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In Vitro Expression of Cytokeratin 19 in Adipose-Derived Stem Cells Is Induced by Epidermal Growth Factor

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Cytokeratin 19 (CK19) is a typical epithelial marker. In this study, we determined whether epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) could enhance CK19 expression in adipose-derived stem cells (ADSCs), thereby inducing the differentiation of ADSCs into epithelial-like cells.

Material/Methods: ADSCs were isolated from perinephric fat, and the expression of CD29, CD90, and CD105 was confirmed. Following isolation, ADSCs were cultured in static medium or medium containing EGF or bFGF.

Results: Flow cytometry revealed that EGF and bFGF could alter mesenchymal stem cell markers as well as the cell cycle of ADSCs. Western blotting and immunofluorescence revealed that after 14 days, EGF treatment enhanced the expression of CK19 in ADSCs.

Conclusions: Our findings offer important insight for the clinical use of ADSCs in the generation of epithelial-like cells in the future.

MeSH Keywords: Adult Stem Cells • Cell Differentiation • Mesenchymal Stem Cell Transplantation

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Background

Skin substitutes are considered effective for the treatment of deep dermal burns and chronic ulcers. Advances in the fabrication of skin substitutes and tissue engineering technology are paving the way for new regenerative medicine [1]. At present, a skin substitute with properties that match the normal skin is lacking. Advances in stem cell biology have contributed to the production of engineered tissue constructs. Among the potential sources of stem cells, adipose-derived stem cells (ADSCs) are a preferred choice for the generation of skin substitutes [2].

ADSCs have many advantages as they can be easily harvested with minimal donor site morbidity and have a differentiation potential similar to other mesenchymal stem cells (MSCs) [3]. Compared to bone marrow stromal cells, ADSCs have higher yields and greater proliferative rates in culture [4,5]. The discovery that ADSCs are not only precursors to adipocytes but are also multipotent progenitors of a variety of cells [6], including chondrocytes, myocytes, osteoblasts, epithelial cells, and neuronal cells [7], creates the potential to treat a variety of tissue defects from a single, easily accessible, autologous cell source.

Studies have shown that epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) can promote the expansion of ADSCs [8–11]. EGF or bFGF may promote the differentiation of ADSCs into adipocytes, osteoblasts, chondrocytes, and endotheliocytes. However, little is known about the effects of EGF and bFGF on the epithelial differentiation of ADSCs. Because cytokeratin 19 (CK19) is expressed in epithelial cells, its positive expression could be indicative of epithelial differentiation [12,13]. In this study, we hypothesized that treatment with EGF or bFGF might induce the differentiation of ADSCs into cells expressing CK19, a phenotype that could be further used for the generation of skin substitutes. Our results showed that EGF treatment enhanced CK19 expression in ADSCs, which may be a feasible approach for epidermal differentiation.

Material and Methods

ADSCs

Perinephric fat samples were obtained from 6 rats (200±20 g) undergoing dissection. Following extensive washing with phosphate-buffered saline (PBS), the adipose tissue was digested at 37°C for 40 min with 0.02% collagenase (Biosharp, Anhui, China). After digestion, centrifugation, and filtration, the supernatant was discarded and the cell fractions were cultured in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C. After 24 h, the non-adherent fractions were removed and the remaining cells were washed with PBS. Cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) with antibodies against CD29, CD31, CD34, CD45, CD90, and CD105 (all from BD Biosciences). Animal care and procedures were approved by the Animal Care and Use Committee of Southern Medical University, and all of the experiments were conducted in accordance with institutional guidelines.

Cell cycle distribution analysis

Cell cycle analyses were performed using propidium iodide (PI) staining and flow cytometry. The cells were detached, centrifuged, and fixed in 70% cold ethanol for 30 min, after which 1 mL 50 μg/mL PI and 20 μg/mL RNase A (Biosharp) were added. The cells were incubated for 30 min in the dark and filtered through a 100-μm nylon mesh to remove clumps. All of the experiments were performed in triplicate.

Induction of CK19-expressing ADSCs

Cells were seeded into 6-well cell culture clusters (Corning, Corning, NY, USA) and cultured for 24 h under static conditions. Following this, cells were cultured in medium with or without EGF or bFGF (both 10 ng/mL; Peprotech, Rocky Hill, NJ, USA). Medium was changed every 72 h and differentiation was assessed during the course of 14 days.

Gene expression analysis

Real-time quantitative fluorescence PCR (qRT-PCR) was performed using standard protocols. Specific primers were designed for each gene based on the GeneBank sequences. RT-PCR was carried out at 95°C for 1 min, 95°C for 15 s, 57/60°C for 40 s, and 72°C for 40 s (28–37 cycles). qRT-PCR was performed on the Applied Biosystems 7500 Sequence Detection System (BD, Michigan, USA). Briefly, 1 μL cDNA was added to 10 μL of 2×SYBR Green PCR master mix (TaKaRa, Dalian, China) and 200 nM of each primer in a total volume of 20 μL. The reactions were amplified over 40 cycles of 95°C for 15 s and 60°C for 1 min. Afterward, a thermal denaturation protocol was performed to determine the number of products present in each reaction. The reactions were typically run in triplicate. The cycle number at which the reaction crossed an arbitrarily placed threshold (Ct) was determined for each gene. The following primers were used: CK19 sense, 5'-TCTTCCTATGGGGGCATGG-3', antisense, 5'-AGGCGATCGTTCAGGTTCTG-3'; GAPDH sense, 5'-TGTGAACGGATTTGGCCGTA-3', antisense, 5'-GATGGTGATGGGTTTCCCGT-3'. CK19 gene expression was normalized to the expression of GAPDH in each sample. Data were analyzed using the 2-ΔΔCt method [14].

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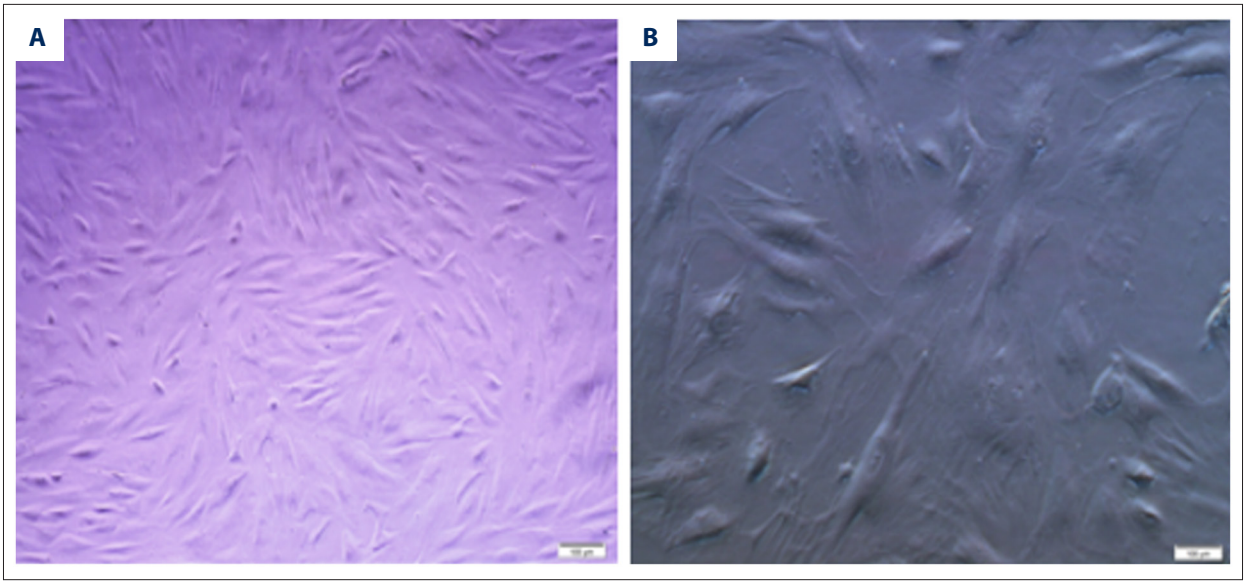

Figure 1. Isolation, culture, and morphological identification of adipose-derived stem cells (ADSCs). Cells were extended and showed long-spindle or polygonal shapes after 48 h of culture. (**A**) Scale bar=100 μM. (**B**) Scale bar=50 μM.

Immunoblotting

Protein samples were denatured at 95°C, electrophoresed, transferred to a polyvinylidene fluoride membrane (Millipore, Stafford, VA, USA), and blocked in 5% bovine serum albumin (BSA; w/v). Then, the membrane was incubated with antibodies against CK19 (1: 1000; Abcam, Cambridge, UK) or glyceraldehyde 3-phosphate dehydrogenase (1: 10 000; Bioworld Technology, Inc., St. Louis Park, MN, USA) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The membranes were washed again and protein bands were detected with the enhanced chemiluminescence system (Pierce, Waltham, MA, USA) and exposed to Kodak XPS film. Bands of interest were quantified by densitometry using Gel-Pro analysis software.

Immunofluorescence

After 14 days of conditioning, the 6-well cell culture cluster was excised and fixed in paraformaldehyde for 30 min. Cells were permeabilized with 0.2% Triton X-100 for 10 min, blocked in 1% BSA for 30 min, and incubated with anti-CK19 antibody (Abcam). After 1-h incubation, cells were washed with PBS, and goat anti-rabbit secondary antibody diluted in 1% BSA (1: 1000) was applied (Fude, Wuhan, China) for 2 h at room temperature. Afterwards, cells were washed twice with Hoechst (1: 20 000) in PBS for 5 min. Samples were examined under a fluorescence microscope (FV1000; Olympus, Tokyo, Japan) and the mean fluorescence intensity of cells was analyzed with Image-Pro Plus software version 6.0.

Statistical analysis

All of the analyses were performed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation and were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test. P values <0.05 were considered statistically significant. Results are representative of an average of at least 3 experiments.

Results

Acquisition and identification of rat ADSCs

After 48 h, the majority of isolated primary ADSCs had already attached and exhibited long-spindle or polygonal shapes. After the first passage, the cell growth rate started to increase, and cells reached 80% confluence after 3–4 days. ADSCs at the third passage exhibited an obvious long-spindle shape and radial growth (Figure 1). ADSC experiments were performed on cells from passages 3–6. Flow cytometry was performed to ascertain the purity of ADSCs. As shown in Figure 2, rat ADSCs were positive for MSC surface markers CD29 (95.1%), CD90 (92.6%), and CD105 (93.2%), but were negative for CD31 (4.18%), CD34 (1.66%), and CD45 (2.09%).

EGF and bFGF altered the MSC markers of ADSCs

To investigate the effects of EGF and bFGF on the stemness of ADSCs, 3 MSC markers (CD29, CD90, and CD105) were monitored by flow cytometry following treatment. As shown in

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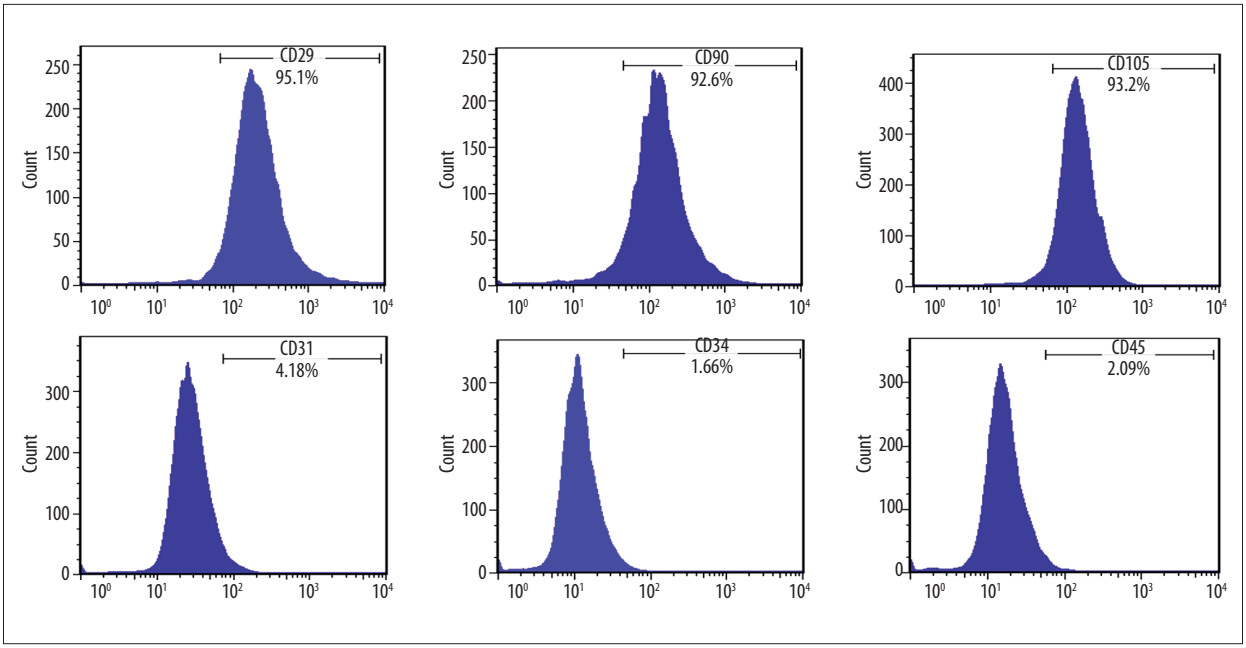

Figure 2. Characterization of ADSCs by flow cytometry. ADSCs expressed the mesenchymal stem cell markers CD29, CD90, and CD105, but were primarily negative for the endothelial marker CD31, and the hematopoietic markers CD34 and CD45.

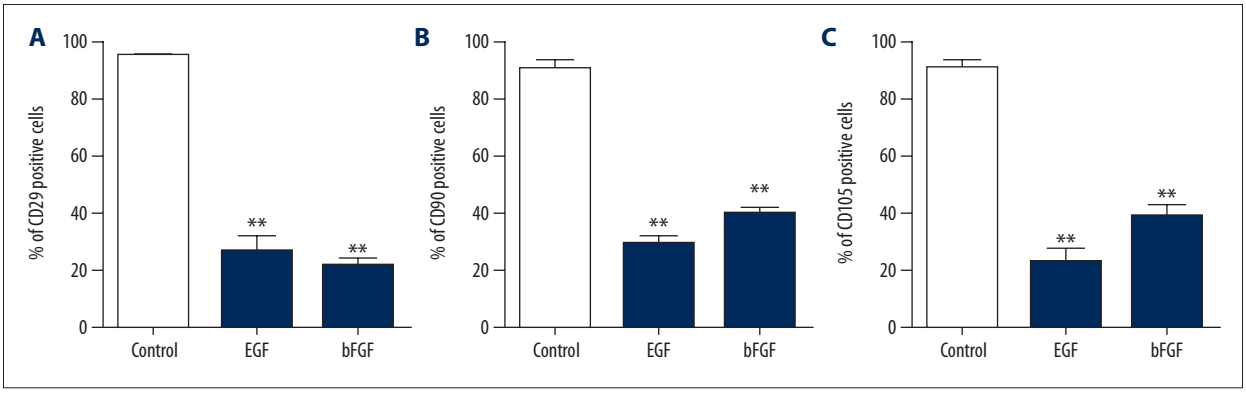

Figure 3. Flow cytometry revealed that epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) treatment altered ADSC markers. Expression of CD29 (**A**), CD90 (**B**), and CD105 (**C**) in ADSCs was analyzed by flow cytometry after 7 days. Data are expressed as the mean ± standard deviation (SD). ** p<0.01 compared to the control.

Figure 3A, after 7 days of culture, the ratio of ADSCs expressing CD29 decreased from 95.1±1% to 28.5±2% when treated with EGF (p<0.01) compared to static medium. Treatment with bFGF produced similar results, as CD29 expression decreased to 21.6±3% (p<0.01). However, as shown in Figure 3B, 92.6±3% of untreated cells expressed CD90, whereas CD90 expression decreased to 29.9±2% following 7 days of EGF treatment (p < 0.01), and decreased to 40.0±2% following treatment with bFGF (p<0.01). On the other hand, as shown in Figure 3B, 93.2±2% of untreated cells expressed CD105, whereas the expression of CD105 decreased to 23.2±5% following EGF treatment (p<0.01), and decreased to 39.4±4% with bFGF treatment (p<0.01). Thus, treating ADSCs with EGF or bFGF resulted in changes in the expression of MSC markers. Although not direct proof of differentiation into the epithelial lineage, these changes indicate the loss of ADSC stemness. These results also suggest that some degree of lineage commitment occurred in cells cultured in medium containing EGF or bFGF.

EGF and bFGF affected the cell cycle of ADSCs

Analysis of the distribution of cell cycle stages in ADSCs (Figure 4) indicated that, compared to static medium, the ratio of (G2+S) phase ADSCs treated with EGF increased from 36.07% to 41.93% (p<0.01), whereas the ratio of (G2+S) phase ADSCs treated with bFGF increased from 36.07% to 44.09% (p<0.01). These data demonstrate that EGF and bFGF stimulated the growth of ADSCs and enhanced their proliferative capacity.

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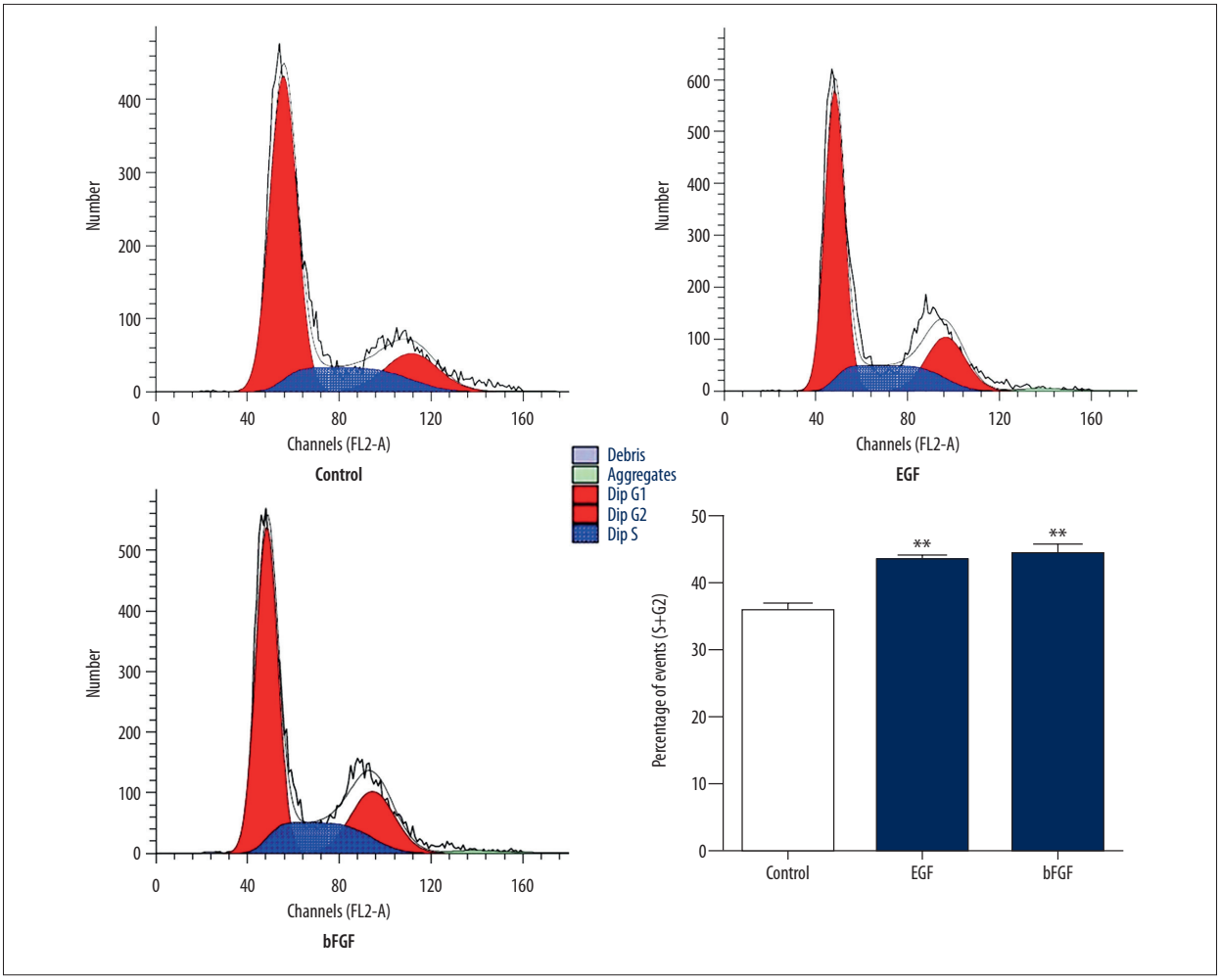

Figure 4. EGF and bFGF treatment altered the cell cycle of ADSCs. The percentage of cell cycle events (S+G2) was analyzed by flow cytometry. Data are expressed as the mean ±SD. ** p<0.01 compared to the control.

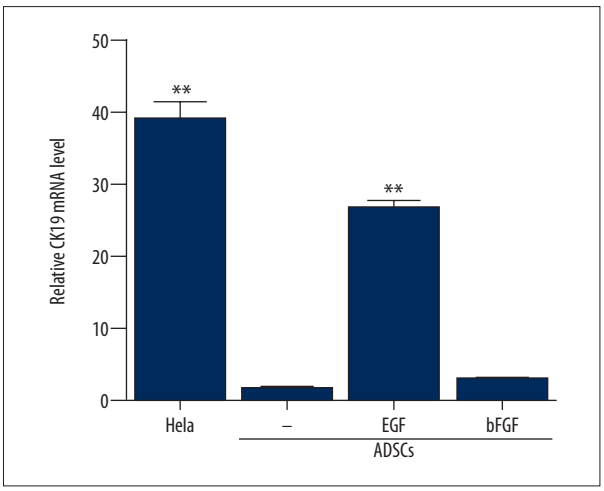

Figure 5. Real-time PCR analysis of the CK19 mRNA level in ADSCs untreated or after 2 weeks of treatment with EGF or bFGF. Data are expressed as the mean ±SD. ** p<0.01 compared to the control.

EGF treatment enhanced the expression of CK19 in ADSCs

Next, the ability of ADSCs cultured in medium with EGF or bFGF to differentiate into epithelial cells was investigated.

The mRNA expression profile of CK19 was studied using qRT-PCR. As shown in Figure 5, the CK19 gene of ADSCs cultured in the presence of EGF was higher than in static medium($p<0.01$), similar to HeLa cells, which was used as a positive control of epithelial cells. There was no obvious difference in CK19 expression between ADSCs treated with bFGF and the control group.

CK19 protein expression in ADSCs was examined by Western blotting. As shown in Figure 6, the expression of CK19 in ADSCs cultured in the presence of EGF for 14 days was higher than that in static medium ($p<0.01$), similar to HeLa cells. There was no obvious difference in CK19 expression between ADSCs treated with bFGF and the control group. Immunofluorescence staining revealed that the expression of CK19 in ADSCs cultured

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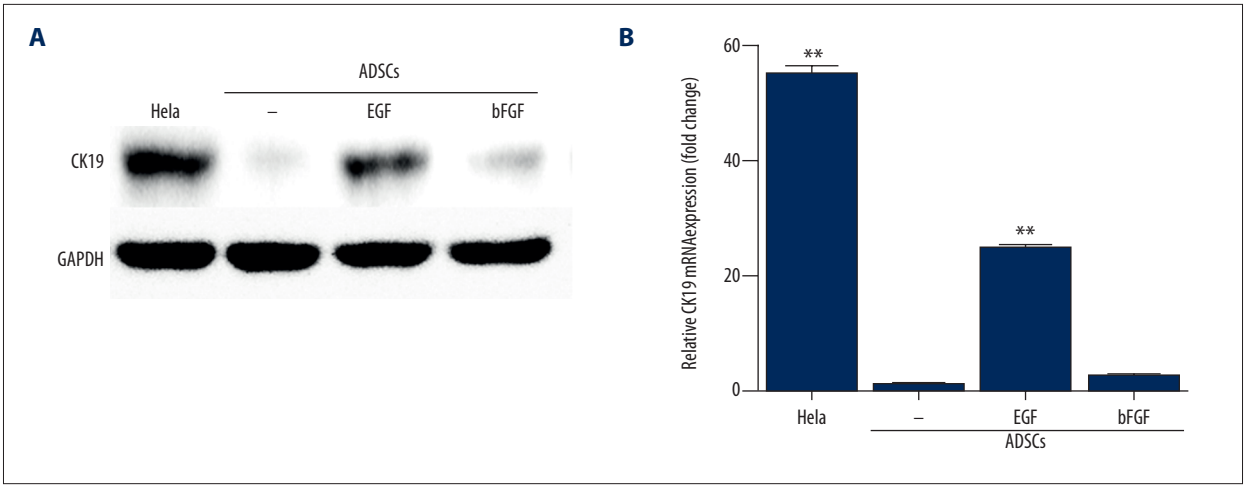

Figure 6. Western blot analysis revealed that EGF treatment increased the expression of CK19 in ADSCs after 2 weeks of culture. HeLa cells were used as a positive control. Data are expressed as the mean ±SD. ** p<0.01 compared to the control.

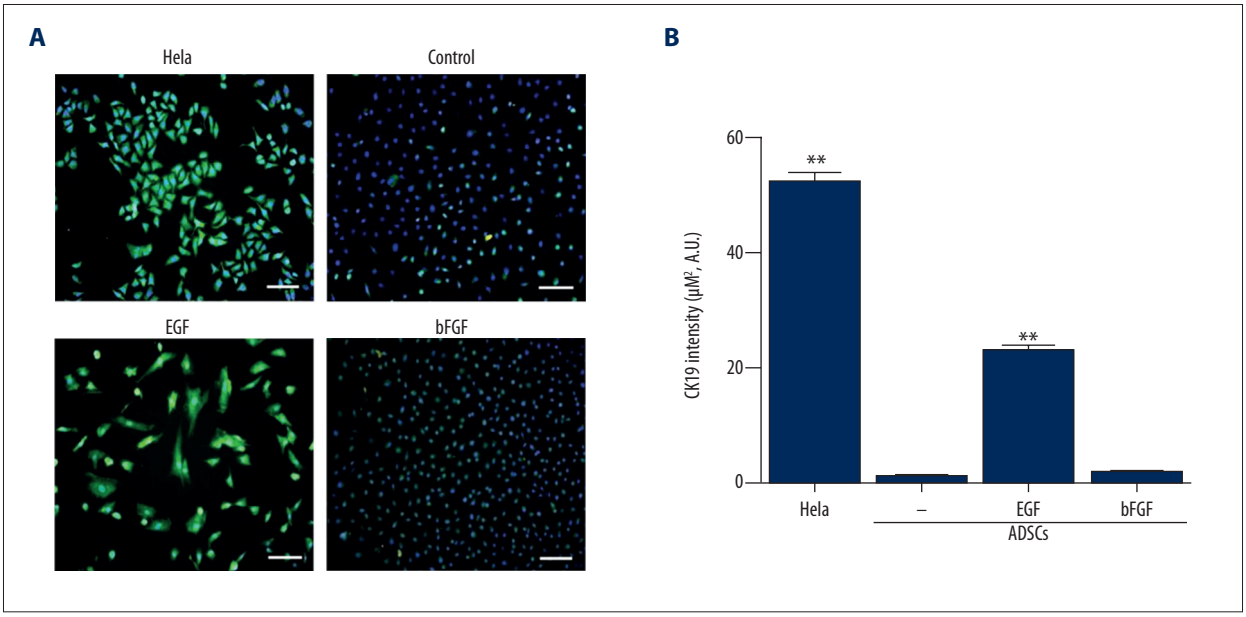

Figure 7. Immunofluorescence staining revealed that EGF treatment increased CK19 expression in ADSCs. Data are expressed as the mean ±SD. ** p<0.01 compared to the control. Scale bar=100 μM.

in medium containing EGF was significantly higher than that in ADSCs cultured in static medium (p<0.01). The expression of CK19 was similar to that in HeLa cells, which are epithelial cells that exhibit high expression levels of CK19. By contrast, CK19 was barely detectable in cells cultured in static medium and medium containing bFGF (Figure 7). These data suggest that ADSCs cultured in medium containing EGF successfully differentiated into CK19-expressing cells.

Discussion

Skin substitutes play key roles in the management of various wound etiologies, which aim to augment wound healing and closure, either temporarily or permanently, depending on the composition of each substitute [15]. It is essential that the transplant graft is able to resist infection, lack antigenicity, and be widely available. In addition, it should be able to provide coverage for the unique characteristics of each wound, including location, depth, underlying etiology, and susceptibility to infection [16].

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Adipose tissue is an abundant, accessible source of adipose-derived MSCs [17]. Many groups have investigated strategies for the application of ADSCs in wound healing with respect to skin tissue regeneration. However, the transplantation of ADSCs is difficult due to their multipotent nature, which is associated with differentiation into a variety of lineages, including adipocytes, chondrocytes, and osteocytes [18]. Transplantation of pre-differentiated cells may be beneficial because they cannot produce undesirable cells.

Following the isolation and culture of ADSCs for 3 passages, flow cytometry was performed to characterize ADSCs by expression of CD29, CD90, and CD105, which are MSC markers expressed by ADSCs. Isolated cells were negative for the endothelial marker CD31. In addition, these cells were negative for the hematopoietic markers CD34 and CD45. Our results are consistent with previous reports [19–22].

Previous studies have demonstrated that EGF and bFGF modulate the differentiation potential of human ADSCs [23,24]. EGF is noted for differentiating effects in many mammalian tissues, and plays a key role in the differentiation of skin appendages and tissue repair [25]. Likewise, bFGF acts as a chemoattractant that enhances migration, angiogenesis, and differentiation of both adipose-derived stem cells and bone marrow stromal cells [26–28]. bFGF is necessary for stem cell proliferation and the maintenance of human ADSC pluripotency [29].

Our results showed that treatment with EGF or bFGF altered the expression of CD29, CD90, and CD105. The ratio of CD29-, CD90-, and CD105-positive cells was significantly decreased in the ADSC population following treatment with EGF or bFGF, which indicates that the ADSCs had differentiated into other lineages. In addition, treatment with EGF and bFGF altered the cell cycle of ADSCs. Cell cycle analysis revealed that EGF and bFGF treatment stimulated the proliferation of differentiating ADSCs. The effects of EGF and bFGF treatment on proliferation were more significant in the early stages of differentiation.

To date, there is no definitive epidermal cell marker. CK19 is considered an acceptable epithelial cell marker, although various different epidermal cell markers have been used in previous studies [30,31]. Thus, CK19 was used as the epithelial cell marker in this study, consistent with previous studies [32,33]. Here, we studied the potential impact of EGF or bFGF on the expression of CK19 in ADSCs. Through qPCR, Western blot analysis, and immunofluorescence staining, the expression of CK19 in ADSCs cultured in medium containing EGF was increased compared to static medium ($p<0.01$), whereas there were no changes in static medium or medium containing bFGF. Our results indicated that EGF induced the expression of CK19 in ADSCs *in vitro*. This result is consistent with the view that EGF can promote the differentiation of ADSCs into an epithelial lineage, while bFGF is necessary for stem cell proliferation and the maintenance of ADSC pluripotency.

Conclusions

Our results showed that EGF treatment enhances CK19 expression in ADSCs, which is considered an indicator of epithelial-like cells. Treating ADSCs with EGF alone may be a feasible approach for epidermal differentiation, and may be useful for the generation of tissue-engineered transplants. Finally, these results may be important for further study of the clinical applications of skin substitutes derived from ADSCs.

Conflicts of interest

None.

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