Isolation of a novel population of multipotent stem cells from epidermal layer of human skin

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Abstract

Fore skin samples were collected after surgical excision from boys below 7 years of age from Deccan Medical College –Hyderabad, in cold saline (4^0 C) in sterile containers. These skin samples were cut into small pieces and incubated in trypsinised phosphate buffer saline (PBS) overnight to dissociate epidermis from the dermis. The epidermal layer which got detached was lifted and taken for preparing the cell suspension. Viability of cells was 88% with PI staining. About 50 µl of cell suspension was aliquoted into vials (10^5 cells/per vial) and incubated with 2 µl of the antibodies. Various biomarkers of stem cells such as CD34 – one of the marker's of skin stem cell, CD 49f & CD29 - common stem cell markers, CD45 Lymphocytic marker, CD90 (Thy-1) skin stem cell marker and CD105 endothelial marker and CD56 (NCAM) neural adherent marker were used . Using these cell surface markers of stem cells, cytometry was performed on a FACS Calibur with sorter (BD Biosciences). These immunophenotypic markers with Cellquest software expressed the stem cells characteristics. FACS analyses gave different profiles of stem cells /progenitors of mesenchymal, hematopoietic and neural progenitors. These expressed profiles indicated that its progenitor might have the multipotent and or pluripotent character. By using embryonic markers, we could select a population of stem cells to give multipotent or pluripotent cells that could have the capacity to regenerate skin cells for application in burn cases, wounds, deep burns and non-healing wounds. In conclusion, we present here for the first time the isolation of pluripotent / multipotent skin stem cells from human foreskin biopsy samples and its potential applications for various applications.

Keywords: Skin Stem Cells; Immunophenotyping; Biomarkers; FACS; Isolation; Epidermis; Antibodies; Multipotent; Pluripotent Stem Cells.

Introduction

Stem cells from the epithelial layer are being isolated due to advances in molecular and cellular biology (Cotsarelis, 2006). It was predicted by several authors that epithelial stem cells have been definitively addressed through new techniques. We know that cells are generated through proliferation that occurs only at the basal layer therefore stem cells must be located there. Based on these proliferative and morphological characteristics, Potten (1974) coined the term epidermal proliferative unit. Hence, the epidermis and hair follicles are the known source of the stem cells.

Several characteristics such as capacity of self-renewal with multipotency have been defined for the epidermal stem cells. The determination of a specific marker or combination of markers that would assist in their direct identification within the epidermis is still the focus of much interest. Surface markers of stem cells in the haematopoietic system such as CD34 (Satterthwaite *et al.*, 1992) and CD133

neural stem cell markers (Uchida *et al.*, 2000) have aided in identification of specific cells with stem cell character, their isolation and purification. Several studies have characterized putative epidermal stem cell with different markers. In general, stemness character is similar in all tissues cells due to the properties of these cells, however whether skin cells also have same profile /properties of surface expression of markers, which relate to stem cells have to be found. It remains to be determined if these markers can be used as a 'footprint' to indicate the 'stemness' potential of the epidermal cell population. The identification and isolation of epidermal stem cells has been the goal in regenerative medicine. Epidermal stem cells from hair follicles and other sources have been widely used for wound healing, even artificial skin has been considered, and cell-based models have been considered for drug testing and for gene therapy. The potential stem cells in current medicine have been well documented (Jamil and Das, 2005; Jamil, 2009).

Pavlovitch *et al* (1991) studied the characters of the homogeneously small keratinocytes ([^{3}H] thymidine label-retaining cells) by the phenotype expression of these proliferating cells, which were approximately 1% of the total basal keratinocytes and consisted of extremely small cells with very little cytoplasm or RNA. These keratinocytes were in the G-0 stage of the cell cycle and did not proliferate rapidly in vivo. 10 per cent of basal cells showed stem cell nature with alpha 6^{bri}10G7^{dim} phenotype (Li *et al.,* 1998). In humans, alpha 6^{bri} 10G7^{dim} phenotype cell-fractions were demonstrated as pure keratinocyte stem cells (Kaur *et al.,* 2000). Later, Tani *et al.* (2000) and others demonstrated high levels of alpha6 integrin and low expression of the transferrin receptor (CD71) in murine organisms. Approximately 8% of *alpha6^{bri}CD71^{dim} cells* were epithelial stem cells (Tani *et al.,* 2000; Webb *et al.,* 2004; Kim *et al.,* 2004; Lorenz *et al.,* 2009). TA cells expressed the phenotype of alpha6^{bri}CD71^{bri} with approximately 60% of basal keratinocytes that were active in cell cycle phase (Tani *et al.,* 2000). Further investigations with the heamatopoietic stem cell marker showed CD34 co-expressed in both slowly cycling (label retaining) cells and keratin-15 positive cells which were basal keratinocytes of follicular origin (Trempus *et al.,* 2003). In human, alpha6^{bright}/CD71^{dim} phenotypic keratinocytes showed stem cells character (Terunuma *et al.,* 2007). In post-primary (cultured cells) neonatal keratinocyte, expression of CD90 phenotype represented the skin stem cells. However, most of the keratinocytes maintained expression profile of alpha6 integrin in culture (Nakamura *et al.,* 2006). Another investigation found a novel cell-surface marker MTS24 in hair follicle keratinocytes progenitor cells (Nijhof *et al.,* 2006). In flow cytometry analysis, Albert *et al.* (2001) showed that cell-surface marker CD34, Sca-1, and Flk-1 (haematopoietic and skeletal muscle stem cells biomarkers) were expressed in non-labeled retaining cells of BrdU labeled mice, these are also important markers for skin stem cells. Beta-1 integrin also expressed highly on label-retaining cells compared to tested markers (Albert *et al.,* 2001). Previously, the human hair follicle bulge was identified as an important niche for keratinocyte stem cells. Bulge cells commonly expressed positive phenotypes such as CD200, PHLDA1, follistatin, and frizzled homolog 1. Other phenotypes expressed by non-bulge keratinocytes were CD24, CD34, CD71, and CD146. Importantly,

CD200+ cells obtained from hair follicle suspensions showed high colony forming efficiency, indicating living human bulge stem cells (Ohyama *et al.,* 2006). K14-GFP-actin expressing alpha6^{low}CD34^{high} and alpha6^{high}CD34^{high} populations formed appreciable numbers of tightly packed, large colonies (>20 mm^2; >10^4 cells) containing cells of small size and relatively undifferentiated morphology (Blanpain *et al.,* 2004). Guo and Jahoda (2009) found that keratinocytes produced from explant cultures expressed skin progenitor markers like p63, K15 and K6, co-expressed with α6 and β1 integrins. In 3-day old cultures CD34, CD133 and K15 antibodies reacted positively but Dsg3^{dim} was downregulated. Mani *et al* (2007) also reported applications of biomarkers like CA125, CEA, AFP and beta HCG in cancers.

The heterogeneity of surface protein profile expression of skin stem cells gives some information about stem cells immunophenotypic markers but more character analysis are needed to isolate primary cells and skin stem cells *in vitro* for proper identification. Hence an attempt was made to identify and sort stem cells from epidermal cells of the skin samples.

Materials and Methods

The project was approved by Institutional Ethics Committee in compliance with National Guidelines regarding the use of human tissue for research purposes. Foreskin samples were collected after surgical excision from boys below 7 years. The skin tissues were transported in sterile tarson tubes containing buffer in ice container. These tissues were immediately processed.

Isolation of epidermal cells
Skin biopsies from healthy volunteer adults (boys below 7years of age) were obtained after surgical procedures; these samples are generally discarded and never used for any purpose. Hence, these skin samples were collected for our studies. Each sample was separately processed for isolation of epidermal cells in aseptic conditions. The skin biopsy samples were washed twice before further processing. The skin tissue after washing was cut into small pieces (approximately 0.75cm^2 area) and incubated in Ca^{++} and Mg^{++} free phosphate buffer saline (PBS) containing 0.25mg/ml trypsin solution in refrigerator and time period required for the dissociation of

epidermis from the dermis was determined. Next, epidermis was detached from the skin tissue, washed and minced into the smallest possible fragments using surgical scissors in hanks buffer medium. The fragments were collected into 50 ml plastic tubes and triturated to produce single cell suspension. Then cells were washed 3 times in PBS by centrifugation at 4000 rpm for 5 min at 4^0C. The cell pellet was collected and suspended in DMEM medium, which contained glucose and growth factors. This cell suspension was stored at 4^0C until further use. This cell suspension was used for cell counting by PI staining and for viability by Trypan blue dye exclusion test. The method for isolating the epidermal cells was essentially as described by Katayama *et al.* (1987) with certain modifications as mentioned above.

Sample processing for Flow Cytometry
Isolated cells were resuspended at 1×10^6 cells in 100 μl of in Ca^{2+}/Mg^{2+}-free PBS buffer containing 0.02% sodium azide and 1% human albumin and divided into 50-µl aliquots containing approximately 1×10^5 cells in each vial, to which fluorescent antibodies or isotype controls were added. The dilutions used were in strict accordance to the protocol as described in the kits, the concentrations used were 1:100 for all samples. Cells were incubated in the dark at room temperature for 20 min and then washed with 2 ml PBS. The samples incubated in the dark for an additional 15 min and then washed again. These cells were resuspended in 200 µl of PBS containing Propidium iodide (1 mg/ml) for viability count.

The antibodies used in this study were obtained from BD Pharmingen: these were CD56/CD3/CD16, CD34, CD 49f, CD29, CD45, CD90 and CD105.

Cytometry was performed on a Fluorescence Activated Cell Sorter (FACS) Calibur (BD Biosciences Pharmingen).

Sample analysis and calibrations by FACS
The isolated cells were first treated with antibodies that have been uniquely coupled with fluorescent dyes and then passed through a laser beam which will separate the cells based on the light emitted, characteristic of the antibody signature of the cell type which in turn be detected by the photocell used to identify the subpopulation of cells. Each Biomarker was separately tagged to the epidermal cell

suspension aliquoted in vials and analyzed in FACS. We also used combination of two markers CD34 with CD49f and CD29 with CD49f along with the cells and these were separately analyzed in FACS. Color compensation was set using calibrated beads, and samples optimized for each fluorochrome. Three-color live gating was used to optimize settings and acquire data. Ten thousand events were collected per sample and analyzed using CELLQUEST Software.

Results

We describe here the results of our efforts to isolate skin stem cells from a novel source - the foreskin biopsy samples from humans. Using the above protocol and the various procedures, we could identify skin stem cells from epidermal cells.

Isolation of epidermal cells
All the six biopsy samples were collected separately in Hanks medium and incubated over night in cold trypsin solution. Epidermis formed a free-floating layer, which was taken for further processing. Cell suspension was prepared as described above, the mixed cell suspension settled at bottom of the vial upon centrifugation. Fibroblast cells did not disassociate from dermis. The average time-required for separation epidermis from dermis was about 22-24 hrs whereas skin biopsy samples incubated in PBS solution as controls took a maximum of 7 days for separation of the two layers. Total yield of epidermal cells were approx. 2.5 million mean cells per biopsy sample measuring about $4cm^2$ area.

Determining the Viability of the cell suspension
Viability of the isolated skin cells was done by (a) Trypan blue dye exclusion method and (b) by Propodium Iodide staining method in FACS. To determine panel adjustments in the FACS system propidium iodide (PI) viability test, dot-plot panel viability clusters were analyzed through PI staining by cell quest software; unstained cells were run as control sample. Dot-plot panel was prepared to analyze the parameters. Further IP stained samples were taken in second panel with dot plot and gated through R_1 and R_2 as shown in the FACS result data sheet using the FACS- software with automatic output. The viable cells did not take up the PI stain, viable cells did not shift towards the R1 gated region, the viable cell count was 88 % by both the methods (Figure- 1).

Immunophenotyping

To identify stem cells from the existing population of cells appropriate surface biomarkers were used and FACS analysis was carried out. The distinct nuclear morphology of stem cells was used for identifying possible contamination of the population from normal differentiated cells. FACS analysis of primary skin epidermal cells which showed stem cells characters as expressed by immunophenotypic markers is shown in the figures (Figure-2 & 3). Primarily the number of clusters was analyzed by forward and scattered profile as they formed two distinct clusters, shown in the figures (figure 2 & 3).

Expression studies using stem cell markers

The protocol for carrying out the stem-cell marker expression studies by FACS has been described in the methods section. FACS data of Immunophenotypic markers profile of isolated epidermal cell is shown in Histogram plot (Figure-2) and bar graph (Figure-4), these are single monoclonal antibodies expressions and Figure-3 represents dual antibodies expressions in dot plot graph. Sample (*n*=6) values of cell quest software are presented in table -1.

(i) CD34 is a known marker for hematopoietic stem cell character as well as a stem cell-marker for many other tissues. The expression of CD34 in test sample cells was 19.60%.

(ii) Similarly beta -1 (CD29) is one of the keratinocyte stem cell marker and its expression was highest - 26.67%.

(iii) The expression of other stem cells markers like CD 49f was 18.10%, CD 90 was 10.86 %, and CD 105 was 1.22%.

(iv) Neural progenitor marker CD 56 (NCAM) was also expressed - 15.08 %,

(v) CD 90 and CD 105 had very low-level expression and this indicates the possible lineage of the mesenchymal progenitors.

(vi) Cells expressing dual markers were: CD34 /CD49f -7.06% and CD49f/CD29 - 6.92% and CD34/CD45 -13%.

Samples that had run up to 10,000 events were taken for counts to express mean values.

Discussion

Identification and isolation of the epidermal stem cells have been goals in regenerative medicine. Here, we discuss our results concerning two major aspects (a) Isolation of skin epidermal cells and (b) Immunophenotyping/characterizing the skin cells for determining the presence of stem cells in the isolated epidermal cells. An analysis of the growth potential of the primary human foreskin keratinocyte cells and their serially cultured cells showed that the cells were smaller than 11 and 20 mμ respectively, were most clonogenic and had the great capacity for colony forming ability. Similar report was also described by Barrandon and Green (1985). Some researchers reported that smaller skin cells showed progenitor/stem cells characteristics (Kim *et al* 2004; Li *et al* 2004; Webb *et al* 2004; Youn *et al* 2004). In our experiments, we found skin stem cells in basal layer as in above studies. These cells were microscopically smaller and interconnected with extra cellular matrix mainly with collagen proteins. Using the modified procedure, we could isolate skin cells, and prepare single cell suspension from tissue layers.

On commitment to terminal differentiation, basal cells exit the cell cycle and subsequently migrate into the suprabasal cell layers. These progenitors are capable of generating both hair follicles and Interfollicular Epidermis (IFE) and lie in the hair follicle bulge (Clayton *et al.,* 2007). We could optimize the methods and standardize with mechanical non-enzymatic trituration method of Panchision *et al.* (2007) and enzymatic dissociation (Rheinwald and Green, 1975; Hybbinette *et al.*, 1999; Walzer *et al.,* 1989; Kitano *et al.,* 1983). The non-enzymatic trituration had the disadvantage as it kills large percentages of cells, which remain as clumps of tissue cells. In enzymatic digestion, it is possible that the crude preparations can cleave important surface antigens and makes the identification of cells more difficult (Panchision *et al.* 2007). Cell surface glycoproteins are important markers to enrich skin stem cells and to determine the lineage characterization *in vitro and in vivo.* Panchision *et al.* (2007) also reported the effects on surface glycoproteins by enzymes in isolation of stem cells. There are several methods, but we have selected the method of Katayama *et al.* (1987) with some modifications and we could separate the skin tissues into epidermis and dermis and we could standardize the collection of the epidermal layer of the skin for our investigation with much ease. Hence, we report here the method of isolating the epidermal cells from the skin tissues. This method minimizes the cell clumps and surface antigen loss and high viability can increase in short time. PI staining test showed approximately 90% viability in our isolation method.

The skin epidermal cells isolated from the epidermis also contain the basal layer cells. Basal layers are known to be a skin germinal layer and persist with skin stem cells. Basal cells were concentrated and removed, undigested tissue cells clump were removed by filtering with 75 micron nylon membrane and skin cells were isolated with intact cell morphology. Under Inverted phase Contrast microscope, 15 μm sized epithelial cells were seen after staining with Haematoxylin and Eosin. These were the immature cells. The isolated skin cells were suspended in DMEM with glucose in the medium and stored in -20°C for longer period and 4°C for shorter period.

Immunophenotypic characterization of skin cells
Cell therapy approach is based on the transplantation of appropriate cells, which must not only be well characterized biologically but should be stem cells that have been isolated from the human adult tissue. The isolation and characterization of skin stem cells from the human skin open up a further interesting therapeutic perspective because of immature and less immunogenic nature. The basal layer zone has a high regenerative potential area, suggesting human foreskin as an ideal source of stem cells which have additional characters of neural crest stem cells (Wong *et al.,* 2006) etc, these are well suited for applications in wound healing or degenerative diseases.

Single human skin biopsy can provide a good source of multiple precursor cells, which were observed in *in* vitro culture system. By inducing growth factors however, these were not analyzed functionally in *in vivo* experiments (Toma *et al.,* 2005; Wong *et al.,* 2006). The main challenge is in the skin regenerative medicine, to isolate viable skin cells for stem cells, further enrichment of epidermal progenitor or stem cells using phenotypic biomarkers can provide maximum enriched population of skin stem cells. Satterthwaite e*t al.* (1992) reported that several characteristics such as capacity of self-renewal with multipotency have been defined for the epidermal stem cells. The determination of specific markers or combination of markers would assist in their direct identification within the epidermis is still focus of much interest.

Hence, we had screened for profile of epidermal cells by immunophenotyping by FACS analysis and quantified. In our methodology, approximately 90% viable cells were present. In immunophenotypic analysis, the isolated foreskin epidermal stem cells expressed the hematopoietic stem cell marker CD34 and it was a universal stem cells marker. This indicates that in the basal layer skin stem cells were present in our samples. These were similar to other identified skin stem cells phenotypic expression however, the quantitative analysis showed 19.60% mean value from adult skin whereas different methods reported 2-10% stem cells present in isolated primary samples (Li *et al.,* 1998).

Evaluation of cell surface markers
Integrin beta-1 (CD29) is a stem cell associated marker and its expression was maintained in follicular epithelial culture colonies (Inoue *et al.,* 2009). Other researchers reported that it was strongly expressed in skin stem cell of cultures and also expressed in primary cells. The percentage of expression of beta-1 immunophenotyping was 26.67%. This indicates that follicular skin stem cells like characters were found in foreskin epidermal cells, similarly CD49f progenitor cell marker quantified as 18.10% in the skin primary cells. Its expression also supports the follicular or bulge type progenitor cells character. Its expression was lower than CD29 marker, 10.86 % cells were expressed by thy -1 (CD 90) marker, which is skin stem marker, non-haematopoeitic stem cell marker, and also a marker for stromal mesenchymal stem cells. This indicates the multilineage type of stem cells present in skin cells. However, one of the endodermic lineage marker (CD105) and also endothelial marker and one of the mesenchymal stem cell marker showed very low level expression (1.22%). This indicates that the isolated cells were maximum ectodermic lineage cells and some mesenchymal cells were present. Other ectodermal related human neural lineage marker CD 56 was expressed as 15.08 % in primary cells. This indicates that neural progenitors were also present in the skin tissue. Isolation of autologous neuronal precursors from skin-derived precursor cells extracted from adult human skin would be a very efficient source of neurons for the treatment of various to regenerate injured nervous systems (Peripheral nervous system and central nervous system) and neurodegenerative diseases. Gingras *et al.* (2007), Fernandes *et al.* (2006) and Hunt *et al.* (2009) reported that neural cells were found in skin in their experiments and they expanded them *in vitro.* It showed that neural precursor cells are present in skin. This supports the plasticity of skin cells to neural cells. However,

we showed direct evidence through the primary cells expression in freshly isolated cells.

Multilineage of stem cells in skin cells
Dual expression of CD90 and CD105 indicated their similarity to MSC derived from bone marrow cells. Similarly found in bone marrow cells (Kassis *et al.,* 2006; Dominici *et al.,* 2006). Nakamura *et al.* (2006) used CD90 (Thy-1) mesenchymal stem cell marker for alternative skin stem cells cell surface marker. CD90 expressed highly in isolated epidermal cells and gave evidence of persistence of skin stem cells. However, the mesenchymal stem cells present in dermal layer cells may be contaminated in the isolated cells.

Dual antibodies, CD34/CD49f, are expressed cells also represents the skin stem cells profile as previously reported *in vitro* by Blanpain *et al.* (2004) and Trempus *et al* (2003). Blanpain *et al* (2004) demonstrated that the CD34+/α6+ were identified from bulge population and showed self-renewing and multipotent skin stem cells by growth and transplantation *in vitro* assays. Similarly, in our study we found dual expressed cells CD34/CD49f, which represent the multipotent skin stem cells profile. In our studies, we found that very few cells expressed CD49f/CD29 markers. Similarly, we found in enriched skin stem cells profile. Tani *et al.* (2000) and Jones *et al.* (1993) reported that they enriched skin stem cells by sorting method that expressed high levels of CD49f or its subunit pair CD29. Fortunel *et al* (2003) reported that Microarray experiments performed by several independent groups also showed CD49f overexpressed consistently in hematopoietic, neural, and embryonic stem cells. The presence of positive profile of the haematopoeitic stem cells (CD34/CD45) supports haematopoeitic cells and were in minor quantity but most of the samples did not express CD14 and CD45 and CD3/CD16 were negative in whole samples.

Meindl *et al.* (2006) have demonstrated that the skin contains CD45$^+$ and CD45$^-$ populations of cells that can be distinguished by the expression of HSC markers such as Sca-1, CD34, and CD117. Moreover, they have further developed a technique, which allowed to purify subpopulations to high purity and viability and to analyze their differentiation capacity *in vitro* and *in vivo*. Furthermore, they have observed that the growing number of antigens available for immunotyping skin endothelium may raise intriguing questions about the significance of

shared gene expression patterns between endothelium and stem or progenitor cells of haematopoietic and nonhaematopoietic tissues. In our investigation, Haematoxilin and eosin staining of isolated skin cells indicated that the blood cells were not contaminated but immunophenotypic heamatopoitic profile was found in those samples. Recently, it has been demonstrated that stem cells in various organs had multipotent nature. For example, bone marrow contains human mesenchymal stem cells, which can be induced in culture to differentiate to adipocytic, chondrocytic, or osteocytic lineages (Pittenger *et al.*1999)

In this investigation, immunophenotypic analyses have shown the different stem cells /progenitor profiles of skin stem cells, mesenchymal, heamatopoitic and neural progenitors. These expressed profiles indicate the possibility of their progenitor having multipotent and/ or pluripotent character and by using embryonic markers, we can enrich them to give multipotent or pluripotent character; that have the capacity to regenerate skin stem cells for applications in burns, wounds and even deep burns and non-healing wounds. Morasso *et al.* (2005) proposed that appropriate treatments could have a broad and complex approach, and may require the targeting of more than one type of cell population. Various studies using biomarkers, expression studies and signaling pathways like those described by Shahi *et al* (2009) increase our understanding of stem cell plasticity, therefore further investigations could lead us to determine if, like other stem cells from various organs, epidermal stem cells can be capable of multipotent differentiation, with the possibility of finding shared and even universal stem cell features. In conclusion, we present the first report of the method for isolating and identifying the stem cells from foreskin using various stem cell markers and characterizing by immunophenotyping using FACS.

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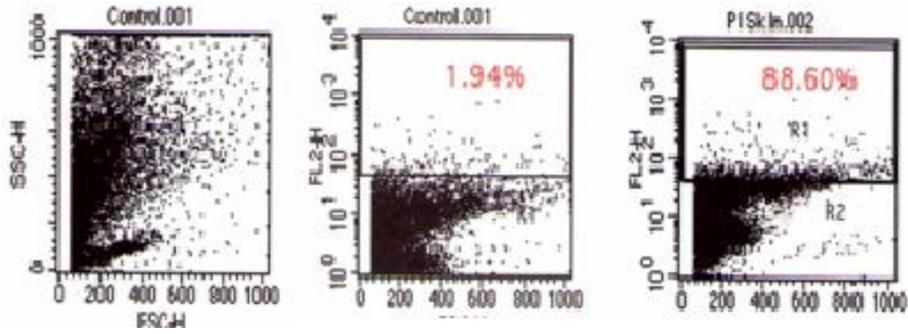
PI stain: Negative = 88.60% (Viable cells)
Control =1.94%

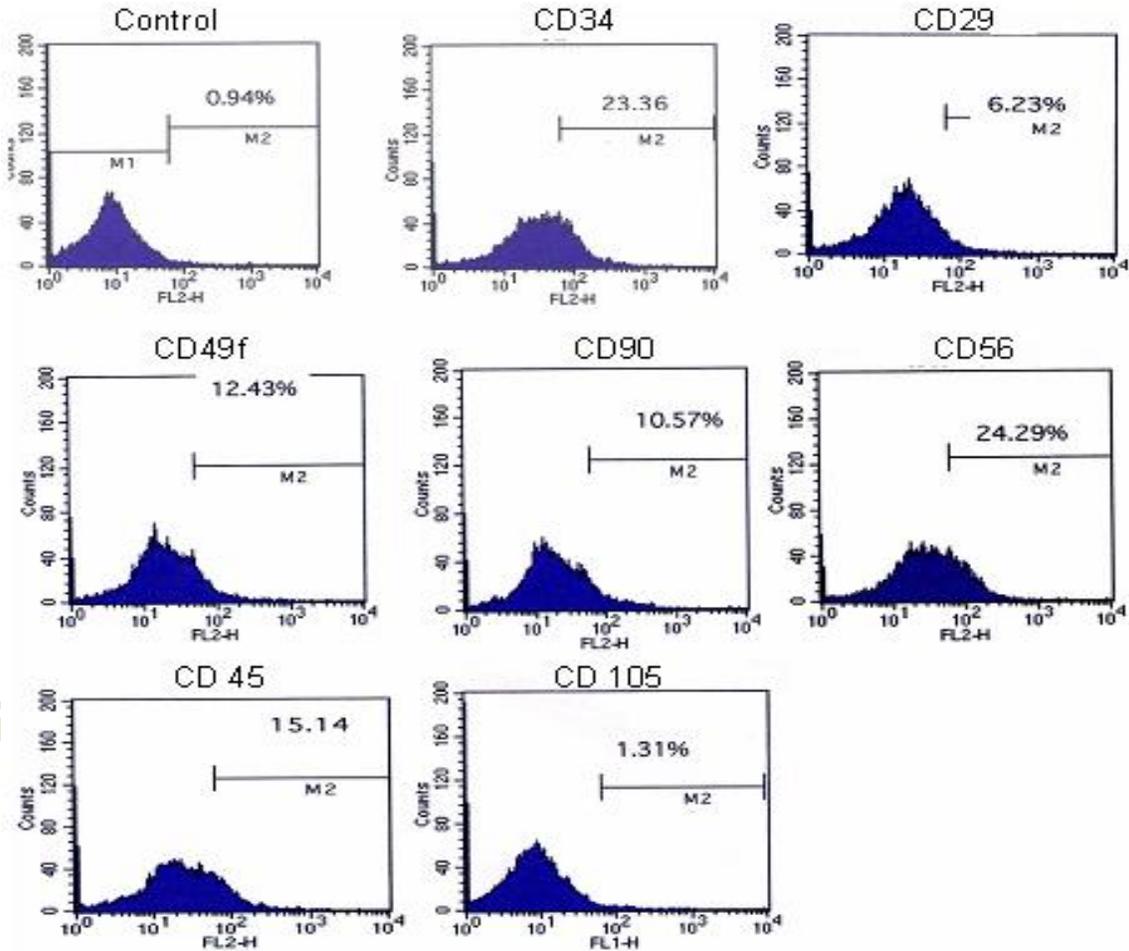**Figure 1: Viability test of epidermal cells by PI-FACS method.**

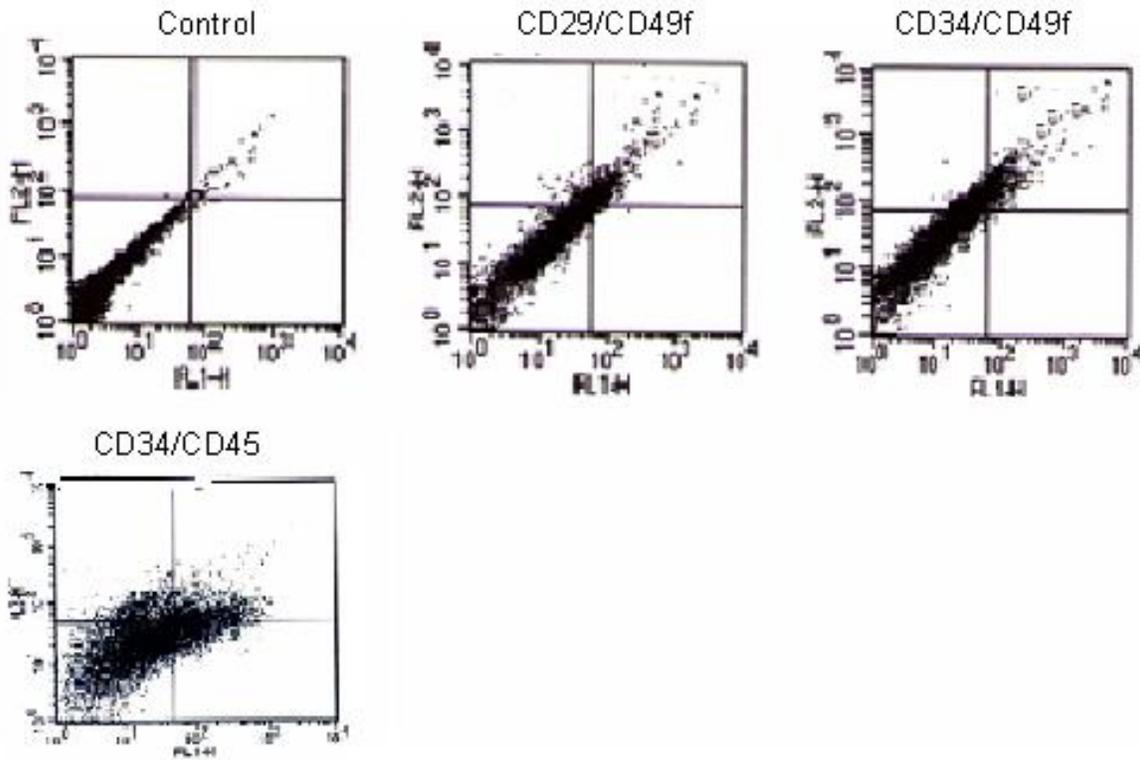**Figure 2: Immunophenotypic expression in dot plot graphs.**

Figure 3: Dual marker immunophenotypic expression in dot plots.

Table 1: Immunophenotypic expression profile of isolated primary skin epidermal cells.

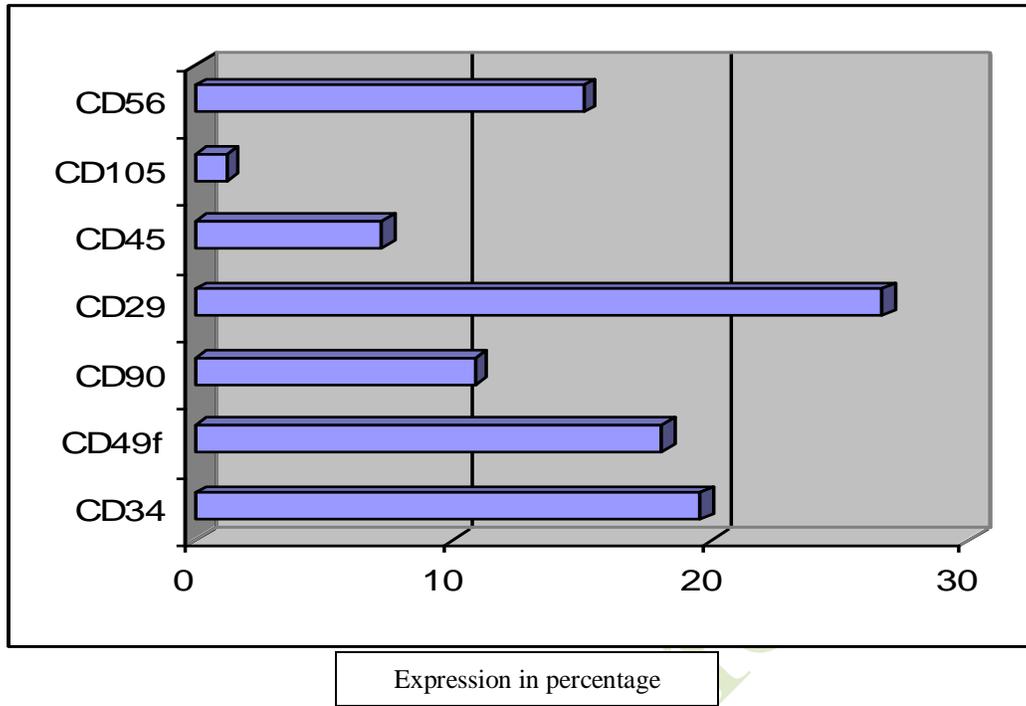| S. No.	CD34	CD49f	CD90	CD29	CD45	CD105	CD56
1	2.95	10.88	3.61	4.24	15.14	3.57	0.14
2	19.25	1.48	5.46	13.78	0.20	0.25	1.49
3	16.87	15.71	6.62	12.79	8.33	0.32	33.21
4	35.21	12.83	20.75	81.07	1.42	0.11	3.14
5	23.36	12.43	10.57	6.23	1,32	1.31	24.29
6	20.01	55.28	18.20	28.04	11.24	1.80	28.25
Mean values	19.60	18.10	10.86	26.67	7.26	1.2	15.08

Expression in percentage

Figure 4: Immunophenotypic expression of epidermal skin cells in bar graph representation.