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RESEARCH ARTICLE

A Comprehensive Comparative Characterization of Keratinocytes; The Significant Wound Healing Agents

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ABSTRACT

Keratinocytes are the main components of skin epidermis constituting more than 90% of it which are responsible to regulate skin regeneration during external or internal injury. These cells can be found in a heterogenic form containing proliferative, terminally differentiated and transit amplifying (TA) cells located in basal layer, outer keratinized layer and intermediate layers, respectively when isolated from an adult skin. Efforts are going on to characterize keratinocytes precisely as in comparison with widely used stem cells, mesenchymal stem cells (MSCs) and their application in cutaneous wound healing as a translational approach in regenerative medicine. In this work, we have applied a comprehensive approach to identify and characterize keratinocytes which are a valuable tool in wound healing. Keratinocytes have successfully been isolated from adult skin and the ratio of proliferative, terminally differentiated and TA cells based on the expression of α6-integrin and CD71 was studied using flow cytometry after 7, 14 and 21 days of their culture in vitro. RT and qRT-PCR was applied to study the change in genetic expression and relativity of cytokeratin markers with the passage of time. PopUp culturing and population doubling time was performed to study the proliferative potential of heterogenic population of cells. Their colonogenicity and wound healing potential were also studied to explain their healing behaviour during the time of injuries. 47% keratinocytes after 7 days, 62% after 14days and 93% keratinocytes after 21 days of culturing. Expression of CD71 was also observed in cells as, 5% after 7 days, 22% after 14 days and 66% of keratinocytes after 21 days were positive for CD71 expression. Gradual increased expression of genetic markers, K10, K14, INV and P63 was observed using qRT-PCR. Studying comparative data, it can be concluded that keratinocytes after 14 days of culturing are better to use for clinical purposes. This comprehensive protocol could be a valuable addition in cutaneous biology helping researchers to identify keratinocytes and to use them for their pre-clinical studies.
Keywords: Keratinocytes, Cutaneous wound healing, Skin regeneration, Cell therapy, terminally differentiated cells, proliferative cells

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Introduction

Human skin is the largest organ of body and a most vital part which protects the body from external fluctuations. Its compromised integrity may cause major disability or even death (1-4). Wound healing is a complex process involving the number of cells and ECM components which is responsible to maintain the skin integrity and keep it in its perfect form (5). Cell based therapies have been recommended a more successful approach in tissue engineering to regenerate damaged skin or to restore skin integrity after being lost during infections or injuries (6). Autologous or cadaver skin are being in practice as a tradition way to repair hard-to-heal or non-healing wounds which cannot be considered as gold standard in wound healing therapies because of the limited availability of cells (7). Mesenchymal stem cells have been recommended informally as the “gold standard” of cell/stem cell therapy but currently facing a variety of clinical barriers such as scaling up production, eliminating cells with tumor-forming potential, and decreasing the time needed for expansion, differentiation, selection/testing and tissue targeted lineage selection etc (8, 9).

Both type of cells, human keratinocytes (hKCs) and mesenchymal stem cells (MSCs) are being used in clinical research and both cells have some advantages as well as some disadvantages for being applied in clinics. MSCs have a specific protocol to characterize them (10, 11) but there is no any comprehensive protocol to characterize keratinocytes and then their comparison with MSCs that can these MSCs be used as alternative to KCs for clinical applications or not? (12-14). In this work, we have used a comprehensive protocol to characterize hKCs identity and we compare their behaviour with Ad-MSCs (adipose-derived MSCs) and proposed the future directions in cell based therapies as a novel route for clinical applications.

Materials and Methods

Preparation of Keratinocytes Growth Medium (KGM): Keratinocytes Growth Medium (KGM) was prepared using DMEM-HG (Gibco, Japan) and Ham’s F12 (Biowest, France) in a ratio of 3:1, respectively. This mixed medium was supplemented with 10% FBS (fetal bovine serum) and other ingredients required for the keratinocytes growth and maintenance such as 30 ng/ml KGF (Sigma, Germany), 10 ng/ml EGF (Sigma, Germany), 0.12 U/ml insulin (Sanofi-Aventis, US), 0.8 lg/ml hydrocortisone (Sigma, Germany), 0.1 nM choleratoxin (Sigma, Germany), 5 lg/ml apo-transferrine (Sigma, Germany), 2 nM 3,3,5 Triiodo-L-Thyronine (Sigma, Germany) and 0.18 mM adenine (Sigma, Germany) as reported in many other studies (15).

Isolation of Cells

Human Keratinocytes (hKCs)

Separation of Epidermis from Human Skin: Human waste skin pieces were washed with 1x PBS containing Pen/Strep antibiotics (1:8) and then rinsed in 70% ethanol followed by 1x Pen/Strep for 4-5 minutes under the sterile conditions of cell culturing hood (JTLVC2, Jal Tajhiz, Iran). Germs free pieces of human skin were washed with 1x PBS and were placed in already prepared sterile dispase solution (0.07 g dispase (Gibco, USA) + 10 ml 1X PBS) at 4°C for overnight and epidermis was separated mechanically using sterile forceps.

Expansion of Epidermis Separated Keratinocytes (hKCs): Mechanically and chemically separated epidermis from human adult skin was subjected to trypsin treatment. Epidermis was placed in trypsin (1X) and incubated at 37°C for 30 minutes. Trypsin treated epidermis was washed using DMEM-HG in a 50 ml falcon tube so that all loosely attached cells should separate from epidermis. The DMEM-HG containing epidermis and separated cells were filtered by 70 µm mesh filter and cells were separated after centrifugation at 400 *g* for 6 min. The supernatant was removed and fresh KGM was added to cells. Cells were singled after vigorous pipetting and then counted using cell counting chamber (haemocytometer) with the help of following formula and then transferred to each T75 flask and were incubated at 37°C for 6 days in 5% CO_2 incubator and medium was refreshed after every 24 hours interval.

$$Cells^{T} = Cells^{C} \times 2 \times V_{ml} \times 10,000 \text{ --------- (Eq. 1)}$$

$Cells^{T}$ = Total number of cells in microtube
$Cells^{C}$ = Total number of cells counter in haemocytometer
V_{ml} = Volume of medium containing total cells in ml

Adipose-Derived Mesenchymal Stem Cells (Ad-MSCs)

Waste lipoaspirate samples (60 ml) from women who had undergone liposuction for beauty purposes, were kindly provided by Dr. Sanjar Mosavi, Razavi Hospital, Mashhad, Iran. 60 ml lipoasparate was mixed with the already prepared 50 ml collagenase solution and kept in a hot water tub for 1 hour at 37°C. Mixture was shaken continuously every 5-10 minutes for one hour and 30 ml DMEM-LG containing 10% FBS (Fetal Bovine Serum) (Pan-Biotech, Germany and Biowest, France) was added and mixed vigorously. Mixture was poured into 50 ml falcon tubes, which were then centrifuged at 800 *g* for 10 minutes. The bottom sediment pallet was separated and washed with the DMEM-LG containing 10% FBS and again centrifuged at 400 *g* for 6 minutes. After the second round of centrifugation, the pellet was separated and cultured into the T75 culturing flasks containing 10 ml culture medium (DMEM-LG) supplemented with 10% FBS. The flasks containing SVF (stromal vascular fraction) were incubated in 5% CO_2 incubator at 37°C for 24 hours. After the first 24 hours, cells were attached to the bottom of culturing flasks as observed by inverted microscope. Cells were washed twice with PBS to remove cell debris and their culture medium was changed. After 5 days, cells were trypsinized and passaged in the same flask to have equally distributed cellular population (16). Cells at passage 2, were taken for characterization.

Comparative Characterization of Cells

Microscopic Observation: Expanded hKCs and Ad-MSCs were subject to microscopic observations for their unique morphology from 48 h up to having confluent monolayer of cells. The monolayer of hKCs was obtained after 6 days whereas Ad-MSCs became confluent after 3 days.

Expression Analysis of Genetic Markers: Monolayer cells (Ad-MSCs and hKCs) were washed with 1X PBS and then lysed using 1 ml TriPure Isolation reagent (Roche, USA). The cell lysate was collected in 2 ml microtubes and chloroform (200 µl/ml) was added and the mixture was shaken vigorously for 15 sec. The mixture was incubated at room temperature for 10 min and then centrifuged at 12000 *g* for 20 min at 4°C. 400 µl supernatant (RNA phase) was separated from the centrifuged mixture and the same volume of isopropanol was added to the separated supernatant and inverted gently several times. This inverted solution was incubated at room temperature for 10 minutes and centrifuged again at 12000 *g* for 10 minutes. The pellet was separated by pouring out the liquid and 1 ml 75% ethanol was added to the microtube (for washing purpose to remove the remaining TriPure salts). The mixture was vortexed vigorously until the attached pellet got separated and then centrifuged at 7500 *g* for 10 mints. After centrifugation, the pellet was subjected to air dry for 1-2 min. 25 µl DEPC-treated water was added to the air dried RNA pellet and its concentration was calculated via RNA quantification protocol of BioTek Microplate Reader (BioTek, USA). These RNAs were subject to DNase treatment and cDNA was synthesized following the guidelines of cDNA synthesis kit (ThermoScientific, Lithuania). Positive expression of K10, K14, P63, INV, K19 nad K18 was studied using RT-PCR in hKCs and qRT-PCR was then performed following the PCR kit instructions (ThermoScientific, Lithuania) to study the expression of *K10*, *K14, INV* and *P63* using human specific primers (Supplementary Table 1) after 7, 14 and 21 days of hKCs and Ad-MSCs culturing to confirm the cellular population as keratinocytes and MSCs as these don't express keratinocyte lineage markers and change in cytokertins expression in hKCs over time.

Expression of Lineage Specific Markers: Expanded hKCs were subjected to flow cytometry to further characterise the heterougenous cellular population using α6-integrin, CD71, INV and P63 antibodies. Change in hKCs heterogeneous population was studied after 7d, 14d and 21d using α6-integrin, CD71 cell surface markers and cells after 14 days showed a stable expression of keratinocytes markers when further expression of INV and P63 was investigated. Ad-MSCs were also subjected to flow cytometry analysis for positive expression of CD44, CD73, CD90, CD105 and negative expression of CD45, CD34, CD11b to identify their population as MSCs. For flow cytometry, the media were aspirated from the flasks

and cells were washed twice with 1X PBS. Cells were trypsinized and transferred to 15 ml falcon tubes and mixed with medium containing 10% FBS to neutralise trypsin. Cell suspension was centrifuged at 400 *g* for 6 min and cells were washed with washing buffer (ice cold PBS containing 5% FBS) twice. Supernatant was aspirated out and cells were counted using cells counting chamber (Marienfeld, Germany) after labelling with trypan blue. 300,000 cells were separated into 1.5 ml microtubes and were incubated with diluted primary antibody solutions (1:100) at 4°C for 1 hour. All tubes were covered with foil to avoid direct light contact and were tapped gently after every 10 min for proper mixing of antibody in cellular suspension. After incubation, cells were washed with washing buffer thrice and were incubated with diluted secondary antibody solutions (1:100) for 1 hour and tapping was repeated again. Washing procedure was repeated for every tube and all tubes were filled with 300 µl washing buffer and were then subjected to analysis using a BD Accuri C6 Cytometer (BD, USA) for studying the expression of mentioned CD markers (17, 18). Cells were permiablized for the purpose of staining for intracellular antibody (CD45, INV and P63). Cells were fixed in methanol and permeabelized using TritonX/Tween20 permeabilizing buffer then incubated with secondary antibodies, washed twice and analysed for the expression of specific antibodies using a BD Accuri C6 cytometer (BD, USA).

Colonogenicity and PopUps Culturing: Keratinocytes have a unique behavior of colony formation and creating PopUp cells (floating alive cells which can be further cultured) whereas MSCs float dead cells and also show colony formation but colongenic behaviour of both cells is different. For this purpose, hKCs at passage 1 were cultured and observed under the microscope for their colonization behavior at a regular time intervals of 24 hours up to 96 hours. When cells became fully expanded and a monolayer of cells was achieved, they entered into a process of stratification for the development of epidermal sheet in the flask. When reaching over confluency of cells, the medium was removed after every 24 hours and centrifuged for the purpose of collection of loosed cells from cellular layers in flask. These loosed cells have been named as PopUp cells and we cultured these cells into another flasks to study their proliferation potential as it has been discussed in the literature (19) that these loose cells may also contain cells having stem cell like properties. These cultured PopUp cells were kept under regular microscopic observations at an interval of 24-48 hours for 21 days to analyze their behaviour and survival capabilities over a long period of time.

***In vitro* Wound Healing Assay (Scratch Assay):** Scratch assay was performed to study the migration behaviour of hKCs and Ad-MSCs. Keratinocytes (after 14 days of culture) have the specific behaviour of developing sheets and to cover the spaces between the colonies. For this purpose, a scratch was made between the mono-layered cells (hKCs and Ad-MSCs) at passage 1. These scratches were kept under culturing for 48 h and migration capabilities of cells were analysed after every 24 h.

Proliferation Potential Analysis (Population Doubling Time (PDT) Calculation: Proliferative potential of hKCs and Ad-MSCs (after 14 days of culture) was determined by calculating population doubling time (PDT). For this purpose, cells were seeded at a density of 3.0×10^4 cells/well in 6-well culture plates. The cells were counted after 48 h, 96 h and 120 h and their quantity was determined using cell counting chamber (Marienfeld, Germany) under the invert microscope. The PDT was calculated using the following formulas (20):

$$\mathbf{PDT} = \frac{\mathrm{CT}}{\mathrm{PDN}} \quad (1)$$

$$\mathbf{PDN} = log\frac{N}{N^o}\ x\ 3.31 \quad (2)$$

CT = Time of cultivation between passages
PDN = population doubling number
N = cell number at the end of the cultivation period
N^0 = cell number prior culturing

Differentiation of MSCs to Multilineage: To investigate the differentiation potential of MSCs towards **osteoblasts**, cells were taken at passage 2, and cultured in 6 well plates at a density of 2×10^4 cells/cm^2. When cells reached to >90% confluency, cells were cultured with differentiation induction medium following the detailed protocol mentioned

earlier (21). Alkaline phosphatase assay was also performed to confirm their differentiation towards osteoblasts according to the same protocol and stained cells were observed under the iX70 invert microscope (Olympus, Japan). Cells were also differentiated towards **adipocytes** when reached >90% confluency, using differentiation induction medium mention earlier (21) and stained cells were observed under an invert microscope (Olympus, Japan) to observe stained differentiated adipocytes.

Histological Study of *In Vitro* Generated Epidermal Sheet from hKCs: hKCs when cultured continuously, they started developing epidermal sheet after 14 days of their culturing and after 21 days, they develop a complete sheet. Cultured cellular sheets (after 21 days of culture) were separated from culturing flask using sterile forceps and then were placed in 4% formaldehyde solution for fixation. Fixed sheets of tissue were embedded in paraffin and longitudinally sectioned to 4 µm using laboratory microtome (PooyanTeb, Iran) and then floated on a water bath and placed in slide racks for staining purpose using already made hematoxylin and eosin solution. Dehydrated stained sections were mounted on glass slides and observed under iX-70 invert microscope.

Results

Morphological Observation

Successfully isolated human Ad-MSCs after 4 days of their 1st passage were found to be spindle-like in morphology and had the potential to generate colonies as shown in figure 1. The morphology as a monolayer sheet of hKCs is also visible in figure 2.

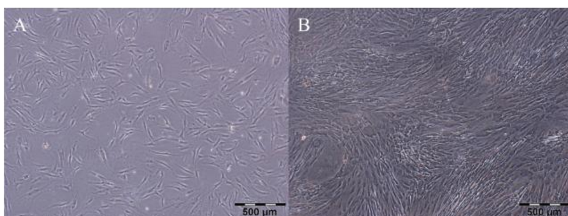


Figure 1. Human Ad-MSCs of primary culture after 2 days (A) and 6 days (B) of first passage.

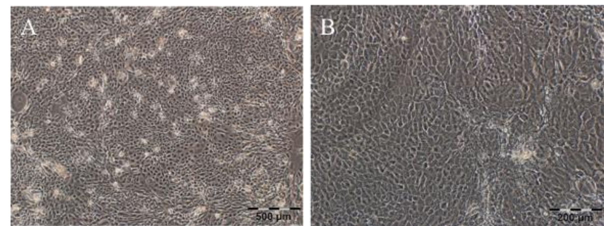


Figure 2. Fully expanded keratinocytes isolated from human adult skin at passage 1.

Expression of Genetic Markers: Identity of hKCs was further confirmed by the positive expression of cytokeratin genes such as K10, K14, P63, INV, K19 nad K18 using RT-PCR (figure 3) and expression of these keratinocyte lineage markers was absent in Ad-MSCs (data not shown).

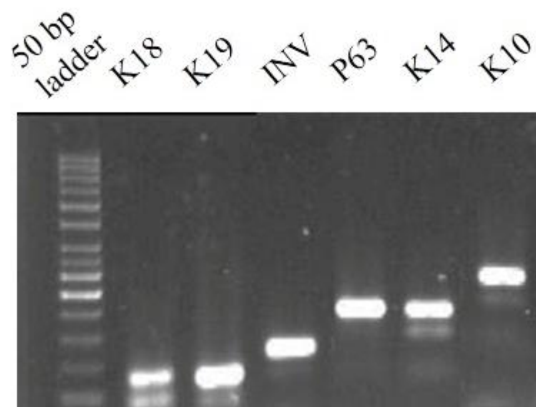


Figure 3. Positive expression of cytokeratin genetic markers in in hKCs using RT-PCR.

Quantitative expression of cytokeratins K10, K14, keratinocyte lineage markers involucrin and P63 was also been found in hKCs after 7, 14 and 21 days of culturing. It was found that as the hKCs remain in culture, they start developing an epidermal sheet and expression of cytokeratins marker also changed along with the change in morphology. After 21 days of hKCs in culturing condition leads to the development of an stratified epithelia (figure 4).

Expression of Cell Surface Markers: MSCs were seen highly positive for CD44, CD73, CD90 and CD105 whereas negative expression of CD45, CD34 and CD11b was observed as analysed by flow cytometry (Figure 5).

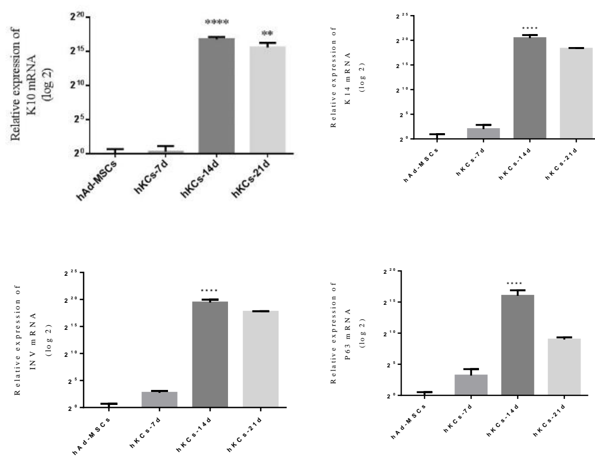


Figure 4. Log2 graphs of quantitative expression of K10, K14, INV and P63 genetic markers in hKCs after 7, 14 and 21 days of culturing *in vitro using* real time PCR.

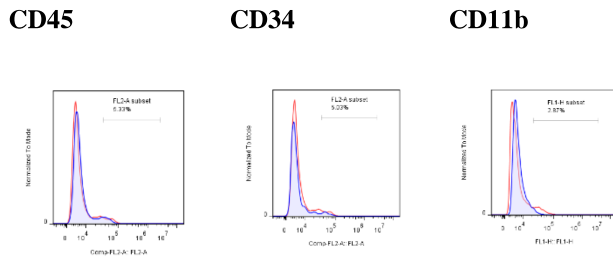
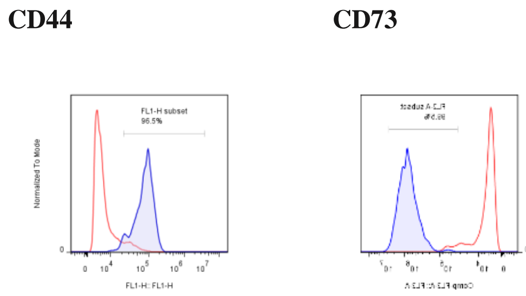


Figure 5-A: Negative expression of CD markers on the surface of Ad-MSCs



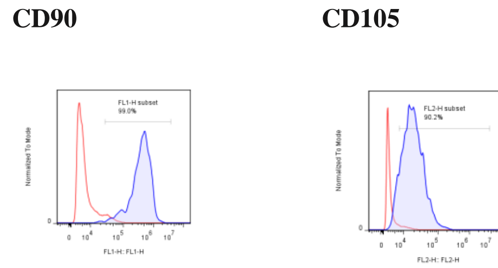
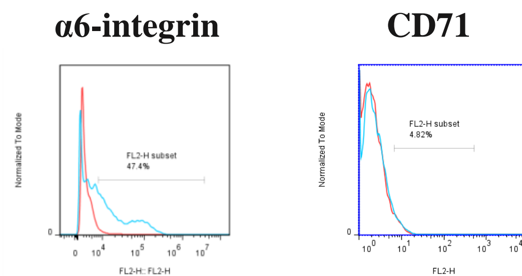


Figure 5-B: Positive expression of CD markers on the surface of Ad-MSCs

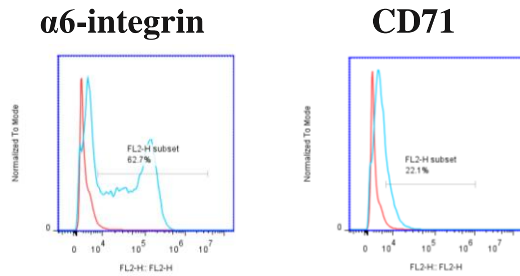
Figure 5. Flow cytometry analysis of cell surface markers in hAd-MSCs at passage 2. The calculated percentages of positive and negative cells are shown in the figures A and B. Figure 5-A is showing the negative expression of CD markers (CD45, CD34, CD11b) whereas figure 5-B is showing the positive expression of CD markers (CD44, CD73, CD90, CD105).

hKCs were also analysed for their lineage specific markers i.e. α6-integrin, CD71, P63 and Involucrin in a triplicate experiments after 7, 14 and 21 days of their culturing *in vitro*. Cells were found positive for involucrin confirming their identity as keratinocytes. Proportion of cells expressing α6-integrin/CD71 were found as 47%/5% after 7 days, 62%/22% after 14 days and 93%/66% after 21 days confirming their heterogeneous population of hKCs as stem cells (α6-integrin^{bright} CD71^{bright}), transit amplifying (TA) cells (α6-integrin^{dim}CD71^{bright}) and terminally differentiated cells (α6-integrin^{dim}CD71^{bright}), as shown in figure 6.

After 7 days of culturing



After 14 days of culturing



After 21 days of culturing

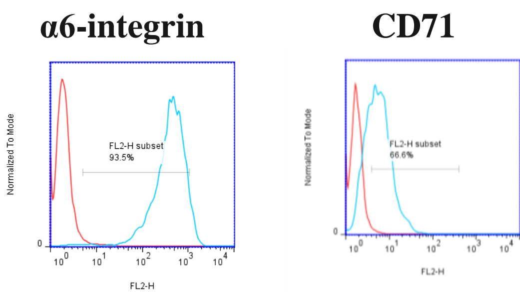


Figure 6-A: Expression of α6-integrin and CD71 in keratinocytes isolated from adult human skin and cultured for 7, 14 and 21 days.

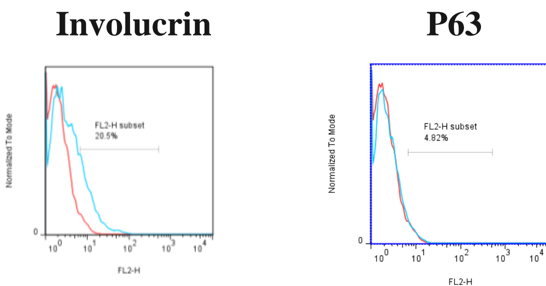


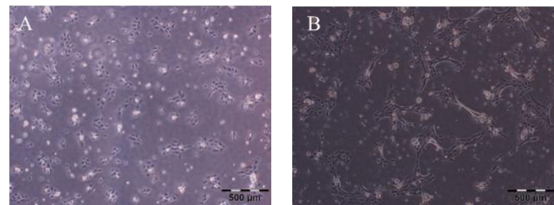
Figure 6-B: Expression of involucrin and p63 in keratinocytes isolated from adult human skin and cultured for 14 days.

Figure 6. Expression of keratinocyte lineage specific markers (α6-integrin, CD71, involucrin and p63) in hKCs using flow cytometry.

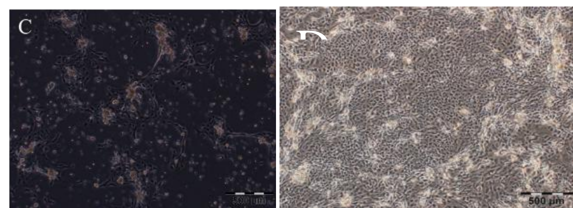
Clonogenic Potential and PopUps formation

Keratinocytes have been found as a heterogenous population of cells when isolated from adult skin containing keratinocytes stem cells, TA cells and terminally differentiated cells (13, 14). The hypothesis of presence of stem cells can further be characterised by studying their clonogenic potential and most recently it has been known that the PopUps of these stem cells can also be further cultured and can generate monolayer of cells (19). It was found out that these cells have high clonogenic potential as were observed by the invert microscope after time interval of 24 h as shown in figure 7. After becoming fully confluent, their floated cells (PopUps) also showed the culturing potential. These cells were found as very low proliferating, which indicated low abundance of stem cells in these cultures. It took about 18 days for the PopUps to reach >90% confluency as shown in figure 7.

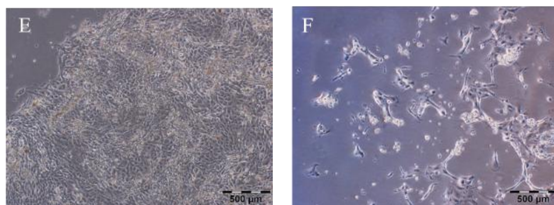
After 1 Day of hKCs Culturing **After 3 Days of hKCs Culturing**



After 5 Days **After 8 Days**

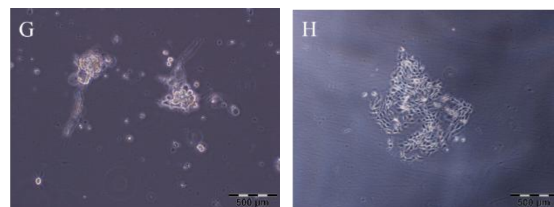


Sprouting Monolayer Sheet of hKCs after 11 Days of Culture



Culturing of hKr PopUps

After 2 days **After 10 days**



After 14 days **After 18 days**

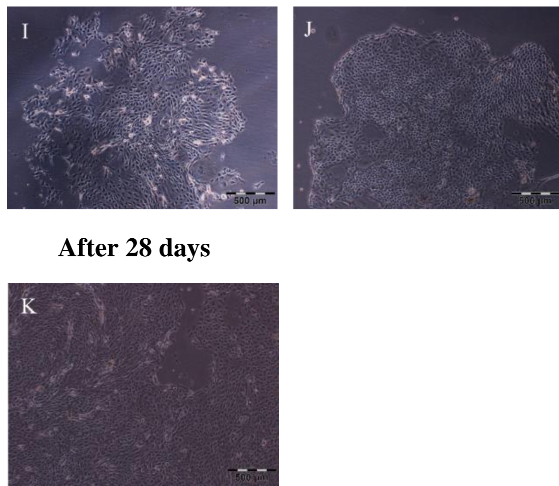
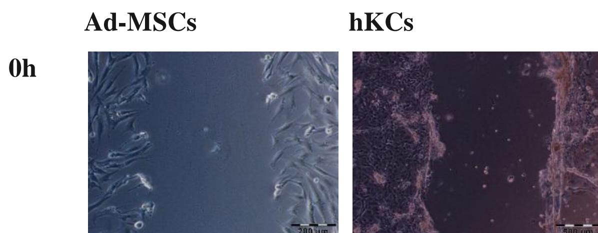


Figure 7. hKCs behavior when isolated from adult skin and cultured *in vitro* over time. Figures A-D are showing the colonogenic potential of hKCs while in culture. Figures E and F are showing the sprouting of cellular sheet and generation of PopUp cells after 11 days of their culturing whereas figures G-K are showing the culturing behavior of separated PopUps (floated cells).

In Vitro Wound Healing

hKCs developed a monolayer sheet after 8 days when cultured in KGM as shown in figure 8-A and they had wound healing potential when their sheet was scratched *in vitro.* It was observed that they healed about 60% of wounds within 48 hours of the scratch as shown in figure 8 in comparision with Ad-MSCs which show very rare migration to heal the wounds *in vitro*.



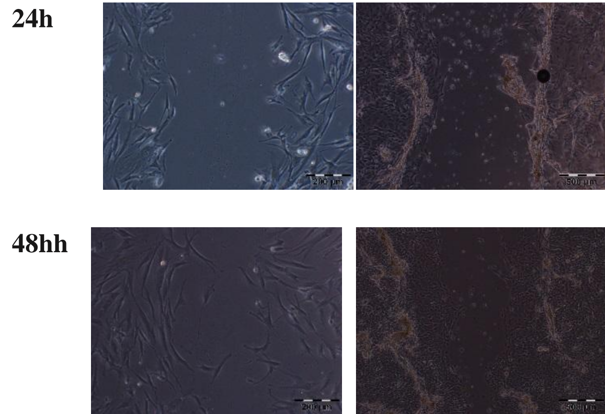


Figure 8-A. *In vitro* wound healing assay showing the migration of cells and healing of wound after the passage of time. hKCs migration is observed after 24 hours of their scratch and after 48 hours, about 60% healed scratch has been shown in figure 3.10-B.

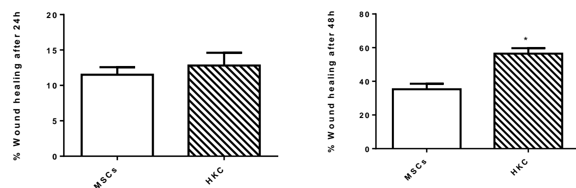


Figure 8-B. *In vitro* wound healing assay showing the migration of cells and healing of wound after the passage of time. hKCs migration is observed after 24 hours of their scratch and after 48 hours, about 60% healed scratch has been shown here.

***In vitro* Cellular Proliferation:** hKCs were found very slow proliferating cells when their population doubling time was calculated using the previously mentioned formula as compared to Ad-MSCs. It was observed that they maintain their original population number until 5 days of their culturing and it could be concluded that these cells take approximately 6 days to reach their original amount of cultured cells. Counting keratinocytes population doubling time is a little bit difficult as the only 40-50% number of cells adhere to bottom of flasks after 48 hours of their culture. We found out the 50% cells after the 48 hours of their culturing and then these were found proliferating very slowly and they doubled their population after 96 hours and continue proliferating until start stratifying and developed sheets as shown in figure 9 whereas in case of Ad-MSCs, they were found doubling their population almost after every 48 hours and continue

proliferating on the same ratio until 96 h as shown in figure 9.

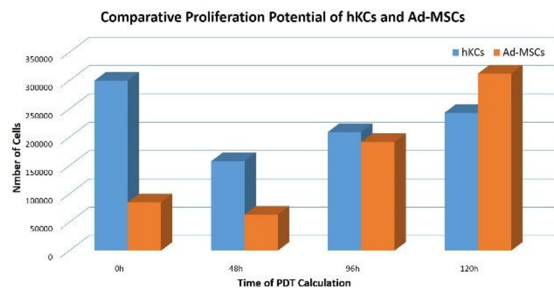


Figure 9. *In vitro* proliferation of hKCs and Ad-MSCs (based on population doubling time (PDT) cultured in T25 flasks and monitored after every 48 hour upto 120 days.

Differentiation Potential Towards Osteoblasts and Adipocytes: MSCs were differentiated to confirm their differentiation potential towards osteocytes and adipocytes in their respective induction media. Their ostegenicity was confirmed by the deposition of calcium in cultures stained by Alizarin Red S and their adipogenecity was confirmed by the presence of lipid droplets after 21 days of induction as shown in figures 10-E and 10-F, respectively. Alkaline phosphatase assay also confirmed the osteogenic differentiation (figure 10-D). Undifferentiated MSCs used as controls are also shown in figures 10-A, 10-B and 10-C.

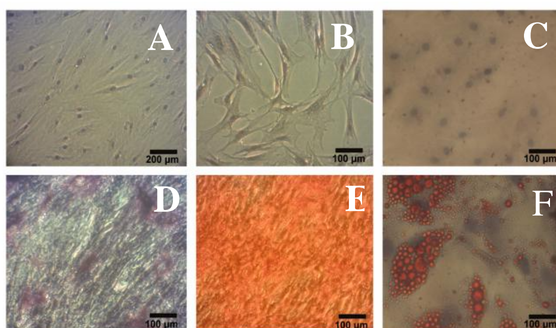


Figure 10. Differentiation of Ad-MSCs to osteoblasts and adipocytes *in vitro*. Figures **'A'**, **'B'** and **'C'** are showing the undifferentiated MSCs as control whereas figures **'D and E'** are showing differentiation of MSCs towards osteocytes as stained with alkaline phosphatase and Alizan Red Solution whereas figure **'F'** is showing adipogenic differentiation of MSCs as stained with Oil O Red staining solution after 21 days of their culturing in osteogenic and adipogenic differentiation media, respectively.

In Vitro Generation of Epidermal Sheet

After 2 weeks of culturing hKCs, they started generating epidermal sheets which is coincided with cease proliferation and start of stratification. After 3 weeks of their culturing, these cells developed into sheets which is clear in T75 culturing flask, isolated in a petri dish and stained with hemotoxylin/eosin as shown in the figure 11. This sheet was further characterised and the cells were shown to be expressing keratinocyte lineage markers (K10, K14, involucrin and P63 using qRT-PCR as shown in figure 11-B.

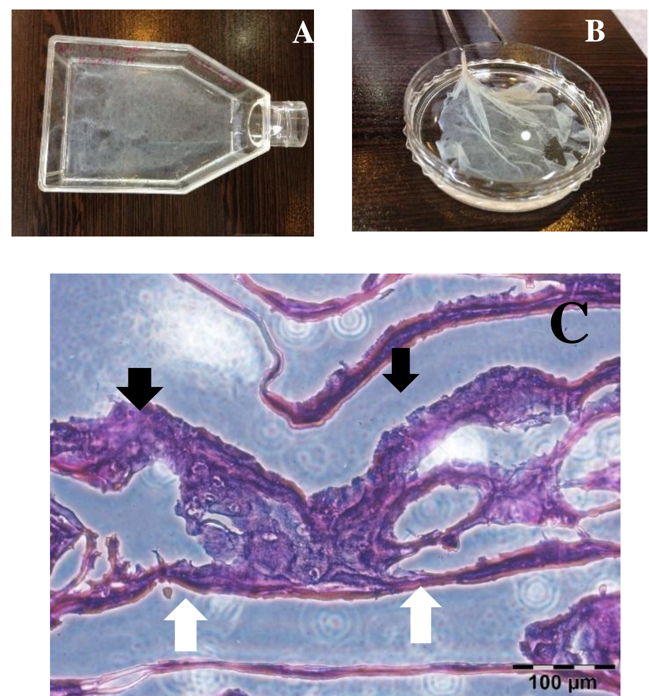


Figure 11. Physical and histological observation of *in vitro* generated stratified epithelia from the culturing of hKCs (after 21 days of culturing) in a T75 flask. **Figure A** is showing the stratified epithelia in T75 flask before separation and **figure B** is showing the separated stratified epithelia in a petridish which was separated from cells culturing flask mechanically using forceps. **Figure C** is histological observation of stratified epithelia. Black arrows are showing the basal cells whereas the white arrows are showing the cornified or

keratinized keratinocytes. Layers of cells are visible between white and black arrows section.

Discussion

Skin cell transplantation, a century old practice from the first graft by Reverdin in 1871 has been facing a number of challenging tasks such as availability of autologous normal skin etc. (22). Clinical researchers continually worked to resolve such challenges and a concept was introduced in 1983 that 'there are epithelial proliferative units (EPUs) under the skin' which are responsible to regenerate the skin in case of injury and rupture (23). In 2002, Potten and Booth rejected the concept of EPUs and introduced the concept of existence of keratinocytes stem cells in skin which are responsible to regenerate the injured skin (24).

First *In vitro* keratinocytes culturing method was introduced in 1975 by Rheinwald and Green (25) and it was focused to culture and characterize keratinocytes before transplanatation and a number of markers have been introduced to characterize keratinocytes identity (26-28). Currently, keratinocytes are defined as the cells of hetergenous population containing basal layer stem cells (α6-integrin^{bright} CD71^{dim}), transit amplifying (TA) cells (α6-integrin^{bright} CD71^{bright}) and terminally differentiated cells (α6-integrin^{dim} CD71^{dim}) (29, 30). Many other researchers have also characterized the human keratinocytes at different stages describing the same phenotype as mentioned above (12-14).

We characterize keratinocytes based on the α6-integrin and CD71 after a time interval of 7 days in culture. After 7 days of keratinocytes culturing, they were found α6-integrin^{bright} CD71^{dim} and an interesting behaviour was observed while increased time of culturing and after 14 days, α6-integrin^{bright} CD71^{medium} expression was observed. Both markers of cell surface were found highly expressed as α6-integrin^{bright} CD71^{bright} showing that after 7 days, there were a number of proliferative stem cells in culture which differentiated to TA cells with the passage of time and after 21 days, more than 60% of cells were TA cells as shown in figure 6 that developed stratified epidermal sheet after 21 days as shown in figure 11. Ad-MSCs were found floated after 7 days and could not remained in culture for 21 days (data not shown).

All cells, Ad-MSCs and hKCs were subject to genetic expression analysis and hKCs shown rare expression of cytokeratins (K10, K14), Involucrin (INV) and P63 after 7 days of their culturing and an increased expression of all these above mentioned markers were found in 14 days and almost same as in cells of 21d culturing as shown in figure 4. Literature has shown that cytokeratins 5/14 and P63 are the markers of basal layer keratinocytes which express themselves in different conditions. P63 is known as the stratification regulator which is responsible to generate stratified epithelia along with the co-expression of cytokerain 14 which is consistent with our results as shwon in figure 4. Cytokeratin 5 dimerize with K14 and make up the cytoskeletan of basal layer epithelial cells (31-33). Ad-MSCs were negative for all keratinocyte lineage markers as shown in figure 4.

While studying the proliferative potential and *in vitro* wound healing, it was observed that keratinocytes are cells having wound healing potential when scratched *in vitro* whereas these cells are not good proliferative when compared with the Ad-MSCs (figures 8 and 9, respectively). Mesenchymal stem cells are playing a useful role in wound-healing strategies and are being applied in both aesthetic and reconstructive surgery (34). These cells have also be found in re-epithelialisation of damaged skin and maintaining the ceulluar population as they reside in the dermis of skin (35), (36-38). In a number of experiments, MSCs have shown very improved wound healing capacity for both acute and chronic wounds. A very promising response has also been observed for non-healing diabetic ulcers when MSCs were applied on the wound surface (39-42).

MSCs being a valuable cells for therapeutic applications are not well approved and have many problems such as escape of cells, non-specific differentiation etc. While comparing both cells, Ad-MSCs and hKCs together, it can be concluded that restrictions that MSCs face in clinical applications, hKCs don't face and vice versa. MSCs have some superiorities over hKCs because of their

immunogenic and paracrine effects. MSCs can be fate determined towards keratinocytes-like cells (KLCs) which may have no any problem, and can be used as the recommended cells for transplantations. Optimum protocols are reqired to generate KLCs and results of their *in vivo* applications can lead to a strong conclusion regarding their validity as promising cells.

Conflict of Interest

Authors declare no conflict of interest with any person or organization.

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