Adipose-derived stem cells express higher levels of type VII collagen under specific culture conditions

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Adipose-derived stem cells express higher levels of type VII collagen under specific culture conditions

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Abbreviations: Col7, type VII collagen; ADSC, adipose-derived stem cell; K14, cytokeratin 14; NHEK, normal human epidermal keratinocyte; HRP, horseradish peroxidase; NHDF, normal human dermal fibroblast; DMEM, Dulbecco's modified Eagle's Medium; ATRA, all-trans retinoic acid; BMP4, bone-morphogenetic protein-4; KSFM, keratinocyte serum-free medium; K5, cytokeratin 5; K10, cytokeratin 10; DAPI, 4,6-diamidino-2-phenylindole; RDEB, recessive dystrophic epidermolysis bullosa

Abstract

Type VII collagen (Col7) is a major component of the anchoring fibrils at the dermoepidermal junction. Adipose-derived stem cells (ADSCs) are a cell population highly useful in regenerative medicine because of their ease of isolation and their potential for multilineage differentiation. Based on the observations that K14 was expressed in undifferentiated ADSCs and the expression was downregulated after differentiation into adipocytes, we speculated that ADSCs are keratinocyte stem/progenitor cells. ADSCs were co-cultured with fibroblasts on type Ⅳ collagen in a medium containing all-trans retinoic acid and bone morphogenetic protein 4. At day 14 of culture in keratinocyte serum-free medium, the cells were harvested and subjected to immunofluorescence, flow cytometry, real-time PCR, and western blotting. Approximately 45% of ADSCs were immunostained positively for anti-human cytokeratin 10, and approximately 80% were stained positively for Col7. Flow cytometry, real-time PCR, and western blotting also confirmed that differentiated ADSCs expressed higher levels of Col7. These findings support the therapeutic potential of ADSCs, not only for wound healing, but also for the correction of Col7 deficiencies.

Key words

adipose-derived stem cell; keratinocyte; type VII collagen; cytokeratin

Introduction

Type VII collagen (Col7), which is synthesized by both basal keratinocytes and dermal fibroblasts in human skin, is a major component of the anchoring fibrils at the dermoepidermal junction. Anchoring fibrils provide a structural attachment between the epidermal basement membrane and papillary dermis. Adipose-derived stem cells (ADSCs) are a cell population highly useful in regenerative medicine because of their ease of isolation and their potential for multilineage differentiation [11]. In this study, we investigated whether ADSCs express Col7 to determine the potential of these cells to provide epidermal cover and rescue basement membrane defects.

Materials and methods

K14 detection in undifferentiated ADSCs by immunofluorescence and flow cytometry

Undifferentiated ADSCs (Lonza Group AG, MA, CHE) were first immunostained for human cytokeratin 14 (K14) using rabbit polyclonal anti-cytokeratin 14 (Covance, NJ,

USA), with normal human epidermal keratinocytes (NHEKs; Kurabo, Osaka, Japan) used as a positive control.

Undifferentiated ADSCs were also incubated with FITC-conjugated anti-K14 antibody (Abcam, Cambridge, UK) and analyzed by flow cytometry.

K14 detection in adipocyte-differentiated ADSCs by real-time PCR

ADSCs were differentiated into adipocytes as previously described [10]. K14 expression before and after the differentiation of ADSCs into adipocytes was evaluated by real-time PCR. Total RNA extracted from ADSCs was converted into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). TaqMan Master Mix (Applied Biosystems, CA, USA) was used to amplify cDNA for 45 cycles on a StepOnePlus System (Applied Biosystems). K14 expression (using primers Hs00265033_m1; Applied Biosystems) was normalized to β-actin levels, and the comparative cycle threshold (Ct) method, using the formula $2^{-\Delta\Delta Ct}$, was used to calculate relative mRNA levels.

K14 detection in adipocyte-differentiated ADSCs by western blotting

K14 expression before and after the differentiation of ADSCs into adipocytes was also evaluated by western blotting after ADSCs were harvested and lysed. Proteins were separated by gel electrophoresis and transferred to a nitrocellulose membrane, which was incubated with primary antibodies against K14 (Covance, NJ, USA). β-actin expression detected using a monoclonal antibody (BioLegend, CA, USA) was used as a loading control. The membrane was then washed, incubated with an anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology, MA, USA), and developed with Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

Differentiation of ADSCs into keratinocyte-like cells

A co-culture system was used to differentiate ADSCs into keratinocyte-like cells [2]. Normal human dermal fibroblasts (NHDFs) (5×10^4 cells/ml) were seeded in the bottom chambers of 6-well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) containing 10% fetal bovine serum for 24 h, while ADSCs (1×10^5 cells/ml) were seeded on 0.4-μm Millicell^R hanging cell culture inserts

(Merck Millipore, Darmstadt, Germany) coated with type IV collagen (Nitta Gelatin, Osaka, Japan) and placed into the plates. All-trans retinoic acid (ATRA) (Sigma-Aldrich, MO, USA) was added at a concentration of 1 μM to the upper chamber, and cells were cultured for three days. Bone morphogenetic protein 4 (BMP4) (R&D Systems, MN, USA) was then added at 25 ng/ml to the upper chamber. After four days of culture, the ATRA- and BMP4-containing media were replaced with keratinocyte serum-free medium (KSFM) (Thermo Fisher Scientific). At day seven of culture in KSFM, ADSCs were removed from the co-culture system and cultured on dish coated with type IV collagen for an additional 14 days in KSFM.

K10 detection in differentiated ADSCs by immunofluorescence and flow cytometry

ADSCs were immuostained for human cytokeratin 10 (K10) using mouse monoclonal anti-K10 (Abcam). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Samples were mounted in Mounting Medium with DAPI H-1200 (Vector Laboratories, CA, USA) to detect fluorescence, visualized under a BZ-X700 microscope (KEYENCE, Tokyo, Japan), and analyzed using Hybrid Cell Count software

(KEYENCE). Five independent samples were analyzed per time point. The average number of positively stained cells was divided by the average number of total cells and multiplied by 100 to obtain the percentage. Undifferentiated ADSCs and normal human epidermal keratinocytes (NHEKs) were used as negative and positive controls, respectively.

Undifferentiated ADSCs were also incubated with FITC-conjugated anti-K10 antibody (Abcam) and analyzed by flow cytometry.

K10 detection in differentiated ADSCs by real-time PCR

K10 expression was confirmed by real-time PCR using the same method used to quantify K14 (using primers Hs01043114; Applied Biosystems).

Col7 detection in differentiated ADSCs by immunofluorescence and flow cytometry

Col7 expression in ADSCs on day 14 after removal from the co-culture system was detected by immunofluorescence. Samples were mounted, visualized, and analyzed using the same method to quantify K10 with primary antibodies against Col7 (Abcam). Five

independent samples were analyzed per time point. The average number of positively stained cells was divided by the average number of total cells and multiplied by 100 to obtain the percentage. Undifferentiated ADSCs and normal human epidermal keratinocytes (NHEKs) were used as negative and positive controls, respectively. Undifferentiated ADSCs were also incubated with FITC-conjugated anti-Col7 antibody (Abcam) and analyzed by flow cytometry.

Col7 detection in differentiated ADSCs by real-time PCR

Col7 expression was confirmed by real-time PCR using the same method used to quantify K14 and K10 (using primers Hs00164310_m1; Applied Biosystems).

Col7 detection in differentiated ADSCs by western blotting (Wet transfer)

Col7 expression in ADSCs was also assessed by western blotting after cells were harvested and lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) in the presence of Protease Inhibitor Cocktail (Sigma-Aldrich). Proteins were separated by 10% Tris-glycine SDS-PAGE (Bio-Rad, CA, USA) under denaturing

conditions and transferred to a nitrocellulose membrane, which was incubated with primary antibodies against Col7 (1:0.8 μg/mL; Abcam). The blot for β-actin was confirmed using a monoclonal antibody (1:2000; BioLegend) as a loading control. The membrane was then washed, incubated with anti-rabbit IgG HRP-linked antibody (1:2500; Cell Signaling Technology), and developed with Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Undifferentiated ADSCs, NHEKs, and ADSCs co-cultured with NHDFs without ATRA and BMP4 were also used as controls. Multi Gauge Software (Fujifilm, Tokyo, Japan) was used for visualization and quantification of bands.

Statistical analysis

Data were analyzed by analysis of variance and are presented as the mean ± standard error (SE) from at least three independent experiments. Statistical significance was analyzed using Student's t-test for each paired experiment.

Results

K14 expression in ADSCs decreased after differentiation into adipocytes

The undifferentiated ADSCs expressed K14 as determined by immunofluorescence microscopy (Fig. 1A) and flow cytometry (Fig. 1B).

Adipocyte-differentiated ADSCs expressed lower levels of K14 than undifferentiated ADSCs as determined by both real-time PCR (Fig. 1C) and western blotting (Fig. 1D). These observations were consistent across three independent experiments performed using ADSCs from three different lots. Based on the present observations that K14 was expressed in undifferentiated ADSCs and the expression was downregulated after differentiation into adipocytes, we speculated that ADSCs are keratinocyte stem/progenitor cells.

45% of differentiated ADSCs expressed K10

The differentiated ADSCs displayed a characteristic polygonal cobblestone shape, showed high levels of desmoglein 3 and cytokeratin 5 (K5) expression as assessed by real-time PCR, and were assessed to be viable by the MTT assay (data not shown).

Approximately 45% (45 ±1.0; n = 5) of the differentiated ADSCs stained positive for K10, although undifferentiated ADSCs did not (Fig. 2A). NHEKs were stained with anti-K10 as positive control (Fig. 2A). ADSCs were also immunostained for human K5 and K14. However, undifferentiated ADSCs as well as differentiated ADSCs were positive for K5 and K14, which are markers specific for the basal layer, although the degree of staining slightly increased after differentiation (data not shown).

Flow cytometry revealed that the differentiated ADSCs expressed K10 (Fig. 2B).

Real-time PCR also revealed that the differentiated ADSCs expressed higher levels of K10 than the undifferentiated ADSCs and ADSCs co-cultured with NHDFs without ATRA and BMP4 ($p<0.05$) (Fig. 2C).

80% of differentiated ADSCs expressed Col7

Approximately 80% (80 ± 2.0; n = 5) of differentiated ADSCs stained positive, although undifferentiated ADSCs showed no Col7 immunostaining (Fig. 3A). NHEKs were immunostained with anti-human Col7 as a positive control (Fig. 3A).

Flow cytometry revealed that the differentiated ADSCs expressed Col7 (Fig. 3B).

Real-time PCR also revealed that differentiated ADSCs expressed much higher levels of Col7 than undifferentiated ADSCs and ADSCs co-cultured with NHDFs without ATRA and BMP4 ($p<0.05$) (Fig. 3C).

Western blotting also revealed that the differentiated ADSCs expressed higher levels of Col7 than the undifferentiated ADSCs and ADSCs co-cultured with NHDFs without ATRA and BMP4 (Fig. 3D).

Discussion

Recessive dystrophic epidermolysis bullosa (RDEB) is caused by mutations in the *COL7A1* gene encoding for Col7 [4]. Thus, patients with RDEB suffer from blistering and repeated wounding of the skin, oral mucosa, and gastrointestinal tract. The use of regenerative medicine to treat severe types of epidermolysis bullosa is beginning to attract attention. In particular, the use of autologous or allogeneic cultured skin grafts is reported to be beneficial for intractable ulcers [1, 3, 6]. Autologous cultured epidermal grafts can be used as permanent coverings, although these cells contain Col7 with the

mutations. Allogeneic skin substitutes, on the other hand, are not permanently adopted. In any case, both of these skin grafts are used as biological dressings. Recently, stem cell therapies for the treatment of RDEB have been developed. The advantages of using ADSCs include their abundance in donors and the ease with which they can be obtained using relatively noninvasive methods such as liposuction. Moreover, ADSCs show low immunogenicity and have powerful immunosuppressive potential [5, 7, 8]. For these reasons, stem cell therapies using allogeneic ADSCs may be applicable for the treatment of RDEB, although anti-Col7 antibodies may be relatively common among RDEB patients [9].

Characterization of keratinocytes and estimations of differentiation efficiencies into keratinocytes are difficult. This study showed the presence of the epithelial basal cell markers K5/14 in undifferentiated ADSCs, and the presence of K10, which is an early marker of epidermal differentiation expressed in all suprabasal cell layers, including the stratum corneum, in differentiated ADSCs. In addition, we showed that ADSCs express higher levels of Col7 under specific conditions. These findings support the therapeutic potential of ADSCs, not only for wound healing, but also for the correction of Col7

deficiencies. However, the method for ADSC administration is still unresolved. The immunogenicity of differentiated ADSCs should be also investigated in future studies.

Acknowledgements

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Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

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Ethical approval

The study protocol was approved by the Ethics Committee of the Juntendo University Graduate School of Medicine. Data were analyzed in a blinded fashion, and procedures were carried out according to the principles of the Declaration of Helsinki.

Informed consent

Informed, written consent was obtained from all study participants.

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Figure legends

Fig. 1.

K14 detection in ADSCs. (A) Undifferentiated ADSCs were immunostained with anti-K14. NHEKs were also immunostained with anti-K14 as a positive control. (B) K14 expression in undifferentiated ADSCs was detected by flow cytometry. (C) K14 expression was measured by quantitative real-time PCR in undifferentiated ADSCs and cells that had differentiated into adipocytes. (D) K14 expression was measured by western blotting (relative to β-actin) in undifferentiated ADSCs and those that had differentiated into adipocytes.

Fig. 2.

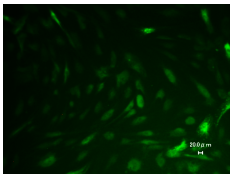
K10 detection in ADSCs. (A) Undifferentiated and differentiated ADCSs were immunostained with anti-K10. NHEKs were also immunostained with anti-human K10 as positive control. (B) K10 expression in differentiated ADSCs was detected by flow cytometry. (C) K10 expression was confirmed by real-time PCR.

Fig. 3.

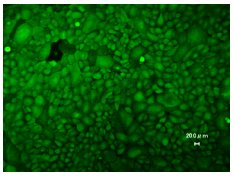
Col7 detection in ADSCs. (A) Col7 expression in undifferentiated and differentiated ADSCs was detected by immunofluorescence. NHEKs were also immunostained with anti-human Col7 as positive control. (B) Col7 expression in differentiated ADSCs was detected by flow cytometry. (C) Col7 expression was confirmed by real-time PCR. (D) Col7 expression was detected by western blotting.

K14

undifferentiated ADSCs

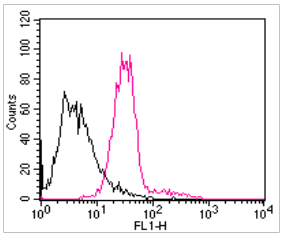


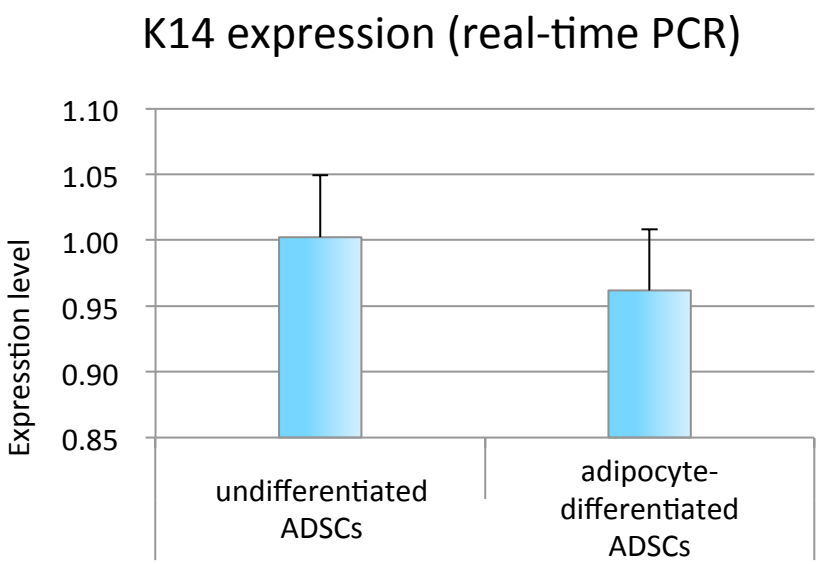
normal human epidermal keratinocytes (NHEKs)

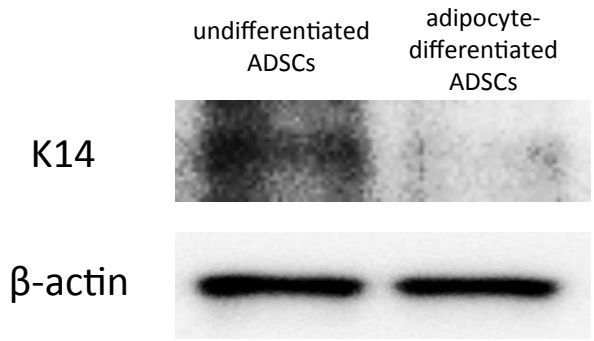


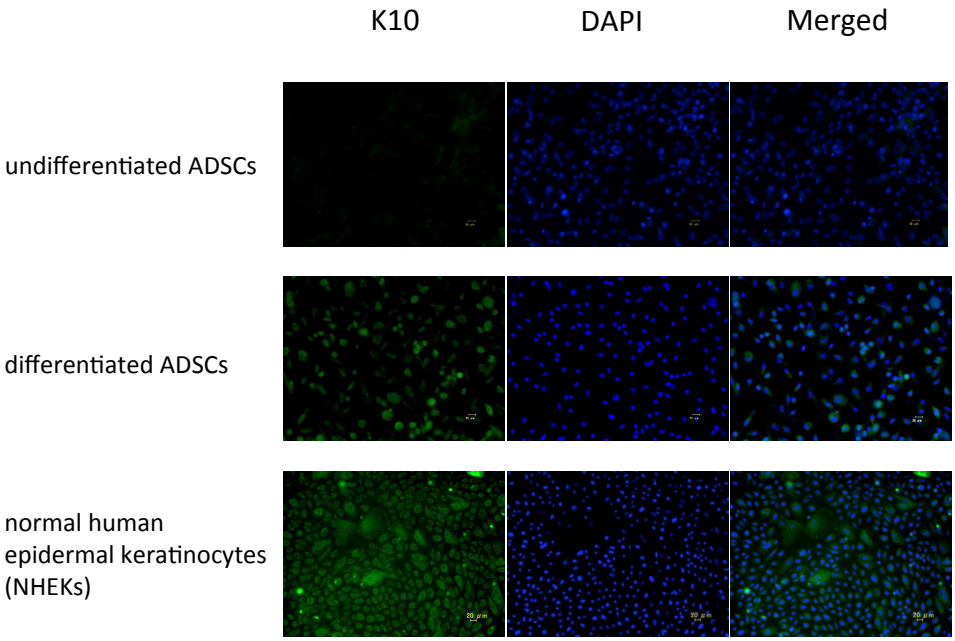
K14

undifferentiated ADSCs



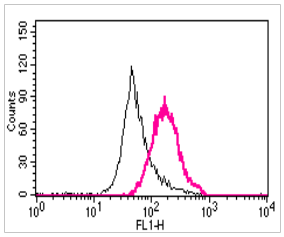

K14 expression (real-time PCR)
Expresstion level
1.10
1.05
1.00
0.95
0.90
0.85
undifferentiated ADSCs
adipocyte-differentiated ADSCs

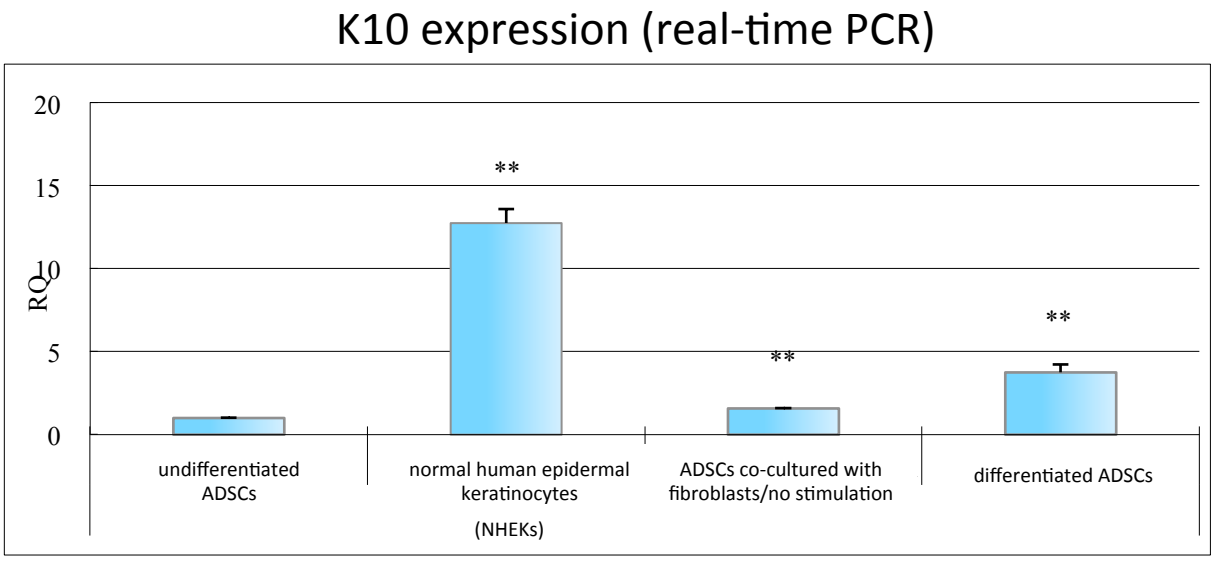

undifferentiated ADSCs
adipocyte-differentiated ADSCs
K14
β-actin


K10
DAPI
Merged
undifferentiated ADSCs
differentiated ADSCs
normal human epidermal keratinocytes (NHEKs)

K10

— undifferentiated ADSCs
— differentiated ADSCs




K10 expression (real-time PCR)
20
15
10
5
0
RQ
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**
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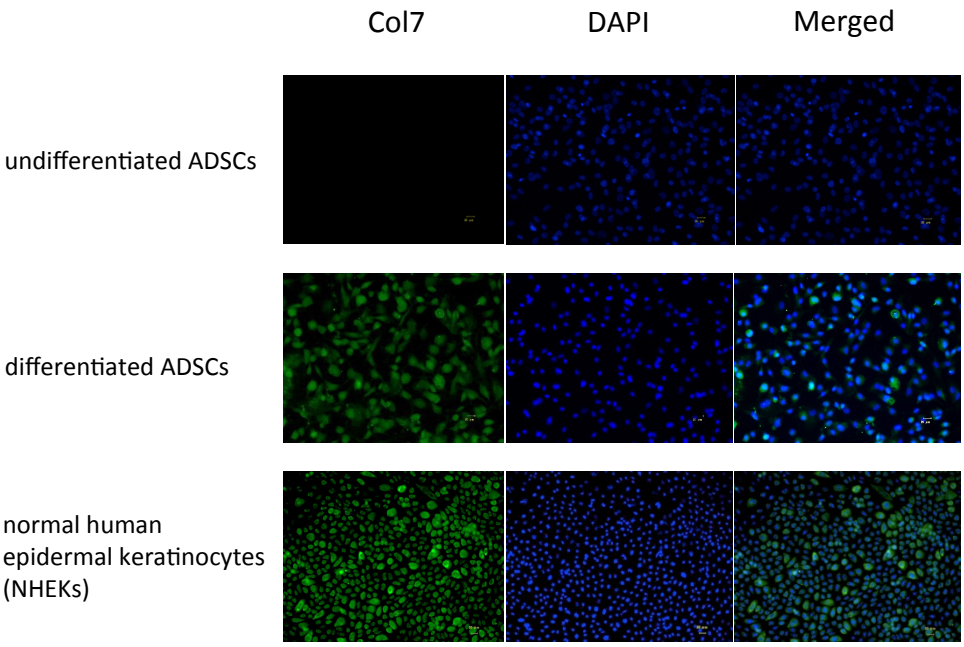
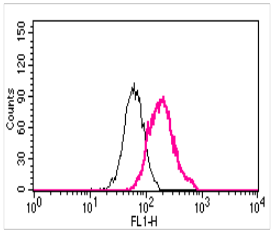
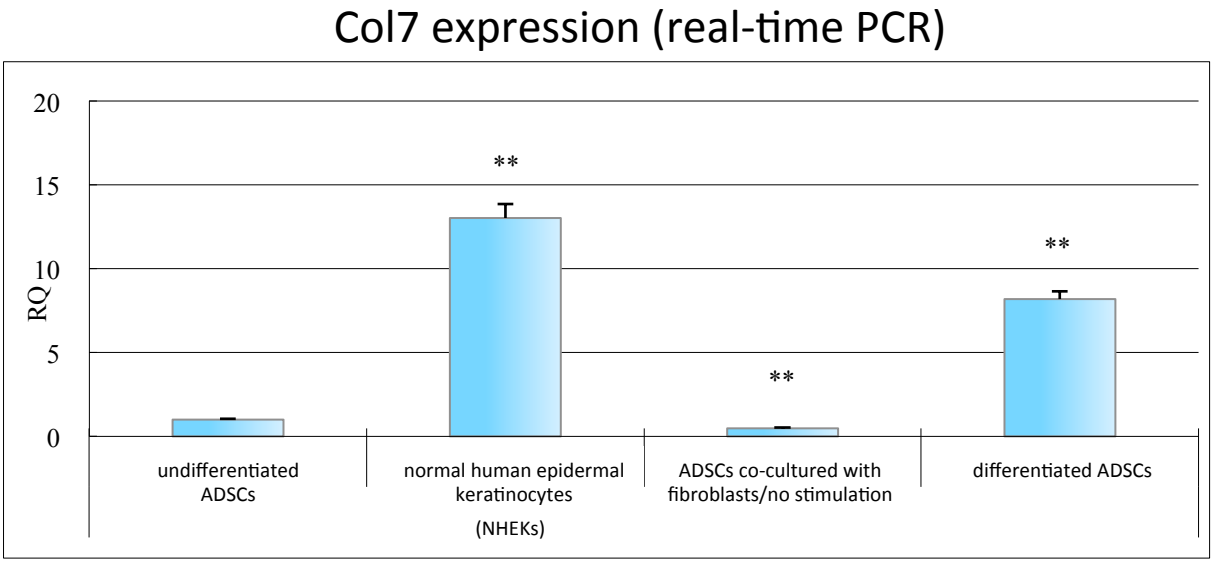
undifferentiated ADSCs
normal human epidermal keratinocytes (NHEKs)
ADSCs co-cultured with fibroblasts/no stimulation
differentiated ADSCs

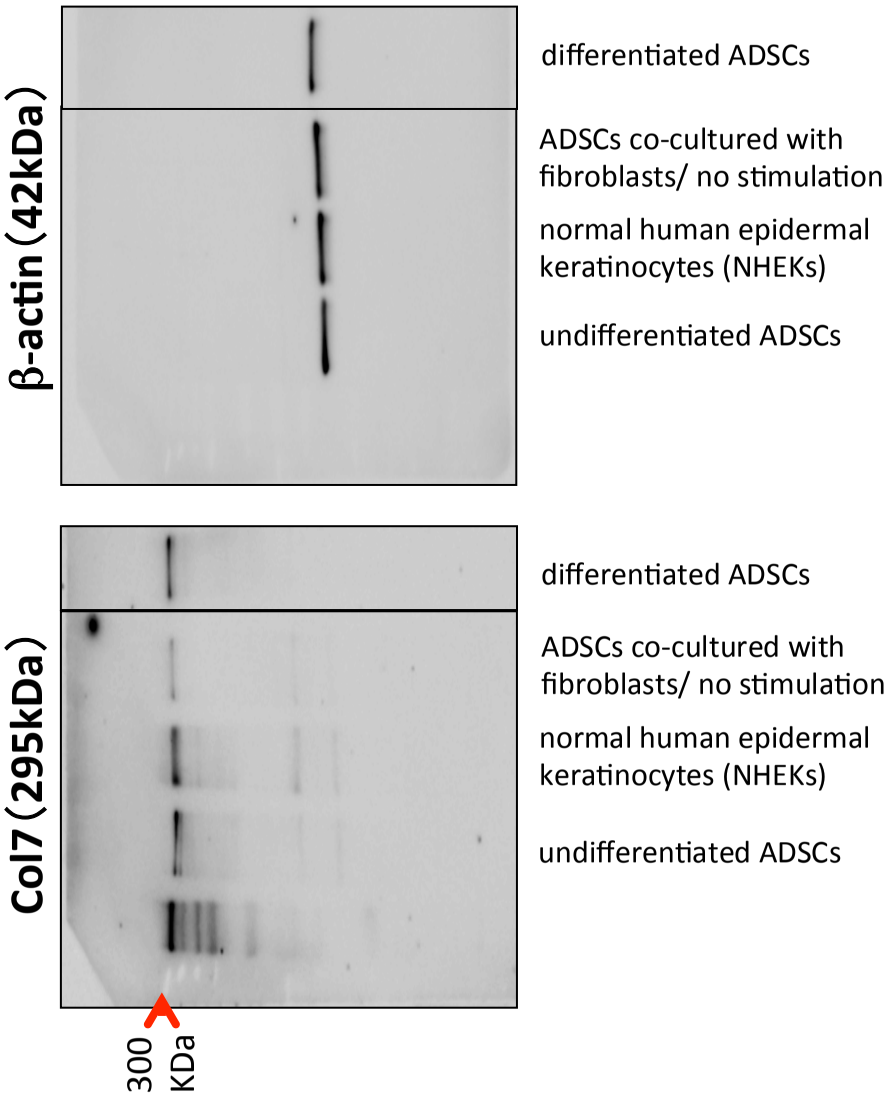

Col7
DAPI
Merged
undifferentiated ADSCs
differentiated ADSCs
normal human epidermal keratinocytes (NHEKs)

Col7

— undifferentiated ADSCs
— differentiated ADSCs




Col7 expression (real-time PCR)
20
15
10
5
0
RQ
**
**
**
undifferentiated ADSCs
normal human epidermal keratinocytes (NHEKs)
ADSCs co-cultured with fibroblasts/no stimulation
differentiated ADSCs


β-actin (42kDa)
differentiated ADSCs
ADSCs co-cultured with fibroblasts/ no stimulation
normal human epidermal keratinocytes (NHEKs)
undifferentiated ADSCs
Col7 (295kDa)
differentiated ADSCs
ADSCs co-cultured with fibroblasts/ no stimulation
normal human epidermal keratinocytes (NHEKs)
undifferentiated ADSCs
300 KDa