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Original Research

Adipose derived mesenchymal stem cells express keratinocyte lineage markers in a co-culture model

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Abstract: Cutaneous wound healing is a complex type of biological event involving proliferation, differentiation, reprograming, trans/de-differentiation, recruitment, migration, and apoptosis of a number of cells (keratinocytes, fibroblasts, endothelial cells, nerve cells and stem cells) to regenerate a multi-layered tissue that is damaged by either internal or external factors. The exact regeneration mechanism of damaged skin is still unknown but the epithelial and other kinds of stem cells located in skin play crucial roles in the healing process. In this work, a co-culture model composed of adipose derived mesenchymal stem cells and keratinocytes was developed to understand the cellular differentiation behaviour in wound healing. Human mesenchymal stem cells were isolated from waste lipoaspirates. Keratinocytes were isolated from neonatal rats skin as well from human adult skin. Both types of cells were cultured and their culturing behaviour was observed microscopically under regular intervals of time. The identity of both cells was confirmed by flow cytometry and qRT-PCR. Cells were co-cultured under the proposed co-culturing model and the model was observed for 7, 14 and 21 days. The cellular behaviour was studied based on change in morphology, colonization, stratification, migration and expression of molecular markers. Expression of molecular markers was studied at transcriptional level and change in cellular morphology and migration capabilities was observed under the invert microscope regularly. Successfully isolated and characterized mesenchymal stem cells were found to express keratinocyte lineage markers i.e. K5, K10, K14, K18, K19 and Involucrin when co-cultured with keratinocytes after 14 and 21 days. Their expression was found to increase by increasing the time span of cell culturing. The keratinocyte colonies started to disappear after 10 days of culturing which might be due to stratification process initiated by possibly transdifferentiated stem cells. It can be concluded that mesenchymal stem cells can regenerate the damaged skin if transplanted to damaged area but for their successful differentiation and enhanced regeneration, they need a population of keratinocytes *in situ* which need further experiments for validation of co-culture model and its potential for being used in clinics.

Key words: Cutaneous wound healing, Keratinocyte lineage, Adipose-derived mesenchymal stem cells, Regenerative medicine, Co-culturing model.

Introduction

Human skin is responsible to protect the human body from being damaged by the fluctuations of external environment. These variations are damaging the skin regularly and skin homeostasis is maintained continuously via a complex regeneration process of cutaneous wound healing. It has been discussed that a number of cellular (keratinocytes, keratinocyte stem cells, fibroblasts, mesenchymal stem cells) and molecular components such as extracellular matrix (ECM) are involved in regeneration of damaged skin and this process is being followed by a strict regulation of a cascade of events involving inflammation, proliferation, differentiation and remodelling to regenerate a multi-layered tissue. All cells involved in healing process are highly intercommunicated to create a healing microenvironment for precise regeneration. Stem cells as being the critical healing agents are either recruited or differentiated for the generation of a multi-layered skin and maintenance of healing microenvironm (1-4). Reliable keratinocyte culturing and characterization techniques (5), better understanding of the molecular mechanisms in the regulation of epidermal stem cells and wound healing (6), techniques to accelerate basement membrane formation and vascularization and induction of mesenchymal stem cells towards keratinocyte-like cells have been studied to explain the complexity of wound healing mechanism but the puzzle yet remains unsolved (7, 8). Cellular proliferation and differentiation have been termed as crucial steps in wound healing and skin homeostasis maintenance (9). A number of other approaches such as cadaver skin grafting, application of skin substitutes, use of growth factors etc have been applied for skin regeneration but post grafting problems including infection, graft rejection, inadequate healing, short shelf life etc. are yet associated problems with available grafting techniques (10).

Stem cells, especially mesenchymal stem cells (MSCs) could be a gold standard to treat cutaneous

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wounds in future. MSCs play a supportive role in almost all kinds of tissues and it has been determined that they can be differentiated towards almost all cell types derived from three germ layers (11-13). These cells have been termed as superior cellular candidates for off-the-shelf clinical and wound healing applications (14, 15). A number of cell based studies have also shown a promising differentiation potential of mesenchymal stem cells to generate keratinocyte-like cells as will be discussed in this paper. We proposed here that cellular proximity and contacts are very important factors in inducing stem cells differentiation and a co-culture model could be a good platform to understand the cellular differentiation behaviour while regenerating the damaged skin. Co-culture models have also been used to induce cells for tissue regeneration, enhanced angiogenesis and production of growth factors like insulin, bone morphogenetic protein 2 (BMP-2), vascular endothelial growth factor B (VEGFB), hypoxia inducible factor-1α (HIF-1α), fibroblast growth factor-2 (FGF-2), and transforming growth factor-β1 etc (16-18). Increasing cell-cell contacts and their crosstalk with the neighbouring cells also induce cellular differentiation and their crosstalk is very crucial in the maintenance of differentiated state (19, 20). Some other factors such as microRNAs and mechanics of cellular organelles also play important roles in stem cell fate determination (21, 22). It has also been well explained that after traumatic injuries, cells release SDF-1α which recruits other cells from the surrounding for healing purpose (23, 24). When a huge part of skin is damaged and there is no availability of surrounding cells, our proposed approach would help to develop the kind of epidermal sheets from MSCs and allografted keratinocytes which will result in the precise differentiation of MSCs and will help to cover the damaged area. In this research, we studied the cellular behaviour and lineage markers at microscopic and transcriptional levels, respectively to analyse stem cell behaviour in a co-culture model from day one to three weeks.

Materials and Methods

Ethical statement

The work on animals as experimental organisms and human tissues were performed under the guidelines of ACECR-Khorasan Razavi Branch for animal and human modelling research. Minimum harm was provided to rats for isolation of keratinocytes using the full anaesthesia protocols.

Isolation and culturing of human MSCs

Human adipose derived MSCs were isolated from the waste lipoaspirates (60 ml) which was treated with already prepared collagenase solution (10mg collagenase) (GIBCO, Japan) + 50 ml of 1X PBS (Phosphate Buffered Saline) + 0.4 ml (BSA) + 0.2 ml $CaCl_2$) at 37°C for 1 h in a shaking water bath (Memmert. Germany) and then mixed strongly with 30 ml DMEM-LG containing 10% Fetal Bovine Serum (FBS). The mixture was centrifuged at 800 g for 10 min and the pallet was separated, washed with DMEM-LG containing 10% FBS and 1% antibiotics (Pen/Strep) and centrifuged again at 400 g for 6 min. The pellet or SVF (stromal vascular fraction) was separated and cultured in T75 culturing flasks containing 10 ml culturing medium (DMEM-LG) supplemented with 10% FBS at 37°C for 24 h in a 5% CO_2 incubator (Memmert, Germany). Cells were washed twice with PBS to remove cell debris after 24 hours and the culturing medium was changed. Cells were trypsinized and passaged 1:1 after 4 days to expand and distribute cellular population equally.

Characterization of Adipose Derived MSCs

Cells at passage 2, were trypsinized and separated for characterization purpose. MSCs characterization was performed by analysing their expression of specific cell surface markers and differentiation assay to investigate differentiation potential towards adipocyte and osteoblasts.

Flow cytometry analysis

To investigate the cell surface markers, cells were subjected to FACS analysis for CD105 (SH2), CD73 (SH3), CD44, CD90, CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. For this purpose, the medium was aspirated from the flasks and cells were washed twice with PBS. Cells were trypsinized and transferred to 15 ml falcon tubes and mixed with medium containing 10% FBS to neutralize 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 h 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™ Flow Cytometer (BD, USA) for studying the expression of cell surface markers (25, 26).

Adipogenic differentiation assay

Trypsinized cells at passage 2 were seeded in 6 well plates at a density of 2 x 10^4 cells/cm^2 and cultured with 2 ml DMEM-LG + 10% FBS. After reaching >90% confluency, cells were cultured with adipogenic differentiation induction medium containing DMEM-LG supplemented with 10% FBS, 1X penicillin-streptomycin, 1 µM dexamethasone, 10 mM β-glycerol phosphate and 100 µM indomethacin for 21 days . The induction medium was refreshed at an interval of 2-3 days, and cells were stained with Oil Red O after 21 days of induction. For staining purpose, induction medium was aspirated out, and cells were washed twice with 1X PBS w/o Mg^{++} and Ca^{++}. PBS was taken out gently and cells were covered with 4% formaldehyde solution (Merck, Germany) and were incubated at room temperature for 30 minutes. Then cells were washed with distilled water twice and were covered with 60% isopropanol for 30 seconds. Isopropanol was also taken out and cells were covered with Oil Red O staining solution and in-

cubated at room temperature for 15 min. Finally, cells were washed with distilled water for several times until getting clear water. Cells were covered with PBS and observed under the iX70 invert microscope (Olympus, Japan).

Osteogenic differentiation assay

To investigate the differentiation potential of MSCs towards osteoblasts, trypsinized cells at passage 2 were seeded in 6 well plates at a density of 2 x 10^4 cells/cm^2 and cultured with 2 ml DMEM-LG + 10% FBS until >90% confluency achieved . Cells were then washed and cultured with osteogenic differentiation induction medium containing DMEM-LG supplemented with 10% FBS, 1X penicillin-streptomycin, 1 µM dexamethasone, 10 mM β-glycerol phosphate and 20 µM ascorbic acid for 21 days. This induction medium was refreshed at an interval of every 2-3 days, cells were stained with Alizarin red solution after 21 days of induction. For staining purpose, induction medium was aspirated out, and cells were washed with 1X PBS twice. PBS was taken out gently from the wells and cells were covered with 4% formaldehyde solution for few seconds and then washed with distilled water. After washing, cells were covered with Alizarin red solution and were incubated for 45 min at room temperature. Finally, cells were washed with distilled water for several times until getting clear water. Cells were covered with PBS and observed under the iX70 invert microscope (Olympus, Japan).

Alkaline phosphatase assay

More than 90% confluent cells in 6 well plate were washed with PBS and then covered with 4% formalin for 1 min. Cells were washed with washing buffer (0.05% Tween 20 in PBS) after fixation and covered with BCIP/NPT substrate solution (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Roche, USA). Plates were covered with aluminium foil and were incubated at room temperature for 5-15 min. Staining process was checked regularly after every 2-3 mins and then washed with water carefully. Cells were covered with PBS and observed under the iX70 invert microscope (Olympus, Japan).

Isolation and culturing of neonatal rat keratinocytes

Separation of epidermis

Neonatal rats (1 or 2 day old) were taken from the animal house (Department of Biology, Ferdowsi University of Mashhad) and were sacrificed by overdosing chloroform under the lab animal treatment guidelines of ACECR-Mashhad Branch. Their skin was separated by using sterile forceps and scalpels and was washed with 1x PBS containing Pen/Strep antibiotics (1:8). For further sterility, the separated neonatal skin was rinsed with 70% ethanol first and then in 1x Pen/Strep for 4-5 minutes under the sterile conditions of cell culturing hood JTLVC2 (Jal Tajhiz, Iran). Germs free pieces of neonatal skin were washed with 1x PBS and were placed in already prepared sterile dispase solution (0.07g dispase (GIBCO, USA) + 10ml 1x PBS) at 4°C for overnight. The epidermis of neonatal skin was then separated mechanically using sterile forceps under sterile conditions.

Isolation and expansion of neonatal rat keratinocytes

Mechanically and chemically separated epidermis from neonatal rat skin was trypsinized to release the loosely attached cells at 37° C for 30 min. DMEM-HG supplemented with 10% FBS and 1% pen/strep was added to neutralize the trypsin and mixture was pipetted many times and released cells were separated using 70 µm cell strainer (Corning, USA) and centrifuged at 400 g for 6 min. Separated cells were cultured in T75 culturing flasks at a density of 2.0 x 10^6 / flask with DMEM-KGM (Keratinocyte Growth Medium) supplemented with 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) and cells were incubated at 37°C in the 5% CO_2 incubator and medium was refreshed after every 2 days. >90% confluent cells were passaged after 6 days of culturing to minimize heterogeneity of cells and were cultured again in the same conditions for expansion purpose.

Characterization of neonatal rat keratinocytes

Expanded neonatal rat keratinocytes were subject to microscopic observations for their unique morphology from 48 h up to having confluent monolayer of cells. The monolayer cells obtained after 6 days were washed with 1x PBS and then lysed using 1 ml TriPure Isolation reagent (Roche, USA). The cell lysate was collected in 2ml 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 12000g for 10 minutes. The pallet 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 pallet got separated and then centrifuged at 7500 g for 10 mints. After centrifugation, the ethanol with dissolved salts was poured out and subjected to air dry for 1-2 mints. 25 µl DEPC-treated water was added to the air dried RNA pallet 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). qRT-PCR was then performed following the PCR kit instructions (ThermoScientific, Lithuania) and expression of *K5*, *K10* and *K14* using rat specific primers (Table 1A) were observed to confirm the cellular population as keratinocytes.

Human keratinocytes (hKr)

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

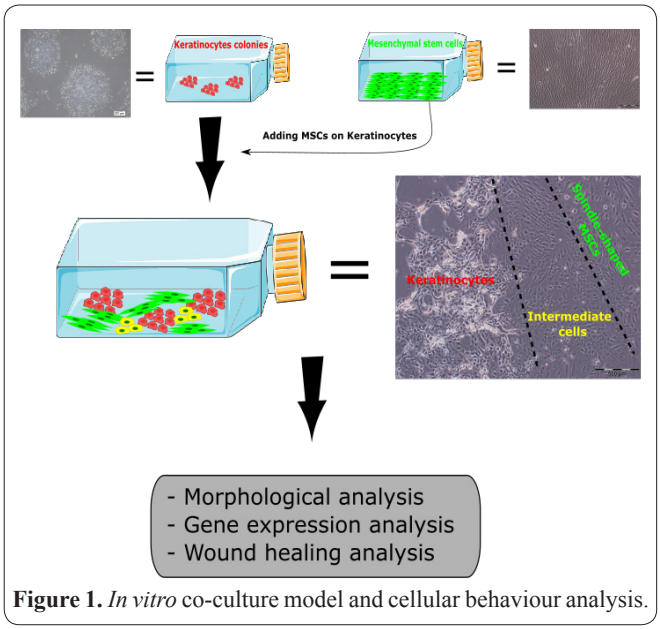

Figure 1. *In vitro* co-culture model and cellular behaviour analysis.

under the sterile conditions of cell culturing hood JTL-VC2 (Jal Tajhiz, Iran). Germs free pieces of human skin were washed with 1x PBS and were placed in already prepared sterile dispase solution (0.07g dispase (GIBCO, USA) + 10ml 1x PBS) at 4°C for overnight. The epidermis of skin was then separated mechanically and characterized using the cytokeratin markers *K10, K14, K18, K19, INV and P63* following the previously described protocol to isolate and characterize keratinocytes (27).

Co-culture model

Neonatal rat keratinocytes (NRKCs) were cultured and expanded in T25 flasks till >90% confluency of cells was achieved. After having a number of fully expanded NRKCs colonies at 14 days of primary culturing, 500,000 hMSCs were seeded and cellular morphology was observed after every 48 h. Both cell types were cultured using DMEM-LG for 7, 14 and 21 days. The entire co-culturing model is illustrated in figure 1.

Cell behaviour analysis

Microscopic observation

Cells in T25 culture flasks were observed every 48 h and the change in cellular behaviour was recorded after 7, 14 and 21 days of their co-culturing using iX70 invert microscope (Olympus, Japan).

qRT-PCR

Total RNAs were isolated from all three groups of co-cultured cells after 7, 14 and 21 days and cDNAs were synthesized and subjected to RT-PCR to observe the expression of cytokeratin markers i.e. *K10, K14, K18, K19, p63* and *Involucrin* using human specific primers (Table 1B) to avoid the expression of these markers in NRKCs. The purpose of using rat keratinocytes and human MSCs was to study the very specific species based expression of cytokeratin markers in human MSCs. A specific annealing temperature of 59° C was assigned to PCR tests which was achieved via gradient PCR to avoid non-specific attachment of primers. The relative expressions of cytokeratin markers were determined using qPCR approach. For this purpose, synthesized cDNAs were mixed with 2x SYBR® Green Real Time PCR Master Mix (Parstous, Iran) in qPCR 8-strip tubes (Gunster Biotech, Taiwan) and the mixture was subject to CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with an annealing temperature of 59° C.

Scratch assay

Scratch assay was performed to study the migration behaviour of induced hMSCs as normal MSCs do not

Table 1. A: Rat specific primers used in this study, **B:** Human specific primers used in this study.

Primer Name	Sequence	Accession No	Amplicon length (bp)
K5	F: CGTGATGTTGAAGAAGGATGTG R: TGAAGTTGATCTCGTCCATTAGG	NM_183333.1	92
K10	F: AATCAAGGAGTGGTACGAGAAG R: TGGCATTGTCAGTTGTCAGG	XM_006247391.2	125
K14	F: AAGTTTGAGACAGAGCAGAG R: GTGGTTCTTCTTCAGGTAGG	NM_001008751.1	153
GAPDH	F: ACAGTCCATGCCATCACTGCC R: GCCTGCTTCACCACCTTCTTG	NM_017008.4	266

Gene	Primer Sequence	Accession No	Amplicon length (bp)
K10	F: CCTTCGAAATGTGTCCACTGG R: CAGGGATTGTTTCAAGGCCA	XM_005257343.2	289
K14	F: TTCTGAACGAGATGCGTGAC R: GCAGCTCAATCTCCAGGTTC	NM_000526.4	189
K18	F: ATCTTGGTGATGCCTTGGACA R: ACTTTGCCATCCACTATCCGG	NM_199187.1	82
K19	F: TGAGTGACATGCGAAGCCAATAT R: GCGACCTCCCGGTTCAAT	NM_002276.4	103
P63	F: CGCCGCAATAAGCAACAG R: GTAGCCTCTTACTTCTCCTTCC	XM_005247844.3	184
INV	F: GGCCCTCAGATCGTCTCATA R: CACCCTCACCCCATTAAAGA	XM_006711300.2	131
GAPDH	F: ACAGTCCATGCCATCACTGCC R: GCCTGCTTCACCACCTTCTTG	NM_001289746.1	266

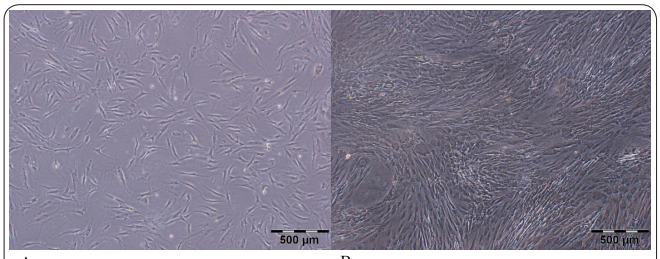

Figure 2. hMSCs of primary culture after 2 days (A) and 6 days (B) of first passage.

show any migration behaviour whereas the keratinocytes 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 cultured for 14 and 21 days. These scratches were kept under culturing for 48 h and migration capabilities of cells were analysed after every 24 h.

Statistical Analysis

GraphPad Prism statistical software was used to draw the bar charts of all triplicate experiments and significant values were determined.

Results

Isolation and characterization of cells

Human mesenchymal stem cells (hMSCs)

Human MSCs were successfully isolated from the waste lipoaspirates and became >90% confluent on culturing in LG-DMEM after 4 days of 1st passage. Cells were found to be spindle-like in morphology and had the potential to generate colonies as shown in figure 2.

Differentiation assay

MSCs were differentiated to confirm their differentiation potential towards adipocytes and osteocytes in their respective induction media. Their adipogenecity was confirmed by the presence of lipid droplets after 21 days of induction and their ostegenicity was confirmed by the deposition of calcium in cultures stained by Alizarin Red S as shown in figures 3b and 3c, respectively. Alkaline phosphatase assay also confirmed the osteogenic differentiation (Figure 3d). Undifferentiated MSCs used as control are also shown in figure 3a.

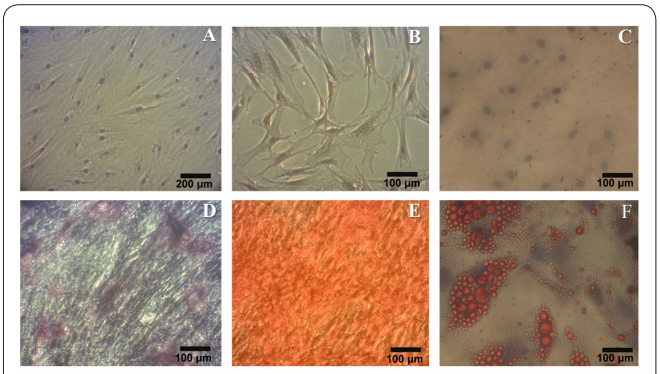

Figure 3. Differentiation of MSCS to adipocytes and osteoblasts *in vitro*. Figures **'A', 'B'** and **'C'** are showing the undifferentiated MSCs as control whereas figure **'F'** is showing differentiation towards adipocytes. Figures **'E'** and **'F'** are showing osteogenic differentiation of MSCs.

Flow cytometry analysis

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 4).

Neonatal rat keratinocytes (NRKCs)

NRKCs were successfully isolated from skin of new born rats (1 or 2 days). Their morphological behaviour was studied from day 4 to day 21. Their growing colonies and finally development of stratified sheet as shown in figure 5A-5F confirmed their identity as being keratinocytes. Their identity was further confirmed by the expression of cytokeratin genes such as *K5, K10* and *K14* using RT-PCR (Figure 5G).

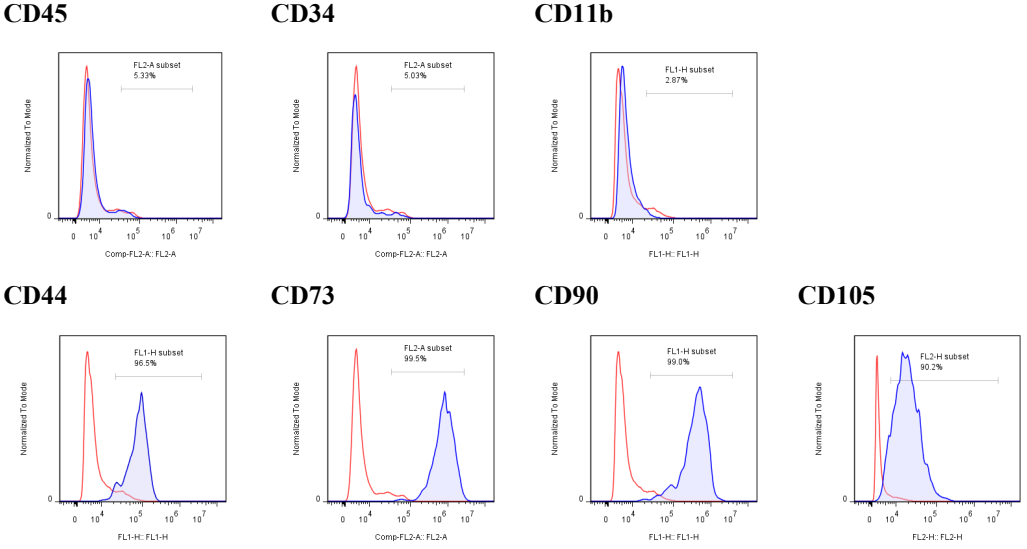

Figure 4. Flow cytometry analysis of cell surface markers in MSCs at passage 2. The calculated percentages of positive and negative cells are shown in figure.

Human keratinocytes (hKr)

hKr were successfully isolated from the waste skin of healthy persons who were subjected for aesthetic surgery and their identity as being keratinocytes was confirmed by the positive expression of cytokeratin genes such as *K10, K14, K18, K19, INV* and *P63* using RT-PCR (Figure 5H), according to the comprehensive protocol described previously.

Cell Behaviour analysis

Microscopic observation of co-cultured cells

Human MSCs were successfully seeded on the fully expanded NRKCs after 2 days of their seeding. It was observed that MSCs occupied all adherent spaces in the flasks surrounding and interacting with the NRKCs as shown in figure 6A, 6B. After 7 days of co-culturing, both cells were observed highly interacting and cellular population was thoroughly mixed inside T75 culture flasks (figure 6C & 6D). After 14 days of co-culture, the cellular populations were highly mixed and the morphology of cells had changed as observed under the invert microscope. At regions with less confluency, the heterogeneous population and intermediate cellular morphology was clearly observed (figure 6F). Co-cultured cells formed a cell sheet of differentiated MSCs showing keratinocyte-like characteristics (figure 6G).

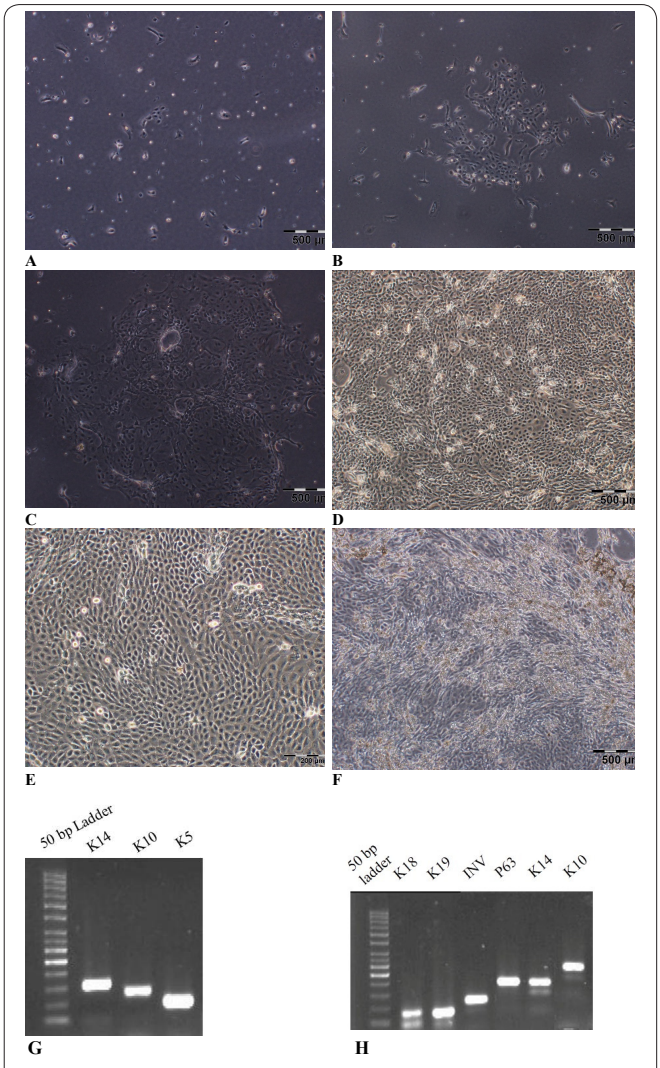

Figure 5. Expansion and characterization of NRKCs. Morphological behaviour of NRKCs after primary culture confirming their identity. **A:** adherence of cells after 4 days of culture, **B:** colony formation of keratinocytes after 8 days of culture, **C:** expansion of keratinocytes colony after 12 days of primary culture and **D:** fully expanded keratinocytes after 16 days of primary culture at 40x and 100x magnifications. **E:** Development of keratinocyte sheet after 21 days of primary culture (**F**) and positive expression of cytokeratins *K5, K10* and *K14* (**G**). **H:** Positive expression of cytokeratin *K10, K14, K18, K19, INV* and *P63* in cultured human keratinocytes.

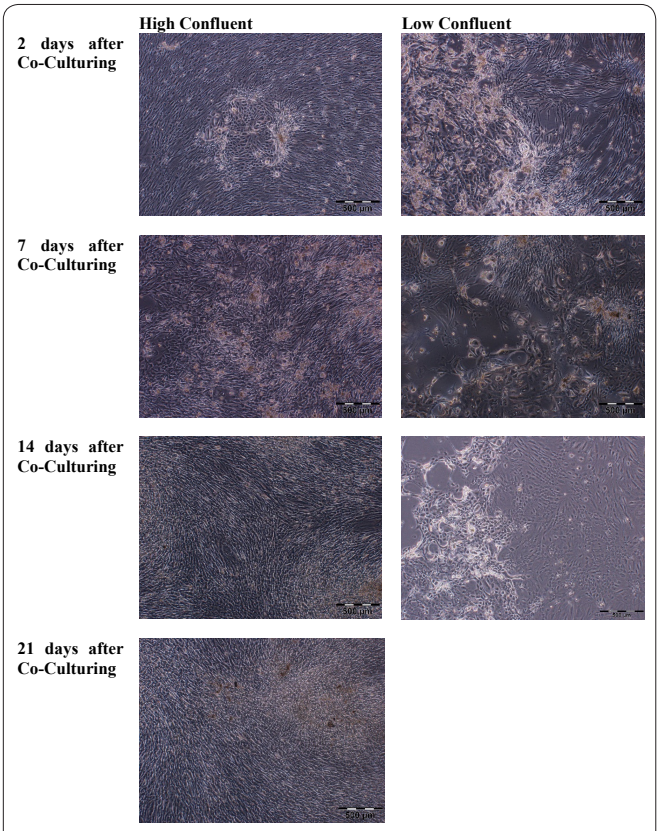

Figure 6. Microscopic observation of co-cultured cells from 2 days to 21 days.

qRT-PCR

Positive expression of cytokeratin gene markers such as *K10, K14, K18, K19, INV* and *P63* was observed when the isolated RNAs of 7, 14 and 21 days of co-culture experiments were subject to RT-PCR analysis (figure 7A). The expression of terminal differentiation marker *INV* and marker of epithelial stratification p63 were also observed as positive. qPCR was performed to further analyse their relative expression in different time intervals and it was observed that keratin 10 and 19 were expressed 10 folds more than human keratinocytes in hMSCs co-cultured for 21 days. K14 was also overexpressed in 21 days experiment only and its expression was very high as compared to all other markers and experiments. A low expression in 7 and 14 days of experiments was also observed but were not significant when compared with the human keratinocytes (figure 7B). Gradual increased expression for INV and P63 was observed in 7, 14 and 21 days of experiments showing their increased differentiation towards keratinocytes and developing epithelial stratification characteristics (figure 7B). K18 showed very different pattern, its expression was very high when MSCs start differentiating towards keratinocytes-like cells and then with time, its expression reduced gradually and in 21 days of experi-

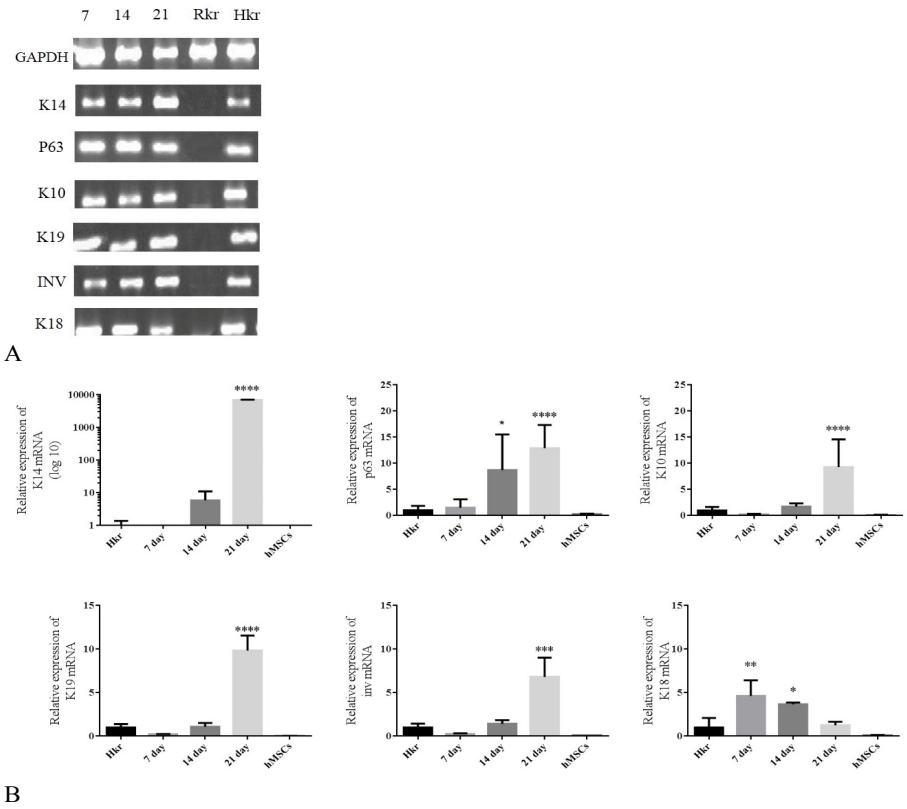

Figure 7. Expression patterns of keratinocyte markers in MSCs when co-cultured with rat keratinocytes for 21 days. **7a:** RT-PCR showing the positive expression of keratinocyte markers in MSCs after 7, 14 and 21 days of experiments. **7b:** The relative expression of *K10, K14, K18, K19, INV* and *P63* in triplicate co-culture experiments. Results were statistically evaluated by using one-way ANOVA with post hoc test. *, P < 0. 01, **, P < 0.001 and ****, P < 0.00001 were considered significant as compared with control (hkr).

ment, it reduced 5 times as compared with the expression in 7 days of experiment.

Scratch assay

MSCs in co-culture experiments showed their induction behaviour as discussed previously in respect of morphological variations and expression of cytokeratin markers. These cells also behaved like keratinocyte cells. These cells showed their induced behaviour to develop sheet and showed that in case of injury or rupturing the integrity of this sheet, they have potential to regenerate it. Having a scratch in the developed sheet was a very difficult task, as the sheet after having a scratch on it, pulled back towards the right and left sides due to the tensile strength present in the sheet. After the 14 and 21 days of co-culturing, the sheet was scratched and it was observed that cells start migrating after 8 h of scratch leaving the margins of ruptured sheet (data not shown). After 24 h of scratch, cells migrated towards the centre of scratch and after 48 h, the whole space of scratch was covered with cells showing its characteristic to regenerate the injury.

Discussion

MSCs have promising therapeutic potential because of their multipotent differentiation capacity and unique immunological modulation. They have several advantages over other cells as they are easy to obtain and culture, and have significant paracrine effects which make them ideal candidates in cell therapy to replace and repair the damaged tissues (28, 29). While understanding

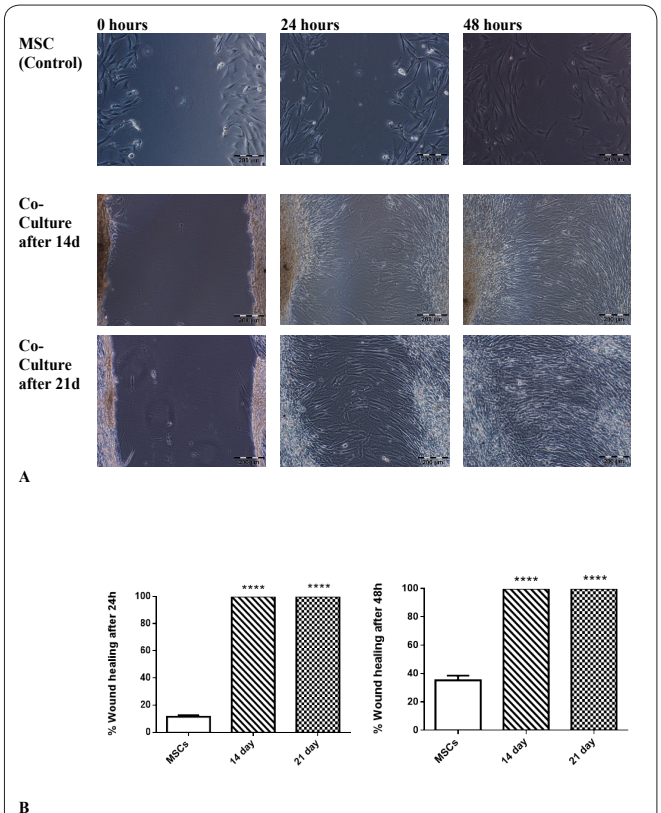

Figure 8. Wound healing assay for the cells in co-culture. **A:** Cellular morphology showing the migration of cells in scratch after 24 and 48 hours. **B:** Bar charts showing the % of wound closure as compared with the untreated MSCs as control. ****, P < 0.00001 was considered significant as compared with control (untreated MSCs).

the complexity of cutaneous wound healing, it has been found that stem cells of skin and surrounding tissues, like MSCs, HFSCs (hair follicle stem cells), Ep-SCs (epidermal stem cells) etc proliferate and diferentiate to maintain the cellular population during wound healing (9, 23, 30, 31). Evidences suggest that MSCs are present in peripheral circulation of wound area (32, 33) and they are known as critical healing agnets, also because of their differentiation potential and very specifically due to the paracrine effects which stimulate the proliferation of other cells such as keratinocytes, endothelial cells, and fibroblasts (34, 35). The exact mechanism of wound wound helaling is unknown but number of clinical studies have been perfomred showing the significant healing effects of MSCs on hard-to-heal or non-healing wounds as given in Table 2.

Re-epithelialisation is known as a very critical event in healing process which requires well organised differentiation of stem cells and epithelial stratification involving cellular migration, recruitment and differentiation (9). A number of evidences have shown that MSCs also reside in the dermis (44, 45) and play significant role in wound healing, as they regulate inflammation, promote angiogenesis, induce proliferation and differentiation of various cells and cellular migration (46). 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 (47-50). MSCs have successfully been applied to treat the specific burns such as radiated burns in a number of *in vitro* and *in vivo* models explaining the potential of MSCs to be applied for burn and other types of acute or chronic non-healing wounds (51-55). Very defined stratified epidermis can be achieved via culturing MSCs with epidermal cells and a number of ECM components (56). Excluding ECM components, MSCs were co-cultured with sweat gland cells for the purpose of enhanced and perfect healing showing the development of skin appendages (56). Some previous studies have also seeded MSCs on artificial skin substitutes (dermal skin substitutes) and were supplied with angiogenic factors like VEGF resulting in the increased vascularization during healing (57, 58).

Considering the importance of MSCs and their interactions with the keratinocytes for precise differentiation, we proposed an *in vitro* co-culture model in this study. We observed that keratinocytes after a long time in culturing conditions, start developing an epidermal sheet which has been applied for clinical purpose in many pre-clinical and clinical studies (59) but these sheets still are facing a number of post-grafting problems. Fibroblasts have also been shown the significant healing potential as these cells help to develop the ECM comoponents required for proper healing (15, 60) but these cells may not define the precise diffrerentiation and healing mechanism as a slight irregularity in cellular fate determination or maintainance may lead to an abnormal healing. These cellular functions are very complex and unique as they are regulating the normal healing process (61-64). The re-epithelialisation process which is very crucial for wound healing depends on the availability of epidermal stem cells for propoer stratification (65-68).

Our proposed model is the combination of MSCs which have many advantages over other clinically appplied cells as discussed earlier and keratinocytes. Keratinocytes were found to develop a sheet after 21 days of culture (figure 5F) whereas MSCs because of the high proliferation potential start developing sheet after 14 days and a thick epidermal sheet was observed after 21 days of culturing (figure 6). These induced cells in co-culture when were subject to molecular characterization, showed a very high expression of cytokeratin markers as compared with the human keratinocytes (figure 7B) showing the clinical potential of induced MSCs in wound healing. Keratinocytes have a high potential of migration to cover the vacant spaces among the different colonies and start developing epidermal sheets. Their migration capabilities have shown as they covered the scratches within 48-56 h showing their wound closure and healing potential (60). Induced hMSCs in our co-culture model showed 100% wound closed when were subject for *in vitro* scratch assay within 24 h and

Table 2. Clinical applications of MSCs to treat hard-to-heal or non-healing wounds.

No of Patients	Type of Cells	Treatment Results	Ref
3	Autologous BM Cells	Enhanced dermal regeneration and non-healing chronic wound closure	(33)
1	Authologus BM aspirate	Reduction in wound size and enhanced vascularity in non-healing wounds	(36)
1	BM aspirates &*in vitro* expanded MsCs	Closing and healing of the non-healing diabetic ulcer	(37)
4	Autologous cultured MSCs	Improved healing of non-healed chronic wounds	(38)
12	Autologous BM-MSCs	Cells loaded in a fibrin spray resulted in the subsequent reduction of chronic wound size	(39)
3	BM aspirates	Complete healing of chronic wounds	(40)
20	*Ex-vivo* expanded MSCs	Cells placed in collagen sponge resulted in complete healing of chronic wounds	(41)
24	autologous cultured BM- MSCs	Accelerated healing of non-healing ulcers	(42)
41	BM-MSCs & BM-MNCs	Improved healing of chronic wounds	(43)

high density of cellular population was observed after 48 h of scratch (figure 8A and 8B).

We concluded that MSCs alone are not the good agents for clinical applications because of the associated post grafting problems but when they co-cultured with keratinocytes (allografted) then it could be the gold standard in wound healing because of its precise fate determination. Further studies are required to findout the signaling pathways involved in their fate determination while in co-cultured and their wound healing potential due to the enhanced differentiation and proliferation potential because fate determined MSCs have high potential of being the wound healing agents to heal hard-to-heal wounds. Furthermore, *in vivo* experiments, preclinical studies and clinical trials are required to confirm their regenerative capabilities. Adcanced techniques are also needed to clarify the fate determination of MSCs in co-culture models before its recommendation as clinically significant model for cutaneous wound healing.

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