Proliferation and Differentiation of Human Hair Follicle Stem Cells on Chitosan-Skin Engineered Template in Vitro

Abu Bakar Mohd Hilmi[#], Mohd Noor Norhayati[*], Ahmad Sukari Halim[*], Zulkifli Mustafa[%], Zamzuri Idris[%], Saidi Jaafar[+]

[#]School of Diagnostic and Biomedicine, Faculty of Health Sciences, Universiti Sultan Zainal Abidin, 21300 Kuala Nerus, Terengganu, Malaysia
E-mail: mhilmiab@unisza.edu.my

[*]Reconstructive Sciences Unit, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
E-mail: norhayati_mnoor@yahoo.com, ashalim@usm.my

[%]Department of Neuroscience, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
E-mail: jeocity_vet@yahoo.com, neurosciceszamzuri@yahoo.com

[+]School of Dental Sciences, Universiti Sains Malaysia, 16150 Kelantan, Malaysia
E-mail: saidijaafar@usm.my

Abstract— Hair follicles repeatedly regress and reconstitute themselves, suggesting the presence of intrinsic tissue stem cells. Among the unique characteristics of adult stem cells isolated from hair follicles is their ability to differentiate into keratinocytes. Study on chitosan skin-engineered templates (SETs) as scaffolds for the proliferation of human fibroblasts have shown the promise of SETs in facilitating skin cell growth in three-dimensional culture. High proliferation in three-dimensional culture using human cells allows the researcher to extensively evaluate the cultivation of desirable cell types on chitosan SETs. Therefore, this study aimed to evaluate the *in vitro* attachment, proliferation and differentiation of hair follicle stem cells (HFSCs) on a chitosan SETs. HFSCs were isolated from human scalp tissues using collagenase type I prior to propagation in supplemented CnT-07 media. The phenotype of the HFSCs was verified using the markers keratin-15 (K15) and CD200, as detected by immunocytochemical staining. The attachment and proliferation of the HFSCs on the chitosan SETs were evaluated using scanning electron microscopy (SEM), an alamar blue assay and a live/dead assay. Subsequently, the HFSCs were differentiated using CnT-2D differentiation media. The cells' differentiation was verified using the markers involucrin and keratin-6 (K6), as detected by immunofluorescence staining. The HFSCs were successfully isolated, proliferated and differentiated according to staining positivity and microscopy imaging. HFSCs are able to proliferate and directly differentiate into keratinocytes on a chitosan SETs, which may facilitate their use in regenerative medicine.

Keywords— hair follicle stem cells (HFSCs); chitosan SETs; pore size; proliferation; differentiation

I. INTRODUCTION

The isolation of hair follicle stem cells (HFSCs) derived from humans has been simplified [1] and has led to a great opportunity for researchers to use HFSCs in differentiation experiments. Many studies have proven that the multipotent HFSCs situated in the bulge, serving as its niche [2], [3] are able to differentiate into sebocytes [4] and melanocytes, neuronal and smooth muscle cells [5] *in vitro*. In addition, a new hair follicle can be formed in reconstitution assays after HFSCs are combined with dermal cells [2]. Human HFSCs in a co-culture model were successfully demonstrated to regenerate tissue to replace wounded or diseased tissues both in acute and in chronic animal models [6]. However, studies of HFSC proliferation and differentiation into keratinocytes *in vitro* using scaffolds are scarce. The differentiated cells, and particularly keratinocytes, that are derived from human HFSCs are potent, especially for use in the clinical setting. Clinically, autograft and allograft skin grafting is still the gold standard for the management of burns and wounds that involve extensive skin loss [7]. This type of treatment leaves a scar and pain at the donor site. Alternative management of burn and wound patients is definitely challenging for plastic surgeons.

The availability of a lot of cultured keratinocytes is certainly crucial for alternative management. Unfortunately, keratinocyte cultures easily undergo senescence [8],

impairing their application to burn and wound management. Another issue in the clinical setting is obtaining human HFSCs that have the high proliferative ability and that are easy to differentiate into keratinocytes [1]. Prior to grafting HFSCs, a delivery system is not necessary if the cells are cultured in a culture vessel before administration to the recipient because the cells must be administered in spray form, which is time-consuming and labor intensive.

Differentiating cells on biocompatible and biodegradable scaffolds is another challenge. A scaffold must lead to extensive cell proliferation, and in the case of differentiation, the scaffold should have properties that support and trigger stem cell differentiation. Chitosan skin-engineered templates (SETs) have been proven to have potential as an applicable scaffold for three-dimensional culture, accelerating fibroblast proliferation [9]. Chitosan is a poly-(β-1, 4-D-glucosamine) and deacetylated derivative of chitin. Among the attractive features of chitosan are its biomechanical properties [10], which are ideally suited for skin tissue engineering [6], [9], [11]. The potential of chitosan in supporting HFSCs and their differentiation potential in three-dimensional culture is yet unexplored. HFSC culture on chitosan is a strategy to avoid technical limitations, including HFSC delivery and transplantation issues. The elimination of these limitations may render HFSCs a potential source of stem cells for cutaneous regenerative medicine and skin tissue engineering [12]. Therefore, our aim was to evaluate the attachment, proliferation, and differentiation of HFSCs cultured on a chitosan SETs for application in regenerative medicine.

II. MATERIAL AND METHOD

A. Preparation of Chitosan SETs

A chitosan SETs was produced from ultrapure medical-grade chitosan powder (Hunza Nutriceuticals Sdn Bhd., Nibong Tebal, Penang, Malaysia) developed at the Advanced Materials Research Centre of SIRIM (AMREC-SIRIM) in Malaysia (Fig. 1). The chitosan SETs production procedures have been described in detail by [9], [13]. The chitosan powder was irradiated via gamma radiation, and the chitosan SETs was sterilized using ethylene oxide gas. Prior to use, the scaffold was quarantined for one month. The chitosan SETs was briefly washed with supplemented CnT-07 media (Cell-N-Tech) prior to cell seeding.

Raw material   Chitosan powder   Chitosan SET

Fig. 1 AMREC-SIRIM producing Chitosan SET originated from prawn shell

B. Morphological Analysis of Chitosan SETs

The top surface and the cross-sectional morphology of the chitosan SETs were observed by scanning electron microscopy (SEM; Quanta 450 FEG, FEI, Netherlands) after specimens were coated with an ultrathin layer of gold in a coating apparatus. Imaging was conducted at a voltage of 20 kV.

C. HFSC Primary Culture and Characterization Assay

HFSCs were obtained following a method outlined in our prior publications [1], [14]. All human scalp tissues used in this study were collected from consenting donors. The study was approved by the Human Ethics Committee of Universiti Sains Malaysia (USM) (USMKK/PPP/JEPeM [212.3(15)]). The cultured cells were grown in supplemented CnT-07 media at 37°C in a 5% CO_2 incubator. The HFSCs were characterized and their phenotype was verified following a method outlined in our prior publications [1], [14]. The HFSCs were grown in a 24-well plate until reaching 80% confluence before fixation in absolute methanol (Sigma) for 15 minutes at 4°C. After that, incubation of the cells in normal goat serum for 20 minutes was performed. Mouse monoclonal anti-human keratin-15 (K15; Abcam Inc., 1:1000 dilution) and mouse monoclonal anti-human CD200 (Abcam Inc., 1:500 dilution) were then used as primary antibodies to validate the HFSCs' phenotype. These primary antibodies against the markers were incubated for one hour. Incubation with a secondary antibody (conjugated to biotin) for 30 minutes was then performed, followed by a 20-minute incubation with streptavidin-horseradish peroxidase. Afterward, the cells were incubated with 3,3'-diaminobenzidine (DAB substrate) for another 20 minutes. Finally, hematoxylin was added to the cells to counterstain the nucleus.

D. Inclusion and Exclusion Criteria

In normal hair cycle, the hair is actively produced because the hair follicle composed of hair follicle stem cells. Therefore, to confirm that the hair follicle stem cells are easily isolated, scalp samples were collected from patients that fulfilled these following criteria: i) Patients age less than 60 years. This criterion was chosen because older people normally presented with hair loss that clearly confirmed the hair cycle was abnormal. ii) Scalp containing hair follicle. Some scalps that were collected but have no hair follicle have been excluded from the isolation procedures. iii) Not receiving radiotherapy on the head. Radiation exposure causes the hair loss and effects the hair follicle function. Meanwhile, patients having a scar on the scalp, androgenic alopecia and excessive open injury on the head were excluded from sample recruitment.

E. HFSC Seeding on Chitosan SETs

HFSC cultures were washed with Dulbecco's modified phosphate-buffered saline (DPBS) (Gibco) and detached using TrypLE™ Express (Invitrogen). The cells were then seeded onto chitosan SETs at a seeding density of 6×10^6 cells/well. The first 20 µl of cells was seeded onto the center of each chitosan scaffold. After three minutes, another 20 µl of cells was seeded onto the chitosan. This process was repeated until 100 µl of cells was seeded. After 30 minutes, 100 µl of supplemented CnT-07 medium was added to support cell proliferation. The cultures were incubated at 37 °C in a 5% CO_2 incubator. After one hour, the chitosan-

cell constructs were transferred to 24-well plates to provide an adequate volume of growth media for cell proliferation. The chitosan-cell constructs were incubated at 37 °C and 5% CO^2. The supplemented CnT-07 media were changed every day to mitigate nutrient depletion. On the third day, the chitosan-cell constructs were used for further experiments.

F. HFSC Attachment on Chitosan SETs

The chitosan-cell constructs were rinsed three times with DPBS for 5 minutes each and fixed with 2.5% glutaraldehyde (Sigma) at room temperature. The samples were then washed three times with PBS, dehydrated using a graded series of ethanol (30-100%), air dried and viewed by SEM. A chitosan SETs incubated with growth media alone was used as the negative control.

G. Proliferation of HFSCs on Chitosan SETs Observed Via Laser Scanning Microscope (LSM)

The chitosan-cell constructs were washed three times for 5 minutes each. A LIVE/DEAD Kit (Invitrogen) was used to investigate HFSC proliferation on the chitosan SETs. A 200 μl solution containing 4 mM ethidium homodimer-1 reagent and 2 mM calcein AM (ratio 2:1) in DPBS was used, as recommended by the manufacturer. Incubation of the chitosan-cell constructs with the solution was performed for 45 minutes at room temperature, after which the scaffolds were viewed using LSM (Zeiss, Germany) with excitation/emission wavelengths of 495 nm/635 nm for ethidium homodimer-1 and of 495 nm/515 nm for calcein AM. A chitosan SETs incubated with growth media alone was used as the negative control.

H. Viability of HFSCs on Chitosan SETs

Chitosan-cell constructs were assessed with the alamar blue test (Invitrogen) for different culture periods of up to 7 days. Alamar blue solution was also applied to the chitosan SETs without cells, and the data were regarded as the control. At different culture times, 20 μl of alamar blue solution was added to each well, and the samples were incubated at 37 °C for 4 hours. The medium (100 μl) from each well was transferred into a 96-well plate (Orange, Belgium) and analyzed using a NanoQuant enzyme-linked immunosorbent assay reader (Tecan, Austria). The absorbance was determined at 570 nm, with a reference wavelength of 600 nm.

I. Differentiation Ability of HFSCs

The differentiation abilities of the HFSCs on the chitosan SETs and on a 6-well chamber plate were evaluated by immunofluorescence staining. A solution at 6×10^6 cells/ml was seeded on the chitosan scaffold, and the cells were differentiated using CnT02-3D induction media (Cell-N-Tech). The differentiation of the HFSCs into epidermal keratinocytes was validated using a mouse anti-human involucrin monoclonal antibody (Abcam, UK) and a mouse anti-human cytokeratin-6 (K6) monoclonal antibody (Abcam, UK). Primary antibody incubation (1:500) was performed at room temperature for one hour. The involucrin- and K6-positive cells were detected by a secondary antibody coupled to fluorescein isothiocyanate (FITC) (Sigma). To stain the nucleus, 4',6-diamidino-2-phenylindole (DAPI) (1:500) (Sigma) was used. All images were viewed by LSM.

J. Statistics

The data are presented as the mean ± standard error of the mean (SEM) for the live/dead assay and as the mean ± standard deviation (SD) for the OD values. The means of the groups in the live/dead assay were compared using the Wilcoxon signed-rank test. A significant difference was considered when $P < 0.05$.

III. RESULTS AND DISCUSSION

K. Microstructure of Chitosan SETs

In the current study, the SEM images of the top surface of the chitosan SETs revealed a smooth, porous surface. Meanwhile, the cross-sectional imaging revealed interconnected pores. The pore size of the scaffold ranged from 44.41 ± 15.11 μm to 145.65 ± 18.21 μm in diameter (Figs. 2A and 2B). The pore size of the chitosan SETs was fabricated via freezing and lyophilization processes. During these process, ice crystals nucleate along thermal gradients, creating a porous structure [9], [15]. The particular porous structure of the chitosan SETs was fabricated with the aim of increasing the dimensions for cell attachment and proliferation. A suitable pore size for a scaffold for skin tissue engineering is between 100 and 200 μm [16]. Another report described a lyophilized collagen-chitosan scaffold with a pore size of 40-100 μm as containing an interconnected structure that increased cell proliferation and concurrently decreased inflammation [17]. Moreover, the high porosity of the scaffold increased tissue regeneration and supported tissue formation. Porosity on a specific scale accelerates specific tissue integration and vascularization. This can be achieved by using a high density of cells to proliferate throughout the scaffold and to ultimately regenerate the specific tissue [11]. In the area of tissue engineering, the function of a scaffold is actually to provide three-dimensional cultivation conditions and to ultimately enhance cell adhesion and proliferation in vitro [9], [15].
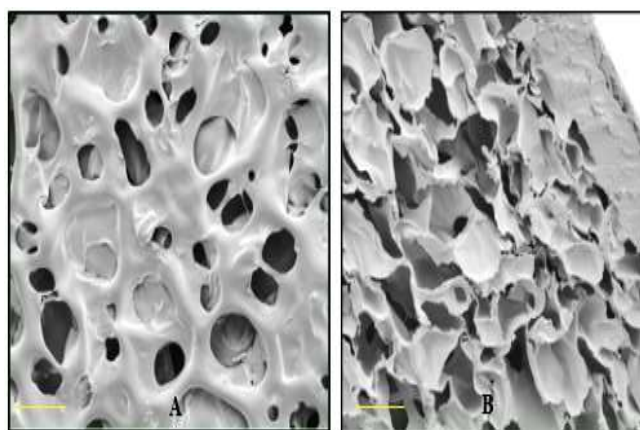
Fig. 2 SEM images of a chitosan SET. Top surface microscopic image of the chitosan SET (A). Cross-sectional microscopic image of the chitosan SET (B). Scale bar 200 μm

There is various types of the commercial produce of biomaterials for the proliferation of skin cells especially for the management of burn patients and wound infections. To

date, researchers from diverse pharmaceutical sector or research centre are still focusing on fabrication of novel biomaterials. The innovation of biomaterial that employs a new polymer or technique for skin replacement product is continuing.

Currently, electrospinning is the latest technology of fabrication for novel biomaterial. In comparison with the conventional method of freeze drying that has been employed for fabrication of chitosan SET, electrospinning producing nanofibrillar scaffold. Nanofiber provides a better substrate for cell adhesion, proliferation and differentiation in 3-D culture [18]. Most importantly, the major skin cells particularly keratinocytes, fibroblasts and endothelial cells were highly grown in this type of scaffold [19]. During skin repair and regeneration, nanofiber leads to increased vascularization, re-epithelialization and enhanced granulation tissue formation. In future, the fabrication process of chitosan SET is suggested to use the electrospinning technique. This new technique will create the novel nanofiber chitosan SET and may show excellent results for future tissue engineering application.

Alternatively, the culture technique of fabrication for skin substitute can be done via the air-liquid interface. It uses special commercially available of culture chamber named as organotypic inserts. The inserts are expensive, however, they lead the differentiation of skin cells into epidermis or dermis rapidly. They allow highly differentiated cells functions and structures compared with the cells cultured under immersion. Cells are seeded onto the permeable membrane of a cell culture insert. Upon reaching confluence, cells are supplied with culture medium to the only basal chamber and exposing the apical surface to air. The structure of skin substitute produced with this technique is almost similar to the native human skin [20].

Natural polymeric scaffolds, including collagen-, hydrogel- or chitosan-based scaffolds, have been the focus, as they have favourable biomechanical properties, significant nutraceutical properties [21], [22] and are both biodegradable and biocompatible [10]. In contrast, synthetic materials are found to generate acidic products that cause on-site inflammation, which is harmful to the surrounding tissue [23]. Furthermore, exudates and a high degree of inflammation may occur if a wound is surrounded by acidic conditions. As a natural polymeric scaffold, the chitosan SETs has suitable water vapor permeability (WVP). The function of WVP is to maintain wound bed moisture; to increase regeneration of the dermis; and most importantly to accelerate wound healing. Additionally, the chitosan SETs has tensile strength, which functions to increase dermal and epidermal regeneration [9], [24].

L. Primary Culture and Characterization of HFSCs

In the current study, an overnight incubation with collagenase type I was performed to release hair follicles from the dermis of human scalps. After 48 hours of cell culture, groups of squamous-like cells were observed to be growing in supplemented CnT-07 media. After two weeks of culture, the grown cells had reached 70-80% confluence (Figs. 3A and 3B). To identify and validate the presence of HFSCs in the cultures, K15 and CD200 were used and showed positivity, indicated by brown color, in the cell membrane (Figs. 3C and 3D). Many studies have shown that K15 is highly expressed in epithelial stem cells from hair follicles [25]-[28]. Furthermore, in [29] show that stem cells expressing CD200 have high colony-forming efficiency. CD200 has been proven to be the best positive surface marker for the human hair follicle bulge.

In this current study, the characterization of HFSCs was performed by examining the surface markers of CD200 and K15. Other negative surface markers such as CD34 and CD271 were not performed. Inoue and co-workers [30] confirmed that bulge cells possess K15-positive, CD200-positice, CD34-negative and CD271-negative markers. Determination of negative surface markers is useful for characterizing freshly isolated human epithelial cells. Negative surface markers for HFSCs are recommended to double confirm the characterization of stem cells. Meanwhile, other positive markers for HFSCs are recommended including nestin [31] and neural crest characteristics [32]. The presence of stem cells with neural crest characteristics and nestin-positive marker in hair follicle is useful to confirm its pluripotency.

To date, there is no commercially available of epithelial stem cells derived from the human hair follicle. The epithelial stem cells or epithelial cells derived from colon, tongue, or other organ are existed but not from the hair follicle. In European countries and USA, there are many centres of stem cells for storage or commercial purpose. As HFSCs are important in skin tissue engineering research, the plan for collaboration with the private sector for the establishment of HFSCs is highly suggested.
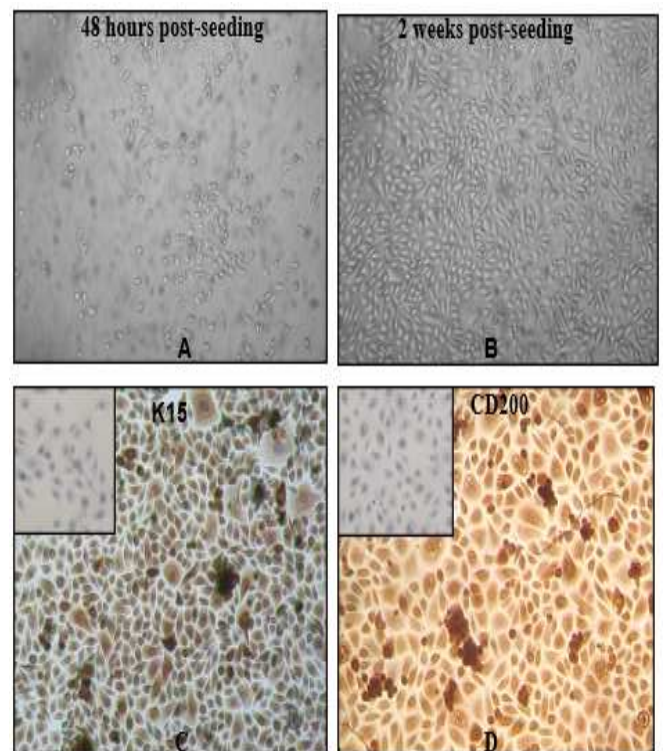

Fig. 3 Primary culture of human HFSCs (A,B). Magnification 100x. Characterization of HFSCs using K15 (C) and CD200 (D). Magnification 100x. Inserts represent negative control

M. Attachment and Proliferation of HFSCs on Chitosan SETs

After 72-hour cultivation, HFSCs attached to and proliferated on the chitosan SETs (Fig. 4A). Live HFSCs predominantly covered the surface of the chitosan SETs, as indicated by green fluorescence (Fig. 4B). Meanwhile, red fluorescent dead cells were minimally found. In addition, a live/dead cell assay of the HFSCs on the chitosan SETs showed significant green fluorescence, indicating that more HFSCs were viable. According to the graph, at 72 hours, the population of living HFSCs on the chitosan SETs was double compared with the population of dead HFSCs (Fig. 4C). The viability assay of the HFSCs on the chitosan SETs is presented in Table 1; this assay demonstrated that the growth of the HFSCs increased over time.
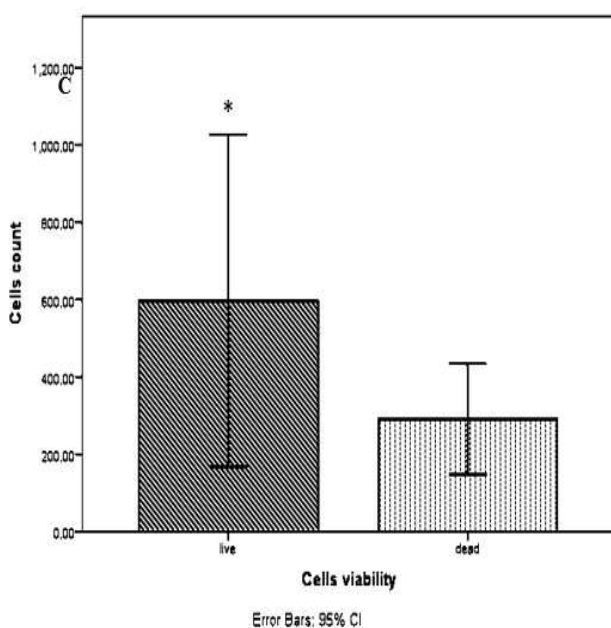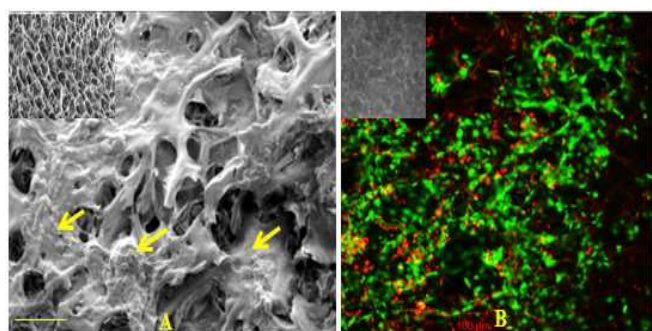

Fig. 4 Analysis of cell-chitosan construct. SEM images of HFSCs at 72 hours post-seeding on the chitosan SET (A). The HFSCs have attached and growth on the surface of the scaffold (arrows). Insert is the chitosan without cells. Scale bar 200 μm. Live/dead assay of HFSCs at 72 hours post-seeding on the chitosan (B). The viable of HFSCs expressed green fluorescent meanwhile the dead cells expressed red fluorescent. Insert is the control. Scale bar 100 μm. Graph of living and dead HFSCs on the chitosan SRT at 72 hours post-seeding (C) (n = 3). $^*P \leq 0.05$

This promising finding of HFSC viability and growth using a chitosan SETs has increased the interest of researchers in employing this scaffold for the fabrication of epidermal substitutes [6], [9], [11]. Chitosan skin substitutes and chitosan SETs have been demonstrated to accelerate wound healing in irradiated rats. Both of these materials have also been found to significantly decrease the size of the scar [6]. In addition to the above finding, the current study suggests that the chitosan SETs supports the initial attachment, spreading and proliferation of HFSCs in vitro. This phenomenon is likely due to close binding between the positively charged chitosan and the negatively charged cells [21]. Other reports have demonstrated that both fibroblasts and keratinocytes attached to and proliferated well on a chitosan-based scaffold [33], [34]. A previous study [35] has successfully created chitosan-based scaffolds that were used to construct an artificial skin bilayer in vitro using a co-culture technique, and other studies demonstrated that the peripheral nerve could also be regenerated [6], [25], [36]. All of the reports have shown that chitosan-based material is a promising scaffold for the attachment and proliferation of mostly skin cells, which support skin regeneration.

Another suggestion to increase the attachment of HFSCs onto chitosan is via co-culture with fibroblasts. The dynamic of co-culture between fibroblast and epithelial cells in skin tissue engineering has clearly shown that the interaction of these two cells regulate skin homeostasis, control the growth, migration, and differentiation of epithelial cells, and establish autoimmune activities. Co-culture between HFSCs and HDFs in the previous study has shown an interaction by which a skin-like structure was produced after HFSCs were overlaid on HDFs. The HFSCs were overlaid on the fibroblasts after the fibroblasts were seeded in chitosan for two weeks and the co-culture was done for a further one week. The high density of cells ($3x10^6/cm^2$) was chosen as it promotes cell attachment, infiltration, and uniform distribution throughout the construct [37]. Co-culture between HDFs and HFSCs may secret various soluble cytokines, growth factors and extracellular matrix components which promote proliferation of epithelial cells.

TABLE I
OD570 VALUE OF HFSCS VIABILITY CULTURED ON THE CHITOSAN SET
(MEAN ± SD, N = 6)

Day 1	Day 3	Day 6	Day 7
0.04 ± 0.02	0.067 ± 0.02	0.115 ± 0.01	0.136 ± 0.01

N. Differentiation of HFSCs on Chitosan SETs

The investigation of HFSC differentiation into keratinocytes on chitosan SETs was performed. The cells on the chitosan SETs were differentiated into epidermal keratinocytes, as indicated by the green fluorescence staining of the markers K6 and involucrin (Figs. 5A and 5B). Stem cells have the ability to differentiate, providing an ideal source for skin replacement products. Stem cells can differentiate into specialized cell types in vitro, as demonstrated by HFSCs, adipose stem cells and bone marrow-derived human mesenchymal stem cells [1], [38], [39]. Previous studies have reported that stem cells proliferated on chitosan-based scaffolds and successfully differentiated into nucleus pulposus (NP)-like cells and osteoblasts [40], [41]. A report by [42] showed that HFSCs adhered, proliferated, and differentiated on a poly-L-lactic acid nanofiber scaffold for peripheral nerve regeneration. Our study aimed to obtain baseline knowledge of the differentiation of HFSCs into keratinocytes, which, in the

future, could be used for skin grafting. The differentiation of HFSCs into epidermal keratinocytes is an alternative way to achieve skin regeneration in a burn patient. Classically, epidermal keratinocytes were isolated from a skin sample and propagated on murine fibroblasts that were first irradiated, known as a feeder layer. The feeder layer supported expansion and proliferation of the cells. The culture duration for the keratinocytes was prolonged until a thick layer that contained two or three sublayers were fabricated, known as a cultured epithelial autograft (CEA) [43]. CEAs are used in the treatment of burn patients and are combined with nylon or polyglactin mesh [44]. In the current study, the use of a feeder layer to grow HFSCs was omitted, providing a simplified technique for differentiated keratinocyte formation. Therefore, HFSCs were cultured on a chitosan SETs as an alternative to feeder layer adaptation.
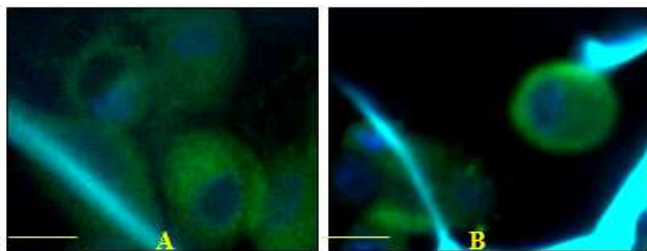

Fig. 5 Differentiation of HFSCs into keratinocytes on the chitosan SET at 72 hours post-seeding. The HFSCs expressed green fluorescent for involucrin (A) and K6 (B). Nucleus stained blue. Chitosan stained light blue. Scale bar 200 µm

Although HFSCs can be grown and differentiated on chitosan SETs, in our opinion, more studies are needed to facilitate the proliferation of cells on chitosan SETs. One effective approach is to use a bioreactor, which enables the circulation of nutrients and eliminates waste during the development and proliferation of HFSCs on scaffolds and which enhances the rate of growth. A bioreactor provides complete monitoring of and control over intrinsic factors [45]. In addition, a bioreactor allows the investigator to determine the roles of specific biological, chemical and physical parameters. TransCyte and Dermagraft are among the products that have been produced using a perfusion bioreactor [46].

Apart of chitosan that supporting the cultivation of stem cells and cells of connective tissue, there are a few scaffolds that support the cultivation of human cells. Collagen, for instance, is the most abundantly used for human skin cells' growth and differentiation. Collagen is easily found in bovine, porcine or fish and as the main resource in tissue component of tendon, dermis, bone and other parts of the bodies. Biocompatibility and biodegradability of collagen increase its application for skin grafting in varies form such as film, paste, gel, and sponge. However, collagen from the mammalian source causes prion disease transmission to human. Another crucial scaffold that mainly used in surgery is fibrin. Fibrin is isolated in human blood and increases the renewal of damage tissue. After mesenchymal stem cells derived from bone marrow are seeded into fibrin, the tissue regeneration has successfully engineered with less scar formation. The limited source of row human blood affects it uses in a the clinical setting. Commercial fibrin kit is available however, patients with severe disease are not affordable to pay due to its high cost. Therefore, chitosan is an alternative scaffold for tissue regeneration based on its low cost, do not introduce transmission to human and highly foster healing process.

IV. CONCLUSION

Our study showed that HFSCs were successfully isolated and further proliferated in three-dimensional cultivation. Human anti-K15 and anti-CD200 antibodies were used for verification of isolated cultures, and a live/dead assay was used to confirm the viability of the HFSCs during cultivation on chitosan SETs. Subsequently, we tested the ability of the HFSCs to differentiate into epidermal keratinocytes after culture in epidermal induction medium. In addition, the current study showed that a chitosan SETs supports the attachment, proliferation, and differentiation of HFSCs, which is promising for application in skin tissue engineering.

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