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The Trans-Differentiation of Keratinocyte: Requisite for Skin Wound Healing and Cosmetic Surgery

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Abstract

Objective: Skin wound healing involves the interaction of epidermal and dermal cells, growth factors, and cytokines. Keratinocyte is the most dominant cell type in the epidermis and plays multiple roles for skin repair function. Cultured human primary keratinocytes are required for the treatment of burns, cutaneous wounds diabetic foot ulcers, and cosmetic therapy.

Methods: Human primary keratinocytes from different age groups were isolated, characterized and efficacy was analyzed. Primary human keratinocytes were purchased from commercially available sources and their trans-differentiation properties were studied.

Results: Serum-free media with high calcium was used to isolate keratinocytes from different donors with varied age groups. The number of keratinocytes isolated from the total population of cells varied depending upon the age. Periodical photographs were being taken for analysis. Quantitative analysis of percentage expression of CK5 and CK10 has been done in keratinocytes and fibronectin and collagen staining has been done in transdifferentiated fibroblast.

Conclusion: Isolation of primary keratinocytes brings the hope that healthy cultured keratinocytes are useful for the wound microenvironment. Trans-differentiation of keratinocytes to fibroblast provides a new angle for understanding the interaction between keratinocytes and fibroblast in cosmetic surgery and wound healing.

Keywords: Skin wound healing; Trans-differentiation; Cultured keratinocytes; Primary human keratinocytes; Dermal fibroblast

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Introduction

Advanced treatment strategies for skin regeneration are the first and foremost step of cutaneous wound healing. Keratinocyte therapy is the integral component of cell-based therapy for burn wounds and other wounds in which cellular material, keratinocyte injected, grafted or implanted into patient for healing of wound repair. Transdifferentiation is the process in which one type of adult tissue undergoes a phenotypic switch and differentiates it from another type of functional adult tissue. This is caused by a change in the expression of a master switch gene and the cells exhibit the functional characteristics. The skin wound repair varies upon the balanced process of cell proliferation and differentiation of epidermal stem cells, keratinocytes and fibroblasts [1]. Advances in medical treatments have facilitated survival rates of severe patients who face difficulties in healing the wound. Primary keratinocytes in the form of cultured epithelial autografts (CEA) were used clinically for the first time in the early 1980s. Since 1984 good results have been achieved while using CEA by several groups. During the process of normal wound healing, the skin allows repairing itself; however extensive burn patients, diabetic ulcers, and road accidents require intervention for tissue restoration. Especially, the role of Keratinocyte in donor site healing and deep burn wound healing is a distinctive event. Several research groups used keratinocyte cells to induce the regenerative capacity of residual cells at the wound surface. Cultured epithelial autograft (CEA) are aseptically processed wound dressings, approved by FDA in 2007 [2]. The average

culture time of epithelial cells has been improved and the long-time interval between biopsy and grafting has been reduced as a result of which cells derived from a small biopsy have been used for enhancement in the speed of re-epithelialization. The main advantage of cultured epithelial autografts is that large burn wounds can be covered with autologous cells derived from a small biopsy. In terms of cosmetic results also, the improvement in the speed of re-epithelialisation has been reported by various groups and CEA seems to have better results when compared to widely meshed autograft. However, the greatest merit of CEA is that it harnesses the potential for grafting of epidermal keratinocytes and it has preserved sufficient proliferative capacity. Taken together, the keratinocyte-based treatment is promising, and it holds the future impact of keratinocyte-mediated cell coverage options, upon which is promising, but more research is needed [3]. Additionally, keratinocyte-based treatments need to be pursued carefully, as the other side of the story invites hypertrophic scarring if over-activation of keratinocytes can subsidize. As the cell culture is an expensive process and the cost-benefit relationship of this method is comprehensively discussed and the potential of graft site will be devoid of any carcinoma or scars with the use of keratinocyte transplantation. The merits of cultured keratinocytes have been reviewed and the results obtained through basic research can be incorporated into the medical treatment of burns [4]. The anticipated results can be acquired through donor site healing as well as skin tissue regeneration. In the light of reality, the appropriate success of healing will be accomplished by means of cultured keratinocyte transplantation. It is remarkable to note that, the process of trans-differentiation of keratinocyte to fibroblast has been proven by using primary human keratinocytes. Also, similar phenomenon was observed in the same way while using mouse and rat keratinocytes [5].

Materials and Methods

Reagents

Primary human keratinocytes (PCS-200-010) were purchased from ATCC collection (ATCC, LGC Standard, and Poland). Human Primary Keratinocyte cell culture media Cascade BiologicsTM EpilifeTM (MEPI500CA) and supplemented with Cascade BiologicsTM Supplement S7(S0175), TrypLE Select, were purchased from GIBCO (GRAND, Island, NY, USA). Antibody cytokeratin 5, (CK5) (A2662) and collagen I (A5786) from ABclonal (ABclonal, USA), Anti-CK10 (ab111447), Fibronectin (ab6328) was purchased from Abcam (Abcam, Cambridge, UK). Dispase II and DAPI (4,6-diamidino-2-phenylindole from Sigma Aldrich (Sigma, USA) [6].

Skin Sample collection & isolation of Keratinocytes

With the approval of the Institutional Ethics committee, normal skin biopsies were collected from the extra surgical discards with written consents from the donor/patients/caregiver. Samples positive for HIV, HbsAg, HCV, microbial contaminated skin samples, known cases of cancer, active jaundice, STDs were excluded from the study. Samples of approximately 2x2 cm from normal (thigh region) tissue was collected and processed for keratinocyte culture. A maximum of 2x2=4 cm2 tissue was washed in phosphate-buffered saline (PBS) and incubated in 10 mL of 2.5mgmL (0.25 %), Dispase II in Keratinocyte-specific basal medium and left for one hour at 37 deg or overnight at 4 °C [7]. Then epidermis was mechanically separated from the dermis with fine forceps and incubated in TrypLE Select from GIBCO (GRAND, Island, NY, USA)) for 5-10 min at 37 °C. Upon cellular dissociation, trypsin activity was reduced by diluting the solution with three volumes of fresh Human epidermal Keratinocyte-specific medium. Keratinocytes were then collected through centrifugation and suspended in Human Primary Keratinocyte cell culture media. The washing step was repeated 4 to 5 times and finally centrifuged at 1100 rpm for 5 min. After removing the supernatant, the cell pellet was re-suspended in a complete keratinocyte culture medium first with 1 ml. Then total cell number was counted and finally re-suspended with 10 to 15 ml. Cells were seeded into a 25 cm2 culture flask (BD Falcon, Stockholm, Sweden). Sub-confluent primary cultures were serially passaged at 1 × 104 cells per cm2. On every second day, the medium was changed throughout the study. The primary culture was sub-cultured into 1:3 when sub-confluence had been achieved. It is remarkable to note that, the isolation methods use serum-free media [8].

Assessment of keratinocyte viability by Trypan blue Dye

Each time, while passaging, the cell pellets were dissolved in the 1 ml culture medium and from the cell suspension; dilutions were made in Trypan blue and were taken for counting. The number of living and dead cells was counted in a haemocytometer, by triplicate samples of cell suspension 10 µl, each mixed with Trypan blue 90 µl. Live cell percentage was more than 90 % every time. Normal Human Keratinocytes were isolated from normal skin samples obtained from operation theatre and primary cultures were Initiated and maintained in a replicative state [9].

Trans-differentiation of Primary human Keratinocytes

The primary human keratinocytes are used, were available commercially from ATCC (PCS-200-010). The keratinocytes were seeded in a T-25, Nunc flask for 24 hours. After keratinocyte morphology appears, media switching is done for dermal fibroblast. DMEM [GIBCO- REF#10569-010] with 10% fetal bovine serum [GIBCO- Ref-25030-01] along with L-glutamine [GIBCO- REF#25030-081] derived the keratinocytes to attain fibroblast-like morphology. The keratinocyte-specific markers such as cytokeratin 5 (CK5), PAR2 were used before the start of transdifferentiation process and after 2 weeks, the dermal fibroblast specific markers such as collagen -I and fibronectin were done [10].

Immunocytochemistry

Human primary epidermal keratinocyte cultures were grown on collagen-coated cover glass. In sub-confluent label fixed in 4% paraformaldehyde in 1× PBS for 15-20 min and permeabilized in PBS/ 0.1% Triton X-100 solution for 5 min. Samples were washed three times with PBS, followed by blocking with 1% BSA (bovine serum albumin) for 15 min and then incubation with primary

antibodies overnight at 4 °C. Dilution of the primary antibody used as per the datasheet provided by the manufacturer. Following three washes with PBS, the samples were incubated with fluorescence-labelled secondary antibodies for 1 h at RT to visualize the antigens. Additional three washes were given with PBS and nuclei of the samples were counterstained DAPI and mounted with Vectashield without DAPI (Life Technologies) and visualized under a Nikon florescence microscope (Nikon, Japan) [11].

Results

Morphological and growth measurements of keratinocytes

The use of commercially prepared various kinds of keratinocyte media such as low-calcium, serum-free medium, the fetal bovine serum-containing medium was reported by several groups. However, this method of isolation is used as serum-free media with high calcium, which is suitable for burn patients. During the isolation of keratinocytes processes, starting from seeding the cells to sub-culturing, the number of cells was counted, and periodical photographs were taken for analysis [12]. The number of keratinocytes isolated from the total population of cells varied depending upon the age of the sample. The total number of cells obtained from 4 sq cm is shown in (**Figure 1**). About 2.7 million cells are obtained from 1 square centimetre of skin approximately. The Keratinocyte became confluent after 7 days and was ready for sub-culturing. Within a week of time, keratinocytes are almost 90% confluent. Live image captured on days 1, 3, and 7 as shown in (**Figure 2**). Sub-culturing, cell counting was done in each passage number from P1 to P2 and the doubling number implicates the doubling time of the keratinocytes was found to be 18-72 hours [13].

Characterization and Potential of Keratinocytes

While characterizing the keratinocyte, the cytokeratin 5 (CK5) expression pattern has been observed. Among five different age group samples, cytokeratin 5 expressions have been observed in the early passage (P1) and it was found that, 86 to 98 % of expression prominently observed and quantitation, which is shown in (**Figure 3A & 3B**). During the subsequent passages, the percentage of CK5 expression is almost being maintained up to passage number 7, which indicates that the keratinocyte population, which was isolated in the proliferative stage, is retaining the multiplying capacity [14]. It predicts that the proliferative keratinocyte may bring better wound healing at the wound site. In addition, the differentiated keratinocyte marker Cytokeratin 10 (CK10) have been observed in late passages (P6-P8) and it was found that CK10 expressing cell number increases from 10-15% only as shown in (**Figure 4A & 4B**), which clearly emphasizes that the differentiation capacity would not be dominated over proliferation. Quantitative analysis of percentage expression of CK5 and CK10 has been done using image-J software [15,16].

Trans-differentiation of Primary Human Keratinocytes

The primary human keratinocytes, obtained from available commercial sources followed a similar pattern of proliferation as primary human keratinocytes isolated directly from human skin. The doubling time was noted and up to passage no 11 and it did not change. The expression of CK5 from early passage to later passage remains almost the same. The collagen I expression was absent in primary human keratinocytes which is a notable point. During 2 weeks of trans-differentiation, changes in the morphology of keratinocytes were observed as fibroblast-like morphology appeared and bright-field images captured on days 1, 3, 7, and 12 are shown in **Figure 5(A, B, C & D)**. The dermal fibroblasts play a critical role in synthesizing the extracellular matrix components (ECM) like collagen I, fibronectin, fibrin which are considered potent protagonists for the healing of wounds.

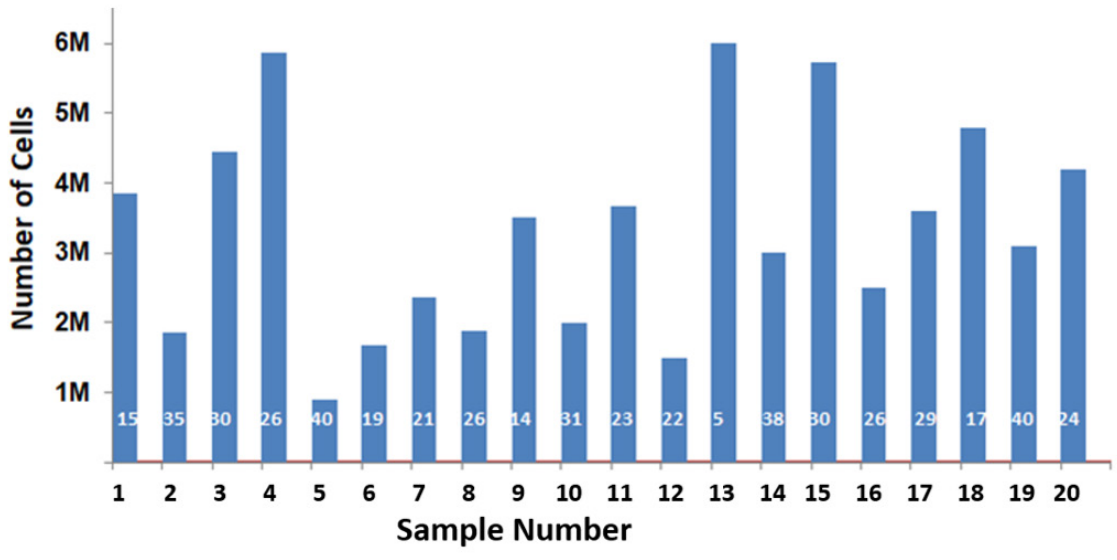

Figure 1 Primary human keratinocyte cells isolated from donor.

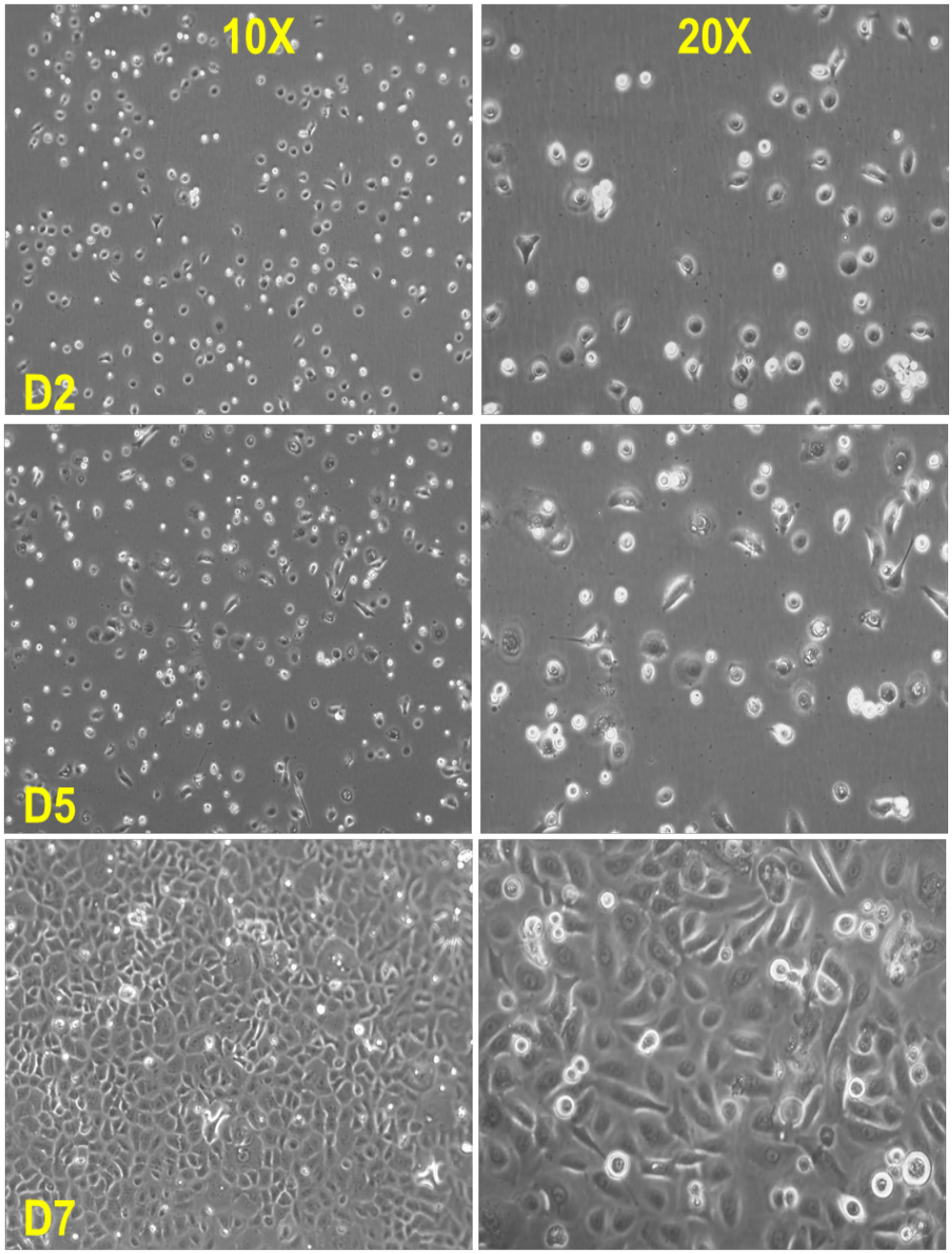

Figure 2 Morphology of keratinocytes shown in different days of culture.

Immunocytological analysis has been done for fibroblast specific markers and expression of the collagen I and fibronectin and High magnification of the fibronectin network both are shown in **Figure 6(A, B & C)**.

Discussion

Keratinocytes play a vital role in wound closure and are the most abundant cell type in the epidermis. They prevent desiccation and provide immunological and barrier mechanisms of defense against potential pathogens. With the discovery of the introduction of cultured autologous keratinocytes new perspective emerged in solving the problem of coverage of extensive burns and diabetic ulcers. The first clinical application of culture Epithelial Autograft (CEA) was performed in 1981 following the successful serial cultivation of keratinocytes from skin biopsy by Green et.al in 1979 [17]. But even after three decades, there are mixed results of the clinical efficacy of the CEA for the closure of deep burn wounds. But keratinocyte culture has been proved beneficial in accelerating the wound-healing when used as an adjunct therapy. Today cultured keratinocytes are used in many forms (spray, sheets, in composite grafts) for wound healing around the world. In 1986 madden et.al reported that allogenic cultured keratinocytes when used in superficial to deep dermal burns resulted in improved wound healing without

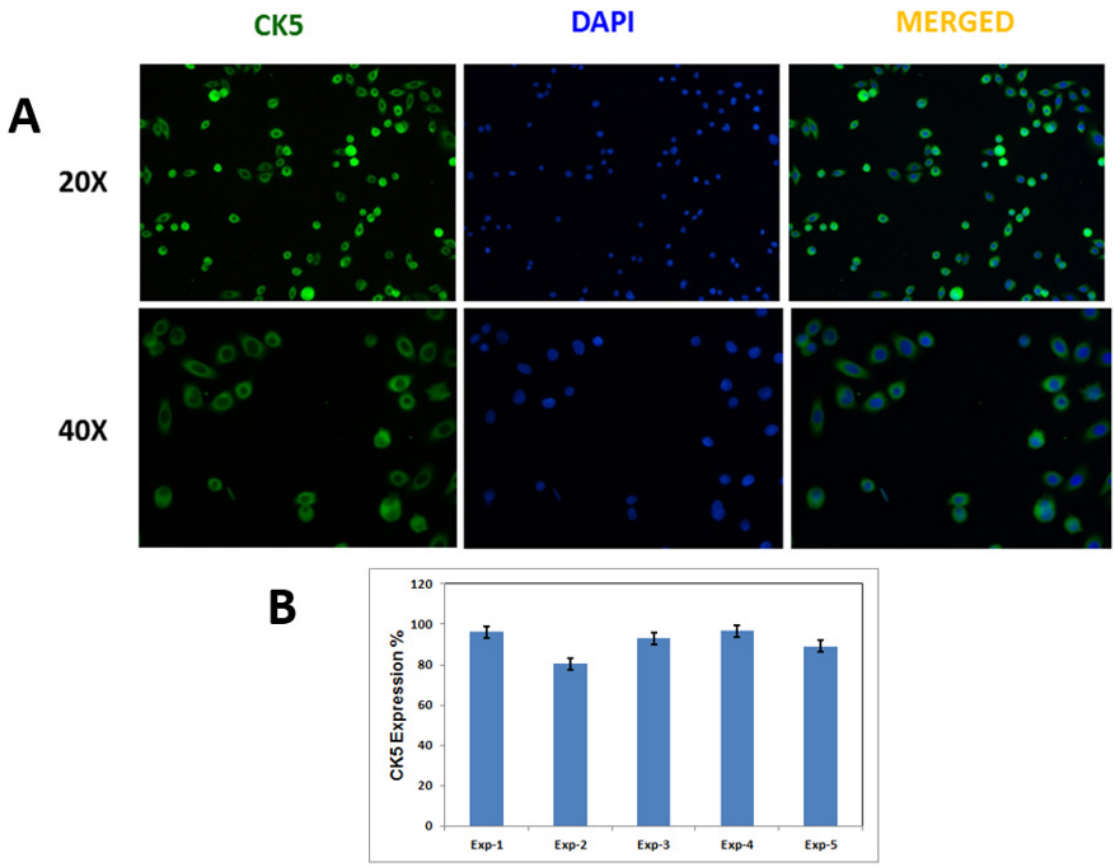

Figure 3 (A,B) Immunocytological analysis of keratinocyte specific marker.

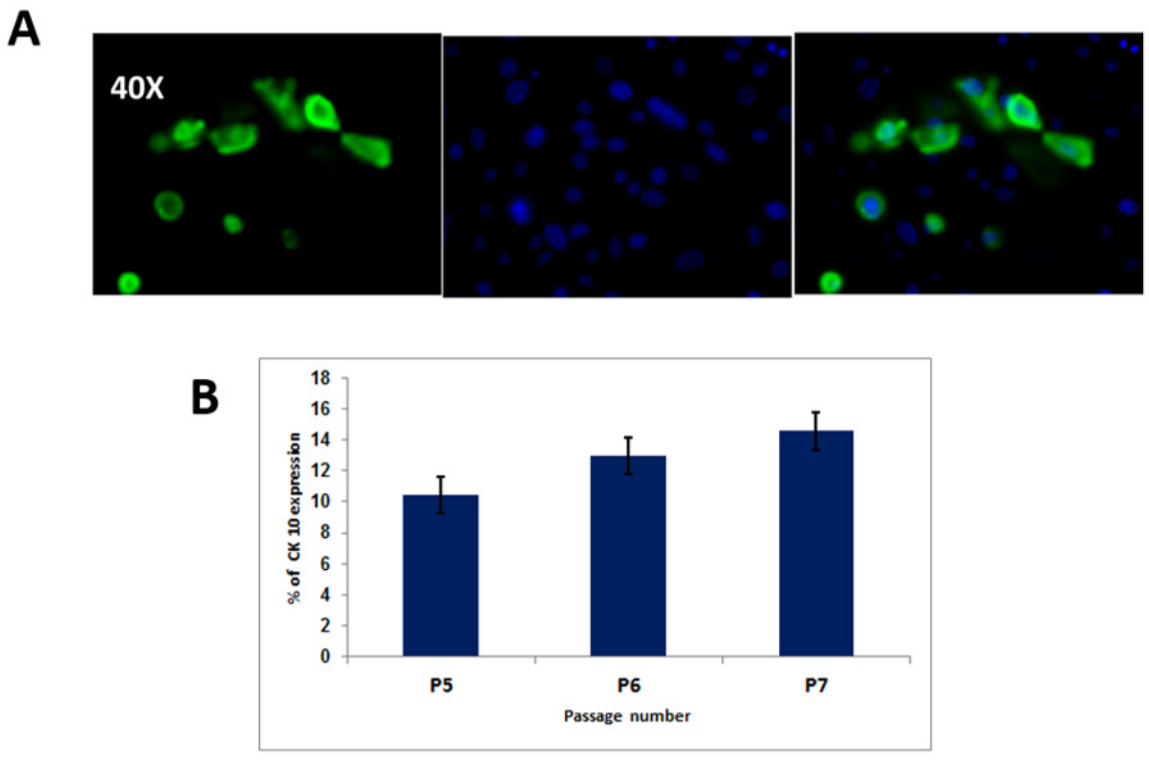

Figure 4. (A, B) Immunocytological analysis of keratinocyte differentiation marker.

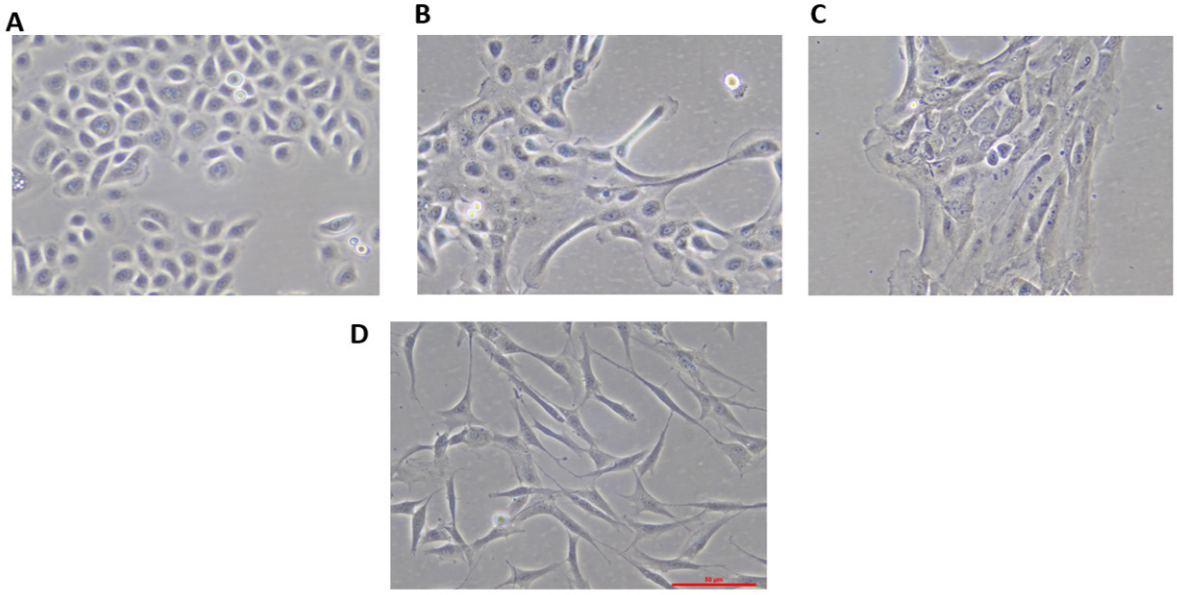

Figure 5. (A, B, C & D) Morphology of human primary keratinocyte during the process of transdifferentiation.

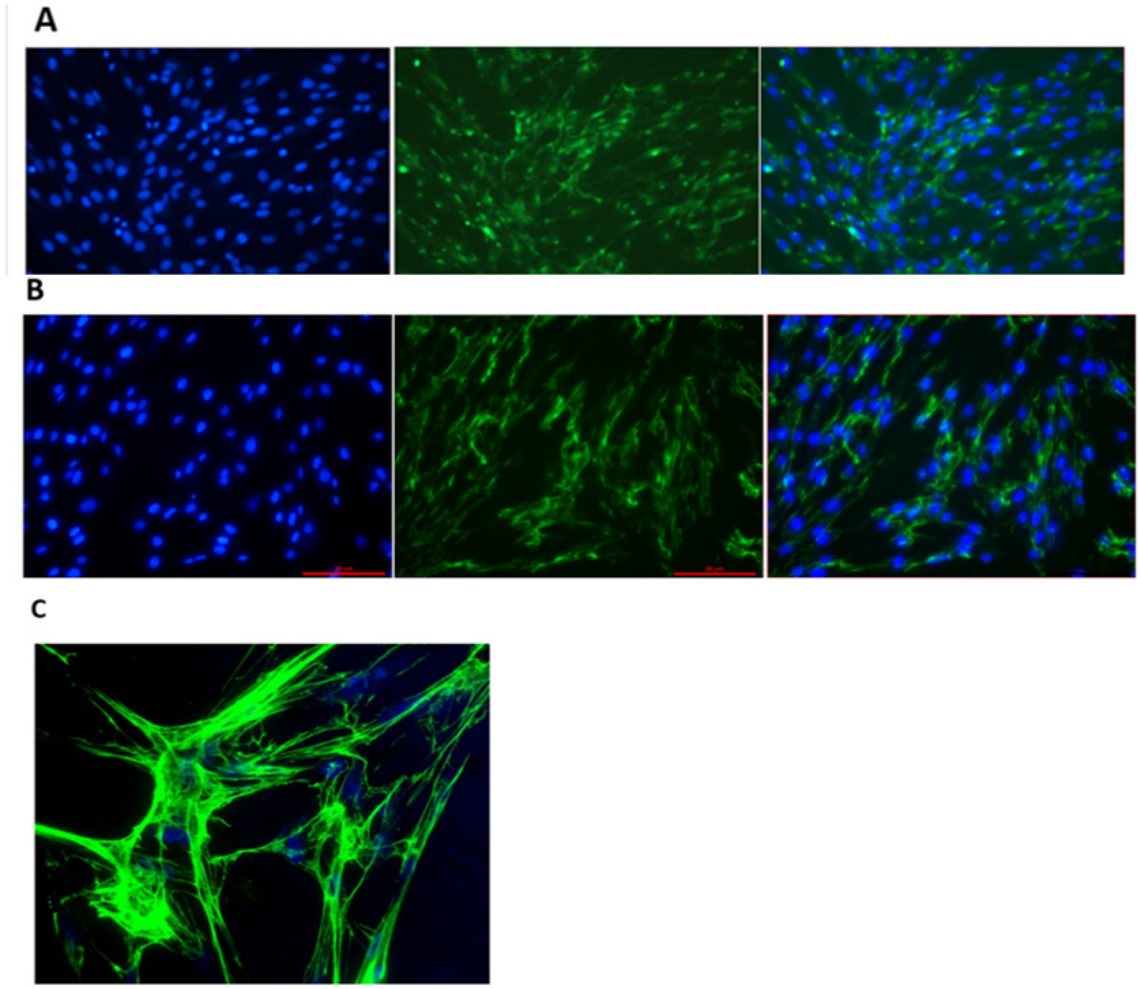

Figure 6 (A, B, C) Specific marker protein expression in Transdifferentiated Fibroblast.

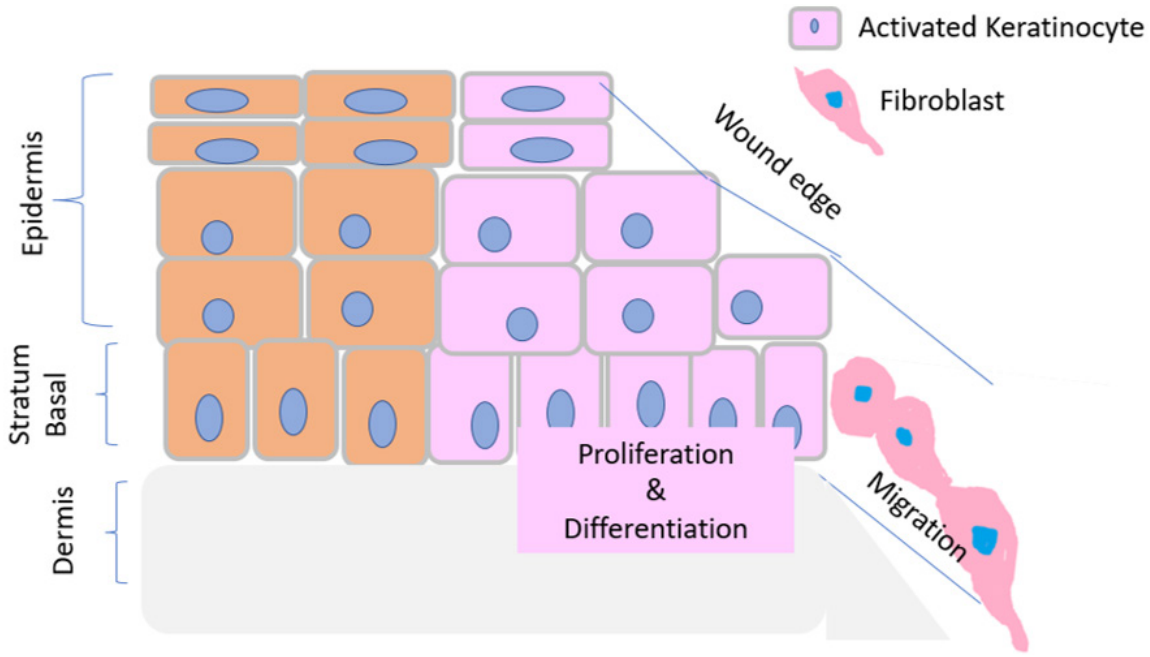

Figure 7 Model of Keratinocyte transdifferentiation during wound healing.

any adverse reaction because of non-immunogenic response. They further confirmed that the allergenic keratinocytes grown in culture do not express HLA-DR antigen [18]. There are studies to show that the allergenic keratinocytes secrete the growth factors required for wound healing. Successful therapeutic use of allogenic keratinocytes over the last two decades indicates that it is safe and effective to use on burn wounds and donor sites. Cultured epithelial autograft (CEA) are aseptically processed wound dressings, approved by FDA in 2007, and CEA is used for patients with deep dermal or full-thickness burns greater than 30% TBSA. Hence serum-free media procedure has been used to culture allogenic keratinocytes which can be used for therapeutic use [19].

Successful serum-free cultures of the keratinocytes were done as per the clinical-grade protocol and sterility of the culture has been maintained throughout different passages and efficacy of the keratinocytes would be predicted as the cells continued to be in proliferative stage. Cytokine activation causes keratinocyte migration in the proliferative phase, leading to closure and restoration of a vascular network. Keratinocytes can also be activated by mu-opioid receptor agonists but the role of these agonists on inflammation and wound closure remains unclear. Nowadays, several commercialized bioengineered skin products derived from autologous cells are available. Clinicians harvest autologous skin, and the company produces a graft-able substrate seeded with the autologous cells for clinical use in approximately 2 weeks. Here, a non-invasive isolation method was used, with pure epidermal cells without any dermal contamination and the isolated keratinocytes continued their proliferative capacity up to later passages like P11. Immunoreactivity of specific epidermal cytokeratin used as a marker for proliferation (CK5) has been confirmed and the differentiation marker (CK10) expression remains under control during the late passages even. Also, there was no use of feeder layer in the culture system and the capacity of keratinocytes in retaining the *in vivo* nature of epidermis. This evaluation of keratinocyte culture happens within a less time period (one week) and it facilitates the development of an enhanced epidermal culture system which would bring hope for wound healing. Improvement of keratinocyte culture method in terms of reducing infection risk and elimination of xenobiotic products indicates the efficacious investigation. In the last two decades, several views and discussions are going on for use of keratinocytes in skin tissue engineering, transplantation of keratinocytes, and making skin substitutes using keratinocytes. In the future, the use of cultures keratinocytes requires frequent use of these, and that may, in turn, guarantee the indications of prescriptions and handing for the successful practice for wound healing in burns and cutaneous wound healing in diabetic ulcers [20-28].

Dermal fibroblasts act as an originator, modulator, and which enable the ECM for the efficient functioning of wound healing. The trans-differentiation of keratinocyte to fibroblast demonstrates that keratinocyte stimulates the potential of keratinocyte to modulate collagen, fibronectin and fibrin synthesis. The production of soluble growth factors by fibroblast is responsible for enhancing extracellular matrix deposition. Advances in cell culture and tissue engineering have made the precise measurement of extracellular matrix components. Synthesis of ECM molecules and proteins is done by homing of fibroblast progenitor cells by migration of keratinocyte progenitor cells towards the dermal layer as a process of transdifferentiation [29].

Keratinocytes are the executors to accelerate the re-epithelialization process whereby activated keratinocytes migrate, proliferate, and differentiate to restore the epidermal

barrier and keratinocyte transdifferentiated to form dermal fibroblast to fill the gap for wound healing as shown in model (Figure 7). Keratinocytes are cells that proliferate rapidly without losing their capability. Trans-differentiation of keratinocytes to fibroblast implicates that delivery of healthy cultured keratinocytes execute as intracellular transmitter system in the wound microenvironment and consequently heal the wound effectively. Further trans-differentiation property of human dermal fibroblast offers a new angle for insight into the interaction between keratinocytes and fibroblast in the wound microenvironment [30].

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Conflict of Interest

The author has declared no conflict of interest.

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