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공배양 기술을 이용하여 생쥐의 지방유래 줄기세포에서 턱밑샘의 분비세포로 교차분화

Transdifferentiation of mouse adipose-derived stromal cells into acinar cells of submandibular gland using the co-culture system

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Abstract

Transdifferentiation of mouse adipose-derived stromal cells into acinar cells of submandibular gland using the co-culture system

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A loss of salivary gland function often occurs after radiation therapy in head and neck tumors, though secretion of saliva by the salivary glands is essential for the health and maintenance of the oral environment. Transplantation of salivary acinar cells (ACs), in part, may overcome the side effects of therapy. Here we directly differentiated mouse adipose-derived stromal cells (ADSCs) into ACs using a co-culture system. Multipotent ADSCs can be easily collected from stromal vascular fractions of adipose tissues. The isolated ADSCs showed positive expression of markers such as integrin beta-1 (CD29), cell surface glycoprotein (CD44), endoglin (CD105), and Nanog. The cells were able to differentiate into adipocytes,

osteoblasts, and neural-like cells after 14 days in culture. ADSCs at passage 2 were co-cultured with mouse ACs in AC culture medium using the double-chamber (co- culture system) to avoid mixing the cell types. The ADSCs in this co-culture system expressed markers of ACs, such as α-amylases and aquaporin 5, in both mRNA and protein. ADSCs cultured in AC- conditioned medium also expressed AC markers. Cellular proliferation and senescence analyses demonstrated that cells in the co-culture group showed lower senescence and a higher proliferation rate than the AC-conditioned medium group at Days 14 and 21. The results above imply direct conversion of ADSCs into ACs under the co-culture system; therefore, ADSCs may be a stem cell source for the therapy for salivary gland damage.

Keywords : Adipose-derived stromal cell, Acinar cell, Co- culture system, Transdifferentiation, Conditioned medium

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목차

Introduction

Salivary glands are essential for the maintenance of a healthy oral environment, and saliva plays an essential role in digestion, lubrication, oral homeostasis, and defense of the oral cavity. Hypofunction of salivary glands and xerostomia often occur after radiotherapy targeting head and neck tumors, which can cause diverse problems such as dry mouth, oral discomfort, periodontal disease, and taste disturbances [1]. The damaged salivary glands can also affect oral mucosal function and sense of taste; therefore, a plan for prevention and treatment should be developed for salivary gland damage [1, 2]. Cell transplantation may be an effective method of treatment for repairing tissue in radio-damaged salivary glands.

In mammals, there are three major salivary glands. The submandibular (SMG) and sublingual (SLG) glands are located under the tongue, while the parotid gland (PG) is located underneath the ear between the upper and lower jaw. These major salivary glands produce approximately 90% of saliva. The components of salivary glands are classified as acinar cells (ACs), ductal cells (DCs), endothelial cells, nerves, and myoepithelial cells; the ACs are categorized as serous, mucous, or seromucous. Mucous cells (MCs) secrete mucin, which contributes to the viscosity of saliva, while serous cells (SCs) secrete many proteins such as α-amylase. Although the component of cells varies in mammals, ACs mainly exist in three major salivary glands [3]. SCs are purely in the PG and MCs are mainly in the SLG, whereas both SCs and MCs exist together in the murine SMG. The PG develops from the oral epithelium of the ectodermal lineage, whereas the SMG and SLG develop from the oral epithelium of the endodermal lineage during

embryonic development. The salivary gland structure develops through mesenchymal-epithelial interaction [4]. Previous studies have suggested that Aquaporin5 (AQP5), α-amylase (Amyl), and mucins can be used as AC specific markers, while Na^+/K^+-ATPase (ATP1a1,NaK), cytokeratin7 (CK7), and cytokeratin19 (CK19) can be used as ductal cell-specific markers in mouse submandibular glands [5, 6].

Recently, adult stem cells have been isolated and applied in cell-based therapies to restore and regenerate salivary gland tissues. Lin and colleagues showed that rat bone marrow stem cells (BMSCs) have the potential to differentiate into ACs at the rate of 30-50% after co-culture with ACs for 1 to 3 weeks [7]. Huang and colleagues proposed that human amniotic epithelial cells (hAECs) can differentiate into ACs, and the cells are positive for mucins after two weeks of co-culture with ACs [8]. Human adipose-derived mesenchymal stem cells (hADSCs) can differentiate into salivary gland cells (SGCs) when transplanted into radiation-damaged salivary glands. In an *in vitro* experiment, 13 to 18% of hADSCs transdifferentiated into SGCs when hADSCs were co-cultured with SGCs [9]. Most studies in the field of transdifferentiateion into SGCs have only focused on the expression of a few specific markers, such as α-amylase and mucins. However, it is yet to be investigated whether those predecessor cells primarily transdifferentiate into the acinar or ductal form of SGCs and whether these SGCs have potential in the context of cell therapy.

ADSCs are considered to be multipotent adult stem cells and are able to transform into various types of cells such as adipocytes, osteoblasts, neuronal, neural, endothelial cells, and hepatocytes [10-15]. ADSCs are a good stem cell source for repairing damaged tissues

and for drug screening, and these cells can be obtained by high yield and are easily collected from stromal vascular fractions (SVFs) of adipose tissues [16]. The outcome is approximately 5,000 cells per gram, compared to 100 to 1,000 cells per milliliter when BMSCs are used [17]. These reports indicate that ADSCs can be efficiently recovered from SVFs, in addition to the advantage of versatile differentiation potential.

Culture of two different types of cells using a double-chamber (co-culture system) is an appropriate method to investigate the communication between two types of cells without mixing them [7, 8]. The communication between different type of cells may include unknown secreted factors which are essential for (trans) differentiation of the cells [18].

We investigated the transdifferentiational potential of mouse ADSCs into ACs after co-culture with ACs from the SMGs. The co-culture system was compared with AC-conditioned medium (ACM) because ACM obtained after culturing ACs may contain various growth factors and signaling molecules originating from the secretory ACs [19]. The factors in ACM may facilitate differentiation of ADSCs into ACs, like the factors secreted by ACs in a co-culture system. To demonstrate the transdifferentiation potential of ADSCs in a co-culture and ACM system and the change of their morphology from mesenchymal to epithelial lineage, we assessed cell proliferation and senescence as well as the expression of AC-specific markers.

Materials and Methods

Animals

Eight-week-old C57BL6 X DBA2 F1-hybrid (B6D2F1) female and male mice were used in the following experiments. All animal works were approved and performed under the guidelines and regulatory standards of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (approval number: SNU-130123-3).

Isolation and culture of ACs

ACs from submandibular glands were obtained from female mice by the explants outgrowth technique without collagenase treatment, as previously described with a few modifications [20, 21]. The thin fascia covering the submandibular glands was carefully removed to expose the glandular tissues. After the glands were dissected and washed 4 times in phosphate-buffered saline (PBS Gibco, Grand Island, NY, USA) with 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), they were cut into 1 mm^3 fragments. The tissue fragments were then cultured at 37°C in a 5% CO_2 atmosphere in a humidified incubator with Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium (1:1 mixture, v/v, Gibco) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Inc., Fort Collins, CO, USA), 20 ng/mL epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), 1% penicillin-streptomycin and 0.1% Fungizone ™(Gibco), here after referred to as Acinar cell medium (AM). Cells

released from the tissues grew to confluence in 4 to 6 days of culture. At approximately 90% confluence, the tissue fragments in suspension were removed along with the medium, and only the adherent cells were cultured for passaging. The cells at the second passage (P2) were used in the following experiments.

Isolation and culture of ADSCs

Murine ADSCs were isolated from inguinal adipose tissues of male mice. Previously described methods [22, 23] were used with a few modifications to isolate the cells. The tissue was digested with 0.05% collagenase type I (w/v, Gibco) in HBSS (WelGENE, Daegu, Korea) for 20-30 min at 37°C and filtered through a 100 μm cell strainer (BD, Franklin Lakes, NJ, USA). The stromal - vascular fractions (SVFs) which contain ADSCs were then separated from fibrous materials and floating adipocytes by centrifugation at 400 X *g* for 8 min. The cell pellet was resuspended in a high-glucose DMEM (WelGENE) supplemented with 10% FBS and filtered through a 40 μ m cell strainer (BD). Initial culture and expansion of the cells from SVFs (SVF-cells) was performed in 6-well plates (Corning, Tewksbury, MA, USA) with high-glucose DMEM supplemented with 1% GlutaMAX™ (Gibco), 20% FBS, 1% Non-essential amino acid (Gibco), 1% penicillin-streptomycin, and 0.1% Fungizone™, hereafter referred to as ADSCs medium (ADSCM). Cells were cultured at 37°C in a 5% CO_2 atmosphere in a humidified incubator and were allowed to adhere for 24 h. The medium was replaced once daily until the cells were 70-80% confluent. The cells adhering to the plate were ADSCs, and second-passage ADSCs were used.

Identification of ADSCs

Multipotency of ADSCs was identified by adipogenic, osteogenic, and neural induction using previously published methods with a few modifications [24]. For osteogenic differentiation, ADSCs were cultured in high-glucose DMEM containing 10% FBS, 0.1 μM dexamethasone (Sigma), 0.05 μM ascorbate - 2 - phosphate (Sigma), 10 mM ß-glycerophosphate (Sigma), 1% penicillin-streptomycin and 0.1% Fungizone™for 2 weeks. After differentiation induction, the cells were stained with Alizarin red (Sigma) for osteogenic cell identification. For adipogenic differentiation, ADSCs were cultured in high-glucose DMEM supplemented with 20% FBS, 1 μM dexamethasone, 200 μM indomethacin (Sigma), 0.5mM 3-isobuthyl-1-methylxanthine (Sigma), 1% penicillin-streptomycin, and 0.1% Fungizone™ for 2 weeks, and then the cells were stained with Oil Red-O (Sigma) for determination of adipogenic differentiation. For neural differentiation, ADSCs were grown in neurobasal medium (Gibco) containing 2 mM GlutaMAX™, 2% B27 serum-free supplement (Gibco), 1% penicillin-streptomycin and 0.1% Fungizone™for 2 weeks, and then the cells were subjected to immunofluorescence (IF) analysis to confirm expression of Nestin (Millipore, Billerica, MA, USA), a neural marker.

Co-culture system

ADSCs were co-cultured with ACs (CCA) from the submandibular glands with AM in a double-chamber system (Fig. 1). ACs were placed on the upper inserts and ADSCs were in the lower culture plate area, as described previously [7, 8]. Briefly, the ratio of cell density was 1:4 for ADSCs and ACs, respectively. ADSCs were

placed at a density of 2 x 10^4 cells/well in a 12-well plate (Corning). Three culture inserts (Corning), each containing 8 x 10^4 ACs, were placed in three of 12 wells (one insert per well). An insert membrane with a pore size of 0.4 μm was used to allow only the transmission of soluble factors and to prevent direct interaction between ADSCs and ACs. The ADSCs in another three wells were cultured in AC-conditioned medium (ACM). ACM, recovered from AMs which were incubated with ACs at P2 for 3 days, was diluted by adding an equal volume of fresh AM before the culture of ADSCs. The ADSCs in the other six wells were divided into two control groups. The cells in each control group were cultured either in ADSCM or AM.

RNA extraction, cDNA synthesis and Reverse transcription-PCR

Total RNA isolation and cDNA synthesis were practiced as described previously [25]. Briefly, total RNA was isolated using the RNeasy™ Mini Kit (QIAGEN, Hilden, Germany) and cDNA synthesis was performed by M-MLV reverse transcriptase (20 U/μL, Life Technologies, Carlsbad, CA, USA). The resulting cDNA was amplified by using AccuPower™ PCR Premix (Bioneer, Daejeon, Korea) with the following sets of primers: Integrin β-1 (CD29): forward 5′-GAGTCCGGTTTTAGGGACCG-3′, reverse 5′-ACTGTGGCATTCGGGATCAG-3′ Cell surface glycoprotein (CD44): forward 5′-TCCCAGGAATGACGTCTCCA-3′, reverse 5′-TCATGATGCTATCCCCAGCAG-3′ Endoglin (CD105): forward 5′-TGTACCCACAAGTAAGTGTCC-3′, reverse 5′-AATGGGAAACTCAAGGCTCA-3′ AQP5: forward 5′-GAGGTGTGTTCAGTTGCCTT-3′, reverse 5′

-TCAAGATGGAGGAGCATGGA-3′ Amyl: forward 5′
-ATGAGCAAGAGATAACCCGAG-3′, reverse 5′
-CAGTGAAAGGATGGGCAGTA-3′ NaK: forward 5′
-GGTAGTGAGTCCTCTTTTGGG-3′, reverse 5′
-TCCCCTACTCCCTTCTCATC-3′ Oct4: forward 5′
-CCGTGTGAGGTGGAGTCTGGAG-3′, reverse 5′
-GCGATGTGAGTGATCTGCTGTAGG-3′ Nanog: forward 5′
-GAAATCCCTTCCCTCGCCATC-3′, reverse 5′
-CTCAGTAGCAGACCCTTGTAAGC-3′ PPAR-g: forward 5′
-CTGTGCAGAATGTCGGGTCT-3′, reverse 5′
-AGGTGTGGCGTACACATCAG-3′ LPL: forward 5′
-CCCTTGTAACTGTTCTGCCC-3′, reverse 5′
-GAAAAACGTACCGTCTGCTG-3′ OC: forward 5′
-GGATACACACACCCTCTTCC-3′, reverse 5′
-GGACAACACATGAGGGAGAC-3′ Runx2: forward 5′
-TACGTGTGGTAGTGAGTGGT-3′, reverse 5′
-TTCCCTCCCCATCTTCTGTT-3′ Col1: forward 5′-
ACTTAAATGGAGGTGCAGGG-3′, reverse 5′
-CTTTCTCATCCAGCAACCCG-3′ Nestin: forward 5′
-GGAGAAGCAGGGTCTACAG-3′, reverse 5′
-AGCCACTTCCAGACTAAGG-3′ Tubb3: forward 5′
-TCCGCCTGCCTTTTCGTCTCTA-3′, reverse 5′
-AGTTGCCGCTGGGGTCTATGC-3′ Oligo2: forward 5′-
AGAGATGCGTTCGTTCCAGAA-3′, reverse 5′-
AAGTACCCGCGCGTTGTG-3′ CK19: forward 5′
-ATTGACAAGTCGAGGGAGGG-3′, reverse 5′
-TCAGCATGGTACGTGTCTCC-3′ CK7: forward 5′
-AGGAGATCAACCGACGCAC-3′, reverse 5′
-GTCTCGTGAAGGGTCTTGAGG-3′ Gapdh: forward 5′

-GCATGGCCTTCCGTGTTCCTA-3′, reverse 5′ -CTTCAGTGGGCCCTCAGATGC-3′. The mixture was first heated at 94°C for 2 min. Amplification was performed for 35 cycles at 94°C for 45 s, 59°C for 30 s, and 72°C for 90 s, followed by 72°C for 5 min. PCR products were further analyzed on 0.8% agarose gels and photographed with BIO-PROFIL X-press Zoom 2000 (Vilber-Lourmat, Marne-la-Vallee, France).

Real time-PCR (qPCR)

For quantification of mRNA levels, qPCR reaction was carried out as previously described [25] with the following sets of primers: E-cadherin (E-cad): forward 5′-CAGGTCTCCTCATGGCTTTGC-3′, reverse 5′-CTTCCGAAAAGAAGGCTGTCC-3′ Epithelial cell adhesion molecule (EpCAM): forward 5′ -GGAGTCCCTGTTCCATTCTTCT-3′, reverse 5′ -GCGATGACTGCTAATGACACCA-3′ Keratin 18 (Krt18): forward 5′-ACTCCGCAAGGTGGTAGATGA-3′, reverse 5′ -TCCACTTCCACAGTCAATCCA-3′ N-cadherin (N-cad): forward 5′-AGCGCAGTCTTACCGAAGG-3′, reverse 5′ -TCGCTGCTTTCATACTGAACTTT-3′ Vimentin (Vim): forward 5′-CGGCTGCGAGAGAAATTGC-3′, reverse 5′ -CCACTTTCCGTTCAAGGTCAAG-3′ Fibronectin (Fn): forward 5′-TTCAAGTGTGATCCCCATGAAG-3′, reverse 5′ -CAGGTCTACGGCAGTTGTCA-3′ AQP5: forward 5′ -AGAAGGAGGTGTGTTCAGTTGC-3′, reverse 5′ -GCCAGAGTAATGGCCGGAT-3′ Amyl: forward 5′ -AACCCAAATAACAGGGACTTTCC-3′, reverse 5′ -GGTAGTTCTCGATACCTCCACTT-3′ NaK: forward 5′

-GGGGTTGGACGAGACAAGTAT-3′, reverse 5′
-CGGCTCAAATCTGTTCCGTAT-3′ CK7: forward 5′
-AGGAGATCAACCGACGCAC-3′, reverse 5′
-GTCTCGTGAAGGGTCTTGAGG-3′ CK19: forward 5′
-GGGGGTTCAGTACGCATTGG-3′, reverse 5′
-GAGGACGAGGTCACGAAGC-3′ Gapdh: forward 5′
-ATGACTCCACTCACGGCAAAT-3′, reverse 5′
-GGGTCTCGCTCCTGGAAGAT-3′. Briefly, qPCR was performed using the ABI PRISM 7500 system and SYBR™ Premix Ex Taq II (Takara Bio Inc, Shiga, Japan). All reactions were conducted with samples prepared in triplicate and were normalized to murine Gapdh as an endogenous control.

IF analysis

All samples were identified by IF analysis. A portion of the cultured cells was plated on a cover glass, fixed with 4% paraformaldehyde (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 20 min, and washed with PBS three times. Then, the samples were permeablized with 0.25% Triton X-100 (Sigma) in PBS for 20 min, washed with PBS three times, and blocked with 2% bovine serum albumin (Sigma) and 0.1% Tween-20 (Sigma) in PBS for an additional 20 min. The samples were incubated with monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:100; Abcam, Cambridge, UK), polyclonal rabbit anti-CK19 antibody (1:100; Abcam), polyclonal rabbit anti-Amylantibody (1:50; Santa Cruz), polyclonal rabbit anti-AQP5 antibody (1:50; Santa Cruz), polyclonal goat anti-NaK antibody (1:50; Santa Cruz), polyclonal rabbit anti-p21 antibody (1:2,000; Abcam) or polyclonal rabbit anti-CDKN2A antibody

(1:1,000; Thermo Scientific, Rockford, IL, USA) as primary antibodies for 16 h at 4°C. Then the samples were washed and incubated with Fluorescein-conjugated anti-goat antibody (1:100; Millipore), Cy3-conjugated anti-rabbit antibody (1:100; Millipore), and Alexa-Fluor 488 anti-mouse antibody (1:200; Life Technologies) as secondary antibodies for 2 h at room temperature. Stained samples were washed and 40,6-Diamidino-2-phenylindole dihydrochloride staining was applied for staining cell nuclei. The stained samples were then observed utilizing a fluorescence microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

Senescence-associated β-galactosidase (SA-β-gal) staining

Cells were stained for SA-β-gal activity following the manufacturer's protocol (Cell Signaling Technology, Danvers, MA, USA). The total number of β-gal$^+$ cells was counted under an optical microscope after all samples were detached by 0.25% trypsin-EDTA (Gibco), and the percentage of β-gal$^+$ cells was calculated.

Statistical analysis

Each experiment was replicated three times. Relative quantification of gene expression was calculated as 2-ΔΔCt. The results of qPCR were statistically analyzed by one-way ANOVA using Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). A statistical difference of $P < 0.05$ was considered significant.

Results

Isolation and Characterization of ADSCs

ADSCs were obtained from the fat pad of mice. The cellular morphology was similar to MSCs such as BMSCs (Fig. 2A). The cells expressed typical surface markers of MSCs, such as CD29, CD44, CD105, and Nanog, a pluripotency marker (Fig. 2B). After 2 weeks of differentiation induction, the cells were positive for Oil-Red-O (the adipocyte marker) Alizarin red (the osteoblast marker), or for IF staining for Nestin, a neural cell marker (Fig. 2C, D and E, respectively). The expressions of adipocyte-specific markers (PPAR-g and LPL), osteoblast-specific markers (OC, Runx2, and Col1) and neural-specific markers (Nestin, Tubb3, and Oligo2) showed that ADSCs can differentiate into other cell types such as adipocytes, osteoblasts, and neural-like cells (Fig. 2F).

Isolation and Characterization of ACs

ACs were obtained by the explant outgrowth technique and *in vitro* culture with AC culture medium. During primary culture of explant tissue from the submandibular gland, the cells were outgrown around the tissue (Fig. 3A). The cells at passage 2 after subculture showed round epithelial cell-like structure, which is characteristic of AC morphology (Fig. 3B). The expression of Amyl and AQP5, AC markers, was detected by RT-PCR and IF staining. DC markers such as (NaK, CK19, and CK7 were also expressed in mRNA and NaK was detected by IF staining (Fig. 3C and D). These findings

demonstrate that the cultured cells possess the characteristics of ACs and DCs.

Change in cell morphology

Morphological changes were monitored after 2 weeks of transdifferentiation induction, and epithelial and mesenchymal cell markers were analyzed in mRNA or protein. ADSCs in the ADSCM group and the AM group were used as controls. All experimental groups were compared with ACs at passage 2. The cells in the CCA and ACM groups did not present increased expression of epithelial markers such as E-cadherin (E-cad), Epithelial cell adhesion molecule (EpCAM), and Keratin18 (Krt18), whereas both cell types showed decreased expression of mesenchymal cell markers such as Vimentin (Vim), and Fibronectin (Fn) than in ADSCM group (Fig. 4A and B). In particular, gene expression in the CCA group was approximately 1.7-fold (Fn) and 1.7-fold (Vim) down-regulated compared to the expression in the ADSCM group. Gene expression in the ACM group was approximately 2-fold (Krt18), 41-fold (EpCAM) and 36-fold (E-cad) up-regulated, and 2.9-fold (N-cad), 4.3-fold (Fn) and 1.6-fold (Vim) down-regulated, compared to the expression in the ADSCM group. Although both ACM and CCA group cells showed changes in gene expression, their mRNA levels did not equate with the levels of these genes in normal cultured ACs at passage 2. IF staining confirmed that E-cad protein was found in the ACM and CCA groups (Fig. 4C). No E-cad protein was detected in negative controls for the ADSCM and AM groups. These results suggest that both the CCA and ACM groups show changes in cellular morphology and gene expression during the process of transdifferentiation from

ADSCs to ACs.

Transdifferentiation of ADSCs into ACs

To transdifferentiate ADSCs into ACs, ADSCs were co-cultured with ACs using a double chamber system [7, 8, 19]. The expression of Amyl, AQP5, NaK, CK19, and CK7 genes was compared among CCA, ACM, and ADSCM groups (Fig. 5). Amyl (3.8-fold) and CK19 (3.5-fold) were significantly up-regulated in the CCA group, while only Amyl (3-fold) was up-regulated in ADSCs in the ACM group, compared to the expression in ADSCM group. The expression of AC and DC marker genes demonstrated that the cells in the CCA group and ADSCs in the ACM group were similar to ACs, not DCs.

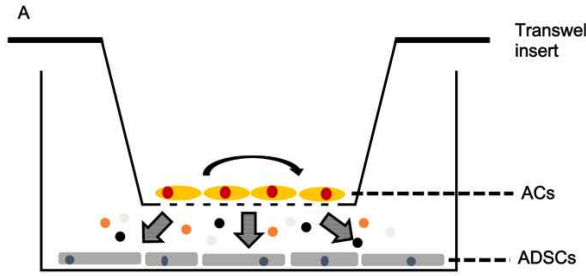
To detect Amyl, AQP5, and NaK proteins in the cells, IF staining was performed at 14 and 21 days after culture. In CCA and ADSCs in the ACM groups, Amyl and AQP5 proteins were detected, though any markers of ACs and DCs were not detected in the ADSCM and AM groups (Fig. 6). The detected level of Amyl and AQP5 proteins was higher on Day 21 than Day 14. These results reveal that the cells in CCA and ADSCs in the ACM groups appear to be similar to ACs in terms of the expression of genes and proteins.

Comparison of senescence and proliferation assays between CCA and ACM group

To select an appropriate and efficient transdifferentiation method, we assessed senescence and proliferation of cells in both experimental groups. The AM group was used as a control for both groups. Proliferation was tested using IF staining for PCNA. The CCA group

had more $PCNA^+$ cells than the ACM group at Day 14. In both experimental groups, the number of $PCNA^+$ cells was remarkably decreased by Day 21 (Fig.7A and B). To detect cellular senescence, we performed SA-β-gal staining and IF staining for senescence markers (p16 and p21). Regardless of experimental group, the percentage of $β\text{-}gal^+$ cells at Day 14 was less than the percentage at Day 21. In particular, the percentage of $β\text{-}gal^+$cells at Day 14 is remarkably less in the CCA group than in the ACM group (Fig. 8A and B). Expression of p16 and p21 proteins increased until Day 21 (Fig. 9). The expression of p21 protein in the CCA group tended to be lower than in other groups.

A



B

	Group (transwell + lower well)
1	No cell + ADSCs Cell in ADSC medium
2	No cell + ADSCs Cell in AC medium
3	ACs + ADSCs Cell in AC medium
4	No cell + ADSCs Cell in AC conditioned medium

Fig. 1 - Cell culture in a double chamber co-culture system. The transwell is comprised of 12 cell culture inserts with a 0.4-㎛pore size to avoid mixing the two cell types. The lower part is a well of the 12-well plate (A). Four experimental groups were designed to evaluate the co-culture system (B). ADSC: adipose-derived stromal cell; AC: acinar cell.

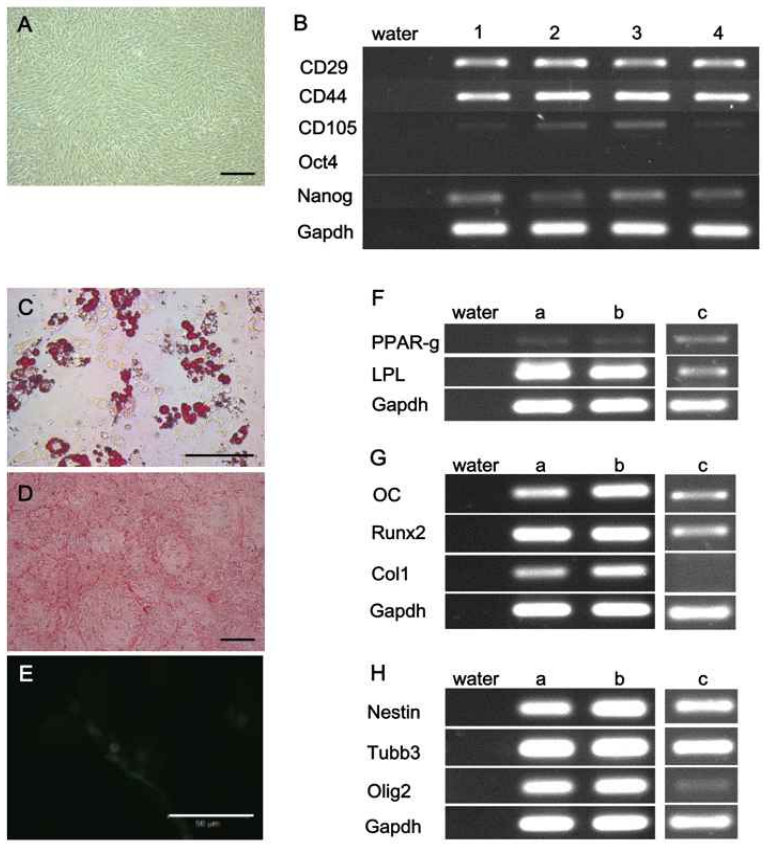


Fig. 2 - Isolation and characterization of adipose-derived stromal cells (ADSCs). Isolated and cultured ADSCs at passage 2 (A). Expression of ADSCs marker genes (CD29, CD44, and CD105) and pluripotency marker genes (Oct4 and Nanog) in the ADSCs (1, 2, 3, and 4) were analyzed by RT-PCR (B). Then, the cells were differentiated into adipocytes, osteoblasts, and neural-like cells after 2 weeks of induction culture. Lineage-specific staining (OilRed-O staining for adipocytes, C; Alizarin red staining for osteoblasts, D; and immunofluorescence of Nestin for neural-like cells, E) and RT-PCR analysis were performed (adipocyte differentiation, F; osteoblast differentiation, G; and neural differentiation, H). a: ADSCs after

differentiation induction; b: murine ESCs after differentiation induction; c: ADSCs at passage 2. PPAR-g: peroxisome proliferator-activated receptor gamma; LPL: lipoprotein lipase; OC: osteocalcin; Runx2: runt-related transcription factor 2; Col1: type-I collagen; Tubb3: tubulin, beta 3 class III; Olig2: oligodendrocyte lineage transcription factor 2; Gapdh: glyceraldehyde 3-phosphate dehydrogenase. Scale bar=100 μm.

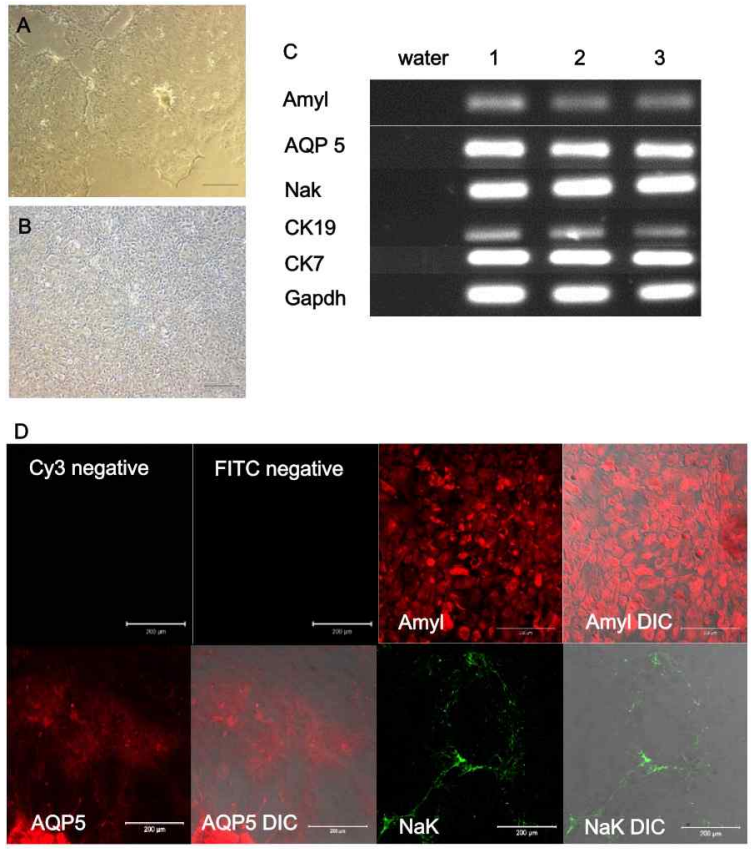


Fig. 3 - Isolation and characterization of ACs. Isolated cells from murine submandibular glands were cultured on a Laminin-coated dish in acinar cell (AC) culture medium at passages 0 (A) and 2 (B). The mRNA expression of Amylase 1 (Amyl) and Aquaporin 5 (AQP5; acinar cell marker), ATP1a1 (NaK; Na^{+}/K^{+}-ATPase), CK19, and CK7 (ductal cell marker) were evaluated using RT-PCR in ACs (1, 2, and 3) at passage 2 (C). The protein expression of AQP5, Amyl, and NaK were detected using immunofluorescence staining (D, Cy3 and FITC negative: negative controls of dye conjugated antibody; DIC: cellular morphology). Scale bar=200 μm.

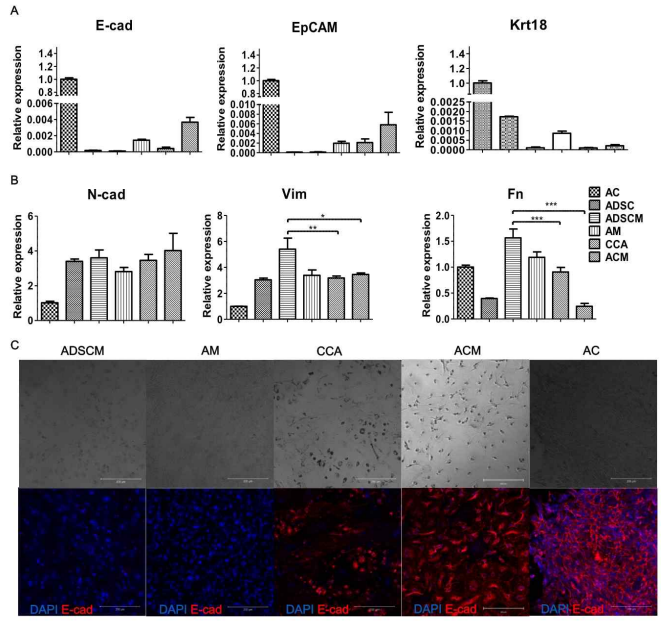


Fig. 4 - Gene expression changes after 14 days of differentiation culture to identify mesenchymal to epithelial transition (MET). Expression of E-cadherin (E-cad), Epithelial cell adhesion molecule (EpCAM),and Keratin18 (Krt18) genes for epithelial cell markers, and N-cadherin, Vimentin, and Fibronectin (Fn) genes for mesenchymal cell markers were evaluated by quantitative PCR. (A and B). The expression change in E-cadherin was demonstrated by immunofluorescence staining in each group (C). ADSCs and ACs at passage 2 were used as controls. AC: ACs at passage 2; ADSC: ADSCs at passage 2; ADSCM: culture in medium for ADSCs; AM: culture in medium for ACs; CCA: Co-cultured ADSCs with ACs in AM; ACM: ACs-conditioned medium. DAPI: nucleus staining (blue); E-cad: E-cad staining (red). Scale bar=200 μm. $*P<0.05$, $**P<0.01$, $***P<0.001$.

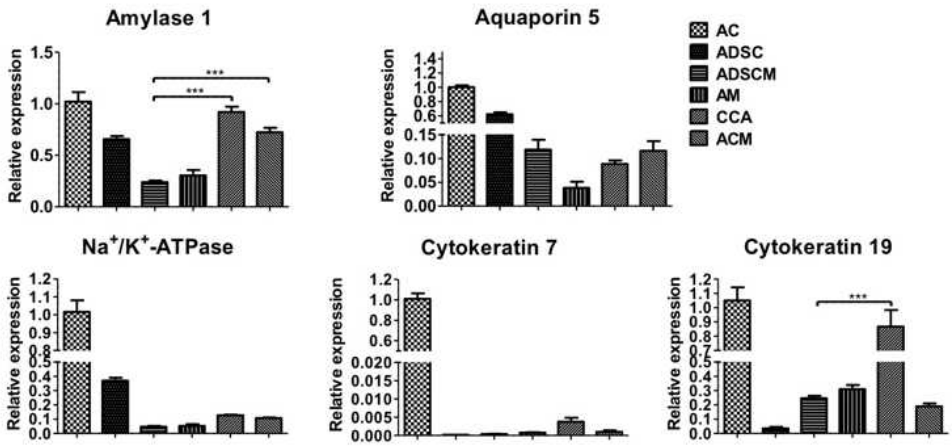


Fig. 5 - Expression of markers for acinar cells (ACs; Amylase 1 and Aquaporin 5) and ductal cells (Na^+/K^+-ATPase, Cytokeratin 7, and Cytokeratin 19) at the mRNA level were analyzed by quantitative PCR after 21 days of transdifferentiation induction culture of adipose-derived stromal cells (ADSCs). ACs and ADSCs at passage 2 were used as controls. AC: ACs at passage 2; ADSC: ADSCs at passage 2; ADSCM: culture in medium for ADSCs; AM: culture in medium for ACs; CCA: Co-culture ADSCs with ACs in AM; ACM: ACs-conditioned medium, ***$P<0.001$.

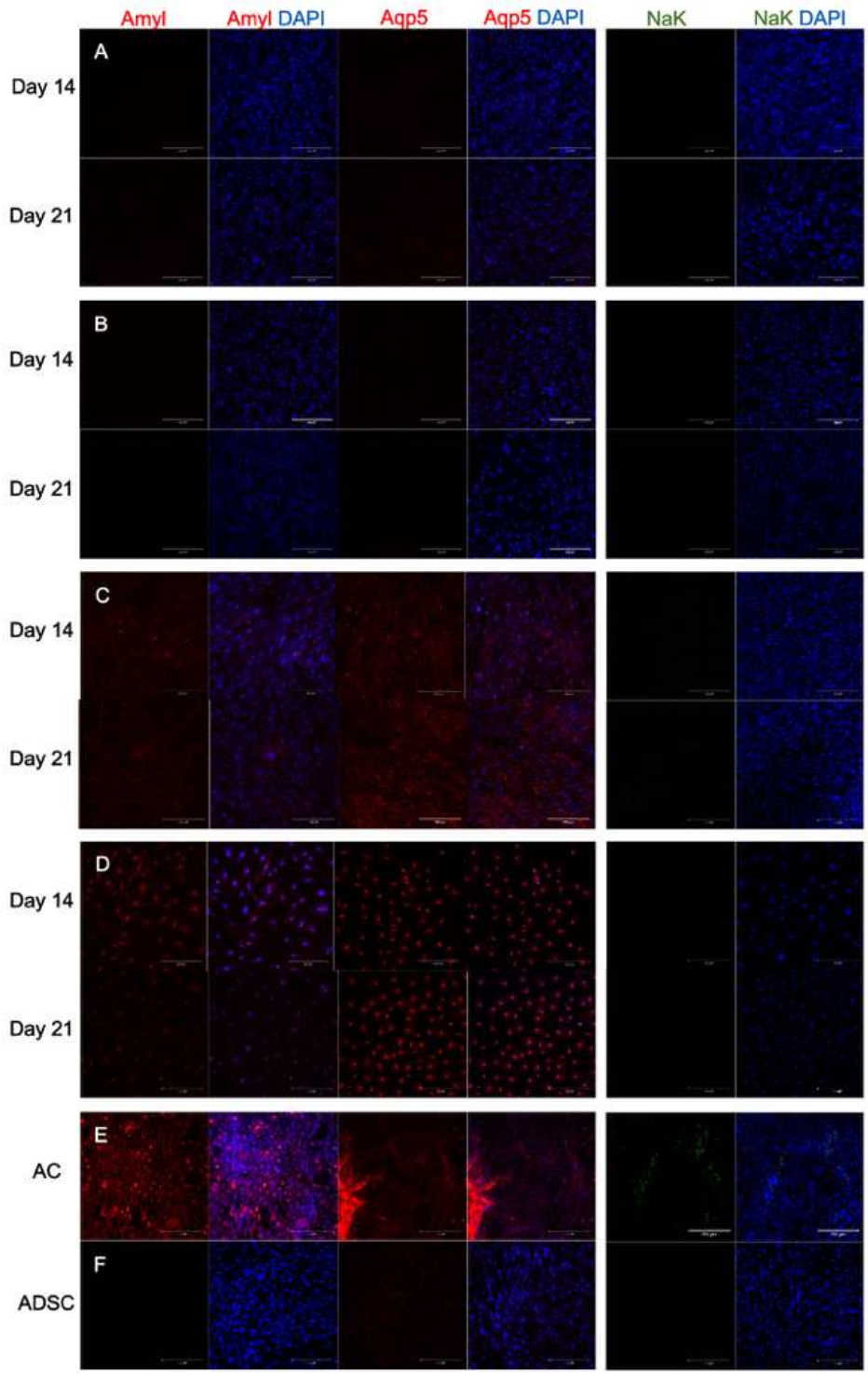

Amyl
Amyl DAPI
Aqp5
Aqp5 DAPI
NaK
NaK DAPI
A
Day 14
Day 21
B
Day 14
Day 21
C
Day 14
Day 21
D
Day 14
Day 21
E
AC
F
ADSC

Fig. 6 - Expression of markers for acinar cells (ACs; Amylase 1, and Aquaporin 5) and ductal cells (Na^+/K^+-ATPase) at the protein level was assessed by immunofluorescence staining after 14 and 21 days of transdifferentiation induction culture of adipose-derived stromal cells (ADSCs). A: Adipose-derived stromal cells (ADSCs) cultured in medium for ADSCs B: ADSCs cultured in medium for ACs; C: ADSCs co-cultured with ACs; D: ADSCs cultured in AC-conditioned medium. ADSCs and ACs at passage 2 were used as controls (E and F). DAPI: nucleus staining (blue); Amyl: Amyl staining (red); Aqp5: Aqp5 staining (red); NaK: NaK staining (green). Scale bar=200 μm.

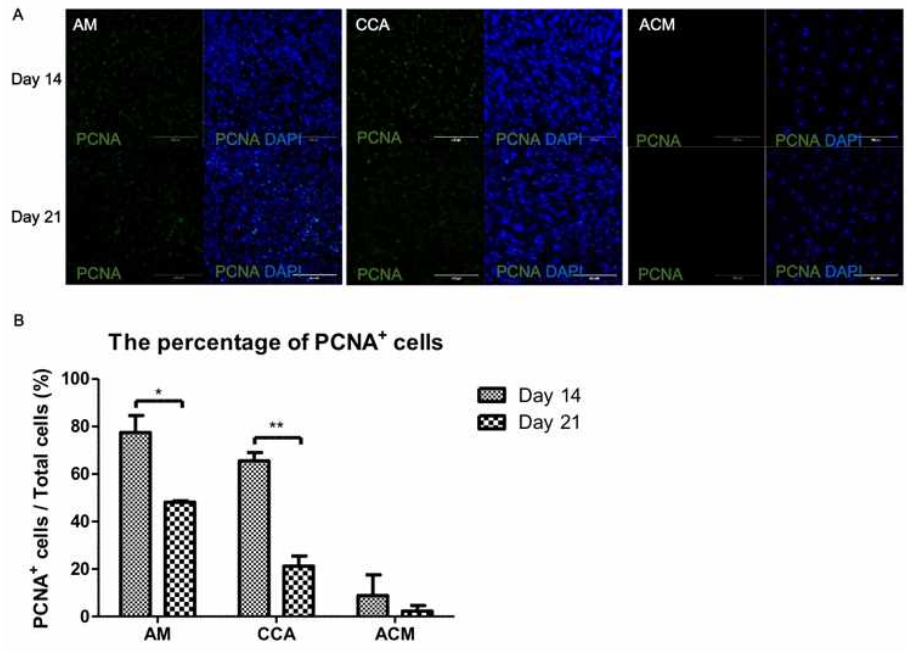


Fig. 7 - Proliferation analysis after transdifferentiation. A: Immunofluorescence for PCNA, a proliferation marker, was detected after 14 and 21 days of transdifferentiation induction culture. AM: Adipose-derived stromal cells (ADSCs) cultured in medium for ACs; CCA: ADSCs co-cultured with ACs; ACM: ADSCs cultured in ACs-conditioned medium. B: Percentage of $PCNA^+$cells in each group. PCNA: PCNA staining (green); DAPI: nucleus (blue), $*P<0.05$, $**P<0.01$.

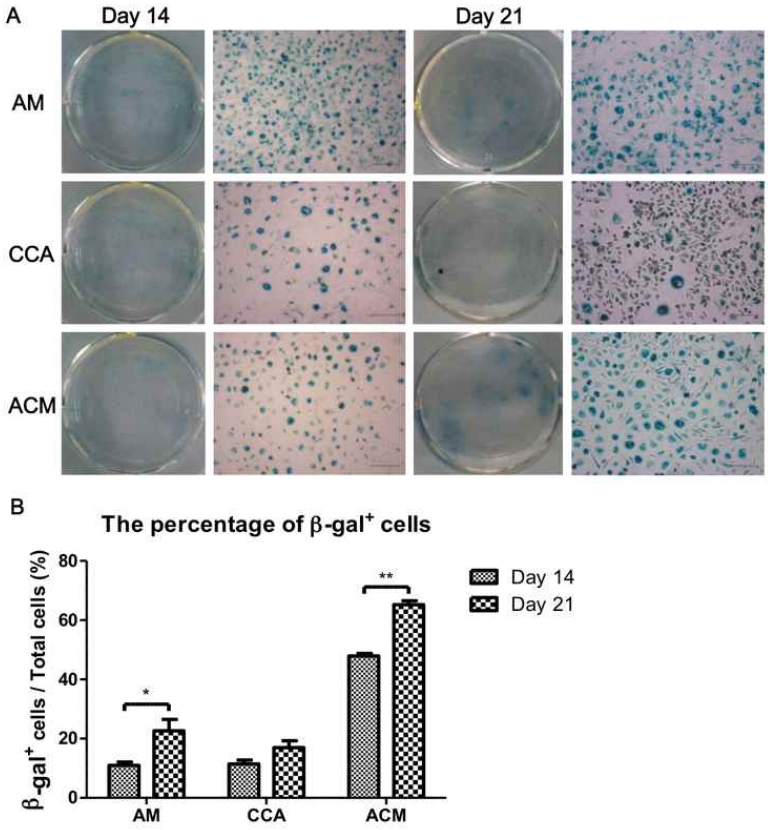


Fig. 8 - Senescence analysis after transdifferentiation. A: SA-β-gal activity was analyzed after 14 and 21 days of transdifferentiation induction culture. AM: Adipose-derived stromal cells (ADSCs) cultured in medium for ACs; CCA: ADSCs co-cultured with ACs; ACM: ADSCs cultured in ACs-conditioned medium. B: Percentage of β-gal^{+} cells in each group. $*P<0.05$, $**P<0.01$.

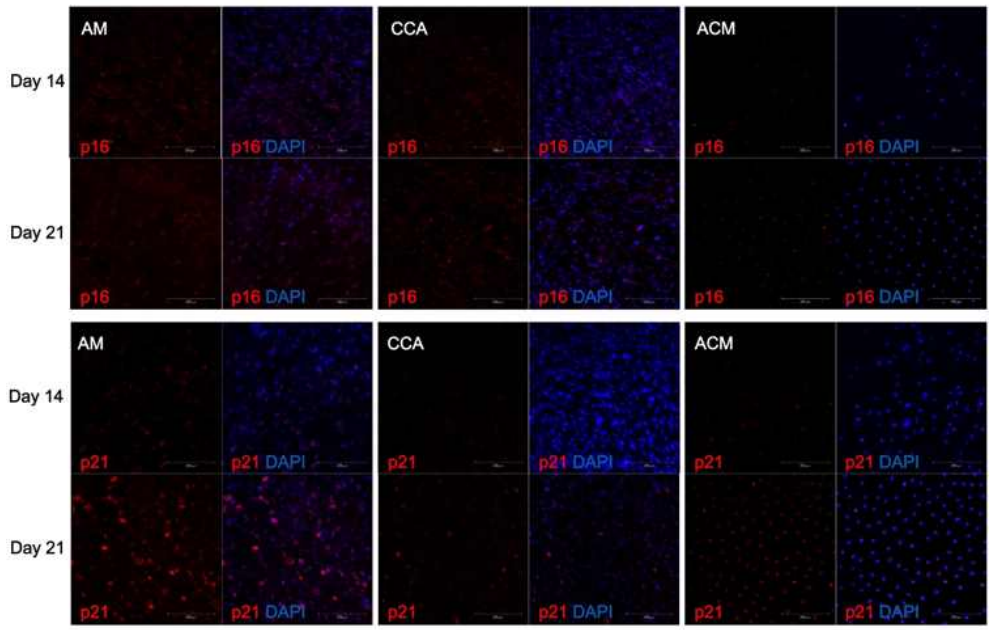


Fig. 9 - Expression of p16 and p21 proteins after transdifferentiation. Immunofluorescence of p16 and p21, the senescence markers, were detected after 14 and 21 days of transdifferentiation induction culture. AM: Adipose-derived stromal cells (ADSCs) cultured in medium for ACs; CCA: ADSCs co-cultured with ACs; ACM: ADSCs cultured in ACs-conditioned medium. p16: p16 staining (red); p21: p21 staining (red); DAPI: nucleus (blue). Scale bar=200 μm.

Discussion

ADSCs can be isolated and proliferated by passaging subcultures without a specific selection process, such as fluorescence-activated cell sorting. Our isolated ADSCs expressed CD29, CD44, and CD105 at the mRNA level, which have been previously reported using various mice strains (BALB/c, C3H, and C57BL/6) [26]. Differentiation into adipocytes, osteoblasts, and neural-like cells shows their potential for differentiation into various cell types. Interestingly, AQP5 was also expressed in the isolated cells at passage 2. This protein is expressed in murine bone marrow-derived MSCs and may play an important role in cellular differentiation [27]. The expression of AQP5 should have a positive effect on differentiation of ADSCs into ACs.

In this study, ADSCs were co-cultured with ACs derived from a mesodermal lineage and could differentiate into ACs, which are endodermal lineage cells. This phenomenon is called "transdifferentiation" [28]. We found an increase in mRNA for epithelial markers (E-cad, EpCAM, and Krt18) and a decrease in mesenchymal cell markers (N-cad, Vim, and Fn) in the CCA and ACM groups when compared with the ADSCM group. We also found an increase in E-cad protein in both groups. These findings indicate that ADSCs may change from mesenchymal to epithelial cells, termed mesenchymal-epithelial transition (MET). This event mainly contributes to the metastatic process in cancer [29] and to early reprogramming of mouse fibroblasts into induced pluripotent stem cells, which is a key process for the initiation of reprogramming [30]. MET might be inevitable because mesenchymal ADSCs must change into epithelial ACs. This alteration was observed more in the ACM group than in the CCA group at Day 14.

The expression of AQP5 and Amyl is essential to confirm the characteristics of ACs. In the gastrointestinal system, Amyl is produced and secreted from the salivary gland and pancreas, and has two typical functions [31]. Firstly, Amyl activity is related to the digestion of carbohydrates into sugars. Secondly, high concentrations of Amyl can reduce the risk for various processes associated with oral health. Oral disease may be affected by a low concentration of Amyl in the oral cavity [31]. AQP5 is mainly expressed in the salivary gland [32, 33], respiratory epithelium [34], lacrimal gland [35] and sweat gland [36]. In salivary glands, it is primarily localized at the apical membrane of ACs [37]. Water permeability is decreased about 65% in parotid ACs and 77% in sublingual ACs in Aqp5-deficient mice [38]. Thus, AQP5 is important in the process of water transfer during saliva secretion from ACs [37]. In the present study, AC-specific markers (Amyl and AQP5) were expressed from Day 14 during the transdifferentiation of ADSCs (Fig. 6). This implies that ADSCs could primarily transdifferentiate into ACs due to unknown factors secreted from ACs in both the CCA and ACM groups. In particular, the number of $AQP5^{+}$ or $Amyl^{+}$ cells in CCA group was higher than in the cells in the ACM group.

The difference between the CCA and ACM groups is not clear, based on the results of AC specific-gene and protein expression after transdifferentiation of ADSCs. Unfortunately, there is no direct comparison between the co-culture system and conditioned medium in the differentiation of ADSCs, though it has been demonstrated that human granulosa cell-conditioned medium is more efficient than a co-culture system in mouse embryo development [39]. In the neuron, both co-culture with BMSCs and BMSC-conditioned medium promote survival and repress apoptosis [40]. Proliferation of tumor cells such

as Eca-109 (esophageal cancer cell line) and A549 (lung cancer cell line) is inhibited by co-culture with human MSCs or by culturing in human MSC-conditioned medium [41]. We compared cellular senescence and proliferation between CCA and ACM. The CCA group has an increased percentage of $PCNA^{+}$ cells at Day 14 than the ACM group. This indicates that the cells in CCA group may be more applicable to cell therapy because they may sustain and grow better after transplantation to damaged tissues. Cellular senescence was assessed by SA-β-gal staining, and senescence is significantly decreased in the CCA group. In addition, the expression of p16 and p21 proteins, which are senescence markers, was also lower in the CCA group. Hence, the cells in the CCA group may be a better choice for cell transplantation to rescue damaged salivary glands.

Conclusion

Our findings indicate that ADSCs can transdifferentiate into ACs using an *in vitro* co-culture system, and these cells can be used for cell transplantation therapy to recover damaged salivary glands. Further studies of AC-secreting factors or signaling pathways are required to develop defined culture conditions for the transdifferentiation of ADSCs into ACs.

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요약(국문초록)

타액선은 타액을 분비하며 이는 구강환경과 건강유지에 필수적이다. 타액선의 기능적 손실은 머리와 목 부근에 발생하는 종양을 치료하기 위한 방사선 치료의 부작용으로 발생한다. 타액선 분비세포를 이용한 세포이식은 방사선 치료의 부작용을 극복할 수 있을 것으로 생각된다. 본 연구는 세포치료를 위한 세포자원으로서 지방유래 줄기세포를 이용, 타액선 유래 분비세포와 공배양을 통해 이를 분화시키고자 하였다. 다분화능을 갖는 지방유래 줄기세포는 지방 주변의 stromal vascular fraction 으로부터 쉽게 분리가 가능하다. 분리된 지방줄기세포는 특정 표지인자인 integrin β-1 (CD29), cell surface glycoprotein (CD44), endoglin (CD105) 및 Nanog의 발현을 통해 확인하였다. 그리고 이 세포들은 지방, 뼈와 신경세포로 분화가 될 수 있는 능력이 확인되었다. 이후 지방줄기세포는 타액선 분비세포와 공배양을 하기위해서 Transwell을 사용하여 실험하였다. Transwell를 이용한 공배양 시스템은 서로 다른 두 종류의 세포가 섞이지 않고 세포 분비인자들만 이동 가능한 배양체계이다. 공배양 처리 후 지방줄기세포에서 타액선 분비세포 마커인 α-amylase와 aquaporin 5가 mRNA와 단백질수준에서 발현되었다. 공배양 뿐만 아니라 타액선 분비세포의 conditioned medium으로 배양한 지방줄기세포에서도 타액선 분비세포의 표지인자가 발현되어 최적의 분화기법을 선별하기 위해서, 세포의 증식성 및 노화수준에 대한 분석을 실시하였다. 그 결과 공배양을 실시한 실험군이 타액선 분비세포의 conditioned medium을 이용한 실험군에 비하여 상대적으로 낮은 수준의 노화현상과 높은 세포 증식력을 보였다. 결론적으로, 지방유래 줄기세포는 공배양체계를 통하여 타액선 분비세포로의 분화가 가능함이 확인되어 침샘 복원을 위한 세포치료 시 유용한 세포자원으로 이용될 것으로 생각된다.

주요어 : 지방유래 줄기세포, 분비세포, 공배양 기술, 교차분화, Conditioned medium

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