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ORIGINAL PAPER

Bilayer Amniotic Membrane/Nano-fibrous Fibroin Scaffold Promotes Differentiation Capability of Menstrual Blood Stem Cells into Keratinocyte-Like Cells

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Abstract

The skin provides a dynamic barrier separating and protecting human body from the exterior world, and then immediate repair and rebuilding of the epidermal barrier is crucial after wound and injury. Wound healing without scars and complete regeneration of skin tissue still remain as a clinical challenge. The demand to engineer scaffolds that actively promote regeneration of damaged areas of the skin has been increased. In this study, menstrual blood-derived stem cells (MenSCs) have been induced to differentiate into keratinocytes-like cells in the presence of human foreskin-derived keratinocytes on a bilayer scaffold based on amniotic membrane and silk fibroin. Based on the findings, newly differentiated keratinocytes from MenSCs successfully expressed the keratinocytes specific markers at both mRNA and protein levels judged by real-time PCR and immunostaining techniques, respectively. We could show that the differentiated cells over bilayer composite scaffolds express the keratinocytes specific markers at higher levels when compared with those cultured in conventional 2D culture system. Based on these findings, bilayer amniotic membrane/nano-fibrous fibroin scaffold represents an efficient natural construct with broad applicability to generate keratinocytes from MenSCs for stem cell-based skin wounds healing and regeneration.

Keywords Skin wound · Scaffold · Stem cells · Amniotic membrane · Fibroin · Menstrual blood · Keratinocytes

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Abbreviations

BM	Bone marrow
ESCs	Embryonic stem cells
K14	Keratin 14
Ks	Keratins
MB	Menstrual blood
MenSCs	Menstrual blood-derived stem cells
MSCs	Mesenchymal stem cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
AM	Amniotic membrane
SEM	Scanning electron microscope

Introduction

The human skin is consisted of an outer layer, epidermis, and an inner layer, dermis, which derived from embryonic germinative layers ectoderm and mesoderm, respectively. The epidermis is an extreme dynamic tissue that contains mainly keratinocytes. This outer layer also contains a

population of unipotent stem cells which divide into transient amplifying cells that undergo few divisions and differentiate into keratinocytes [1]. The skin provides a dynamic barrier and protects the body from the exterior world. Therefore, immediate repair and rebuilding of the epidermal barrier is crucial after wound and injury [2]. The skin, the organ of body exposed to environment undergoes injuries and insults for a lifetime. Therefore, disruption of the integrity of skin, be related to disease or injury, may lead to serious conditions or even disability and death [3–5].

Wound healing is the restoration of anatomic and physiologic integrity of skin. Sometimes repair and regeneration process of skin fails to progress through a well-ordered and appropriate procedure to form anatomic and functional continuity. Current techniques and approaches including antibiotic treatment, pressure relief of decubitus areas, and surgical debridement are taken to manage the wounds but are suboptimal due to lacking in providing functional, structural, and cosmetic satisfaction [6, 7]. The introduction of bioengineered skin substitutes has developed the strategy for treating of large area injuries and difficult condition [8]. Despite worthy progresses in constructing of skin substitutes, there is uncertainty concerning their effectiveness and safety in comparison with other wound healing methods [9]. Therefore, current attempts focus on regeneration rather than replacement. The demand to engineer scaffolds that actively promote regeneration of damaged areas of the skin has been increased.

Over past years major effort has been done to produce scaffolds suitable for tissue engineering using biodegradable and biocompatible synthetic or natural polymers [10]. Scaffold with great advantages such as suitable cell attachment capacity and desired mechanical strength has a very critical role in tissue engineering. Developing scaffolds with three-dimensional organization which mimics the structure and function of ECM proteins and promotes cell adhesion, cell-biomaterial interactions, and cell proliferation and differentiation gives promise to re-establish the tissue integrity and provides mechanical support in skin regeneration [11–13]. Engineering and fabrication of appropriate dermal scaffolds by incorporating stem cells create a desired microstructure to overcome most of serious skin conditions.

Silk fibroin, a natural protein, has applied in biomedicine and regenerative studies [14]. Silk as a biomaterial product of silkworm or spider has a core of fibrous protein and surrounding proteins [15]. The impressive cytocompatibility and malleability of silk fibroin materials make silk an excellent scaffold that supports the attachment, proliferation and differentiation of cells. Because of these unique properties, silk fibroin has been proposed as a popular starting material for tissue engineering scaffolds used in the repair and regenerate of different tissues including skin [14].

On the other hand, human amniotic membrane (HAM) has been used in the case of large skin ulcers. Basement membrane of AM can act as a mechanical scaffold which promotes cellular proliferation, survival, and regeneration. To mimic the native extracellular matrix in the aspects of high surface area-to-volume ratio and high porosity and also to provide the suitable mechanical support and stiffness, silk fibroin nano-fibers can be electrospun on the basement membrane side of de-epithelized amniotic membrane.

Nowadays, stem cells due to their ability to self-renew and differentiate into various cell types are central to the field of regenerative medicine. Subsequently, they enhance angiogenesis by releasing cell-specific growth factors and cytokines [16]. According to the origin of the stem cells, scientists categorize them into 2 types: embryonic stem cells (ESCs) and adult stem cells (ASCs). Because of limitation in use of ESCs due to low availability, tumorigenicity potential, and ethical concerns, ASCs are preferred source for clinical and tissue engineering applications [17]. Of the various types of ASCs that have been mentioned in the literature, two types are ideal cell resources to tissue engineering: bone marrow-derived mesenchymal stem cells and adipose-derived stem cells. Despite the described advantages in use of these cells, the harvesting and isolation protocols are invasive and fraught with donor site morbidity and also need to do surgical operation. Newly defined stem cells, menstrual blood-derived stem cells (MenSCs), derived from endometrium shedding monthly through menstruation fill the vacuum left by the lack of easy accessible and high potent stem cells [18]. MenSCs with a unique population of cells show high rate of cell proliferation and have the capacity to differentiate into various functional cells such as osteocytes, cardiomyocytes, endothelial cells, neuronal cells, hepatocytes, and adipocytes [15, 18, 19].

In this study, we investigated the potential of MenSCs to generate keratinocytes when seeded on bilayer amniotic membrane/nano-fibrous fibroin scaffold. The in vitro generation of keratinocytes from MenSCs using scaffold may provide an excellent model for designing the skin substitute in wound-healing strategies.

Materials and Methods

Harvesting and Cultivation of MenSCs

MenSCs were isolated from menstrual blood (MB) which collected from 25 to 35 aged healthy females on second day of menstrual cycle via Diva cup (Diva international Co., Lunette, Finland). All donors were tested for blood-borne viruses such as HCV, HBV, HPV and HIV and also for herpes simplex and chlamydia. The negative reported cases were referred to study. All volunteers signed informed consent

form approved by Medical Ethics Committee of Avicenna Research Institute. The MB was then transferred into Falcon tubes containing 2.5 µg/ml fungizone (Gibco, Scotland, UK), 100 µg/ml streptomycin, 100 U/ml penicillin (Sigma), and 0.5 mM EDTA in PBS. Steps for isolation and culture of stem cells within MB were performed as described in our previous studies [20–22].

Multi-lineage Differentiation of MenSCs into Osteoblasts, Chondrocytes and Adipocytes

The differentiation ability of isolated MenSCs into osteoblasts, chondrocytes and adipocytes was evaluated prior to assessment of keratinocyte differentiation potential. For osteogenic differentiation, the cells were induced by DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), 0.1 mM Dexa, 10 mM b-glycerophosphate, and 50 mM ascorbate–phosphate (all from Sigma-Aldrich). After 2 weeks, the calcium deposition of cultured cells was identified by alizarin red staining (Sigma-Aldrich). For chondrogenic differentiation, the cells were treated with a medium containing 2% FBS, 100 mg/ml sodium pyruvate, 20 ng/ml transforming growth factor-b3 (TGFb3), 50 ng/ml bone morphogenetic protein 6 (BMP 6), 100 nM Dexa, 1_ ITS + 1 (ITS + bovine serum albumin and linoleic acid), and 50 mg/ml ascorbic acid (Sigma-Aldrich) for 3 weeks. Medium changes were performed twice weekly, and chondrogenesis was assessed by Alcian blue staining for determination of glycosaminoglycan deposition. For adipogenic differentiation, cells were treated with DMEM supplemented with 10% FBS, 1 mM Dexa, 10 mg/ml recombinant human insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 200 mM indomethacin (all from Sigma-Aldrich) for 2 weeks. Adipogenic-induced cells were stained for fat vacuoles using oil red O staining.

Undifferentiated cells were stained in the same manner as the control.

Isolation of Keratinocytes

The isolation and culture of human keratinocytes was performed based on previous reported protocol [23]. Briefly, foreskin samples were collected from healthy newborn males aged 2–10 months who circumcised. All donors' parents signed an informed consent form accepted by the Medical Ethics Committee of Avicenna Research Institute. The samples were transferred into the tubes previously contained 7.5 µg fungizone (Gibco), 300 µg/ml streptomycin, and 300 U/ml penicillin (Sigma-Aldrich) in HBSS (Gibco). Transferring of the foreskins to the cell culture room took only few minutes. After washing and removing all unwanted particle by PBS under sterile condition, the sample was cut into small pieces and then placed into one Falcon tube containing 4 ml dispase (Gibco), 100 U/ml penicillin, and 100 µg/mL streptomycin. To separate the epidermis from dermis, the tube was incubated at 4 °C overnight. Then separate the epidermis from dermis into petri dish contained 2.5 mg/ml PBS. The epidermal section was cut to very small pieces, and next 2.5 mL trypsin EDTA 0.04% (Gibco) was added and finally incubated in 37 °C for 15 min. Afterward the solution transferred to 5 mL DMEM with 10% PBS. Extracted cells were centrifuged at 200 g for 5 min at 14 °C. At the next day, the two layers of the skin were easily separated from each other using narrow-tipped instrument. The desired layer, epidermis, was chopped into several pieces, and then 2.5 ml of the digestion solution (trypsin/EDTA) was added and incubated at 37 °C in 5% CO_2 incubator for 20 min. Afterthat, the digestion solution was inactivated using 5 ml DMEM supplemented with FBS (10%). After pipetting up and down, the solution was passed through 70-µm mesh filter and at the next step centrifuged at 200 g for 10 min. The cell pellet was suspended in keratinocytes specific medium, EpiLife medium (Gibco), and then placed into flask which was coated by coating matrix kit (Gibco) to enhance the cell attachment to the flask. The isolated cells were maintained at 37 °C in a humidified 5% CO_2 incubator to allow cells attachment. To reach confluence, medium was changed twice a week.

Fabrication of Bilayer Composite Scaffolds

The bilayer scaffold was produced according to a previously published paper [24]. Amniochorionic membranes were collected from healthy mothers, with no infectious disease, undergoing elective cesarean section to give birth at Iran-Mehr Hospital (Tehran, Iran). Amniochorionic membranes were dissected free of the placenta and washed in cold phosphate-buffered saline (PBS) several times to remove blood residue. Afterward, amniotic membrane (AM) was separated from chorion and de-epithelized as described by Hopkinson et al. [25]. In brief, the amniochorionic membrane was incubated in PBS for 25 min, thrice, with gentle agitation for the spongy layer to swell. Next, AM was cleaned from spongy layer using scraper and forceps and ultimate incubation in PBS for 30 min. AM de-epithelization was carried out in thermolysin solution (125 µg/ml—in PBS) for 9 min at room temperature, after which samples were rinsed with PBS for 15 min, several times, with gentle agitation to remove residual cellular debris. De-epithelized AM samples were lyophilized and stored at − 20 °C for future use.

Degummed silk fibers were provided from STERCO. (Tehran, Iran) and dissolved in 9.3 M lithium bromide solution (10 wt%) at 55 °C for 5 h. The resultant solution was dialyzed through dialysis membrane (12 kDa cutoff value) in ultrapure water and lyophilized.

Finally, to produce bilayer scaffold, silk fibroin nano-fibers were electrospun on the basement membrane side of de-epithelized AM. AM was affixed on aluminum foil on a cylindrical collector, which was rotated at 1500 rpm. Extracted silk fibroin was dissolved in formic acid (99.9%) at a concentration of 20% (wt/v) and electrospun at working distance of 80 mm, flow rate of 0.2 ml/min, and high voltage of 18 kV. The bilayer scaffold was incubated in methanol (99.98%) for 1 h to regenerate silk fibroin nano-fibers, rinsed by distilled water, and air-dried.

Microstructure Study of the Bilayer Scaffold and Cell-Seeded Scaffolds

The microstructure of the bilayer scaffold and cell-seeded scaffolds was studied by scanning electron microscope (SEM). Scaffolds without cells need no preparation, whereas cell-seeded scaffolds were prepared accordingly; culture medium was decanted, and scaffolds were rinsed with PBS twice, fixed in 2.5% glutaraldehyde for 2 h, rinsed with PBS, and incubated in 1% osmium tetroxide for 1.5 h. The solution was decanted, and samples were left at room temperature to dry overnight and stored in a desiccator. Proper pieces of each sample were mounted on aluminum sample holder and coated with gold using EMITECH SC7620 sputtering machine at 1 kV and 10 mA for 120 s. Gold-coated samples were visualized by means of AIS2100 SEM (Seron Technology, Korea) at 20 kV.

Differentiation of MenSCs into Keratinocytes

To induce MenSCs differentiation into keratinocytes, the seeded MenSCs over scaffold were co-cultured with foreskin keratinocytes. For this purpose, the bilayer scaffolds were put in 12-well plates, drenched in filtrated 70% ethanol for 2 h, and washed with PBS. Then the scaffolds were immersed in DMEM overnight before cell seeding. At the next day, the MenSCs cultured in flasks were trypsinized, counted, and plated at a density of 2×10^4 cells/cm^2 over the surface of bilayer scaffolds. By incubation of the seeded scaffolds at 37 °C for 2 h, cells were diffused into and adhered to the scaffold before the addition of medium to each well. To avoid drying of the scaffold at the first 2 h of incubation, 50 µl of Epilife culture medium (Gibco) supplemented with human growth factor was gradually added to each well every 30 min.

One day later, transwell with 0.4-µm pores (ThinCert, Greiner Bio-one) was placed in the wells containing MenSCs seeded scaffold. Then foreskin-isolated mature keratinocytes were planted onto the surface of transwell. To co-culture the cells, the plate was kept at 37 °C in a humidified 5% CO_2 incubator for 2 weeks. Medium was refreshed twice a week.

To evaluate efficacy of scaffold in MenSCs differentiation, the MenSCs was induced into keratinocyte lineage by designing an indirect scaffold-free co-culture system. To achieve this purpose, MenSCs were cultured in 24-well plate containing Epilife medium for 24 h. Then, the 0.4-µm-pore transwells (ThinCert, Greiner Bio-one) were inserted into the wells. At the next step, foreskin-isolated keratinocytes were seeded on transwell. This co-culture system was also continued for 2 weeks.

Immunostaining Assay

To assay the expression of specific markers in foreskin-isolated keratinocytes and in vitro differentiated keratinocyte, immunofluorescence staining was performed as following: In both culture systems, the cultured cells were fixed through 20-min incubation in PBS containing 4% paraformaldehyde (Merck, Germany) at room temperature. The fixed cells were quenched with FBS and then permeabilized using 0.02% Triton X-100 (Merck, Germany). Immunostaining of the cells was continued through incubation of primary antibodies against K14 (mouse monoclonal; 1:100, Abcam, UK) and involucrin (rabbit polyclonal; 1:50, Abcam, UK) at 4 °C overnight (16–18 h). After washing by TBS/BSA (Tris-buffered saline, containing BSA), incubation of secondary antibodies including FITC-conjugated goat anti-rabbit IgG and FITC-conjugated sheep anti-mouse IgG (Avicenna Research Institute) was performed for 45 min at room temperature in dark place. Nuclei were stained using 4′,6 diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The cells were inspected and photomicrographed using a fluorescence microscope (Olympus BX51 microscope, Tokyo, Japan).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

QRT-PCR was performed to analyze the expression profile of keratinocyte genes including P63 and involucrin. Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Valencia, USA) as described in the manufacture's data sheet. To perform transcription reversely a mixture of 2 µg purified RNA, 1 µl SuperScriptTM II Reverse Transcriptase (200 U) (Life Technologies, CA, USA), 20 pM dNTP Mix, 20 pM N6 random hexamer, 2 µl dithiothreitol (0.1 M), 4 µl 5 × first standard buffer, and 1 µl RiboLockTM RNase inhibitor (all from Fermentas Inc) was prepared and to complete the reaction was put in a thermocycler (Eppendorf, Germany) (25 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min). Then, 1 µl of cDNA was mixed with 12.5 µl reaction master mix (Amplicon, Copenhagen,

Denmark) and 1 µl of each primer (Table 1). After initial denaturation at 95 °C for 10 s, PCR amplification was continued at 95 °C for 5 s and 60 °C for 30 s for 40 cycles. Finally, dissociation stage was performed at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The amplified genes were sequenced by 3130 Genetic Analyzer (Applied Biosystems, CA, USA). Mean efficiencies and crossing point values for all genes were determined by LinRegPCR (version 11.0). Relative expression levels were calculated using ΔC_T method normalized to value for GAPDH in differentiated MenSCs with undifferentiated cells by REST 2009 software (available at http://www.gene-quantification.info). Comparative expression levels of all genes in differentiated cells were calculated to undifferentiated cells. PCR products were verified by melting curve analysis or 2% agarose gel electrophoresis.

Statistical Analysis

All experiments were performed using cells at passages 2–4 from 3–5 donors. All measurements were taken in triplicate. Statistical analysis of real-time PCR data was performed using REST freeware according to formula presented by Pfaffl et al. [26].

Results

Characterization of Multi-lineage Differentiation Capability of MenSCs

Calcium deposition and mineralization was pronounced in cells differentiated into osteoblasts, as shown by alizarin red staining. In addition, cells differentiated into chondrocytes could produce GAG as demonstrated by alcian blue staining. Moreover, the formation of oil vacuole in differentiated cells proved MenSCs ability in adipogenic differentiation (Fig. 1).

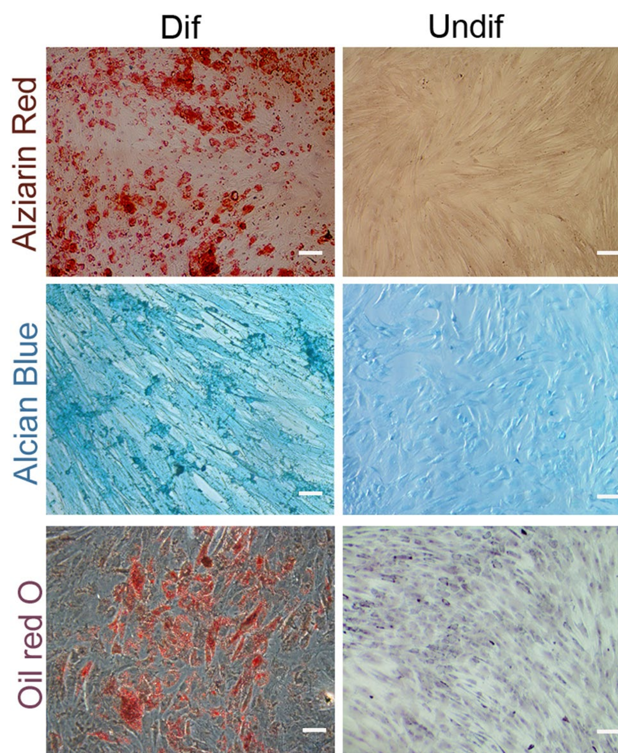


Fig. 1 Phenotypic characterization of isolated MenSCs. MenSC differentiation into osteoblasts, chondrocytes and adipocytes, judged by alizarin red, alcian blue and oil red O staining, respectively; right panel shows staining result of undifferentiated cells. Scale bar: 100 µm (Color figure online)

Characterization of Isolated Human Keratinocytes

Enhanced attachment of the isolated cells from foreskin is achieved by using tissue culture flask coated by matrix proteins. Using fresh foreskin tissues and completing the isolation protocol without delay considerably facilitated the attachment of desired cell to culture flask and consequently increased the cell proliferation rate. In an appropriate condition, we could get on average 2.5×10^6 mononuclear live cells from each 1 cm^2 of foreskin sample. Four days after single cell seeding, the isolated keratinocytes reached 70–80% confluency and exhibited polygonal

Table 1 Primers used in the experiments

Gene	Sequence	Product size (bp)	Annealing temperature (°C)
P63	F 5′-TCAACGAGGGACAGATTGCC 3′	129	60
	R 5′-CAACCTGGGGTGGCTCATAA-3′		
Involucrin	F 5′-CTCTGCCTCAGCCTTACTG-3′	166	55.9
	R 5′-CAGTGGAGTTGGCTGTTTCA-3′		
GAPDH	F 5-CTCTCTGCTCCTCCTGTTCG-3′	114	60
	R 5′-ACGACCAAATCCGTTGACTC-3′		

and cobblestone morphology (Fig. 2). The isolated cells showed typical morphology until passage 6 but at further passages their apperance changed into flat and thin shape, and some granules were seen around the nuclei (Fig. 2). Further analysis of foreskin-derived cells confirmed their identity. Immunostaining of specific markers including involucrin and K14 confirmed their expression at the protein level (Fig. 3).

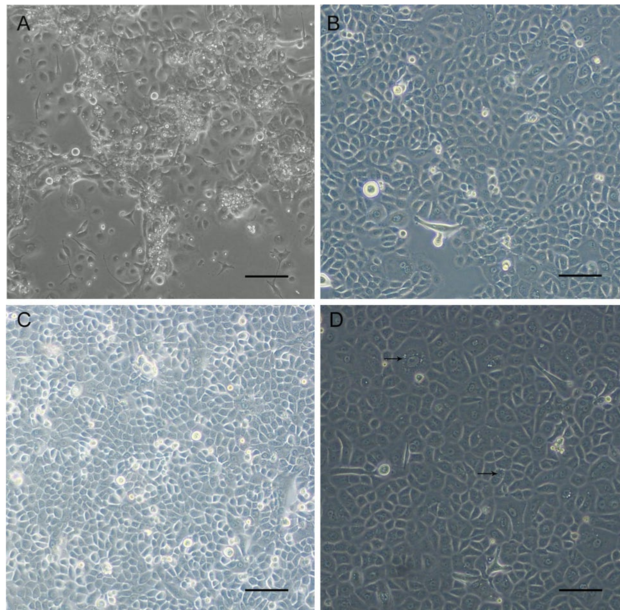


Fig. 2 Morphological assessment of foreskin-derived keratinocytes. The isolated keratinocytes reached 75–80% confluency at day 4 after single cell seeding with polygonal morphology. Although the cells kept their normal morphology at higher passages (**a** passage 0; **b** passage 3; **c** passage 5), accumulation of granules in their cytoplasm appeared by passing culture time (**d** passage 7; arrows). Scale bar: 50 µm

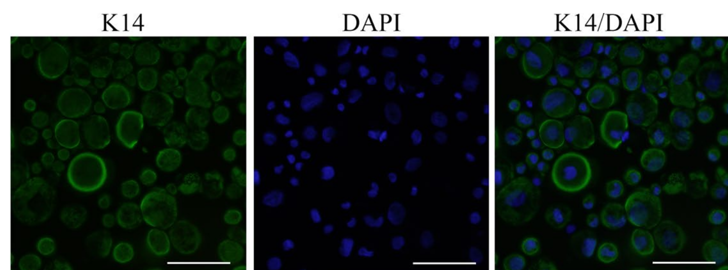
Morphological Assessment of Prepared Scaffold and MenSCs on Scaffold

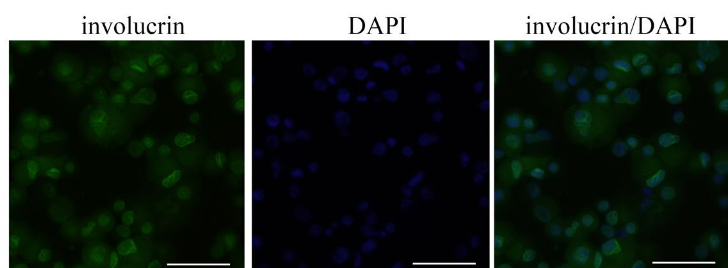
The SEM images revealed that the electrospun fibroin had the bead-free non-woven morphology (Fig. 4a). The diameters of 100 randomly taken fibers were measured, and the average diameter was calculated as 249 ± 116 nm. In order to investigate the potential of amniotic-based scaffolds to provide a three-dimensional structure for cell attachment, proliferation and migration, cellular morphology and cell–matrix crosstalk were studied after 21 days of cell cultivation using SEM (Fig. 4b). The cells fully spread on scaffold, penetrated, and grow inside the pores in the direction of fibers.

Immunofluorescence Staining of Keratinocytes Markers

At the end of the induction period, immunostaining assay was done to detect the expression of keratinocytes markers, K14 (Fig. 5) and involucrin (Fig. 6). The results showed that despite of undifferentiated MenSCs, both differentiated cells on scaffold and 2D culture system could express these markers. There was no significant difference in expression intensity of K14 protein between differentiated cells on 3D and 2D culture system; however, the expression of this marker in both systems was less than foreskin-derived keratinocyte (Fi.5). Nonetheless, the expression intensity of involucrin was higher in differentiated cells in 3D culture compared to 2D culture system, but it was, respectively, lower than foreskin-derived keratinocyte (Fig. 6).

Fig. 3 Immunostaining of keratinocyte specific markers, K14 and involucrin in foreskin-derived cells. Immunostaining showed the expression of K14 and involucrin isolated from foreskin. Nuclei were counterstained with DAPI. Scale bar: 50 µm





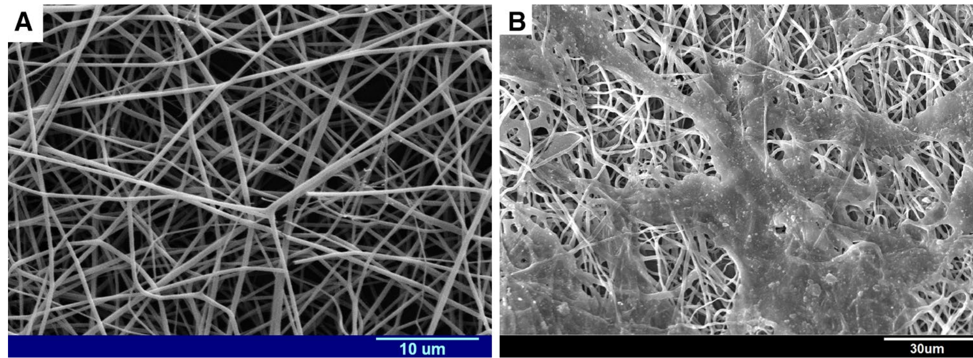


Fig. 4 Morphological assessment of fabricated bilayer scaffold with and without MenSCs. **a** The SEM image of the cell free-scaffold. **b** MenSCs attachment and expansion on the scaffold. Scale bar: 50 µm

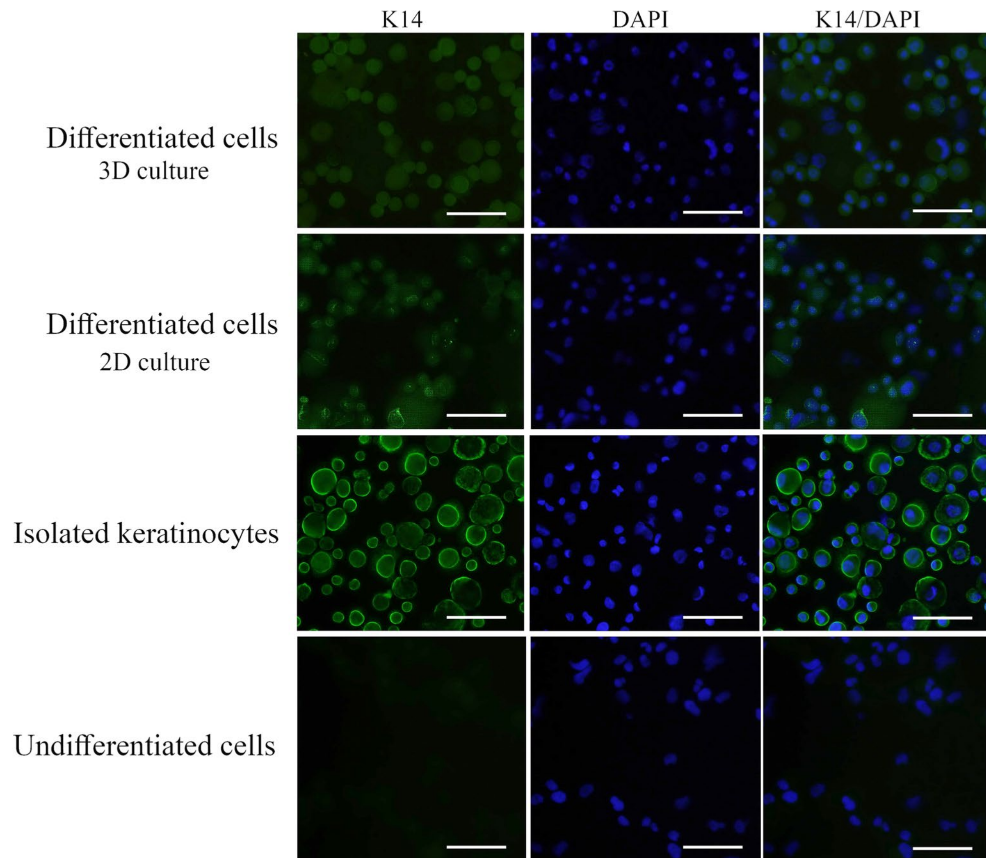


Fig. 5 Expression of K14 protein in differentiated cells in both 3D and 2D culture systems. Immunostaining assay showed the expression of K14 in the differentiated cell by using both 2D and 3D culture system. The foreskin-derived keratinocytes and undifferentiated cells were used as a positive and negative controls, respectively. Nuclei were counterstained with DAPI. Scale bar: 50 µm

Expression Profile of Keratinocytes Specific Genes

Both differentiated cells in 3D and 2D culture system expressed keratinocyte genes judged by quantitative RT-PCR analysis (Fig. 7). The expression levels of these genes were significantly higher in differentiated cells in both 3D (512/17 for P63 and 5495/92 for involucrin) and 2D (10/24 for P63 and 2/94 for involucrin) culture systems in reference to undifferentiated MenSCs. The up-regulation levels of p63 (512/17 folds, $p = 0.001$) and involucrin (5495/92 folds, $p = 0.001$) in differentiated cells over bilayer scaffold were greater than those of differentiated cells in 2D culture system (10/24 folds, $p = 0.001$ and 2/94 folds, $p = 0.001$, respectively).

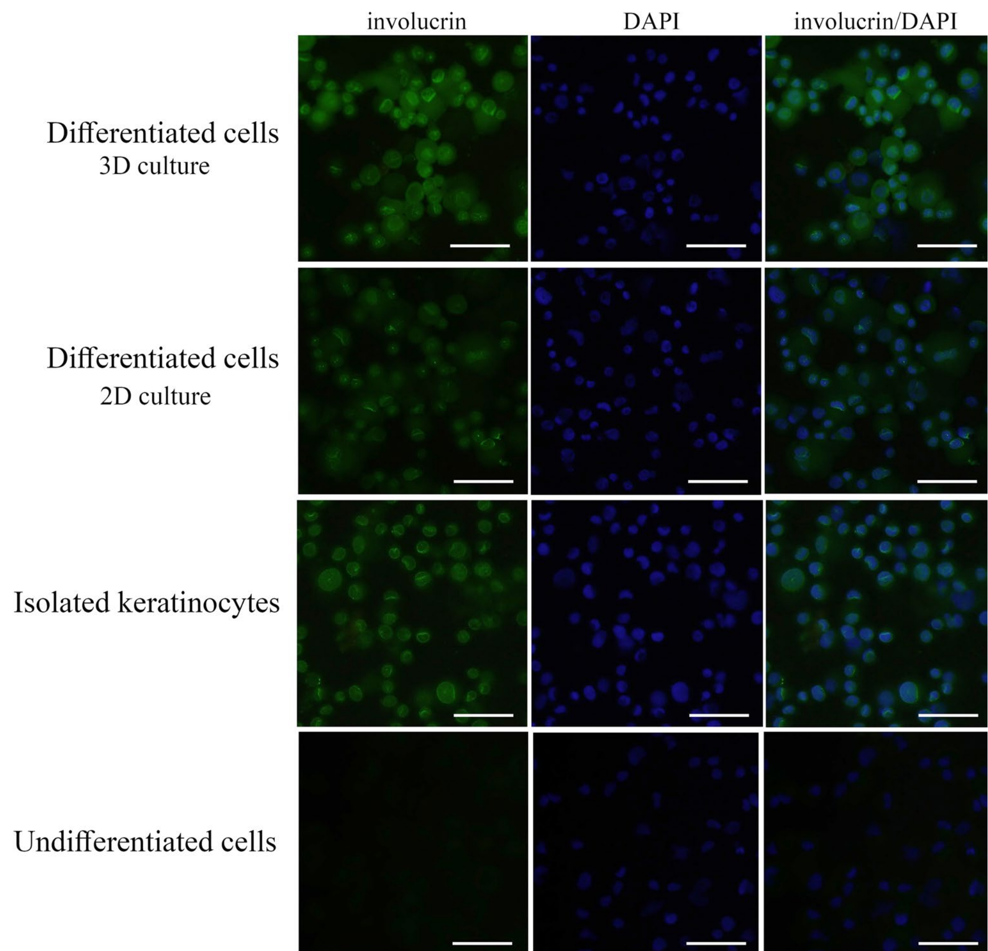
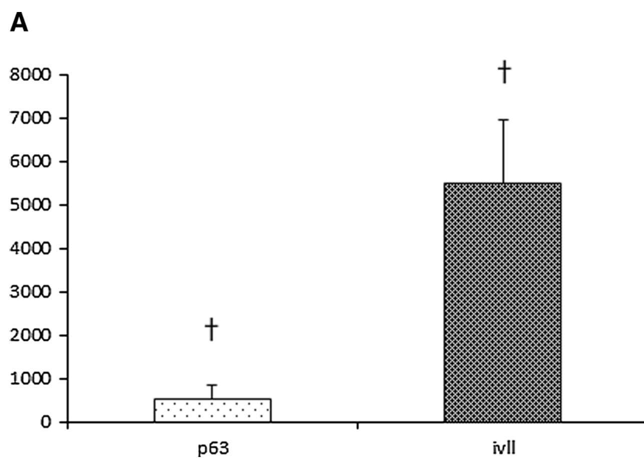


Fig. 6 Immunofluorescent staining of keratinocyte specific marker, involucrin. Differentiated cells and foreskin-derived keratinocytes (as positive control) showed remarkable staining of involucrin in both 2D and 3D culture system. Undifferentiated cells were used as negative controls, respectively. Nuclei were counterstained with DAPI. Scale bar: 50 μm



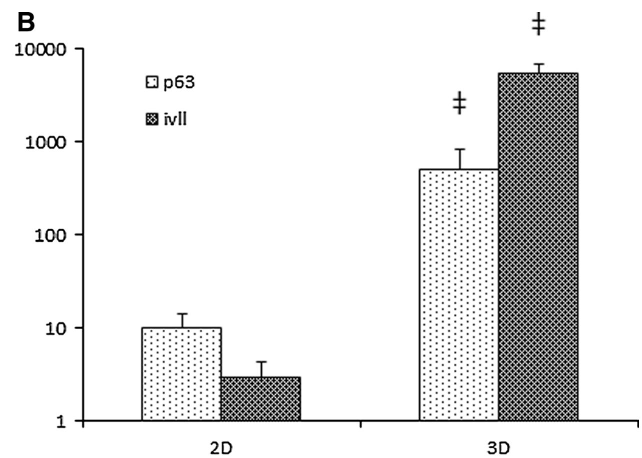


Fig. 7 Real-time PCR analysis of keratinocyte specific markers. **a** Data obtained from real-time PCR assay of differentiated cells were normalized to corresponding GAPDH and calculated in reference to undifferentiated cells. Results revealed the significant up-regulation of the p63 (512/17 folds, $p = 0.001$) and involucrin (ivll) (5495/92 folds, $p = 0.001$) in MenSCs seeded over bilayer scaffolds. **b** It is also shown that the differentiated cells over bilayer composite scaffolds express the keratinocytes specific markers at higher levels when compared with the cells differentiated using a 2 D inducing system. †indicates significant difference between differentiated and undifferentiated cells ($p \leq 0.05$), ‡indicates significant difference between 3D and 2D culture systems ($p \leq 0.05$)

Discussion

Although various efforts have been made for regeneration of skin wounds and insults, finding an excellent substitute for injured skin had remained highly challenging because of immune rejection in the case of skin transplantation from a donor, lacking enough patients own skin to cover the extensive wounds, and unsatisfactory and temporary synthetic dressing [27, 28]. An appropriate strategy to repair skin wound is using cultured keratinocytes especially in extensive acute or chronic burn wounds. While this cell-based therapy was used for wound closure and verified to be lifesaving, slow growth and proliferation rate of cultured keratinocytes and long time needed to get the sufficient amount of desired cells have limited the application of this approach [28]. Recent studies on stem cell-based therapy provide an alternative way and new hope to overcome skin lesions.

In recent years, problems with well-known stem cell sources such as low availability, difficult access or limited proliferative ability have impelled scientists to take advantage of MenSCs in cell therapy and regenerative medicine [31].

At this point, no data are available on the potential of MenSCs to generate keratinocyte. The findings of our study are essential for evaluation of stem cell therapy using MenSCs as new approach for wound healing. We showed previously that MenSCs possess some markers of mesenchymal stem cells such as CD44, CD73, CD29, and CD90 but fail to express STRO-1 [19, 20]. Moreover, due to high expression level of BMSCs markers such as CD146, CD166, CD105, and vimentin in parallel to OCT-4, the latter being an embryonic stem cell marker, we suggested a dual characteristic for MenSCs [31]. Here, for further characterization of MenSCs, the immunophenotypic properties and capacity of MenSCs to differentiate into osteoblasts, chondrocytes and adipocytes were demonstrated prior to assessment of the MenSCs potential to differentiate into keratinocyte.

After MenSCs characterization, we showed that MenSCs possess the potential to differentiate into keratinocyte-like cells. We revealed that the MenSCs can differentiate into keratinocyte-like cells in both 2D and 3D culture systems. More interestingly, we found that keratinocyte-like cells derived from MenSCs seeded over silk fibroin nano-fibers bilayer composite scaffolds express specific markers at higher levels when compared with 2D inducing culture system. To improve the proliferation rate, cell attachment, and efficacy of differentiation condition, we fabricated bilayer composite scaffold to mimic the natural microarchitecture of extracellular matrix of skin. This bilayer skin substitute possesses good mechanical strength, high elasticity, and high cell harboring capacity. We also previously showed that bilayer amniotic membrane/nano-fibrous fibroin scaffold improved the integration with cultured cells in vitro which appear to be suitable matrix for skin tissue engineering [24]. Usage of such scaffold may provide some advantages over only cell injection into the injured tissue. These 3D scaffolds as substitutes of damaged tissue such as skin provide a transitional support to cell proliferation, colonization, and migration [29, 30]. For these reasons, seeding of the stem cells on bilayer amniotic membrane/nano-fibrous fibroin has been investigated to provide a strategy in skin wounds regeneration. In our study, the keratinocytes-like cells derived from MenSCs seeded over bilayer scaffold successfully over-expressed the keratinocytes genes when compared with differentiated keratinocytes-like cells in 2D culture system. These findings introduced the MenSCs as novel alternative cell source in stem cell-based skin regeneration. At the first time, Meng et al. [18] introduced MenSCs as a novel stem cells source in endometrium which shed monthly through menstruation. These remarkable stem cells revealed high cell growth and proliferation rate and the potential to differentiate into various cell types. Expanding in quantities without chromosomal abnormalities, non-immune rejection possibility, and lack of tumorigenicity following transplantation makes this biological women waste an appropriate resource in cell therapy applications [31]. Recent studies report the differentiation of MenSCs into different cells such as chondrogenic, muscular, hepatic, neuronal, and cardiac cells [32–34] encouraged us to investigate their potential to change into keratinocytes especially using a three-dimensional culture system which mimics the skin biological scaffold. To provide an in vivo condition with realistic mechanical properties which are comparable with epidermis and a sufficient cocktail of inducers to derive keratinocytes, we cultured the MenSCs on nano-fibrous bilayer composite and in the presence of human foreskin-derived keratinocytes using an indirect culture system.

Keratinocytes, the main cells in the outermost layer of skin, epidermis, are difficult cells to isolate and grow and show low rate of proliferation following time-consuming multi-step isolation and cultivation procedure [23]. Phenotypic and morphological characteristics of human keratinocytes as a polygonal cell with a cobblestone pattern in the flasks [35] were observed in isolated and cultivated epidermal cells in present study. To assess molecular profile of isolated cells, we confirmed the expression of the keratinocytes specific markers including P63, an early keratinocyte marker, and involucrin, a late marker of keratinocytes.

Based on previous investigations, the cells could affect on each other in indirect co-culture system through secretion of cytokines [36]. Therefore, we hypothesized that foreskin-derived keratinocytes can influence the seeded MenSCs to differentiate into keratinocytes by producing sufficient inducing and growth factors. Obtained results in our study

confirmed the derivation of keratinocyte-like cells from MenSCs after 2-weeks of indirect co-culturing.

Based on our results and the advantages of MenSCs over other kinds of adult stem cells even embryonic stem cells (ESCs), and considering the more efficient results by differentiating MenSCs over bilayer scaffolds, we can introduce the MenSCs as novel and excellent alternative in skin regeneration therapeutic programs. There are some studies showing in vitro derivation of keratinocytes from ESCs. Coraux and colleagues demonstrated the in vitro differentiation of keratinocytes from murine ESCs seeded on extracellular matrix. They could show the enriched preparation of keratinocytes was able to form a stratified epithelium as an epidermal equivalent [37]. Another group demonstrated the potential of human ESCs to differentiate into basal keratinocytes that are fully functional. The expression levels of specific markers in newly formed cells were similar to those in basal keratinocytes. ESCs-derived keratinocyte formed the stratified epidermal layer when seeded on an artificial matrix. Expression of keratinocytes late markers such as involucrin was detected in the keratinocytes of constructed pluristratified epidermis [35]. The expression of involucrin in MenSCs-derived keratinocytes in our study showed the efficacy of co-culturing of MenSCs seeded on bilayer amniotic membrane/nano-fibrous fibroin scaffold. Involucrin as a late marker of keratinocytes plays a critical role in keratinization of skin. Since the expression of involucrin is restricted to suprabasal and upper layers of epidermis, involucrin-positive cells which obtained in our investigation definitely belong to the epidermal lineage.

More interestingly, investigators introduced murine iPSCs as a novel source of keratinocytes. Generated keratinocytes also expressed the markers belong to basal keratinocytes [38]. Differentiation of human iPSCs into keratinocytes was shown by another report [39]. The challenges facing the usage of ESCs and iPSCs limit their application in clinical therapeutic procedures. Because of concerns such as the possibility of immune rejection, tumorigenicity after transplantation, and ethical issues in the usage of ESCs and iPSCs, the researchers have focused on using adult stem cells (ASCs) as a safe alternative. So derivation of various kinds of cells from ASCs isolated from mature tissues such as bone marrow, adipose tissue, umbilical cord, and peripheral blood was reported.

Based on accumulative data, we showed that MenSCs have enough potent to generate keratinocyte-like cells. Derivation of keratinocyte-like cells from an easily access source without technical intervention introduces a valuable resource to overcome the limitation of using stem cells in clinical application to regenerate injured skin. Moreover, bilayer amniotic membrane/nano-fibrous fibroin scaffold is a novel, accessible, and natural construct which improves MenSCs trans-differentiation into keratinocytes. Therefore, MenSCs seeding over the fabricated scaffold could be introduced as an appropriate substitute for further animal studies and also clinical trials on healing of acute and chronic wound lesions.

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Compliance with Ethical Standards

Conflict of interest The authors declare no commercial or financial conflict of interest.

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