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Differentiation of Human Adipose-derived Stem Cells along the Keratocyte Lineage *In vitro*

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Abstract

Purpose: To evaluate differentiation of human adipose-derived stem cells (hASCs) to the keratocyte lineage by co-culture with primary keratocytes *in vitro*.

Materials and Methods: A co-culture system using transwell inserts to grow hASCs on bottom and keratocytes on top in keratocyte differentiating medium (KDM) was developed. hASCs that were cultured in complete culture medium (CCM) and KDM were used as control. After 16 days, hASCs were examined for morphologic changes and proliferation by cell count. qRT-PCR and flow cytometry were used to detect the expression of aldehyde dehydrogenase 3 family, member A1 (ALDH3A1) and keratocan.

Results: hASCs became more dendritic and elongated in co-culture system relative to CCM and KDM. The doubling time of the cells was longer as differentiation progressed. qRT-PCR showed a definite trend towards increased expression of both ALDH3A1 and keratocan in co-culture system despite statistically non-significant p-values. Flow cytometry showed significantly increased protein levels of ALDH3A1 and keratocan in co-culture system relative to CCM group ($p<0.001$) and even relative to KDM group ($p<0.001$ for ALDH3A1 and $p<0.01$ for keratocan).

Conclusion: The co-culture method is a promising approach to induce differentiation of stem cell populations prior to *in vivo* applications. This study reveals an important potential for bioengineering of corneal tissue using autologous multi-potential stem cells.

Keywords: Human adipose-derived stem cells; Co-culture system; Keratocyte; Bioengineered cornea

Introduction

Corneal diseases are a major cause of vision loss and blindness, affecting more than 10 million people world-wide [1]. Stromal opacity is the main cause of corneal blindness. The stroma is the middle layer of the cornea and represents 90% of corneal thickness. It is extremely well organized, consisting of keratocytes, collagen fibers and glycosaminoglycans. Currently, the "gold standard" of treatment for corneal blindness is penetrating keratoplasty or keratoprosthesis; however, there is a discrepancy between supply and demand for human donor corneas. Furthermore, there are relatively high rates of postoperative complications associated with these treatments, which has prompted appreciable clinical interest in the development of a suitable replacement for corneal transplants. Tissue engineering of a living cornea created by integrating biomaterials with cells is a new hope for corneal blindness cure [2]. In the effort to find a proper cell source for keratocytes, some studies showed human adipose-derived stem cells (hASCs) are able to differentiate to keratocytes *in vitro* and *in vivo* [3-5]. Our preliminary data [5] showed that hASCs on a modified hyaluronic acid scaffold could survive in rabbit corneas up to 10 weeks; furthermore, they maintained their morphology and began secreting cornea specific proteins. These results led us to design experiments that would show whether hASCs exposed to keratocytes before implantation would be more successful. Other groups have shown that co-culturing multi-potential stem cells such as bone marrow-derived mesenchymal stem cells with differentiated cells would facilitate the differentiation process [6-12]. Therefore we decided to investigate the effectiveness of co-culturing adipose-derived stem cells with adult keratocytes *in vitro*. The primary focus of this study is to find a more effective method of inducing the differentiation of adipose-derived stem cells to keratocytes.

Materials and Methods

Human keratocyte isolation and culture

Human corneas were obtained from Louisiana Southern Eye Bank. Donor human corneas that were not suitable for transplantation without corneal ulcer and opacity or history of previous corneal surgeries were used. Under a dissecting microscope (Zeiss Opmi 6s/S3, Oberkochen, Germany), the Descemet's membrane, including the attached corneal endothelium, was stripped from the stroma and the epithelium was debrided. Corneal stromas were incubated with 2.4 U/ml Dispase II (Roche Diagnostics, Pleasanton, CA) overnight at 4°C and then minced into ~2 mm cubes and digested up to 3 hours at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Grand Island, NY) containing 1 mg/ml collagenase type L (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/ml testicular hyaluronidase

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(Sigma-Aldrich). Thereafter, stromal cells were cultured in keratocyte growth medium (KGM) containing DMEM/F12 (Invitrogen-Gibco) with 10 ng/ml fibroblast growth factor 2(FGF2, Sigma-Aldrich), 1X insulin, transferrin, selenium (ITS, Invitrogen), 1 mM ascorbic acid-2-phosphate (Sigma-Aldrich), and 100 unit/ml penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO). The cells were incubated at 37°C with 5% humidified CO_2, and the medium was changed every 3~4 days [13]. When 80%–90% confluence achieved, they were trypsinized, subcultured and used for co-culture as described below.

Cells of passage 4 were examined under microscope and harvested for RNA isolation. Gene expression of keratocan, ALDH3A1, cadherin 5 (CDH5), and alpha smooth muscle actin (αSMA) was examined by RT-PCR and DNA electrophoresis.

Human adipose-derived stem cell culture

Adult human adipose-derived stem cells (hASCs) were isolated from lipoaspirates as previously described [14]. Briefly, human subcutaneous adipose tissue samples that were obtained from liposuction aspirates were treated with type I collagenase at 37°C for about 60 minutes. After digestion, the samples were centrifuged to separate the stromal vascular fraction (SVF) from primary adipocytes. SVF was washed, plated, and the cells were expanded. Cryopreserved hASCs were thawed and cultured in complete culture medium (CCM) containing DMEM/F12, 10% FBS (Atlanta Biologicals Lawrenceville, GA) and 1X Antibiotic-Antimycotic (Invitrogen-Gibco). The cells were incubated in a humidified atmosphere at 37°C with 5% CO_2, and the medium was changed every 3~4 days. Cells from two different donors between passages 3-4 were used for the experiments.

Colony-forming unit-fibroblasts (CFU-F) assay

For CFU-F assay, 100 hASCs were plated on a 10 cm dish in CCM and cultured for 14 days. Plates were then washed three times with PBS and stained with 3% crystal violet (Sigma-Aldrich) to visualize the colonies [15].

Osteogenic and adipogenic differentiation of hASCs

Human adipose-derived stem cells were cultured in 6-well plates in CCM until 90% confluence achieved. For osteogenic differentiation, the media were replaced with osteogenic differentiation media containing 1 nM dexamethasone (Sigma-Aldrich), 20 mM β-glycerol phosphate (Sigma-Aldrich), 50 μM ascorbate 2-phosphate (Sigma-Aldrich) and 50 ng/ml L-thyroxine (Sigma-Aldrich). The osteogenic differentiation media were changed every 3~4 days. After three weeks cells were fixed in 10% formalin for 1 hour at 4°C and stained for 10 minutes with 40 mM Alizarin Red (pH 4.1, Sigma-Aldrich) to visualize calcium deposition. For adipogenic differentiation, the media were replaced with adipogenic differentiation media containing of 1 μM dexamethasone (Sigma-Aldrich), 5 μg/ml insulin (Sigma-Aldrich), 0.5 μM isobuytlmethylxanthine (Sigma-Aldrich) and 50 μM indomethacine (Sigma-Aldrich). The adipogenic differentiation media were changed every 3~4 days. After three weeks, cells were fixed in 10% formalin for 1 hour at 4°C, stained for 10–15 minutes at room temperature with Oil Red O (Sigma-Aldrich) to detect neutral lipid vacuoles. Images were acquired on Nikon Eclipse TE200 with Nikon Digital Camera DXM1200F using Nikon ACT-1 software version 2.7. The hASCs were cultured in CCM for 3 weeks and stained with Alizarin Red and Oil Red O as controls.

hASCs and keratocytes co-culture system

The hASCs (passage 3~4) were plated into fibronectin-coated 6-well plates (Millipore, Billerica, MA) at a density of 7.5×10^4 cells/well in 2 ml keratocyte differentiation medium (KDM) containing advanced DMEM (Invitrogen-Gibco) supplemented with 10 ng/ml FGF2, 0.1 mM ascorbic acid-2-phosphate, 1% heparin-stripped, platelet-poor horse serum (HSHS, Sigma-Aldrich) and 100 unit/ml penicillin (Invitrogen-Gibco), 100 μg/mL streptomycin (Invitrogen-Gibco). 24 mm transwell inserts with 0.4 μm diameter pores (Corning, Corning, NY) were placed into the 6-well plates, and human keratocytes were inoculated into the inserts at a density of 1×10^5 cells/insert in 1.5 ml KDM. hASCs and human keratocytes were co-cultured in this transwell system at 37°C in a 5% CO_2 humidified atmosphere. Half of the culture medium was replaced every 3rd day with KDM. On day 16, the cells were examined under microscope and harvested for cell counting, RNA isolation or flow cytometry. hASCs cultured in CCM or KDM alone were used as controls.

RNA isolation, PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells using an RNeasy mini kit (Qiagen, Valencia, CA) following the instructions provided by the manufacturer. Total RNA concentration was measured using NanoDrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE), and the quality was assessed by the 260/280 and 230/260 ratios. The RNA was first treated with DNase I (Invitrogen) and then converted into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The reaction mixture contained 1000 ng of RNA with 4 μl 5X iScript reaction mix, 1 μl iScript reverse transcriptase, and nuclease-free water with a total reaction volume of 20 μl. The samples were subsequently run on a PTC-200 Peltier Thermal Cycler (MJ Research, Ramsey, MN) at 25°C for 5 minutes, 42°C for 30 minutes, followed by 85°C for 5 minutes.

For PCR reactions, target genes were amplified in a 20 ml reaction volume for 35 cycles using the following settings: 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, 1 min at 72°C and a final extension step of 5 min at 68°C on a PTC-200 Peltier Thermal Cycler. The oligonucleotide primer (5'–3') sequences specific for the genes examined are as follows GAPDH CAGCGACACCCACTCCTC-CACCTT (forward) and CATGAGGTCCACCACCCTGTTGCT (reverse); CDH5 CAGCCCAAAGTGTGTGTGAGAA (forward) and CGGTCAAACTGCCCATACTT (reverse); αSMA CCCAGC-CAAGCACTGTCA (forward) and TCCAGAGTCCAGCACGATG (reverse). PCR products were run on a 1.0% agarose gel, and images were captured using the ImageQuant LAS 4000 System (GE Healthcare Life Science, Piscataway, NJ).

The qRT-PCR reactions were performed as previously described [16] with slight modification. Briefly, each reaction mixture contained 1 μl of commercially available TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems, Foster City, CA), 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems), 4 μl cDNA template, and 5 μl RNase-free water with a total volume of 20 μl. Human keratocan (KERA) and aldehyde dehydrogenase 3 family, member A1 (ALDH3A1) mRNA levels were assayed using Taq-Man Gene Expression Assays Hs00559942_m1 and Hs00964880_m1 (Applied Biosystems), respectively. All samples were normalized to 18S rRNA (Hs03928985_g1, Applied Biosystems) content. The reaction was performed at 50°C for 2 minutes, 95°C for 10 minutes followed by a 40-cycle two-step PCR (95°C for 15 seconds and 60°C for 1 minute) using the CFX96 Real-Time System (Bio-Rad).

Flow cytometry

For hASC characterization, aliquots of hASCs (P0) were incubated

with either PE- or FITC-conjugated antibodies for human CD29, CD105, CD45 (eBioscience, San Diego, CA), CD44, CD73, and CD90 (BD Biosciences, San Jose, CA) at room temperature for 30 minutes. The samples were washed and then analyzed by FACScan (FACScalibur; BD Biosciences) with CellQuest software. To analyze keratocan and ALDH3A1 expression, cells were harvested, washed with PBS and fixed and permeabilized using IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA) following the instructions provided by the manufacturer. Cells were then stained with rabbit anti-human keratocan or ALDH3A1 primary antibody (1:100) followed by FITC-conjugated goat anti-rabbit secondary antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA). The samples were washed three times with PBS and were then analyzed by Beckman Coulter Gallios flow cytometer (Beckman Coulter, Brea, CA).

Statistical analysis

All values are presented as means ± SEM. The statistical differences were determined by ANOVA, followed by Bonfferoni post-hoc multiple comparison tests. Statistical significance was set at $p<0.05$.

Results

Verification of adult keratocytes in KGM

Figure 1A shows the spindle-shaped morphology of keratocytes in KGM after 4 passages. RT-PCR demonstrated that keratocytes expressed ALDH3A1, CDH5, and keratocan. These cells did not express α-SMA (Figure 1B). CDH5 was selected as a specific keratocyte marker since Scott et al. [13] showed CDH5 was expressed only by keratocytes not by fibroblasts. Human lung fibroblasts treated by transforming growth factor beta (TGF-β) were used as positive control for α-SMA.

Characterization of hASCs

Human ASCs were plated at very low density (100 cells in 10 cm dish) to allow colony-forming unit-fibroblasts (CFU-Fs) formation. Two weeks later, the cells were able to generate CFU-Fs (Figure 2A). The colony forming efficiency was 20.78 ± 2.32% (N=9).

To demonstrate the multi-potential characteristics of hASCs, cells were induced to differentiate into osteogenic and adipogenic lineages. Figure 2B shows the cells were induced into an osteogenic lineage confirmed by Alizarin Red staining, and figure 2C illustrates hASCs were induced into an adipogenic lineage. Figures 2D and 2E show

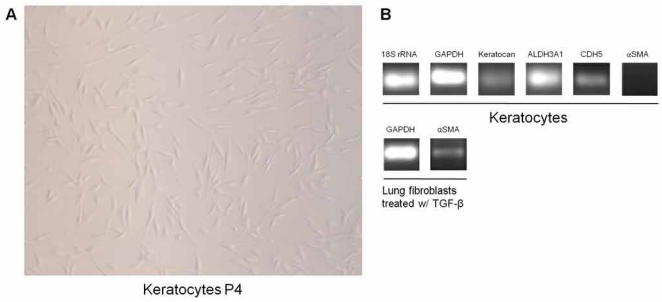

Figure 1: Verification of adult keratocytes in KGM.

A) Phase-contrast image of keratocytes after four passages in KGM. Original magnification 4x. **B)** Gene expression of ALDH3A1, CDH5, and keratocan in passage 4 as demonstrated by RT-PCR. Keratocytes had negative expression of α-SMA. Lung fibroblasts treated by TGF-β were used as positive control for α-SMA expression.

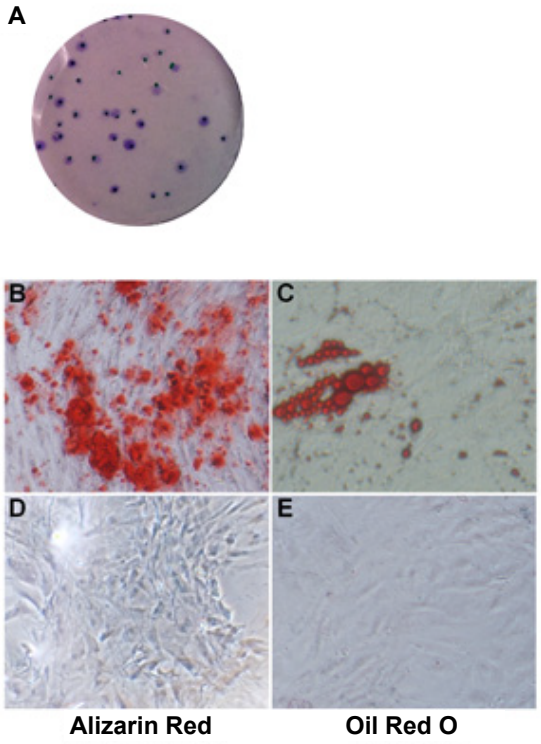

Figure 2: Characterization of hASCs.

A) hASCs were seeded at low density and incubated in CCM for 14 days to allow CFU-Fs formation. The colonies were fixed and stained with crystal violet. **B-C)** Representative images showing osteogenic (Alizarin Red staining) and adipogenic (Oil Red O staining) differentiation of hASCs, respectively. **D-E)** Representative images showing hASCs cultured in CCM for 3 weeks as controls and stained with Alizarin Red and Oil Red, respectively. Original magnification 4x for B and D and 20x for C and E.

hASCs that were cultured in CCM (control medium for differentiation) for 21 days did not stain with Alizarin Red and Oil Red O, respectively. ASCs of passage 0 were analyzed for surface markers by flow cytometry. The cells were found positive for CD29, CD105, CD73, and CD90 and negative for CD44 and CD45 (Table 1).

hASCs morphology and proliferation in co-culture system

ASCs became more dendritic and elongated in co-culture system relative to CCM and KDM as shown in figures 3A, 3B and 3C. The hASCs started to grow slower and to become more keratocyte-like as differentiation progressed. After 16 days of culturing, we obtained $18.65 \pm 3.83 \times 10^4$ ASCs in CCM, $13.11 \pm 3.27 \times 10^4$ ASCs in KDM, and $6.89 \pm 2.45 \times 10^4$ ASCs in co-culture system. One-way ANOVA with Bonferroni adjustment demonstrated significantly decreased growth of ASCs in co-culture relative to KDM and CCM ($p<0.001$) and also in KDM relative to CCM ($p<0.01$) (Figure 3D).

qRT-PCR for markers of differentiation

Figures 4A and 4B show the result of qRT-PCR for keratocan and ALDH3A1 gene expression in hASCs grown in 3 different culture systems: CCM, KDM, and co-culture in comparison to keratocytes cultured in KGM. Gene levels were normalized to 18S rRNA level. Level of gene expression of keratocan was 1.00 ± 0.26 in CCM, 4.57 ± 3.31 in KDM, 10.69 ± 8.76 in co-culture system and 20.87 ± 9.57 in keratocytes, and for ALDH3A1 was 1.00 ± 0.09, 3.01 ± 0.94, 4.54 ±

	CD29	CD105	CD45	CD44	CD73	CD90
Donor 1	99.11	97.54	9.26	11.31	89.57	89.52
Donor 2	99.25	98.72	5.51	5.39	95.07	84.91

Table 1: Summary of cell surface antigen markers of hASCs (passage 0) from two different donors that were used in the experiments. The numbers are percentages of cells that showed surface markers.

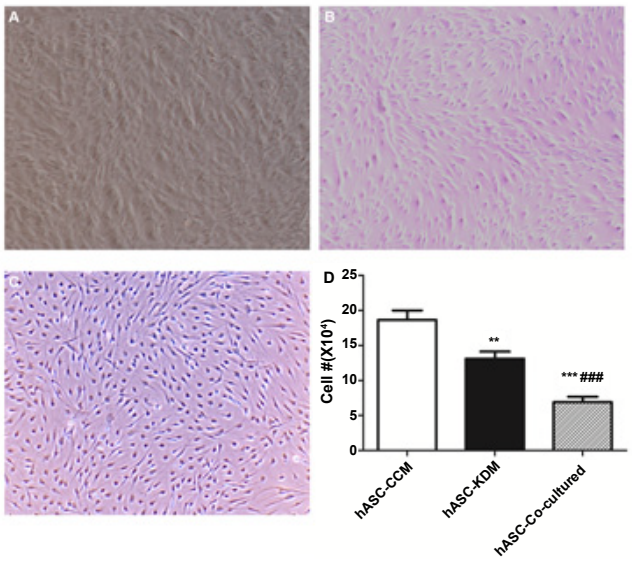

Figure 3: Phase-contrast images and cell counts after 16 days in three different culture systems.

A) hASCs that were grown in CCM. **B)** hASCs that were grown in KDM. **C)** hASCs that were grown in co-culture system. **D)** Cell counts in different culture systems on day 16 showed hASCs started to grow slower as differentiation progressed. Original magnification 4x for A-C. Significance was defined as ** and *** for p <0.01 and p <0.001, respectively, compared to hASC-CCM group; significant difference between hASC-KDM group and hASC-Co-cultured group was denoted by ### for p <0.001.

1.33, and 311.20 ± 57.76, respectively. Although the p-value between 3 groups in one-way ANOVA test was not statistically significant, there was a definite trend towards increased production of both ALDH and keratocan in co-culture system.

Flow cytometry for markers of differentiation

Figures 5A and 5B show the results of flow cytometry for keratocan and ALDH3A1 in 3 different culture methods. The results indicate that protein levels of keratocan and ALDH3A1 were significantly higher in co-culture and KDM relative to CCM ($p<0.001$) and also in co-culture relative to KDM ($p<0.05$ for keratocan and $p<0.001$ for ALDH3A1).

Discussion

The corneal stroma contains multi-potent adult stem cells, which express ABCG2, BMi1, CD166, C-kit, Notch1, PAX6, and Six2 [17].

Previous studies have shown that it is possible to both culture and differentiate these cells into keratocytes in order to produce a full thickness corneal stroma [18,19]. However, obtaining sufficient numbers of stromal stem cells by sub-culturing has been challenging. Moreover, keratocyte stem cells are neither sufficiently numerous nor easy to acquire, and their extraction requires several donor corneas [3].

hASCs were selected as a stem cell source for the generation of keratocytes, for several reasons. First, adipose tissue is readily harvested in relatively large quantities with minimal risk. In addition, the yield of mesenchymal stem cells (MSCs) from adipose tissue is 100 to 500-fold greater than that from bone marrow [20-22]. ASCs also have immunomodulatory properties similar to bone marrow-derived MSCs, as previously described in literature [23-25]. Furthermore, Arnalich-Montiel et al. [3] demonstrated differentiation of hASCs in rabbit corneal stroma, and Du et al. [4] showed hASCs could differentiate to keratocytes *in vitro*. Our preliminary data [5] showed hASCs on modified hyaluronic acid scaffold could survive in rabbit corneas up to 10 weeks, maintain their morphology, and start secreting cornea specific proteins in the vicinity. Other groups showed co-culturing of multipotential stem cells such as bone marrow-derived mesenchymal stem cells with differentiated cells would facilitate the differentiation process [6-12]. Therefore, the effectiveness of co-culturing adipose-derived stem cells with keratocytes *in vitro* was examined for the first time, and demonstrated a significant increase in production of cornea-specific proteins such as ALDH3A1 and keratocan in a co-culture system relative to CCM ($p<0.001$) and KDM (Figure 5). As Du et al. [4] showed, our study also confirmed increased production of ALDH3A1 and keratocan in KDM relative to CCM. However, co-culture system significantly increased the protein levels of ALDH3A1 and keratocan compared to KDM. Although there was a trend towards increased mRNA levels of ALDH3A1 and keratocan in co-culture relative to KDM and CCM, p- values were not significant. This can be explained by different time points of RNA expression and post-translational modifications to protein production nevertheless in our study PCR and flow cytometry were assessed simultaneously on day 16.

Both keratocan and ALDH3A1 were used as markers of the

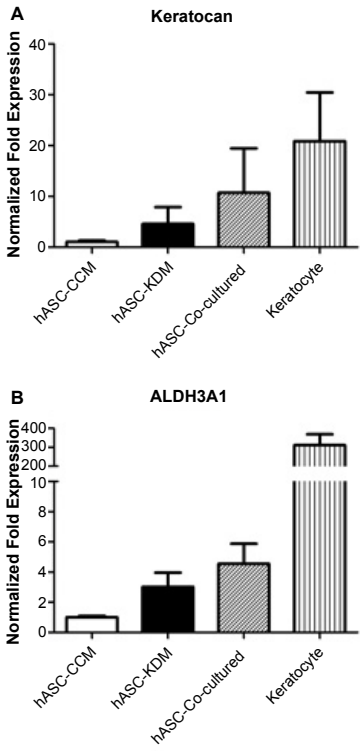

Figure 4: Results of qRT-PCR in 3 different culture systems after 16 days.

A) qRT-PCR for keratocan. **B)** qRT-PCR for ALDH3A1. Gene levels were normalized to 18S rRNA level.

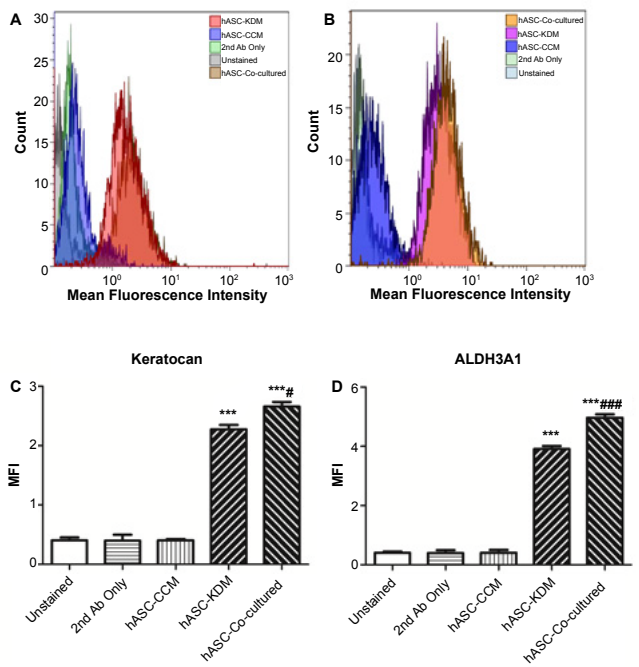

Figure 5: Results of flow cytometry in 3 different culture systems after 16 days.

A) Mean fluorescence intensity (MFI) histogram for keratocan. **B)** MFI histogram for ALDH3A1. **C,D**) Quantified MFI of keratocan and ALDH3A1 respectively. Significance was defined as *** for p <0.001 compared to hASC-CCM group; significant differences between hASC-KDM group and hASC-Co-cultured group were denoted by # and ### for p <0.05 and p <0.001, respectively.

keratocyte phenotype; however, assessment of ASC differentiation to functional keratocytes requires production of the highly organized, transparent extracellular matrix (ECM) of the corneal stroma. Thus, more detailed molecular characterization of ASC-derived keratocyte-like cells and the extracellular matrix surrounding the cells is necessary. The data also showed that hASCs grew slower in the presence of keratocytes. To elucidate the mechanisms of action more comprehensive investigations and longer follow-up are necessary.

In conclusion, the results provide more evidence of potential differentiation of hASCs to keratocytes *in vitro* in a novel co-culture system. Although clinical application of multi-potential stem cells needs long-term evaluation for tumorigenesis and mutagenesis, this study reveals an important potential for bioengineering of corneal tissue using autologous multi-potential stem cells.

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Conflict of Interest

None of the authors has any financial or personal interest in any materials and methods that presented in this study.

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