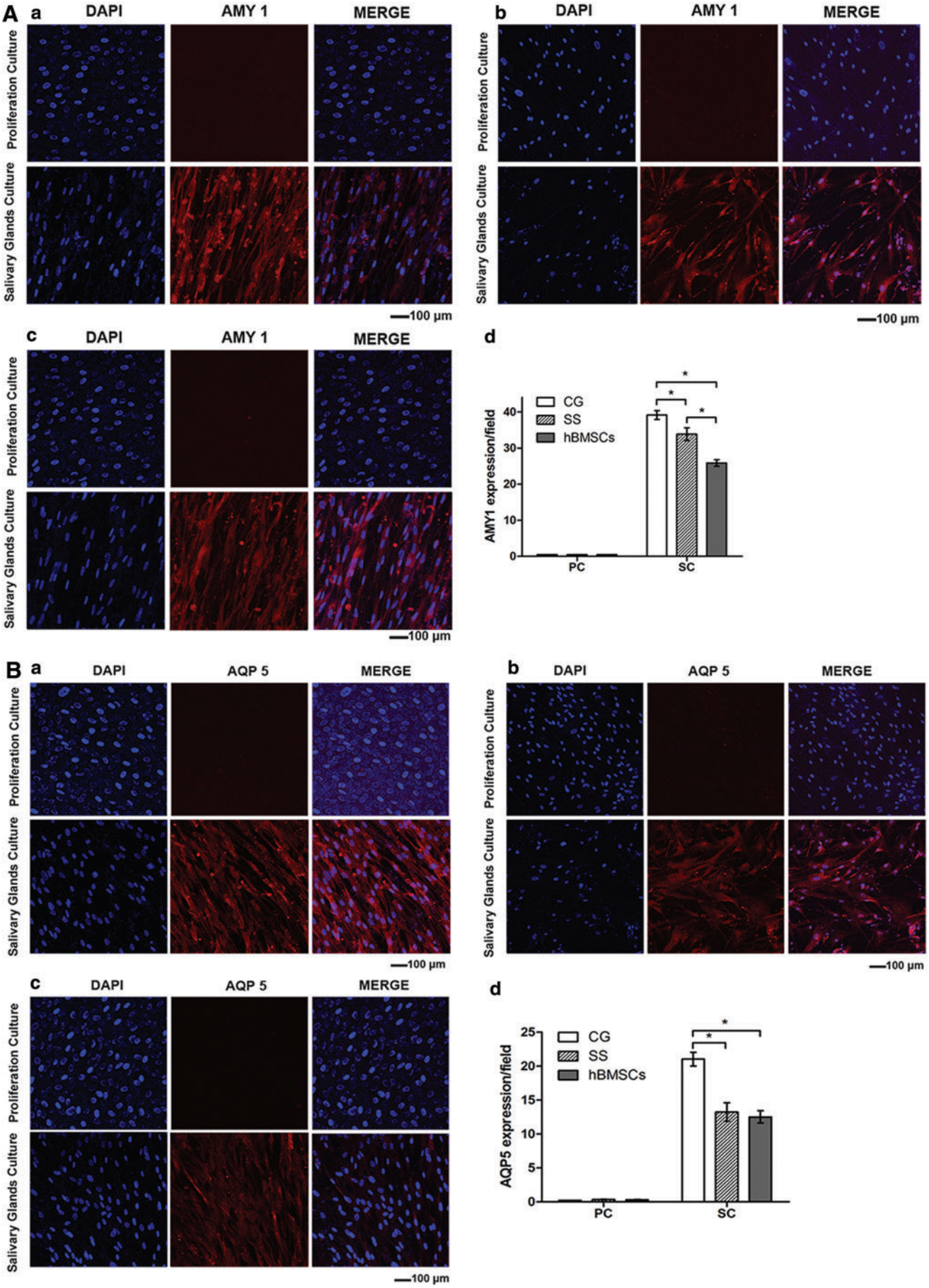


FIG. 5. Adipogenic differentiation potential of the labial gland stem cells. **(A)** The cellular morphology change and Oil Red O staining results after the 21-day adipogenic induction: no stem cells in the three noninduction groups differentiated into any adipocyte, whereas adipocyte-like cells and adipose droplets were observed in the adipogenically induced groups, and the number of the red-stained adipose droplets was highest in the human adipose-derived MSCs (hASCs) group but lowest in the SS group. **(B)** The real-time PCR detection results of the adipogenic gene expression: The levels of the CEBPα and PPARγ gene expression in the induction groups were higher than those in the noninduction groups, and the levels of the labial stem cells in the induction groups were significantly lower than those in the adipogenically induced hASCs group. There were statistically significant differences in the adipogenic gene expression between the SS group and the control group. $*P<0.05$; $**P<0.01$; $***P<0.001$. Color images available online at www.liebertpub.com/scd

FIG. 6. Differentiation potential of labial stem cells into salivary gland epithelioid-like cells. **(A)** The AMY1 expression was observed by immunofluorescence staining, with DAPI being presented by blue staining and AMYI being presented by red staining, 100×. **(a)** The control group of the labial stem cells, **(b)** the hBMSCs group, **(c)** the SS group of the labial stem cells, and **(D)** the semi-quantitative detection of the AMY1 expression levels indicated that the levels were highest in the control group of the labial stem cells and lowest in the hBMSCs group, and the levels in the SS induction group of the labial stem cells were lower than those in the control induction group of the labial stem cells. $*P<0.05$. **(B)** After the 7-day induction for differentiation into salivary gland cells, AQP5 expression was detected by immunofluorescence staining, with DAPI being presented by blue staining and AQP5 being presented by red staining, 100×. **(a)** The control group of the labial stem cells, **(b)** the hBMSCs group, **(c)** the SS group of the labial stem cells, and **(d)** the semi-quantitative detection of the AQP5 expression levels indicated that the levels were highest in the control group of labial stem cells, and the levels in the hBMSCs induction group and the SS induction group were lower than those in the control induction group of the labial stem cells. $*P<0.05$. **(C)** After the 7-day induction for differentiation into salivary gland cells, the ZO1 expression was detected by immunofluorescence staining, with DAPI being presented by blue staining and ZO1 being presented by red staining, 100×. **(a)** The control group of the labial stem cells, **(b)** the hBMSCs group, **(c)** the SS group of the labial stem cells, and **(d)** the semi-quantitative detection of the ZO1 expression levels indicated that there were statistically significant differences in the expression among the three induction groups, and the levels of the ZO1 expression were highest in the control group of the labial stem cells but lowest in the SS induction group. $*P<0.05$; $**P<0.01$. **(D)** Real-time PCR detection results of the expression differences of salivary gland cell genes: The gene expression levels in the induction groups were statistically significantly higher than those in the noninduction groups. The expression levels of the *AMY1*, *AQP5*, and *ZO1* genes in both the SS induction group and the hBMSCs induction group were significantly lower than those in the control induction group of the labial stem cells. $*P<0.05$; $**P<0.01$. Color images available online at www.liebertpub.com/scd



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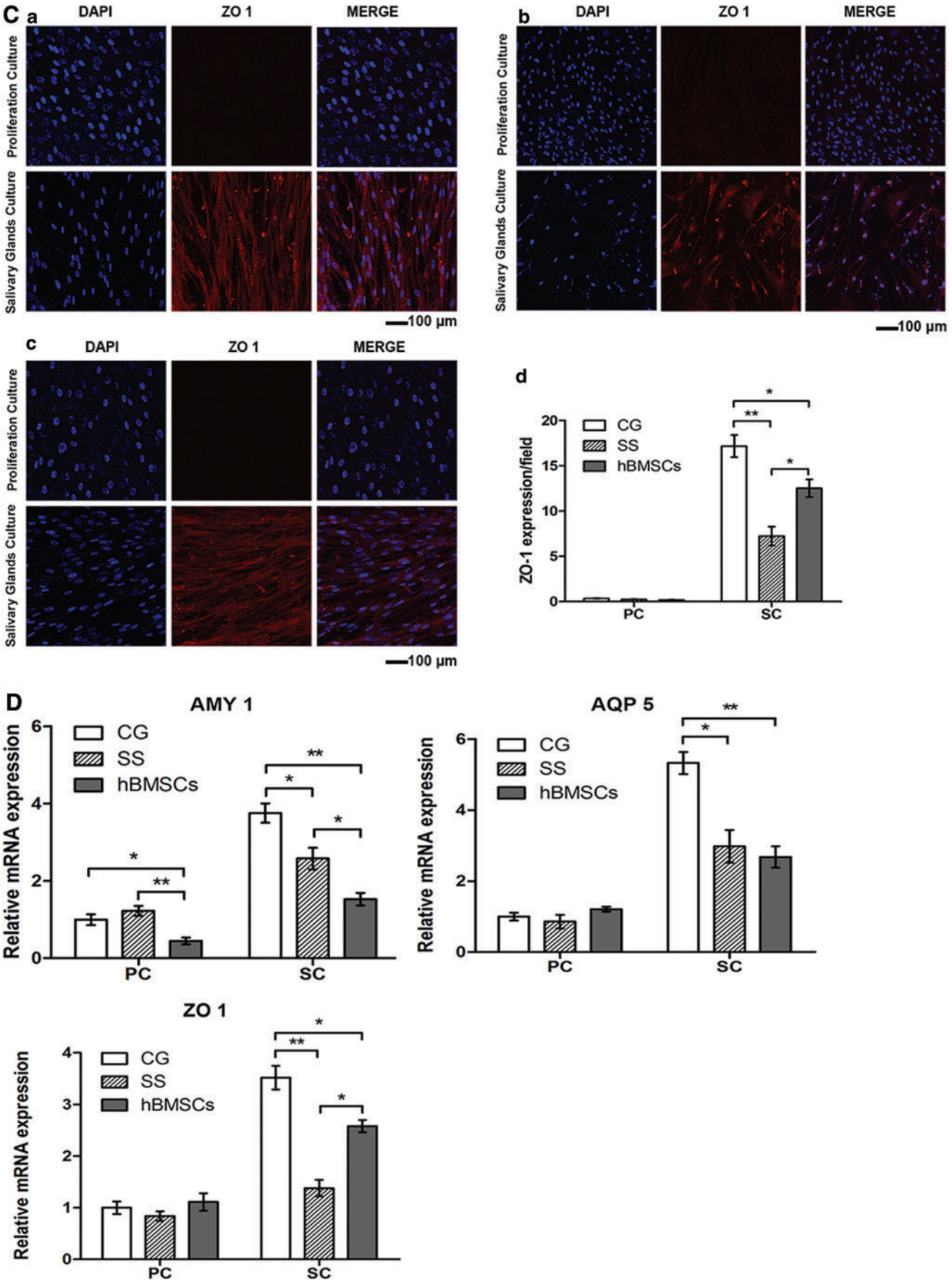

FIG. 6. (Continued).

Differentiation potential of labial stem cells into salivary gland epithelioid-like cells

Immunofluorescence staining results. After a 7-day induction culture to induce differentiation into salivary gland epithelioid-like cells, immunofluorescence staining was used to detect the expression of proteins (AMY1, AQP5, and ZO1) relevant to salivary gland cells (Fig. 6A–C). AMY1 expression, visualized as red-stained granules, was observed in the cells of the three induction groups, but not in the noninduction groups (Fig. 6Aa, Ab, and Ac). The levels of AMY1 expression in the two labial stem cell induction groups were significantly higher than those in the hBMSCs. There were no statistically significant differences in AMY1 expression levels between the SS and control groups ($P < 0.05$), as shown in Fig. 6Ad.

Figure 6B shows the AQP5 expression in the cell groups. AQP5 expression was observed in the induction groups, but not in the noninduction groups (Fig. Ba, Bb, and Bc). AQP5 expression levels were highest in the induction control group of the labial stem cells, and there were no statistically significant differences in AQP5 expression levels between the SS group and hBMSCs ($P > 0.05$), as shown in Fig. 6Bd.

Figure 6C shows the ZO1 expression in the cell groups. ZO1 expression was observed in the induction groups, but not in the noninduction groups (Fig. 6Ca, Cb, and Cc). There were statistically significant differences in ZO1 expression levels among the three induction groups, and the ZO1 expression levels were highest in the induction control group of the labial stem cells but lowest in the SS group ($P < 0.05$), as shown in Fig. 6Cd.

Real-time PCR detection of salivary gland cell genes. After a 7-day induction of differentiation into salivary gland epithelioid-like cells, real-time PCR was used to detect the expression of salivary gland cell genes (*AMY1*, *AQP5*, and *ZO1*) in each group (Fig. 6D). The PCR results were consistent with the immunofluorescence staining results, and the gene expression levels in the three noninduction groups were lower than those in the induction groups. The expression levels of *AMY1* and *ZO1* genes were statistically significantly different among the three induction groups ($P < 0.05$) and highest in the control induction group of the labial stem cells. The levels in the SS induction group were significantly lower than those in the control induction group of labial stem cells. The levels of *AQP5* gene expression were highest in the control induction group of the labial stem cells, and there were no statistically significant differences in *AQP5* gene expression between the SS induction and the hBMSC induction groups ($P > 0.05$).

Discussion

The pathogenesis of SS is characterized by the destruction of the salivary glands and abnormal infiltration of lymphocytes. There is currently no definitive cure for SS, and its treatment mainly focuses on reducing the symptoms. Thus, current treatments do not repair the destroyed glands, or reverse lymphocyte infiltration. Stem cells have effects on tissue repair and immunomodulation [16–18]. Stem cells have been effective in the treatment of SS; however, the underlying mechanism is unclear [10].

Stem cells from different tissues exhibit different differentiation and immune regulation abilities [19,20]. The labial minor salivary glands have a shallow location, are easy to obtain, and are widely used for tissue sampling in gland biopsy. Labial gland stem cells could be obtained by the isolation and culture of labial gland tissues [21]. If we suppose that SS is a kind of stem cell function–deficient disease, then the presence and functionality of stem cells in their salivary glands are likely to be abnormal. There have, however, been no reports on this topic. In this study, we isolated and cultured labial gland stem cells from healthy individuals and patients with SS, and we studied the proliferation and growth characteristics, surface marker expression, and multilineage differentiation, to elucidate the mechanisms of SS pathogenesis and explore new therapeutic options for SS.

We isolated and cultured labial gland stem cells by using the monolayer method, and the resulting cells had the basic characteristics of stem cells, including spindle-shaped cellular morphology and an S-shaped proliferation curve. We also observed positive expression of CD29, CD44, CD73, CD90, and CD105—surface markers specific to human stem cells—and negative expression of CD34 and CD45, which are typically hematopoietic markers, also negative for the salivary gland epithelium markers (CD49f and CD117). Thus, the isolated and cultivated stem cells were, indeed, human labial gland MSCs, which exhibited previously reported characteristics of salivary gland stem cells [11,12,21,22].

According to our results, despite there being no differences in the surface marker characteristics and proliferation curves of labial gland stem cells from patients with SS and those from healthy subjects, the colony-forming efficiency of the cells from patients with SS was significantly lower than that of the control cells. Thus, the proliferation capability of low-density single stem cells from SS patients is likely impaired, and merits further investigation.

Multilineage differentiation potential is also an important stem cell characteristic. In this study, we induced differentiation of salivary gland stem cells from patients with SS and healthy individuals into osteoblasts, adipose cells, and salivary gland epithelial cells, and we compared the differentiation with that of human adipose-derived and bone marrow MSCs. After osteogenic induction, we observed *RUNX2* protein and gene expression in the early stage of osteogenic differentiation, specific ALP expression in the middle stage, *OCN* protein and gene expression in the middle-late stage, and formation of mineralized nodules in the late stage of osteogenic differentiation [23]. We, therefore, observed the osteogenic differentiation potential of the SS stem cells at every stage of osteogenic differentiation, which can fully explain the osteogenic differentiation potential of stem cells [24].

The experimental results indicated that after a 7-day osteogenic induction of the labial gland stem cells, the expression levels of *ALP*, *RUNX2* (protein and gene), and *OCN* (protein and gene) in the SS induction group were lower than those in the normal control induction group. Further, the osteogenic differentiation capability of the labial gland stem cells was significantly lower than that of the bone marrow MSCs. Since there is no literature reporting the functions of stem cells from SS patients, we can only speculate that the multi-directional differentiation potential of the labial gland stem cells from SS patients may be related to the pathogenesis of SS, so the stem cells have been damaged or dormant like in other cases [11].

After a 21-day adipogenic induction, polygonal cells similar to adipose cells were observed among all the three groups, although the cells obtained from the human adipose-derived MSCs were larger than those obtained from the human labial gland stem cells, indicating that the labial gland stem cells could differentiate into adipose cells as reported in other similar papers [11,21,25]. The adipogenically induced human adipose-derived MSCs formed the largest quantity of adipose droplets, whereas the adipogenically induced SS group had the lowest abundance of adipose droplets.

Adipogenic gene expression results indicated that the labial gland stem cells from patients with SS had a lower adipogenic differentiation capability than did labial gland stem cells from healthy controls, and the adipogenic differentiation capability of the labial gland stem cells was lower than that of human adipose-derived MSCs. This also revealed the shortcomings of the labial gland stem cells from SS patients, which is worthy of further study, and may be the reason that stem cells are effective in treating SS [26,27].

The labial gland stem cells were also induced to differentiate into salivary gland epithelioid-like cells. Previous studies indicated that human marrow MSCs could be induced to differentiate into salivary gland epithelioid-like cells [28]. We, therefore, used human marrow MSCs as a control group.

After a 7-day induced differentiation into salivary gland epithelioid-like cells, we examined the expression of proteins (eg, AMYI, AQP5, and ZO1) characteristic of salivary gland cells [29]. The immunofluorescence staining results indicated that the protein expression levels in the induction groups were higher than those in the noninduction groups, proving the successful differentiation of labial gland stem cells into salivary gland epithelioid-like cells. The expression levels of AMYI, AQP5, and ZO1 proteins in the SS and hBMSC induction groups were significantly lower than those in the normal labial gland group. The expression of AMYI in the SS induction group was higher than that in the hBMSCs, the expression of AQP5 in the SS induction group was not significantly different from that in the hBMSCs, and the expression of ZO1 in the SS induction group was lower than that in the hBMSCs.

The results of the differentiation into salivary gland epithelioid-like cells indicated that the labial gland stem cells from patients with SS had a lower differentiation potential than that of labial stem cells from the nonpatients. The hBMSCs had a lower potential for differentiation into salivary gland epithelioid-like cells than the human labial gland stem cells did. In our results, although the labial gland stem cells were induced to differentiate into the salivary gland epithelioid-like cells, morphological changes were not observed. Only the differences in gene and protein expression showed that the stem cells had a tendency to differentiate into the salivary gland cells, but they did not completely differentiate into salivary gland epithelial cells, so the location of protein expression might be different from epithelial cells [30].

In this study, the labial gland stem cells isolated from the labial gland had multilineage differentiation potential. These cells, which had a lower capability of osteogenic differentiation than bone marrow MSCs and a lower capability of adipogenic differentiation than adipose-derived MSCs, possessed a relatively high capability of differentiation into salivary gland epithelioid-like cells.

Labial gland tissues may be obtained by tissue biopsy and expanded in vitro. The in vivo application of labial gland stem cell multilineage differentiation capability merits further investigation, which could help in the recovery of functions lost due to radiotherapy or xerostomia [31]. Compared with large salivary gland stem cells and bone marrow MSCs, minor salivary glands of the labial mucosa could be considered an easily accessible, and abundant source of stem cells with potential usefulness in glandular tissue regeneration [21].

Labial gland stem cells could, therefore, have advantages over stem cells from other sources for salivary gland repair and regeneration. In this study, labial gland stem cells obtained from patients with SS showed multiple defects compared with normal labial gland stem cells, which could shed light on the mechanisms of SS pathogenesis, thereby facilitating the improvement of therapeutic methods for the treatment of SS.

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Author Disclosure Statement

No competing financial interests exist.

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