STUDY 3C
Rapid Isolation of Integrin Rich Multipotent Stem Cell Pool and Reconstruction of Mouse Epidermis Equivalent
3C.1. Introduction

The epidermal stem cells (EpSCs) in mammalian skin are the multipotent keratinocyte stem cells that construct the stratified epidermis as well as other tissues of ectodermal origin. In the epidermis, EpSCs form columns of spiny (spinous), keratohyalin producing (granular), and keratinized (cornified) cells sequentially arranged in suprabasal cell layers to construct and maintain the homeostasis of the tissue. EpSCs are located in the basal layer and in the bulge area of hair follicles. These are either quiescent or slow-cycling and small in numbers. EpSCs are actuated to form daughter stem cell exhibiting unlimited proliferation potential or the transient amplifying (TA) cells showing limited proliferation potential and keratohyalin producing property.

In literature, a considerable interest has developed to reconstruct epidermis equivalents using preferably the embryonic stem cells (eSCs) or the induced pluripotent cells (iPSCs) (Bilousova et al. 2011; Ghadially 2012; Vollmers et al. 2012). Apparently deviant in nature, this approach is hypothetically expected to regulate tissue reconstruction by reprogrammed cells and the derived TA cells designed for a secondary and possibly the non-optimal potential of their gene expression profiles. Consequently, such an abnormality may determine expressions of vital factors like integrin, keratins, CD34, p63 required for maintenance of stem cell characteristics. Theoretically, the incongruous potential of eSCs or iPSCs can sub-optimally regulate homeostasis, repair, and maintenance of the engineered tissue by affecting limit of stem cell-cycling potential and/or the length of cell doubling time. We recommend use of EpSCs to dispel these inadequacies and reconstruct bioengineered epidermis product potentially by natural means to accommodate the desired applications; however in literature, there is a paucity of such endeavours. A literature search for epidermis reconstruction using multipotent stem cells yielded inadequate results.

Ordinarily, the pure culture of EpSCs (> 90%) is obtained after enrichment by a combination of FACS and collagen-fibronectin adhesion based methods (Poojan and Kumar 2010; Poojan and Kumar 2011). However, this approach is labour-intensive
and results in a poor yield of EpSCs, this restriction makes it painstaking to develop true skin equivalents. Furthermore, use of FACS is often discouraged for probable involvement of mechanical forces in loss of stem cell-adhesive properties after several passages and for subsequent loss of their characteristic features like self-renewal, quiescence, and differentiation potential finally influencing tissue homeostasis (Vollmers et al. 2012). Low harvest of stressed EpSCs can instill structural and functional abnormalities in new tissue. The unstressed EpSCs can be sorted also by the multiple passage method (Barrandon and Green 1987), however the process is prolonged due to leisurely enrichment of EpSCs and thereafter the epidermis reconstruction.

We worked on the use of exclusively the adhesion enrichment procedure to allow (a) sufficient and rapid enrichment of unstressed and intact EpSCs, (b) reconstruction of the epidermis in a natural manner, and (c) expectantly stability of the homogeneity of gene expression pattern and the tissue homeostasis. Keratinocyte stem cells show strong adhesive properties with collagen and fibronectin (Watt and Jones 1993). The reconstruct thus prepared would be label-free and compatible with natural epidermis. Use of exclusively EpSCs rich preparations has never been attempted earlier to reconstruct epidermis equivalents (Li et al. 2008; Nowak and Fuchs 2009; Ghadially 2012). We have done a comparative study of EpSCs enrichment methods using FACS vis-à-vis matrix-adhesion procedure to obtain a substantially greater yield of authentic EpSC from neonate mice keratinocyte isolates and have examined their stem cell characteristics by analysing the specific biomarkers using immunocytochemistry and immunoblotting techniques. With a known seeding density, epidermis have been reconstructed exhibiting replicable and validatable results. The histological differences, if any, have also been studied in homeostasis of natural vs reconstructed mammalian epidermis. The results validate the physiological lineage of EpSCs enriched by matrix-adhesion approach.

3C.2. Materials and Methods
The EpSCs were isolated from BALB/c neonate mice epidermis and cultured as per our protocol (Poojan & Kumar 2010, 2011). BALB/c mice pups (2-3d old neonates) were
procured from IITR (CSIR), Lucknow. Chelex-100 resin (BioRad Laboratories, CA catalogue No. 1422832); Collagen type IV (Sigma-Aldrich, Cat. No. C5533); Dispase (Invitrogen, Cat. No. 17105-041); DMEM (Invitrogen, Cat. No. 31600-026); Fibronectin (Sigma-Aldrich, Cat. No.F1141); KSFM (Invitrogen Cat. No. 10725018); Stempro Accutase (Invitrogen, Cat. No. 25200-056); Millicell 12mm insert (Millipore, Cat No. PIHP01250); Hoechst-33342 (Sigma-Aldrich, Cat. No. B2261) were procured from the respective sources. Povidine-Iodine solution (Betadine) containing 0.5% w/v available iodine was a commercial product of Win Medicare Pvt Ltd New Delhi and procured locally.

For preparation of dermal fibroblast conditioned media, Ca\(^{2+}\)-free MEM (Cat. # 11380-037) containing 0.05mM CaCl\(_2\), 9% Chelexed FBS, 1% antibiotic-antimycotic mixture (penicillin 100U/ml, streptomycin sulfate 100μg/ml, and amphotericin-B 0.25μg/ml) was used. Fibroblast were isolated from mice neonate skin explants and cultured as described earlier (Poojan & Kumar 2010). The Growth Promoting Medium (GPM) was prepared afresh by mixing 3:1 (v/v) KSFM (without Ca\(^{2+}\)) and fibroblast-conditioned medium; chelexed FBS (9%), antibiotic/anti-fungal mixture (100μg streptomycin, 100U Penicillin, amphotericin-B 0.25μg/ml), CaCl\(_2\) (0.05mM), bovine pituitary extract (50μg/ml), EGF (4ng/ml) were also added. The media were membrane-sterilized (Stericup 0.22 micron Millipore) before use.

### 3C.2.1. Neonate epidermis keratinocytes isolation

BALB/c mice neonates were sacrificed and skin excised. Tissue was placed in 70% ethanol for a minutes and washed extensively with Ca\(^{2+}\)/Mg\(^{2+}\)-free-PBS. Specimens were incubated with dispase overnight at 4ºC; and the next day, epidermis was peeled off from dermis. Single cell suspension was prepared by gently shaking the epidermis. The released cells were harvested after removing the tissue debris with 40μm nylon membrane. Cells were pelleted (300×g/5min/ 4ºC) and washed twice with PBS (Ca\(^{2+}\)/Mg\(^{2+}\)-free)-1% BSA to avoid loss of cells by unspecific binding to FACSaria-tube-surface. Cells (5×10⁶/ml) were resuspended in GPM -1mM HEPES.
3C.2.2. FACS Aria based EpSC sorting
Stem cells were identified in keratinocyte isolates by dye exclusion method. Cell suspension in GPM-1mM HEPES was incubated with 5μg/ml Hoechst-33342 (90min, 37°C). Cells were pelleted (300xg, 10min, 4C), resuspended in GPM containing 10μg/ml Propidium iodide (PI), and left on ice until sorting. FACS Aria cell sorter with FACSDiva software (Bectone Dickinson) was used (setting 20PSI pressure and the pressure difference of 0.8). For each sample, multiple sets of 50,000 events in list mode were acquired. Debris and PI positive cells were gated out (Figure 3C.2). The equipment was reconfigured so as to set the non-rectilinear sort gates as shown in the Bitmap Histogram (Figure 3C.2, A). The LASER power configuration for PI and Hoechst dye detection was 370mW and 190mW respectively; λ max for Hoechst dye fluorescence determination was 355nm excitation & 450/50nm emission and for PI 488nm excitation & 575/25nm emission. Cells sorted out into two sets of population i.e. EpSC showing least uptake and TA cells showing most uptake of Hoechst dye (Figure 3C.3, C). Data were collected using linear amplification in list-mode (Figure 3C.3). EpSC thus obtained were cultured in GPM (Poojan and Kumar 2011).

3C.2.3. Collagen-fibronectin matrix adherence based EpSC sorting
The neonate keratinocytes, prepared in GPM as above, were pelleted and resuspended in GPM (sans HEPES). Cells were seeded in collagen+fibronectin coated flasks and incubated (37°C, 5% CO₂) for 10min. The media were replaced with fresh GPM and EpSC thus enriched were cultured for next three days. Media was changed on fourth day and afterward on every alternate day. Cells at this stage can be cultured, passaged, studied, or cryopreserved. For investigations, the matrix adhered cells were trypsinized after 3rd passage. EpSCs were pelleted (300g/5min/4°C) and resuspended in GPM as above for identification and characterization.

3C.2.4. EpSC characterization and primary cell culture
Both the FACS Aria and matrix-adherence enriched EpSCs were examined for positive (gain of function) or negative (loss of function) biomarkers by immunocytochemistry. The sorted EpSC were cultured in GPM (37°C, 5% CO₂). After 3rd passage, cells were
characterized by detection of stem cell biomarkers. If needed, these EpSc could be cryo-preserved also without significant loss of stem cell characteristics (Poojan and Kumar 2010; Poojan and Kumar 2011).

For immunocytochemistry based characterization, medium of the cultured cells was poured off; and cells were fixed with 4% PFA (paraformaldehyde) in PBS for 10min at RT. After washing with PBS, these cells were refixed in methanol (10min, -20°C), and rewashed three times in PBS-T (PBS-0.1%Tween20). The unspecific binding sites of culture flask were blocked by PBS-1%BSA-0.1%Tween20, and the fixed cells were incubated at 4°C overnight with primary antibodies of respective biomarkers. Cells were washed with PBS, and incubated (2h, RT) with 1:200 diluted secondary antibody (rabbit anti-mice-Alexafluor-conjugated, Invitrogen). The immunofluoro-stained cells were rinsed with PBS-T and mounted with Vectashield solution containing Hoechst dye. Antibodies of Beta-1-integrin, (rabbit polyclonal), p63, CD34, k14 and k15 (mice monoclonal) were diluted 1:200. The fluorescence exhibiting cells were spotted and documented using fluorescence microscope.

For EpSC characterization using the immuno-blotting technique, cell lysates were prepared in ice-cold Celllytic-M-10mMNaF-1mMNa3VO4-1mMPMSF-1%Protease inhibitor cocktail (Sigma Cat P8340). Lysates were centrifuged (15,000xg, 15min, 4°C) and protein content determined by Bradford assay using BSA as standard reference. An aliquot of (40mcg) protein was electrophoresed (10% SDS-PAGE) and transblotted onto PVDF membrane. After blocking the unspecific binding sites (1h, 5% fat-free dry milk in TBS-T (25mM Tris-HCl pH7.6-150mM NaCl-0.1%Tween 20), the protein blots were incubated overnight (4°C) with primary monoclonal antibodies diluted 1:1,000–1:5,000 in 1% fat-free dry milk in TBS-T for k-14, integrin-beta-1, p63, CD34. Membranes were washed three times with TBS-T by gentle shaking. The blots were incubated (4°C, 4h) with secondary peroxidase-conjugated anti-mice and anti-rabbit antibodies (diluted 1:1,000 in 1% fat-free dry milk in TBS-T) with gentle shaking. The markers were detected using enhanced chemi-luminescence kit (ECLPlus Western blotting kit, Pierce) and quantified by densitometry using VersaDoc (BioRad USA)-Quantity-one.
3C.2.5. Mice skin epidermis reconstruction

The reconstructs were prepared from EpSC at 3rd passage. EpSC were trypsinized with 0.05% trypsin/ 1mMEDTA. The enzyme was inactivated using soybean trypsin inhibitor (stock, 250 mg/L in PBS). Cells were harvested and resuspended (0.5 x10^6 cells/ mL) in GPM. EpSCs (0.2 x10^6 suspended in 400µL GPM) were placed in inserts (12mm) held in six-well plate and were incubated till 100% confluence. The confluent cell growth was ensured in primary culture before exposing (15h) to 2mM Ca^{2+} (physiological levels) in Epidermis Reconstruction Medium (ERM).

The cell suspension was dispensed drop by drop avoiding cell damage. Confluent cell growth on the inserts was checked after 4d by Eosin & Hematoxylin staining in one of the inserts. After ensuring confluent cell growth, the medium was aspirated from both inside and outside area of the inserts. The inside area was rinsed with 0.4mL and the outside with 4mL of wash medium (HBSS with 1% chelexed FBS). An aliquot of 0.4ml and 3mL ERM was added to inside and outside area respectively. Inserts were incubated further (15h, 5% CO₂, 37°C). Afterwards, ERM (4 mL) was added exclusively to the outside area of inserts in order to initiate the airlift cell culture. From this point onwards, the surface of the reconstruct starts being exposed to the air. ERM in outside area was replaced on alternate days until the skin reconstruct was ready to harvest. For histology, the media was aspirated both from inside and outside area of inserts. Droplets of leftover medium was removed from the surface of the reconstructs using sterile cotton swabs and avoiding the injury or any insult to the new tissue. After removing inserts from six well plate using forceps, the reconstruct (including the polycarbonate filter) was excised by tracing the circuit closer to the edge and using a scalpel blade. The excised tissue was slid off into a petri dish and saved for histology. The paraffin blocks were made by putting one half of the reconstruct in a histology cassette between 2 black TBS biopsy papers. Whole cassette was immersed in 10% formalin (>4h). The cassette was stored in 70% ethanol (4°C) until ready to process for paraffin embedding. After tissue fixation, the immunohistochemical procedure was similar to immunocytochemical procedure as described earlier.
3C.2.6. Fibronectin-collagen matrix preparation for stem cell adhesion

Collagen (10µL aliquot of stock solution 3mg/ml in 75mM ammonium acetate) and Fibronectin (10µL aliquot of stock solution 1mg/ml) were added into 980µL DMEM+25mM HEPES to get a final concentration of 30µg/ml collagen and 10 µg/ml fibronectin in 1ml for each T25 flask. The collagen -fibronectin-HEPES containing DMEM (1ml) was placed in each flask and sterilized under UV overnight in the Laminar Flow hood at RT. Coating-solution was aspirated from flasks and the coated-surface was washed with PBS three times before seeding stem cells (FACS sorted or freshly isolated keratinocytes).

3C.3. Statistical Analysis

Each experiment was repeated three times and mean (±SE) values used for data analysis.

3C.4. Results & Discussion

3C.4.1. Multipotent EpSC enrichment

The EpSCs enriched over FACS Aria displayed a keratinocyte assortment profile between FSC and SSC detectors as shown in Figure 3C.1, A. PI tagged cells formed a large group of cells that were mostly differentiated. In contrast, PI excluding cells formed a small group of cells clustering separately and forming around 95% of the isolated keratinocytes (pocket P-1 in Figure 3C.1, B). PI excluding cells were further analyzed for percent enrichment of stem cells. P-1 cells, when profiled between FSC and Hoechst dye fluorescence detectors, disclosed a group of small cells with dye exclusion properties (pocket P-2 in Figure 3C.1, C). This cluster grouped nearly 60% of dye excluding keratinocytes. Cells clustered in P-2 were small in size and displayed intact status of dye exclusion trait. Simultaneously, another group of cells omitting uptake of Hoechst dye was found to cluster in pocket P-3 accumulating only 17% of cells (Figure 3C.1, C). These cells were relatively large in size. P-3 cluster of cells was taken as large stem cells programmed already and initiated to differentiate and deviate from stem cell characteristics. Thus, yield of EpSC population by FACS Aria based enrichment procedure was only 60%.
Sorting exclusively over the collagen and fibronectin matrix yielded results as illustrated in Figure 3C.2. The FACS based analysis of these cells revealed assortment profile as shown in Figure 3C.2, A displaying approximately 99% of cells flocking in pocket P-1 and reporting intact status of Hoechst dye exclusion property accumulating in pocket P-2 (Figure 3C.2, B). This approach yielded 40% extra enrichment compared to FACS Aria based sorting (Figure 3C.3). Hoechst dye exclusion by EpSCs occurs due to overexpression of the dye transporter protein and this approach selects exclusively stem cells (Stern et al. 2008). Hoeschst based dye exclusion method for isolation of hematopoietic stem cells has been used earlier also but only in combination with flowcytometry. The strong and rapid adhesive property of EpSCs (Watt and Jones 1993) has been used to sort rapidly the iPSc over fibronectin and laminin matrices as reported recently (Singh et al. 2013).
Figure 3C.3  Comparison of matrix-adhesion or FACS sorted neonate EpSC isolates for cell yield and culture confluence; (a) neonate keratinocyte isolates before attachment, (b) greater seeding density of matrix-adhesion sorted EpSC after attachment (d) after 4d in culture, and (e) after 10-14d in culture; (c) lesser seeding density of FACS sorted neonate EpSC after post matrix-adhesion, (f) after 4d in culture. 400X M

The keratinocyte harvest from neonate mice skin yielded a large number of cells (Figure 3C.3, A). Adhesion enriched EpSCs were greater in number concentration as seen in Figure-3C.3, B than the FACSaria enriched EpSCs (Figure 3C.3, C). Adhesion enriched EpSCs propagated swiftly within 24h of seeding (Figure 3C.3, D); and displayed cuboidal morphology and an increased proliferation rate after 48h of culture. Adhesion enriched EpSCs gained confluence within a week (Figure 3C.3, E); which was 2 weeks earlier than FACSaria enriched EpSCs (Figure 3C.3, F). Rapid growth of adhesion enriched EpSC is valuable for regenerative medicine.

The matrix-adhesion based EpSC enrichment provided several advantages. Most importantly, it allowed the use of known seeding densities of EpSCs and negligible counts of TA cells deemed necessary to initiate epidermis reconstruction in natural manner for authentic and replicable results. It avoided flowcytometry procedures minimizing cellular stress and risk of bacterial contamination for EpSC primary culture. Adhesion based enrichment avoided the use of DAPI, Hoechst dye, or BrdU labels. It
lets EpSC retain its normal biochemical profile and maintain the conformations and richness of cell surface proteins to facilitate their interaction for better anchoring and physiology. Broadly, matrix-adhesion supported EpSC enrichment substantially improved success rate and reproducibility. These attributes are important and desirable in pure stem cell culture required in a wide variety of applications like regenerative medicine, target-cell toxicity studies, skin disease investigations, and related mechanistic aspects.

3C.4.2. EpSC characterization
Matrix-adhesion enriched EpSC were examined for stem cell surface markers; the results are illustrated in Figure 3C.4. EpSCs were found to test positive for specific markers like cytokeratin K-15 and, K-14, p63, beta-1-integrin, CD34 as shown in Figure 3C.4, A thus substantiating the evidence for stemness of EpSC. Whereas all the studied markers showed a similar level of expression in EpSC enriched by either mode (i.e. flowcytometry or matrix-affixation), the analysis of their protein product showed greater expression of integrin (Figure 3C.4, A and 3C.4, B). This observation has provided evidence for isolation of integrin rich EpSCs in adhesion based enrichment procedure. The increased level of expression seemed to be the result of greater enrichment and authenticity of the isolated EpSCs.

Integrins are the expressions of hetero-dimeric transmembrane receptors (Watt 2002; Margadant et al. 2010), and are important for EpSC to organize hemi-desmosomes (Tsuruta et al. 2011) and allow them to interact with ECM components and anchor stem cells into basal layer. A variety of integrins like α- and β-integrin and its subunits such as α6 integrin and β4 integrin are putative epidermal stem cell markers.
Figure 3C.4  EpSC biomarker expression after enrichment by FACS/ matrix-adhesion method illustrated after (a) western blot or (b) immunocytochemical staining. 1000X M
3C.4.3. EpSC lineage affirmation

Sorted by either of the approach as described later, the EpSC were tested for their potential to reconstruct epidermis in the airlift cell culture. The $\text{Ca}^{2+}$ exposure shock by skin reconstruction medium (SRM) induced the differentiation and reconstruction. Using the matrix-adhesion sorted EpSC, the tissue formation was rapid in 2 weeks as displayed in Figure 3C.5; histology revealed the formation of distinguishable strata of basalae, spinosum, granulosum, corneum.

The corneum was found to be composed of cornified keratinocytes and was located on the uppermost surface of the tissue. Underneath, Stratum granulosum was found to be arranged with the grainy layer of keratohyalin producing cells that promote dehydration, aggregation, and cross-linking of keratins. Stratum spinosum was found to be located under the granulosum layer. It consisted of keratinocytes forming the spiny layer. Stratum basalae was observed to form the innermost layer of basal cells that were EpSCs arranged vertically.

In the reconstruct, suprabasal keratinocytes were layered horizontally and were relatively more in numbers. S. granulosum was multilayer thick (6-7 cell layer and invaginated in intact or stripped epidermis; and 10-12 cell layer thick and uninvaginated in the reconstruct). S. corneum was made of multicellular layer thickness, was uninvaginated and dehydrated; however, corneum was relatively thin and hydrated.

![Figure 3C.5](image)

Figure 3C.5 Comparative histology of epidermis in intact, stripped, and reconstructed form showing Stratum basalae ( ), Stratum spinosum ( ), Stratum granulosum ( ), and Stratum corneum ( ).

Study of tumor initiation toxicity in skin carcinogenesis using epidermal stem cell
in the reconstruct and seemed to be of value in regenerative medicine. Using the FACS sorted EpSC, the tissue formation was found to be comparatively slower which apparently seemed to be due to the less concentrations of assumably stressed EpSCs

![Image](https://example.com/image.png)

**Figure 3C.6** *p63 localization in (A) Stratum basale of the reconstructed (\[\rightarrow\]) and (B) Stratum basale as well as hair-bulges of intact epidermis (\[\longrightarrow\]).

The stem cells in the basal layer showed vertical orientation. The sequentially differentiated layers of keratinocytes showed horizontal orientation and were found to be located in between basal and stratified layers (Figure 3C.5). Multiple layers of differentiating keratinocytes were found to lie in this strata of cells. Thus epidermis reconstruct prepared from adhesion enriched EpSC created epidermis showed the basal layer stratum germinativum of epidermal stem cells and multiple layer (stratum corneum) of differentiating oval cells orienting outwardly. The results of the immunohistochemical verification of epidermis are displayed in Figure 3C.6. As evident, the stem cell markers were found to be located in the basal cell layer and were untraceable in the uppermost layer of the reconstruct (Figure 3C.6, A). These features were similar to *in vivo* epidermis tissue as illustrated in Figure 3C.6, B.

Our study has demonstrated a radically improved (99%) yield of label free EpSC using the matrix-affixation method and the stem cells are capable of multiple passages. The reconstruction of epidermis is comparatively rapid than FACSsorted cells and histologically matching to natural epidermis making it a better tool for investigative dermatology and toxicology. Epidermis reconstructs are needed in a variety of applications like regulatory toxicology in drug development, delivery of molecules of therapy, regenerative medicine for biotherapy, and tissue engineering [Itoh et al. 2013;
Vorsmann et al. 2013). Presently, the epidermis reconstructs are available commercially. A few of these are ECVAM approved as the test systems and are generated by keratinocyte pool. These are accepted for use in regulatory toxicology; however, there is a critical inadequacy about the (a) undefined number concentrations of slow cell-cycling EpSCs in the keratinocyte isolates used for reconstruction and (b) inclusion of both stem cells as well as TA cells in the employed isolates.

The contentious issue is the sub-optimal communication among small number concentrations of stem cells and the large number concentrations of TA cells by slow and the interrupted processing of cellular microenvironment information and control of tissue homeostasis via insufficient secretion and interaction of signalling molecules with cell surface receptors and eventually dysregulation of key pathways like cell adhesion, cell signalling, cytoskeleton, cytokine-cytokine receptor interactions. The sub-optimal number concentrations of EpSCs possibly will hinder and delay tissue reconstruction. The ambiguity and lack of information regarding the seeding density and gene expression profile of EpSCs in commercial preps may well contribute uncertainty and batch to batch variation in stem cell characteristics and physiology of the reconstructs for use in regulatory toxicology. The discrepancies might well lessen the degree of confidence and render the test system occasionally inappropriate and unreliable. The apprehensions are lucid in view of the complex biochemistry of EpSC (Eckert et al. 2013) involving cell-cycle phase regulated expressions of a vital biomolecules. These uncertainties at present are however only speculative and need further investigations.