



ISOLATION OF HAIR FOLLICLE STEM CELL AND ITS THREE-DIMENSIONAL PROLIFERATION ON POROUS CHITOSAN SCAFFOLD

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ABSTRACT

The bulge region of hair follicle is the reservoir for multipotent adult stem cells, the hair follicle stem cells (HFSCs). The acquiring of a three-dimensional (3D) construct can further enhance the multipotent features of HFSCs in human skin tissue engineering. However, the proliferation pattern of the HFSCs in the 3D porous chitosan scaffold has not been thoroughly investigated yet. This study aims to isolate HFSCs from human scalp tissue and to further investigate its 3D proliferation potential in a 3D porous chitosan scaffold. HFSCs were isolated from human scalp tissues after being digested in collagenase type-I prior to the culture in CnT-07 growth media. The morphological and biological characteristics of cultured HFSCs were observed using inverted light microscopy and confocal laser scanning microscopy, respectively. The HFSCs were then verified using keratin-15 (K15), β 1-integrin and keratin-19 (K19) markers in immunocytochemical assay. In the 3D proliferation of HFSC, the cells were seeded on the chitosan construct and the growth of cells was evaluated at 24, 48 and 72 hours using a live/dead cell assay. HFSCs were successfully isolated and grown in the CnT-07 growth media. The cultured HFSCs were positive to the presence of K15, β 1-integrin and K19 antibodies. The HFSCs attached and have proliferated on the 3D porous chitosan scaffold. Human HFSCs can be isolated and propagated *in vitro*. They can be verified by the presence of K15, β 1-integrin and K19 markers. The proliferation potential of HFSCs in the 3D porous chitosan scaffold may further provide insights for human skin tissue engineering in the future.

1.0 Introduction

Unethical issues that often limit clinical potential of human embryonic stem cells have currently shed light into the research on adult stem cells from adipose, bone, blood and hair follicle. However to date, identification and isolation of human adult stem cells remains a challenge in the regenerative medicine. Various studies have suggested that the bulge region of hair follicle contains [³H] thymidine label-retaining cells (LRCs), which is a marker for the presence of

anagen follicular papilla signals during the initiation of follicle growth cycle [1, 2]. Taylor *et al.* [3] reported that epidermal regeneration in a wounded mouse involved the migration of HFSCs from the bulge region. These multipotent bulge cells are able to differentiate into sebocytes [4], melanocytes, neuronal and smooth muscle cells [5]. Furthermore, HFSCs can regenerate into new hair follicles when combined with neonatal dermal cells in the reconstitution assays, which may allow hair re-growth [6].

Chitosan is a partially deacetylated derivative of natural chitin [7] that is often being used as a dressing material in wound management [8, 9] and as a scaffold in tissue engineering [10, 11, 12]. The usage versatility of chitosan as a biomaterial is mainly attributed to its excellent biocompatibility [8, 9, 13]. Chitosan oligosaccharides have a stimulatory effect on macrophages, with both the chitosan and chitin have been reported to be able to attract neutrophils in accelerating wound healing *in vitro* and *in vivo* [14]. Yet, to the best of our knowledge, there is no thorough investigation on the proliferative activity of HFSCs in a 3D porous chitosan scaffold. Therefore in this study, we aimed to isolate the residing stem cells in hair follicle bulge from human scalp tissue and to investigate the 3D proliferation potential of the HFSCs on a porous chitosan scaffold.

2.0 Materials and Methods

2.1 Isolation and cultivation of HFSCs

The study was approved by the Human Ethic Committee of Universiti Sains Malaysia (USM/KK/PPP/JEPeM (212.3[5])). Six scalp samples were obtained from the patients undergoing surgical procedures with informed consent. Scalps were briefly rinsed with 70% alcohol (Sigma) and washed in Dulbecco's modified phosphate-buffered saline (DPBS) (Gibco). Scalps were then cut into small pieces and incubated in a dispase (2.4 Unit/mL) (Gibco) at 37 °C for 4 hours followed by the separation of epidermal layer from the dermal layer. The dermal fragments with the intact hair follicles were further digested in collagenase type-I (Gibco) (200 Unit/mL) overnight at 37 °C. The cells were seeded in the mixtures of CnT-07 growth medium (Cell-N-Tech) and Dulbecco's modified eagle medium (DMEM) (Gibco) (1:1), 5% of fetal bovine serum (FBS) (Gibco) and 1% of antibiotic-antimycotic (Gibco) for 24 hours before being cultured in the CnT-07 growth medium. The cultured cells were incubated at 37 °C in a 5% CO₂ incubator. The cells were replenished every 3 days with the CnT-07 growth medium.

2.2 Immunocytochemical analysis of HFSCs

HFSCs were seeded in a 24-well plate at a seeding density of 1×10^4 cells/mL. Upon reaching 70% of confluence, the cells were fixed in an absolute methanol (Sigma) at 4 °C for 15 minutes followed by the addition of a normal goat serum into each well. Mouse monoclonal anti-human K15 (Abcam Inc, dilution 1:1000), mouse monoclonal anti-human K19 (Abcam Inc, dilution 1:500) and mouse monoclonal anti-human β 1-integrin (Abcam Inc, dilution 1:500) was each added to the cells and incubated for one hour. This was followed by a 30-minute incubation of a secondary antibody reagent (conjugated to biotin) before being further incubated with the streptavidin horseradish peroxidase for 20 minutes. The

3,3'diaminobenzidine (DAB Substrate) was added and the cells were incubated for another 20 minutes. The nucleus was then counterstained with hematoxylin.

2.3 HFSCs on porous chitosan scaffolds

Chitosan powder (Hunza Nutraceuticals, Malaysia) with a molecular weight of 634,000 kDa and a degree of deacetylation of 89% was used in this study. To prepare a 2% (w/v) chitosan solution, chitosan powder was dissolved in 1% (v/v) acetic acid. Chitosan solution was then adjusted to a pH of 6.2 by 0.2M sodium bicarbonate before being freeze-dried at -20 °C in a graded porous mould. This was followed by a 24-hour lyophilization process in order to fabricate a chitosan sponge. The porous chitosan scaffolds were sterilized using ethylene oxide and they were further quarantined for a month before use. The porous chitosan scaffolds were then washed in a DPBS prior to cell seeding.

HFSC cultures were rinsed with 10 mL of DPBS and were detached using TrypLE™ Express (Invitrogen). The cells were centrifuged for 5 minutes at 300g, before being suspended in CnT-07 growth media. The cells were counted using a haemocytometer and were seeded onto the porous chitosan at a seeding density of 1×10^5 cells/well. The cultures were incubated in the CnT-07 growth medium at 37 °C in a 5% CO₂ incubator.

2.4 Cell proliferation in porous chitosan scaffold

Cell proliferation in the porous chitosan scaffold was determined using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen) at 24, 48 and 72 hours. The porous chitosan scaffolds seeded with HFSC were washed three times using a DPBS for 5 minutes each followed by a 45-minute incubation in 200 μ L DPBS solution containing 2 mM calcein AM and 4 mM ethidium homodimer-1 reagents at room temperature. The cells stained with fluorescence were viewed using a confocal laser scanning microscope (CLSM) (Leica Confocal Microscope), at an excitation wavelength of 488 nm and a reference wavelength at 568 nm.

3.0 Results

Dermal scalp with intact hair follicles was completely digested after an overnight incubation in the collagenase type-I. At 48 hours post-seeding, small groups of squamous-shaped cells appeared in the culture grown in CnT-07 media. The cells have reached 70-80 % confluence in about two weeks time of culture (Figure 1).

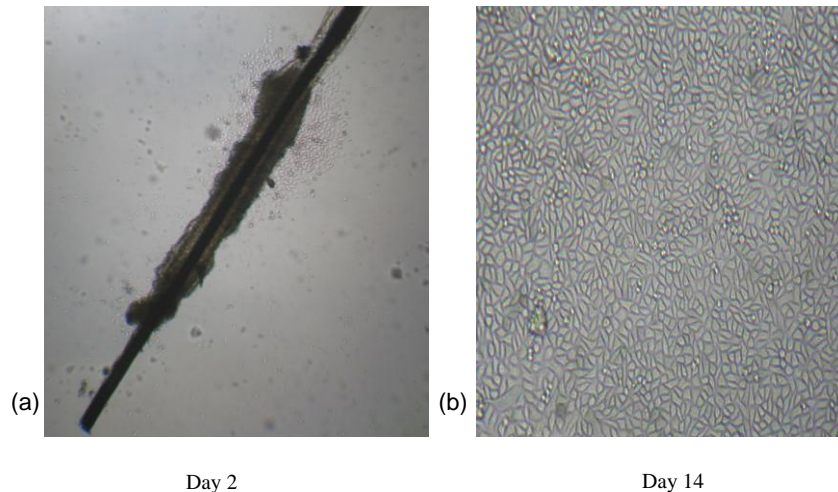


Fig. 1 Primary culture of human HFSC. (a) 48 hours post-seeding (40x) (b) 2-week old of culture (100x).

K15, β -1 integrin and K19 antibodies were used as the cell markers to identify the presence of HFSCs in cultures. In this study, the cultured cells were stained brown, showing that K15, β 1-integrin and K19 were expressed in the cultures,(Figure 2).

In the 3D cell proliferation of HFSCs, a significant number of viable cells (emitted in green fluorescence) have been observed on the porous chitosan scaffold. However, only a few dead cells emitted in red color were present on the porous chitosan scaffold throughout the experiment. This data further suggests that HFSCs have attached and proliferated on the porous chitosan scaffold (Figure 3).

shown the *in vitro* differentiation potential of HFSCs into skin substitutes [16]. However, the attachment and proliferation of HFSCs on the 3D porous chitosan scaffold have not been thoroughly researched. In this study, the dermal scalp with intact follicles was digested in collagenase type-I for the dislodgement of hair follicles from the scalp tissues. Removal of the epithelial tissues prior to the dermal digestion can minimize the cross contamination consisting of epithelial cells and bulge cells in the cultures. The hair follicles released from the dermal scalp in this study have visibly attached to the flask within the first 24 hours, and is usually followed by an outgrowth of adherent squamous-shaped cells from the hair follicles.

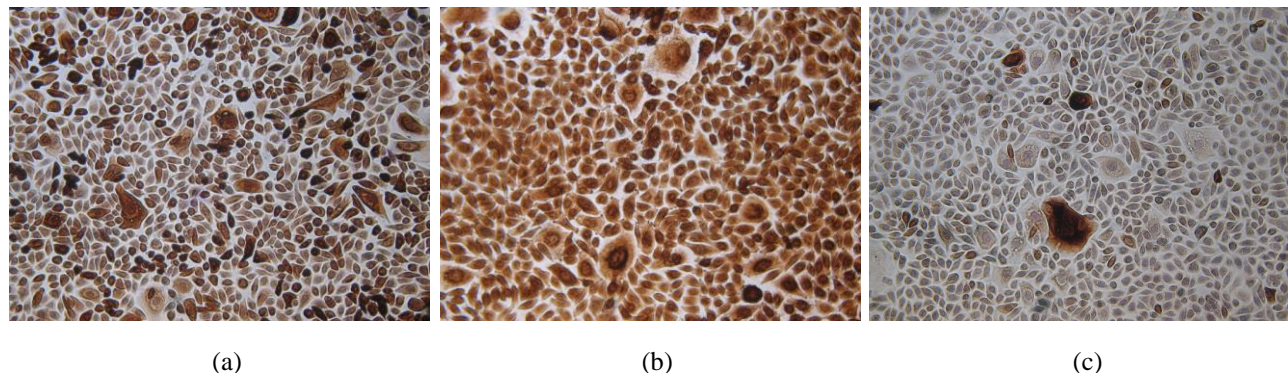


Fig. 2 Immunocytochemical staining of cultured HFSCs (a) K15 (b) β 1-integrin (c) K19. (200x).

4.0 Discussion

Many have reported that hair bulge is a niche area for the presence of HFSCs [1, 2, 15]. Previous studies have further

Nevertheless, the follicles were found detached after a several growth medium replenishments, leaving only the squamous-shaped bulge cells in the cultures. The use of an enzymatic digestion in the isolation of cells may avoid the bulges from being damaged by mechanical force [16]. In a study by Wang *et al.* [16], only 30% of the hair bulges attached to plastic culture plate at 24 hours post-seeding before a few colonies of

cells were able to branch from the bulge area in serum-free keratinocyte medium. However for the first time in this study, CnT-07 growth medium has successfully supported the growth of the isolated HFSCs which has not been previously reported.

corroborates our previous study [26]. Chitosan is a promising biomaterial that can be fabricated into various porous structures for tissue regeneration *via* lyophilization of a chitosan solution with acetic acid. In the attempt to grow the HFSCs in a 3D structure, we have successfully demonstrated the proliferation of HFSCs on the porous chitosan scaffold,

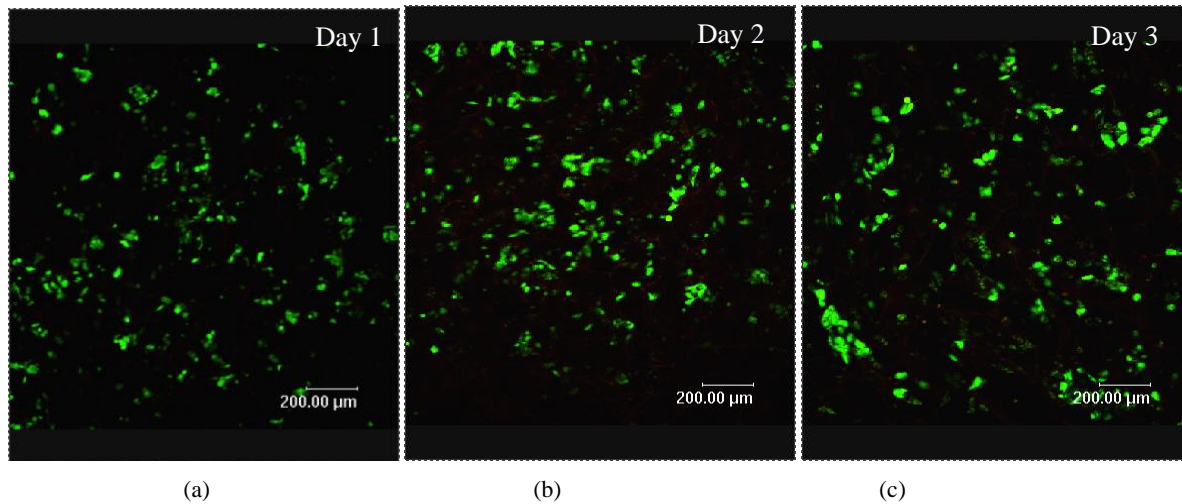


Fig. 3 Immunocytochemical staining of cultured HFSCs (a) K15 (b) β 1-integrin (c) K19. (200 \times).

Several studies have reported that K15 is a significant marker for bulge stem cells in mice and humans [16,17,18,19]. Michel *et al.* demonstrated that K19-positive-cells were also the [3H]thymidine-label-retaining cells, suggesting that K19 can be a positive marker for skin stem cells derived from hair follicles. They further proved that K19-positive-cells expressed high level of β 1-integrin [20] which was also in agreement with Akiyama *et al.* [21]. In addition, Kloepper *et al.* [17] suggested that useful positive markers for human bulge cells are usually K15, K19 and CD200.

In this experiment, K15, β 1-integrin and K19 were used as the cell surface markers for the validation of HFSC cultures. The immunocytochemical analysis proved that K15, β 1-integrin and K19 were expressed in the cultures, which corroborates most recent findings [16,22,23,24]. HFSCs are usually involved in the forming of hair follicles and epidermal cells [3]. Bioengineered skin made up of HFSCs has significantly improved wound healing within 2 weeks by forming the dermis and epidermis [16].

The chitosan scaffold is purposely made in a 3D porous structure to provide space and guide cellular proliferation and differentiation as well as helping cellular organization and eventually facilitate the tissue development *in vitro* and *in vivo*. 3D porous structure of a scaffold is an important requirement in tissue engineering [25,26]. Liu *et al.*, [27] suggested that a scaffold with pore size ranging from 100 to 200 μ m is suitable for skin tissue engineering which further

indicating that it is not only biocompatible but potentially be useful in skin tissue engineering, as in agreement by a few reports [8,9]. Chitosan biomaterials have been mostly reported to support cell adhesion and proliferation of skin dermal fibroblasts [28] and keratinocytes [29]. However in recent studies, 3D proliferation involving stem cells such as adipose stem cell in chitosan has further revealed that not only cells proliferated well but the stem cell characteristics and its pluripotency were also maintained in the 3D construct [30,31].

In this study, a conventional Petri dish culture system was used to propagate the HFSCs in 3D chitosan. Although it has achieved to some extent of success, a new and an improved way to propagate and enhance 3D proliferation of cell in tissue engineering is through the use of a bioreactor such as spinner flask, rotating, and perfusion systems. The bioreactor system can enhance mass transfer by supplying good fluid flow, provide mechanical forces that guide tissue development, and allow better control over culture conditions [32]. However, further research is underway to refine the multipotency of HFSCs in the 3D chitosan scaffold for tissue engineering.

5.0 Conclusion

HFSCs have been successfully isolated from human hair follicles and they can be grown in CnT-07 growth medium. HFSCs were verified by the presence of β 1-integrin, K19, and

K15 antibodies, which can be the cell markers for the characterization of HFSCs. The cell ingrowth of HFSCs on the chitosan porous scaffold further shed light into the promising skin tissue engineering in the future. However, further studies are underway to unravel the multipotency of HFSCs associating with the use of chitosan porous scaffold.

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