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Using paracrine effects of Ad-MSCs on keratinocyte cultivation and fabrication of epidermal sheets for improving clinical applications

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Abstract Recent advances in wound healing have made cell therapy a potential approach for the treatment of various types of skin defects such as trauma, burns, scars and diabetic leg ulcers. Cultured keratinocytes have been applied to burn patients since 1981. Patients with acute and chronic wounds can be treated with autologous/allograft cultured keratinocytes. There are various methods for cultivation of epidermal keratinocytes used in cell therapy. One of the important properties of an efficient cell therapy is the preservation of epidermal stem cells. Mesenchymal Stem Cells (MSCs) are major regulatory cells involved in the acceleration of wound healing via induction of cell proliferation, angiogenesis and stimulating the release of paracrine signaling molecules. Considering the beneficial effects of MSCs on wound healing, the main aim of the present study is investigating paracrine effects of Adipose-derived Mesenchymal Stem Cell (Ad-MSCs) on cultivation of keratinocytes with focusing on preservation of stem cells and their differentiation process. We further introduced a new approach for culturing isolated keratinocytes in vitro in order to generate epidermal keratinocyte sheets without using a feeder layer. To do so, Ad-MSC conditioned medium was applied as an alternative to commercial media for keratinocyte cultivation. In this study, the expression of several stem/progenitor cell (*P63*, *K19* and *K14*) and differentition (*K10*, *IVL* and *FLG*) markers was examined using real time PCR on days 7, 14 and 21 of culture in keratinocytes in Ad-MSC conditioned medium. P63 and α6 integrin expression was also evaluated via flow cytometry. The results were compared with control group including keratinocytes cultured in EpiLife medium and our data indicated that this Ad-MSC conditioned medium is a good alternative for keratinocyte cultivation and producing epidermal sheets for therapeutic and clinical purposes. The reasons are the expression of stem cell and differentiation markers and overcoming the requirement for feeder layer which leads to a xenograft-free transplantation.

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Besides, this approach has low cost and is easier to perform. However, more in vitro and in vivo experiments as well as safety evaluation required before clinical applications.

Keywords Keratinocyte culture · Epidermal sheet · MSCs · Paracrine effects · Wound healing

Introduction

The rise in the incidence of obesity and diabetes has increased the burden of treating chronic wounds and more than 1% of people are affected by pressure and diabetic foot ulcers during their lifetime. In addition, more than 11 million people worldwide are injured by thermal burns annually (Peck 2011; Sun et al. 2014). Decreased function of the injured cells in the wound region including keratinocytes and fibroblasts leads to delayed wound repair or non-healing chronic wounds (You and Han 2014). Thus, cell therapy can be a promising therapeutic strategy for treatment of both acute and chronic wounds and different kinds of skin defects (Tan et al. 2014; You and Han 2014). No surgical processes and donor-site morbidity and reduced scar size are some advantages of cell therapy (Atiyeh and Costagliola 2007; Liu et al. 2008; You and Han 2014). The cells currently applied in clinical therapy include keratinocytes, fibroblasts and mesenchymal stem cells (MSCs) in the various applications (Liu et al. 2008; Tan et al. 2014).

About 95% of cells found in the epidermis are keratinocytes. The epidermis is a major site of self-renewal in a basal to superficial direction resulted from combined activity of potent stem cells and transient amplifying cells (Atiyeh and Costagliola 2007; Esteban-Vives et al. 2015; Zuliani et al. 2013). Keratinocytes form basal, spinous, granular, and cornified layers of the epidermis that correspond to progressive stages of differentiation. Mitotically active keratinocytes including epidermal stem cells, transient amplifying cells and post-mitotic cells expressing differentiation markers are organized in the basal layer. The specialized cell–extracellular matrix junction of these cells is called hemi-desmosomes and they are associated with the basement membrane. Terminal differentiation occurs when the transient amplifying cells lose their ability to adhere to the basement membrane. In both keratinized and non-keratinized epithelial tissues cytokeratins, a diverse group of intermediate filament proteins, are expressed (Papini et al. 2003; Wikramanayake et al. 2014). Several different types of keratins are expressed in human skin with roles in differentiation and tissue specialization and maintaining the overall structure integrity of epithelial cells. Keratin 5 and keratin 14 (K5, K14) are expressed in basal cells while the spinous cells express keratin 1 and 10 (K1, K10). The presence of keratin filaments, profilaggrin and loricrin is a characteristic of cells in the granular layer. In addition to granular layer, involucrin is expressed in the upper layer of human skin (Ojeh et al. 2015; Wikramanayake et al. 2014).

Based on the proliferative potential of keratinocytes and according to morphological criteria, three types of colonies including holoclones, paraclones, and meroclones are generated from a single keratinocyte. Clonogenic keratinocytes including holoclones and meroclones initiate cultured keratinocyte growing colonies and paraclones, keratinocytes with restricted growth potential, initiate aborted colonies (Atiyeh and Costagliola 2007; Ojeh et al. 2015; Papini et al. 2003). The holoclones have an extensive self-renewal potential which is maintained during the lifetime and is able to generate a differentiated progeny. These colonies express high levels of αβ1 integrin, K14, K19 and P63 (Papini et al. 2003; Pellegrini et al. 1999, 2001; Senoo et al. 2007; Suzuki and Senoo 2012) and give rise to both meroclones and paraclones (Atiyeh and Costagliola 2007; Green 2008). The meroclones contain transient amplifying cells and have a high proliferative rate only for a limited period of time. Dramatically reduced expression of P63 was illustrated in meroclones as they leave the stem cell niche (Atiyeh and Costagliola 2007; Pellegrini et al. 1999). The paraclones, originating from transient amplifying cells have a very limited growth potential. Paraclones are believed to be post-mitotic committed cells and they have a short replicative life span. The high expression of the terminal differentiation marker, involucrin, was observed in paraclones (Barrandon and Green 1987; Mommers et al. 2000; Ojeh et al. 2015; Wikramanayake et al. 2014). The transition from holoclone to meroclone and then to paraclone is an irreversible unidirectional process that occurs slowly during aging (Atiyeh and Costagliola 2007; Ojeh et al. 2015; Papini et al. 2003).

In vitro keratinocyte culture has been used in many clinical applications such as treatment of ulcers, burn patients and wound healing via tissue engineered skin (Atiyeh and Costagliola 2007; Boyce 2001; Esteban-Vives et al. 2015; Gerlach et al. 2008; Jones et al. 2002; Kjartansson et al. 1991; Mcheik et al. 2014). Rheinwald and Green introduced the first successful and routine method for in vitro cultivation of keratinocytes in 1975 (Rheinwatd and Green 1975). In vitro expansion of epithelial keratinocytes, forming stratified and coherent layers is the most commonly used technology for producing a graftable epithelium (Atiyeh and Costagliola 2007; Boyce 2001; Esteban-Vives et al. 2015; Gerlach et al. 2008; Jones et al. 2002; Kjartansson et al. 1991; Mcheik et al. 2014). Since first clinical application of these epidermal sheets by O'Connor et al. (1981), cultured epithelial sheets which are enriched with proliferative keratinocytes have been successfully applied in autografts for skin replacement in full and deep partial-thickness burn injuries, chronic wounds and so on (Cirodde et al. 2011; De Corte et al. 2012; Dragúňová et al. 2012). Either cultured keratinocytes or keratinocytes suspension can be applied in tissue engineering in combination with other types of cells or scaffolds for improvement of wound healing (Bisson et al. 2013; Germain 2002; Hartmann et al. 2007; Supp and Boyce 2005). One limitation of epithelial culture is that inappropriate conditions of cultivation result in decreased potential of proliferation and early differentiation of these cells (Bisson et al. 2013; Esteban-Vives et al. 2015; Fusenig and Worst 1974). Considering the small size of the initial skin biopsy obtained from the patient, culture conditions maintaining the proliferative capabilities of the keratinocytes and allowing late terminal differentiation are essential (Bisson et al. 2013; Scuderi et al. 2009). Cultured epithelial autografts (CEA), used in clinical applications for treatment of wounds and burn injuries can be produced in 3 ways: Epidermal sheets produced using human feeder layer which have no risk of animal viruses or other components (Auxenfans et al. 2009; Bisson et al. 2013; Bullock et al. 2006; Jubin et al. 2011; Sun et al. 2004). In another approach, 3T3 cells are used as feeders to support keratinocytes expansion (Guerra et al. 2004; Pellegrini et al. 1999; Rheinwatd and Green 1975) which make the provided sheets xenograft (Esteban-Vives et al. 2015). Use of serum-free and xenograft-free media, containing growth factors and collagen/fibrin matrix has also been proposed as an alternative approach for keratinocyte culture (Esteban-Vives et al. 2015; Izumi et al. 2007; Kim et al. 2004; Larderet et al. 2006). MSCs are self-renewing and expandable stem cells which are able to differentiate into adipocytes, osteoblasts, and chondrocytes. Adult adipose tissue and bone marrow have been proven to be reliable abundant sources of MSCs. MSCs can control the reduction of inflammation, induction of cell proliferation and migration, development of angiogenesis and releasing paracrine signaling molecules which are involved in accelerating wound healing (Ojeh et al. 2015; Sun et al. 2014; Teng et al. 2014). In non-healing wounds, MSCs can reduce the excessive T cells and increase neutrophil infiltration (Falanga et al. 2007). MSCs paracrine signaling, especially including the growth factors such as PDGF (platelet derived growth factor), VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), keratinocyte growth factor (KGF), TGF-ß1 (transforming growth factor beta-1) and mitogens can control key regulatory cells involved in wound healing such as endothelial cells, fibroblasts, and keratinocytes and accelerate the wound repair (Kim et al. 2007; Lee et al. 2012; Rehman et al. 2004).

Considering these great beneficial paracrine effects of MSCs, in this study we aimed to verify the possibility of using MSC conditioned medium for keratinocytes culture and fabrication of an epidermal sheet suitable for grafting and therapeutic use. The results were compared with keratinocytes cultured in serum- and feeder-free conditions.

Materials and methods

MSC isolation from adipose tissue and culture

After obtaining informed consent, adipose tissues of healthy women undergoing elective liposuction were collected to isolate MSCs. Following washing these tissues three times with phosphate-buffered saline (PBS) containing penicillin–streptomycin (Biowest), they were incubated in 0.1% collagenase type I (Gibco) at 37 °C for 1 h. Then fetal bovine serum (FBS) was added to final concentration of 10% to inactivate the enzyme and centrifuged at 800 g for 10 min. The pellet was immediately resuspended in Dulbecco's Modified Eagle's medium (DMEM)

and transferred to flasks containing DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Naderi-Meshkin et al. 2016).

MSC characterization

In order to characterize the cultured Ad-MSCs, mouse anti-CD44 polyclonal antibody, rabbit anti-CD34 polyclonal antibody, mouse anti-CD90 monoclonal antibody, rabbit anti-CD11b polyclonal antibody, mouse anti-CD73 monoclonal antibody (all from Novus Biologicals, Littleton, Colorado, USA), rabbit anti-CD105 polyclonal antibody and rabbit anti-CD45 polyclonal antibody (all from Bioss Inc, Woburn, MA, USA) were applied. 1×10^6 cells at passage 4 were resuspended in 100 µl cold PBS, containing 5% FBS and after 1 h incubation with mentioned antibodies or isotype-matched control the events were analyzed by flow cytometry (BD Accuri, USA) (Naderi-Meshkin et al. 2016).

To investigate the differentiation potential of Ad-MSCs towards osteoblasts and adipocytes, they were induced in specific conditioned media for 3 weeks. Alizarin Red S and Oil Red O dyes were used to stain the differentiated cultures towards osteoblasts and adipocytes respectively. The Osteogenic medium includes DMEM-LG (low glucose) supplemented with 10% FBS, 1 µM dexamethasone, 10 mM β-glycerol phosphate and 20 µM ascorbic acid (Sigma) and the adipogenic medium was composed of DMEM-LG supplemented with 10% FBS, 1 µM dexamethasone, 10 mM β-glycerol phosphate and 100 µM indomethacin. Moreover, alkaline phosphatase activity assay kit (Sigma) was also applied to study the differentiation of the cells to osteoblasts (Naderi-Meshkin et al. 2016).

Keratinocyte culture medium preparation

Two different media, a control and a test medium, were provided for the cultivation of keratinocytes. For control groups, EpiLife medium supplemented with defined growth supplement S7 (Gibco) was used (De Corte et al. 2012).

The test medium was composed of Ad-MSC conditioned medium supplemented with specific factors required for keratinocyte culture. Conditioned medium was prepared by collecting the media from several Ad-MSC cultured flasks at passage numbers 2 and 3 and filtered before use.

The test medium was composed of 300 ml Ad-MSC conditioned medium containing 4.5 g/l glucose and 25 mM HEPES (4(2-hydroxyethyl)-1-piperazine ethane sulphonic acid) (Biowest), mixed with 100 ml Ham's F12 medium with L-glutamine and 25 mM HEPES, without $NaHCO_3$, FBS was added to a final concentration of 10%, and supplemented with 10 ng/ml EGF (epidermal growth factor) (Sigma), 0.1 nM choleratoxin (Sigma), 0.8 mg/ml hydrocortisone (Czech Pharma), 0.18 mM adenine (Sigma), 5 mg/ml transferrin (Sigma), 2 nM 3,3,5 Triiodo-L-Thyronine (Sigma) and 0.12 U/ml insulin (Sigma) (Dragúňová et al. 2012; Wong et al. 2013).

Keratinocyte isolation and culture

Tissue samples (3–4 cm^2) were obtained from the abdominal skin with the approval of the relevant ethical committee and after getting the patients' written consents.

For isolation of human epidermal keratinocytes, the skin was rinsed in PBS for several times and after removal of subcutaneous fat, the remaining tissue was cut into 1 cm × 0.5 cm pieces. After overnight incubation of the samples treated with 2.5 mg/ml dispase (Gibco) at 4 °C, the epidermis was detached from the dermis with forceps and transferred into 5 ml 0.05% trypsin (Gibco). The epidermis was incubated at 37 °C for 30 min and the cloudy solution was then pipetted up and down vigorously to further release the epidermal cells. To remove the remaining cell clusters and epidermis pieces, a 70 µm cell strainer (BD Biosciences) was positioned on top of a 50 ml conical tube through which the cell suspension was passed and then centrifuged for 10 min at 250 g. The supernatant was removed and living cells were resuspended in control and test culture media separately and homogenized by pipetting. Cells were then seeded at a density of 5×10^4 cells/cm^2 and after 48 h, the media were changed for the first time and then every other day (De Corte et al. 2012; Wong et al. 2013).

Preparation of keratinocyte sheets

Keratinocytes were allowed to reach confluency before harvesting. After formation of several layers (a multi-layer), the sheet was ready to harvest. Choleratoxin was omitted from the test medium 12 days after culture. At the point of harvesting on

day 21, the medium was removed and the sheet was rinsed three times in PBS. Dispase solution was added (10 ml per sheet) and incubation allowed to proceed at 37 °C. Sheets started to detach from the edges of the flasks in approximately 5–10 min (Dragúňová et al. 2012).

Flow cytometry

The evaluation of cell surface markers of cultured keratinocytes was performed by using several antibodies including P63-α (D2K8X) rabbit monoclonal antibody (CST, USA) and rat anti-human CD49f (α6 integrin chain) (1:100) antibodies (BD Biosciences, USA).

Real time PCR analyses

RT-PCRs were performed on 3 separate biological samples obtained from different people, with 3 replicates in each experiment, and data related to one sample are shown as the representative.

Total RNA isolation and reverse transcription

After extraction of total RNA from all groups of cells after 7, 14 and 21 days using TriPure (Roche Diagnostics, Germany), they were treated with DNase I enzyme (Thermo Fisher Scientific, USA), in order to remove any possible genomic DNA contamination and cDNAs were then synthesized following manufacture's instruction (TaKaRa, Japan).

Real time PCR analyses

Quantitative expression of the stem cell and differentition markers was performed using SYBR Green Master Mix (Pars Tous, Iran) on cultured keratinocytes in a thermal cycler (CFX-96, Biorad, USA) following the manufacturer's protocol. The sequences of applied primers are shown in Table 1. The efficiency of primers was assayed through generating a standard curve in 10 fold dilution of cDNA and all the efficiencies were in a range of 92–100%.

Histological evaluation of epidermal sheets

In order to perform hematoxylin and eosin staining (H&E staining), the detached sheets were placed in 4% formaldehyde solution to be fixed. Dehydration was performed by gradually increasing ethanol concentrations and then using xylene. Following embedding samples in paraffin they were sectioned with the thickness of 4 μm using a microtome (BioTek, USA). After floating in a water bath, the prepared slides were placed in slide racks for staining using hematoxylin

Table 1 Primer sequences used for quantitative real time PCR

mRNA targets	Oligonucleotides(5′ → 3′)	Product size (bps)	References
GAPDH	F: GGAAGGTGAAGGTCGGAGTC	101	(Gomez-Sanchez et al. 2013)
	R: GTCATTGATGGCAACAATATCCACT		
Keratin 10 (*K10*)	F: AGCATGGCAACTCACATCAG	126	(Umeki et al. 2014)
	R: TGTCGATCTGAAGCAGGATG		
Keratin 14 (*K14*)	F: TTCTGAACGAGATGCGTGAC	189	(Racila et al. 2011)
	R:GCAGCTCAATCTCCAGGTTC		
Keratin 19 (*K19*)	F: TGAGTGACATGCGAAGCCAATAT	103	(Gozgit et al. 2007)
	R: GCGACCTCCCGGTTCAAT		
Involucrin (*IVL*)	F: GGCCCTCAGATCGTCTCATA	131	(Kolev et al. 2008)
	R: CACCCTCACCCCATTAAAGA		
P63	F: CGCCGCAATAAGCAACAG	184	Designed
	R: GTAGCCTCTTACTTCTCCTTCC		
Filaggrin (*FLG*)	F: AGGAATACAGTCACGTGGCA	168	Designed
	R: CTTCCGTGCTGAGAGTGTCT		

(5 min) and then eosin solution (3 min) and after washing they were viewed under a light microscope (Olympus, Japan: IX70) and photogaphed.

Statistical analysis

Statistical analysis was performed using the SPSS 21.0 statistical package (SPSS, Chicago, IL) and GraphPad Prism software. All data were expressed as mean ± SEM and analyzed by *t* test.

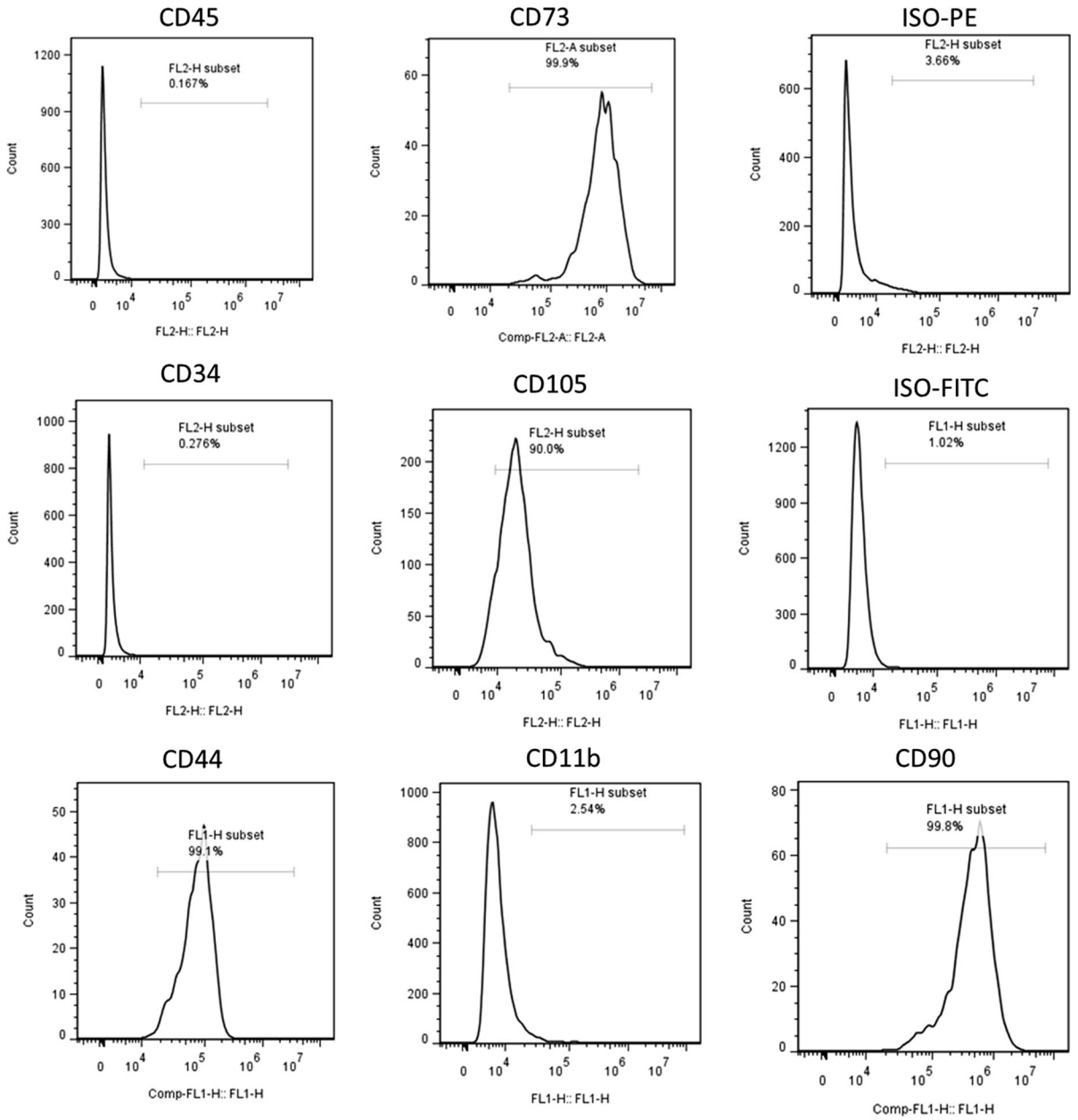

Fig. 1 Characterization of Ad-MSCs. Flowcytometry analysis indicated that more than 90% of cultured cells expressed CD44 (99.1%), CD73 (99.9%), CD90 (99.8%) and CD105 (90.0%), whereas a small portion of them expressed CD11b (2.54%), CD34 (0.27%) and CD45 (0.16%). ISO-FITC and ISO-PE were considered as controls. (FITC: fluorescein isothiocyanate; PE: phycoerythrin)

Results

Characterization of the isolated Ad-MSCs

Evaluating the expression of cell surface antigens for Ad-MSCs at passage 4, indicated that > 90% of cells were positive for CD44, CD73, CD90 and CD105 expression, while they were negative for CD11b, CD34 and CD45 markers (Fig. 1). Osteogenic and adipogenic differentiation of these cells were assessed using Alizarin Red S and Oli Red O staining (Fig. 2) and they authenticated calcium precipitation of osteocytes and accumulation of lipid vacuoles in differentiated adipocytes, respectively.

Morphological characterizations of cells cultured with Ad-MSC conditioned medium

Isolated human epidermal keratinocytes could be successfully expanded in Ad-MSC conditioned medium. Microscopic studies showed that these cells could reach 80% confluency after 5–7 days. Formation of epidermis was observed in cultured keratinocytes since day 7 and stratified epidermis was formed on day 21 (Fig. 3).

Evaluating the expression of stem/progenitor and epidermal differentiation markers in cultured keratinocytes

The expression of stem/progenitor and epidermal differentiation markers was investigated in keratinocytes cultured in test medium on days 7, 14 and in harvested sheets on day 21. In order to confirm the results, the expression of these markers were compared with the cells cultured in control EpiLife medium.

Expression of P63, K19 and K14 as epidermal stem/progenitor markers in cultured keratinocytes

The expression of basal layer markers including *P63*, *K19* and *K14* was analyzed by real time PCR. The results indicated that P63 was expressed in all time points, however, its expression was higher on day 21 compared to day 7 (Fig. 4A, a). In comparison to control, the expression of this marker has significantly increased on days 7 and 21 (Fig. 4B, d).

Hence, *K19* is a stem/progenitor cell marker (Pontiggia et al. 2009), a decrease in its expression on days 14 and 21 is expected and our results confirmed this behavior (Fig. 4A, b). Expression of *K19* on days 7 and 21 in cells cultured in test medium was significantly higher than the EpiLife controls (Fig. 4B, e).

Cultured keratinocytes grown in test medium expressed higher amounts of *K14* in initial days of culture compared to those on middle and final days of culture (Fig. 4A, c). Higher level of expression of this marker was observed compared to EpiLife-control during three different days of cell harvesting but the difference was significant only on day 21 (Fig. 4B, f).

Cultured keratinocytes express K10, IVL and FLG as epidermal differentiation markers

K10 is an early marker of differentiation expressed in all suprabasal layers of the epidermis (Wikramanayake et al. 2014). The results showed higher expression of *K10* on days 14 and 21 comparing to day 7 in cultured keratinocytes in test medium (Fig. 5A, a). It also indicated lower expression of *K10* in keratinocytes cultured in test medium on day 7 and a significant higher expression on day 14 compared to its EpiLife-control (Fig. 5B, d).

Involucrin which is expressed in the cornified layer of stratified squamous epithelium and granular layers of human skin is the terminal marker of epidermal differentiation (Wikramanayake et al. 2014). *IVL* expression was decreased after 14 and 21 days as compared to day 7 (Fig. 5A, b). In comparison to EpiLife-control, *IVL* expression in cells cultured in test medium increased on day 7, while there were no significant differences on days 14 and 21 in comparison to their EpiLife-control (Fig. 5B, e).

Filaggrin is expressed only in well-differentiated keratinized epithelial cells, an intermediate filament associated protein. It is thought that filaggrin aggregates keratins during the terminal differentiation of epidermal keratinocytes (Wikramanayake et al. 2014). There was no significant difference between expressions of this marker on different days of cell harvesting (Fig. 5A, c). Reduced expression of *FLG* in test group relative to controls was observed on days 7, 14 and 21 relative to their EpiLife-controls (Fig. 5B, f).

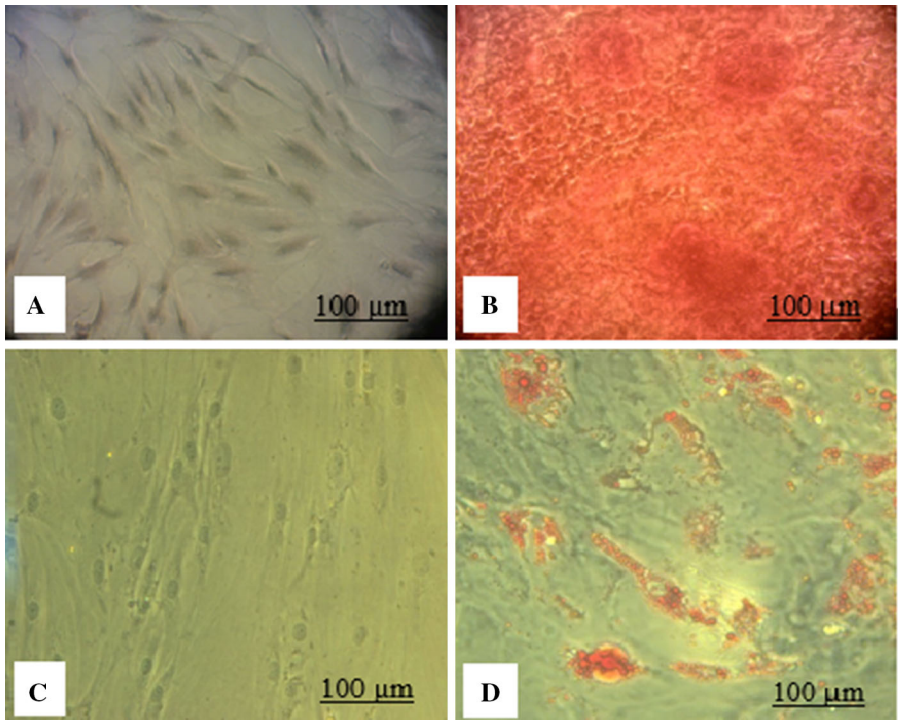

Fig. 2 Osteogenic and adipogenic differentiation of Ad-MSCs. Ad-MSCs were cultured in osteogenic and adipogenic media. **A**, **B** Alizarin red S staining of Ad-MSCs as controls and cells cultured in osteogenic media. **C**, **D** Oil red O staining of Ad-MSCs as controls and cells cultured in adipogenic media. Scale bar = 100 µm. (Color figure online)

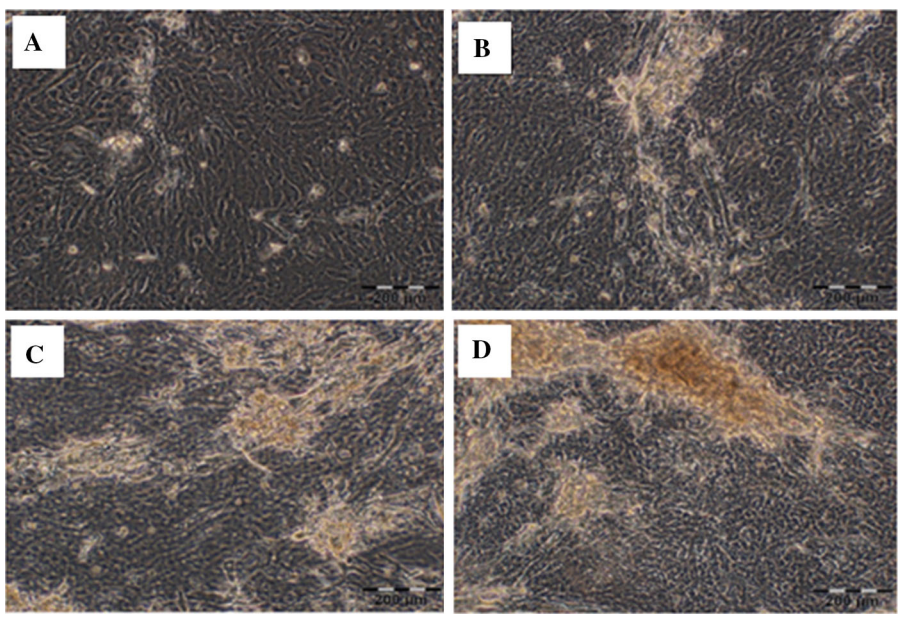

Fig. 3 Human epidermal keratinocytes cultured in Ad-MSC conditioned medium. Cultured cells were observed under an inverted microscope on days 5 (**A**), 7 (**B**), 14 (**C**) and 21 (**D**) after seeding. Scale bar = 200 µm

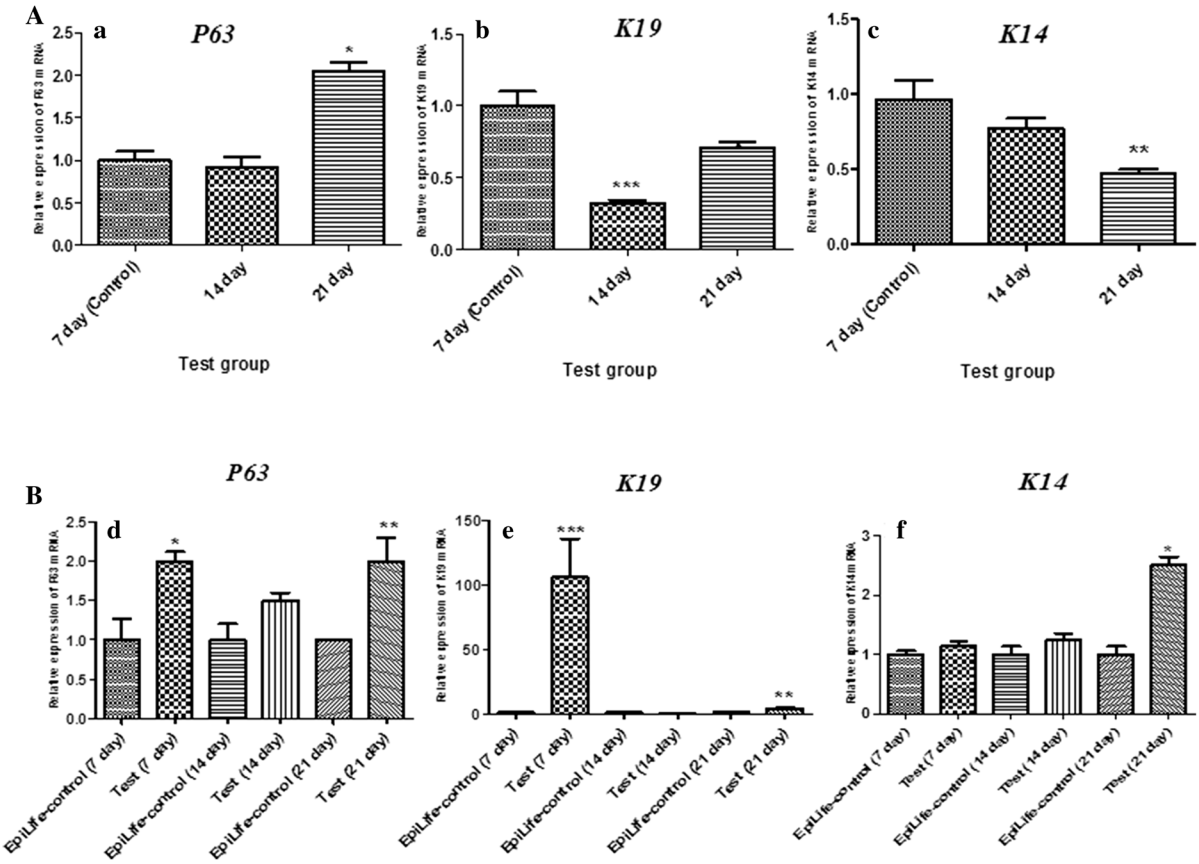

Fig. 4 **A** mRNA expression level of *P63* (**a**), *K19* (**b**) and *K14* (**c**) in test groups on days 7, 14 and 21 using real time PCR. **B** Comparing expression patterns of *P63* (**d**), *K19* (**e**) and *K14* (**f**) in test and EpiLife-control groups on days 7, 14 and 21 using real time PCR. The values are expressed as means ± SEM (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Flow cytometry analysis of stem/progenitor markers

Cells cultured in test medium were immunostained for stem/progenitor markers including P63 and α6 integrin chain on days14 and 21 and then analyzed by flow cytometry. Stem/progenitor cells in epidermis express integrins including α6 integrin which is the transmembrane core component of focal adhesion. These adhesions have roles in membrane assembly, cell substratum adhesion and cell proliferation (Fuchs 2008). Flow cytometry analysis for P63 represented that the expression of this marker has increased during days 14 (7%) to 21 (15%) in cells treated with Ad-MSC conditioned medium while the increase of this marker in EpiLife-control group was from 0.4 to 5% on similar days (Fig. 6).

Furthermore, the results indicated that the expression of α6 integrin on days 14 and 21 of culturing in test medium were 83 and 28%, respectively, while the expression of this marker in EpiLife-control group decreased from 81 to 2% during these days (Fig. 7). This observation confirmed that the expression of α6β1 integrin is more stable in test groups compared to the controls.

Development of transplantable epidermal keratinocyte sheets using Ad-MSC conditioned medium

Human epidermal keratinocytes are able to proliferate eventually forming a stratified epidermal cell layer (Rheinwatd and Green 1975). The ability of epidermal keratinocytes to proliferate in Ad-MSCs conditioned

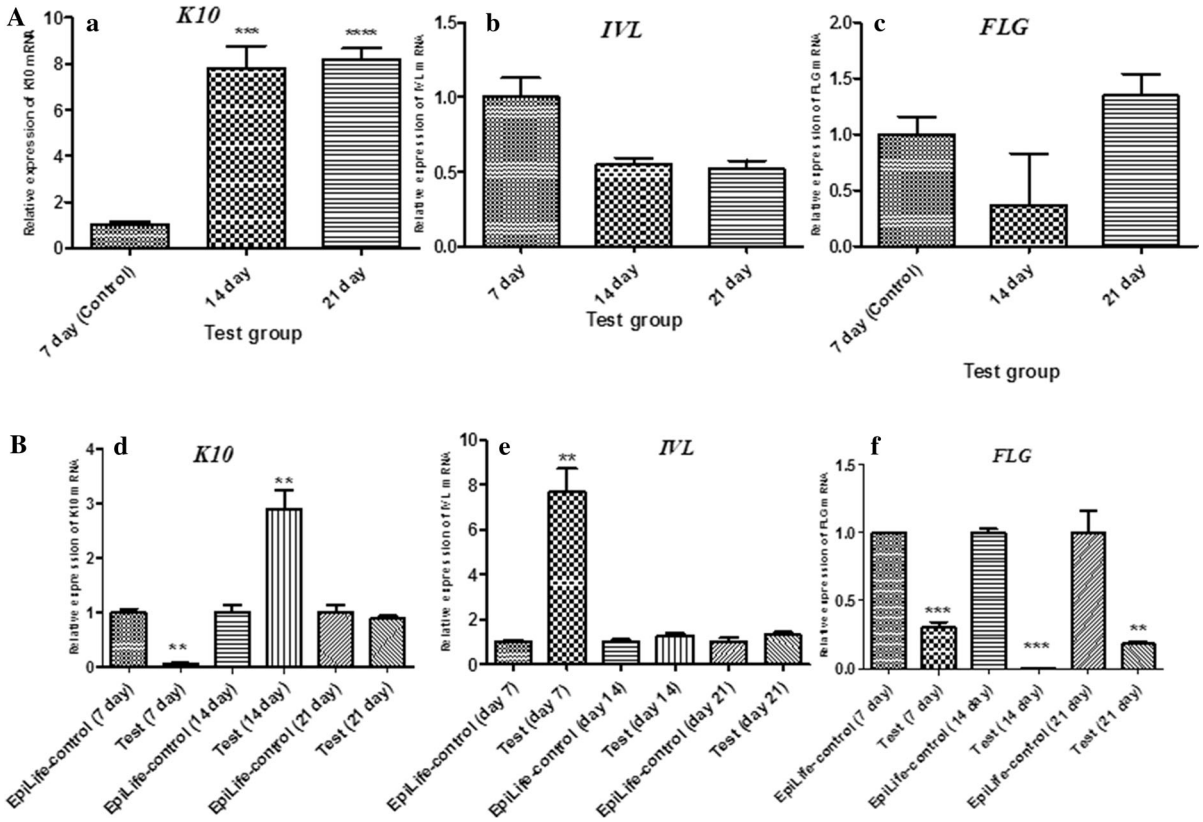

Fig. 5 **A** Expression pattern of *K10* (**a**), *IVL* (**b**) and *FLG* (**c**) in test groups on days 7, 14 and 21 using real time PCR. **B** Comparing expression patterns of *K10* (**d**), *IVL* (**e**) and *FLG* (**f**) in test groups on days 7, 14 and 21. The values are expressed as means ± SEM (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$

medium to develop a transplantable sheet was confirmed through culturing the cells for 21 days.

The epidermal keratinocyte sheet successfully formed as stratified squamous epithelium was harvested after 3–4 weeks of culture. H & E staining indicated a multi-layered epidermis. The high number of cells in basal layer shows that the fabricated sheets have the ability to proliferate and self-renew. Epidermal keratinocytes cultured in our conditioned medium could fabricate a stratified epithelial cell sheet after 17–21 days (Fig. 8).

Discussion

Wound healing is a process which consists of four sequential phases occurring in the presence of various cell types including keratinocytes, fibroblasts, melanocytes and endothelial cells (Ojeh et al. 2015; You and Han 2014). Impaired healings of acute wounds and delayed chronic wounds indicate the necessity for the development of alternative therapeutic approaches (Sun et al. 2014; Zuliani et al. 2013). Cell-based therapy is a promising approach addressing the problems of skin autografts such as limited healthy donor sites in extensive burns and donor-site morbidity (Lootens et al. 2013; Sun et al. 2014; You and Han 2014; Zuliani et al. 2013). Currently cultured autologous/allogenic epidermal cell-based therapy is used to increase wound healing rate and achieve maximum wound repair. The effective application of allogenic and autologous keratinocytes in acute and chronic wounds such as large burns and diabetic foot ulcers has also been investigated by many studies (Lootens et al. 2013; Moustafa et al. 2004; Sun et al. 2014; You and Han 2014).

In clinical applications, the main aim of cell isolation from donor tissue biopsy is the preservation of valuable stem/progenitor cells, which addresses the need for a viable source of cells that can accelerate re-

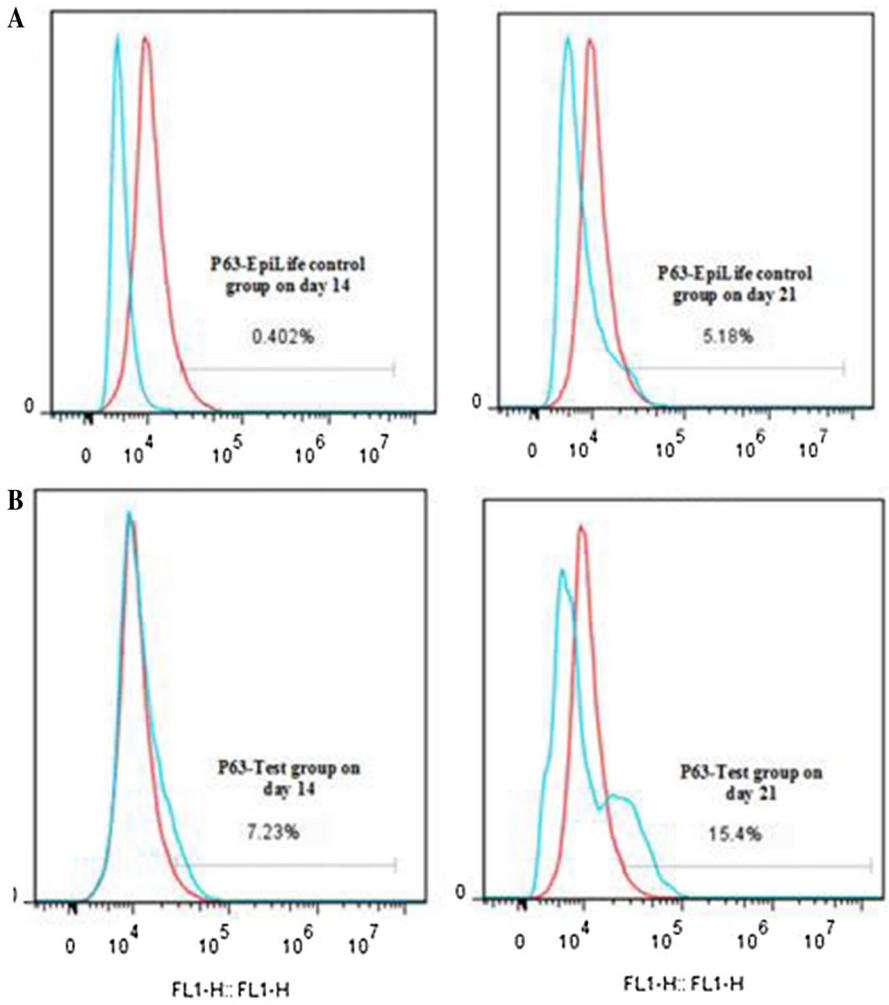

Fig. 6 Flow cytometry analysis of cultured keratinocytes for P63 on days 14 and 21, **a** EpiLife-control group. **b** Test group. (Color figure online)

epithelialization and improve wound repair maximally (Bisson et al. 2013; Ojeh et al. 2015). The paracrine effects of MSCs in wound healing have been studied by several groups both in vitro and in vivo and it has been shown that MSC conditioned medium enhances epithelialization and accelerates wound repair. Both bone marrow (Chen et al. 2008; Gurtner et al. 2008; Hocking and Gibran 2010; Maxson et al. 2012; Smith et al. 2010) and adipose-derived MSC conditioned media were reported to be effective after injection in wound region (Hocking and Gibran 2010; Kim et al. 2007; Lee et al. 2009).

In the present study, we successfully developed a method for cultivation of keratinocytes and fabrication of an epidermal sheet using the paracrine effects of Ad-MSCs which resulted in better maintenance of stem/progenitor cells and delayed terminal differentiation.

Holoclone is one of the most important colonies in basal layer expressing high levels of P63, K19, K14 and $\alpha 6\beta$ integrin. P63, a homologue of P53, is a transcription factor regulating the self-renewal capacity of stem cells with essential roles in epithelial proliferation and development. In addition to epidermal lineage commitment, it is also required for epidermal differentiation and basal membrane formation (Gazel et al. 2003; Koster 2010; Laurikkala et al. 2006; Senoo et al. 2007; Truong and Khavari 2007). Downregulation of P63, while the basal stem cells reach the suprabasal region, leads to proliferation arrest and makes it the best candidate for suppression of proliferation in epithelial stem cells (Fuchs 2008; Koster 2010).

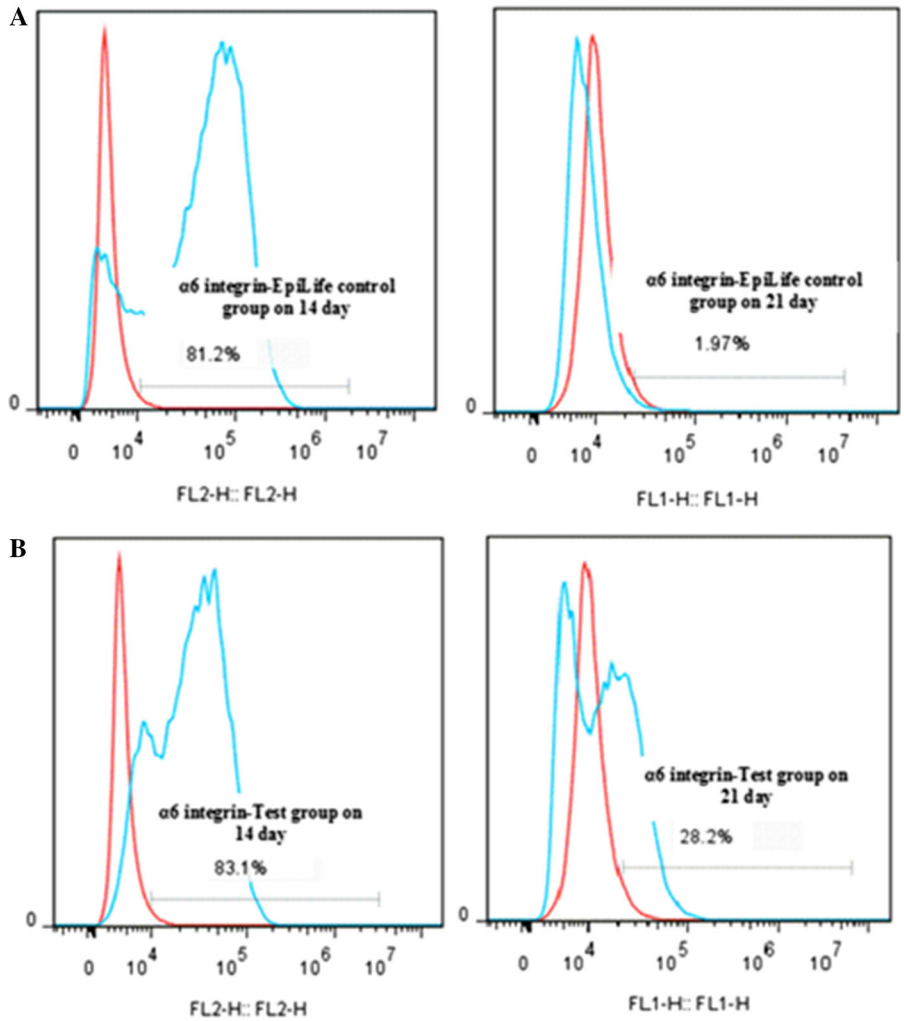

Fig. 7 Flow cytometry analysis of cultured keratinocytes for α6 integrin on days 14 and 21. **a** EpiLife-control group. **b** Test group. (Color figure online)

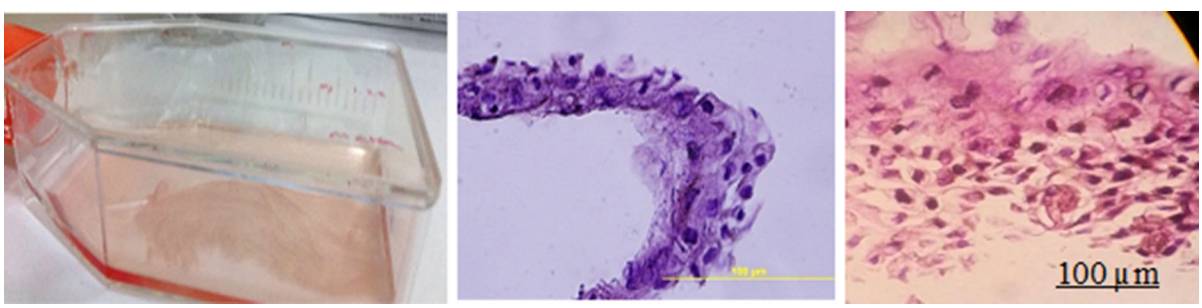

Fig. 8 Fabrication of human epidermal keratinocyte sheets. Harvested human epidermal keratinocyte sheets were cultured in test medium. Macroscopic view (left) and H&E-stained sections (right) of harvested cell sheets are shown. (Color figure online)

Keratin bundles including cytokeratin 19 (K19) and cytokeratin 14 (K14) are expressed in basal keratinocytes as well as P63. K19 is a marker of epidermal homeostasis whose expression indicates a functional epidermis resulting in a successful transplantation (Damanhuri et al. 2011; Michel et al. 1996; Pontiggia et al. 2009).

Epidermal keratinocytes produce large amounts of K14 which is the main structural protein in mammalian cells. It has been demonstrated that K14 makes a scaffold for correct assembly of K1 and K10 (Byrne et al. 1994; Franke et al. 1982).

In a study by Papini et al. (2003) an approach for culturing normal human epidermal keratinocyte stem cells was developed. Immunocytochemistry evaluation of K19 and P63 markers indicated the stemness of keratinocytes has been preserved for a long time (Papini et al. 2003). Moreover, Zuliani et al. (2013) showed that the expression of K14 and integrins in fetal keratinocytes makes them a potential source of cells for transplantation (Zuliani et al. 2013). In another study on the importance of the isolation and identification of keratinocyte progenitor cells (KPCs), expression of K14 and P63 was used for identification of KPCs (Nair and Krishnan 2013). Further, the expression of K14 and K19 as a stem cell niche indicator was confirmed in the basal epidermal layer and epidermis of 22-week fetal skin (Tan et al. 2014).

In the present study, expression of these epidermal stem/progenitor cell markers was studied. The results of real time PCR analyses on days 7, 14 and 21 indicated that keratinocytes cultured in Ad-MSC conditioned medium could express stem/progenitor markers including *P63*, *K19* and *K14*. The presence of these markers from the first to the final days of culture, with the higher expression on day 7 for *K19* and *K14* and also day 21 for *P63*, confirmed their high capability in proliferation, self-renewal and maintenance of stemness properties during 3 weeks of culture.

Significant higher expression of these markers on day 21 compared to EpiLife-control leads to better maintenance of stemness status in keratinocytes treated with Ad-MSC conditioned medium. Since K14 is a prerequisite for correct assembly of K1 and K10, its expression helps in the formation of the epidermis.

Analyzing flow cytometry results revealed that there was a similar increased pattern of P63 expression in both test and EpiLife-control groups. It was increased from 7% on day 14 to 15% on day 21 in the test group, while the level of this marker reached to 5% on day 21 in EpiLife-control group. These results confirm the presence of keratinocyte stem/progenitor cell and basal proliferative layer in our introduced approach.

Integrin α6 is another keratinocyte stem cell marker highly expressed in basal layer progenitor cells. αβ1 integrins compose the focal adhesions required for assembly of the basement membrane, cell proliferation and adhesion (Fuchs 2008). Regarding the expressions of α6 integrin on days 14 and 21 in cells cultured in test medium and their comparison with EpiLife-controls, it was concluded that use of Ad-MSC conditioned medium helps the maintenance of higher numbers of keratinocyte stem cells until the last day of culture. The high level of integrin is also associated with greater proliferation potential.

As epidermal keratinocytes start to differentiate toward the surface, leaving the basement membrane is accompanied with the synthesis of secondary keratin K10 in these cells. IVL and FLG, are also expressed in these progressive terminal differentiating cells. (Wikramanayake et al. 2014).

In a study conducted by Nair and Krishnan the expression of *K10*, *IVL* and *FLG* was investigated to confirm the full differentiation of keratinocytes via flow cytometry and real time PCR (Nair and Krishnan 2013). Tan et al., indicated that fetal keratinocytes could generate a multilayered epithelium, this was achieved by evaluating the expression of K10 and IVL as differentiation markers (Tan et al. 2014).

Similarly, *K10*, *IVL* and *FLG* were examined in our experiments as differentiation markers. Gradual increase of K10 during culture of cells with conditioned medium indicates the process of epidermis formation and initiation of suprabasal layers establishment. The lower expression of this marker on day 7 in test group compared with EpiLife-control, showed that differentiation in these cells initiated at the proper time.

Involucrin as a marker for epidermal terminal differentiation, is mostly expressed in the upper spinous and granular layers of human skin (Kadoya et al. 2014; Wikramanayake et al. 2014).

Expression of *IVL* in different days of the study showed that terminal differentiated keratinocytes

existed in our cells treated with test medium and a well-stratum epidermis was formed.

A marker of epidermal differentiation is an intermediate filament associated protein, called filaggrin expressed only in well-differentiated keratinocytes (Kadoya et al. 2014; Wikramanayake et al. 2014). Reduced keratinocyte proliferation associated with filaggrin expression leads to a post-G1-phase arrest (Presland et al. 2001). The advantage of our approach may be the significant lower expression of *FLG* in comparison with EpiLife-control.

Similar results were obtained by a study, indicating that the expression of FLG was decreased in keratinocytes on day 15 due to cell cycle arrest (Zuliani et al. 2013).

Cultured epithelial sheets enriched with proliferative keratinocytes have been successfully applied in auto/allografts for skin replacement in acute and chronic wound healing.

In 2015, another study on a novel feeder-free method for fabrication of epidermal sheets, a stratified epidermal cell sheet was produced. The efficiency of this method was compared with feeder layer-based method through evaluating gene expression of stem/progenitor markers such as *P63* and epidermal differentiation markers including *K10*, *IVL* and *FLG* and the results revealed that well-differentiated sheets were fabricated by their novel culture method (Nakajima and Takeda 2014).

The cultured keratinocytes with Ad-MSC conditioned medium could form a keratinocyte sheet after 21 days. Keratinocyte sheets fabricated through our feeder-free method showed expression of epithelial stem/progenitor cell markers (*P63, K19* and *K14*) and epidermal differentiation markers (*K10*, *IVL* and *FLG*). Expression of epithelial stem/progenitor cell markers indicates high proliferation and self-renewal potential in these epidermal sheets. Investigations on *K19* expression in keratinocytes involved in skin substitutes in vitro and in vivo have indicated a functional epidermis with high expression of K19 leads to a successful transplantation (Michel et al. 1996; Pontiggia et al. 2009; Sun et al. 2014). In another study, it was shown that formation of a stratified epidermis resembling a normal epidermis, 12 weeks after transplantation, was associated with expression of some keratinocyte stem cell markers including K19 and integrin α6 chain (Webb et al. 2004).

Therefore, the presence of these markers suggests that our medium can maintain epithelial stem and progenitor cells during culture and in fabricated sheets. Moreover, expression of epidermal differentiation markers revealed that epithelial stem/progenitor cells differentiated so well and formed epidermal layers.

In vitro and in vivo studies have demonstrated that the paracrine effects and mitogenic factors secreted from MSCs are involved in the attraction of macrophages and endothelial cells as well as stimulating the proliferation of keratinocytes, dermal fibroblasts and endothelial cells. MSC conditioned medium is composed of various growth factors, cytokines and chemokines such as VEGF, PDGF, bFGF, EGF, KGF and TGF-β. Among these, EGF is involved in the proliferation of keratinocytes, while TGF-β is an important inducer in the migration of these cells (Chen et al. 2008; Hocking and Gibran 2010; Ishimoto et al. 2002; Maxson et al. 2012).

In conclusion, our study provides evidence that Ad-MSC conditioned medium leads to high expression of stem/progenitor markers and similar expression of differentiation markers in cultured keratinocytes as compared to EpiLife-control, which restores the proliferation and migration of these cells. The fabricated epidermal sheets from these keratinocytes are promising candidates for pre-clinical applications. Other benefits of this approach are overcoming the requirement for feeder cells which makes it xeno-free as well as reducing the costs of cell sheets cultivation. Regarding the Ad-MSC immunomodulatory properties, the keratinocytes cultured in this medium may be safe enough to transplant.

Further in vivo and in vitro studies to fully confirm the effects of MSC conditioned medium on wound repair and validate the clinical safety of the fabricated cell sheets by evaluating tumorigenicity and chromosomal abnormality are required befor clinical applications.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no financial or personal conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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