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Hair & skin derived progenitor cells: In search of a candidate cell for regenerative medicine

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***Background & objectives*: Skin is an established tissue source for cell based therapy. The hair follicle has been introduced later as a tissue source for cell based therapy. The ease of tissue harvest and multipotent nature of the resident stem cells in skin and hair follicle has promoted basic and clinical research in this area. This study was conducted to evaluate skin stem cells (SSCs) and hair follicle stem cells (HFSCs) as candidate cells appropriate for neuronal and melanocyte lineage differentiation.**

***Methods*: In this study, SSCs and hair follicle stem cells (HFSCs) were expanded *in vitro* by explant culture method and were compared in terms of proliferative potential and stemness; differentiation potential into melanocytes and neuronal lineage.**

***Results*: SSCs were found to be more proliferative in comparison to HFSCs, however, telomerase activity was more in HFSCs in comparison to SSCs. Capacity to differentiate into two lineages of ectoderm origin (neuronal and melanocyte) was found to be different. HFSCs cells showed more propensities towards melanocyte lineage, whereas SSCs were more inclined towards neuronal lineage.**

***Interpretation & conclusions*: The study showed that SSCs had differential advantage over the HFSCs for neuronal cell differentiation, whereas, the HFSCs were better source for melanocytic differentiation.**

Key words Differentiation - hair follicle stem cells (HFSCs) - skin stem cells (SSCs)

The neural crest is a transient embryonic tissue that gives a wide array of adult cell types and tissues including melanocyte and neuronal cells[1]. Some neural crest stem cells bypass differentiation signal and persist within crest-derived tissues. The hair follicles have been shown to harbour pluripotent neural crest stem cells[2-4] and these can be differentiated into melanocytes, neuronal cells, adipose cells and other lineages[5].

In skin, apart from melanocyte and keratinocyte stem cells[6,7], dermis harbours dermal stem cells (DSCs)[8,9] and skin derived progenitors (SKPs)[10-12]. Both are of neural crest in origin and are capable of differentiating into melanocyte and neural lineages. Furthermore, SKPs are more close to the neuronal cell lineage, while, DSCs are more close to the melanocytes progenitors[13].

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The skin stem cells (SSCs) are in clinical set up for a long period of time and many cell based applications are there for the management of vitiligo, burn and other pigmentary disorders. Some investigators have used hair follicle stem cells (HFSC) for cell based clinical needs, especially in vitiligo[14-16]. We undertook this preliminary study to evaluate the HFSCs in comparison to SSCs to establish a candidate cell for the differentiation into melanocyte and neuronal lineage. These both being originated from neural crest cells could be exploited for research and therapeutic purpose.

Material & Methods

This study was conducted in Stem Cell Facility, All India Institute of Medical Sciences (AIIMS), New Delhi, India. The study protocol was approved by the Institute Ethics Committee and Institutional Committee for Stem Cell Research. Tissue samples were collected from patients after taking their written informed consent. Hair and skin tissues were collected from the patients undergoing surgical management for vitiligo at AIIMS, New Delhi. Five sample of each skin and hair follicle were collected.

Hair follicle tissue processing: Hair follicles isolated by punch biopsy method were transported in the transport media [consisted of 1x Dulbecco's modified Eagles medium (DMEM) supplemented with 150 U/ml of penicillin and 150 µg/ml streptomycin and amphoterecin B 2.5 µg/ml] within 30 min of tissue harvest to the laboratory in ice pack at 4°C. The fatty tissue and dermal part were removed from the hair follicles by enzymatic treatment. To remove the dermal and fattay tissue, hair follicles were incubated in 10 mg/ml dispase enzyme (Invitrogen, NY, USA) overnight at 4°C. Individual hair follicles rooted in the dermis were plucked with the help of an epilation forceps.

Skin tissue processing: Pigmented healthy skin tissues were transported to the laboratory in cool packs. The fatty tissue intact with the skin tissue was removed with the help of a scalpel blade. To further remove the epidermis from dermis, skin tissue sample was incubated in 10 mg/ml dispase overnight at 4°C. Chopped small pieces of epidermis of approximately 2 x 2 mm size were kept intact for the purpose of explant culture.

In vitro expansion of skin stem cells (SSCs) and hair follicle stem cells (HFSCs) by explant culture method: One explant in case of skin was skin tissue measuring approximately 2 x 2 mm in size and in case of hair tissue individual hair follicles were used as explants. One explant/cm^2 area was cultured according to the modified Rheinwald-Green system[17] consisting of 3:1 DMEM and Ham's F12 nutrient mix (Sigma, USA), supplemented with 10 per cent foetal bovine serum (Hyclone, USA), 10 ng/ml epidermal growth factor (PeproTech, USA), 2.8 µg/ml hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin (Sigma, USA), 10 ng/ml cholera toxin (Millipore, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) over fibronectin (Sigma, USA) coated culture dish (BD Falcon, USA).

Characterization of cells expanded from skin and hair follicle tissue for stemness: The SSCs and HFSCs were characterized for the expression of stem cell markers by immunofluorescence. The markers used for the immunofluoresence studies were cytokeratin (CK) 19, CK 15 and β1 integrin.

Cell proliferation study by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay: Rate of cell proliferation was calculated by the MTT assay[18]. The SSCs and HFSCs were separately cultured in a 96-well culture plate (5,000 cells/well) with culture medium. MTT assay was done on day, 3, 5, 7, 10, 12, and 15; and 50 µl of MTT reagent (5mg/ml in PBS) was added to the wells in triplicate and incubated for 3.5 h at 37°C. After incubation, medium was removed and 300 µl of dimethylsulphoxide (Sigma, USA) was added to each well to dissolve the formazan crystals. The coloured solution (200 ul) was transferred to 96-well plates and read at 570 nm in a plate reader (EL 800, Biotek, USA).

Culture of immortalized human foreskin fibroblast (I-HFF) cell lines: I-HFF cells were maintained in growth medium containing Iscove's modified Dulbecco's medium (IMDM) (Sigma, USA) supplemented with 10 per cent foetal bovine serum (Hyclone, USA), 2 mM/ml L-glutamine (Gibco/Invitrogen, USA), 1 per cent non-essential amino acids (NEAA) and 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco/Invitrogen, USA).

Expression level of telomerase reverse transcriptase (TERT) gene transcript expression in SSCs and HFSCs by quantitative real time polymerase chain reaction (qRT-PCR)[19]: The relative expression of *TERT* gene transcript in SSCs and HFSCs was studied in comparison to the I-HFF cell line. SYBR green (KAPA Biosystems, South Africa) chemistry was used for

relative quantification of the gene expression. Relative gene expression was evaluated by $2^{-\Delta\Delta Ct}$ method[20]. ΔCt was obtained by subtracting the $Ct_{Reference}$ from Ct_{target} ($\Delta Ct = Ct_{target} - Ct_{Reference}$). ΔΔCt was obtained by subtracting $\Delta Ct_{Control\ cells}$ from ΔCt_{Target} cells ($\Delta\Delta Ct = \Delta Ct_{Target} - \Delta Ct_{Control\ cells}$). Realplex 4 epgradient Mastercycler (Eppendorf, Hamburg, Germany) was used for setting up the relative quantification experiments. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as the reference gene. The realplex software was used to analyze the data.

The primers used for the study were as follows: reference gene *GAPDH* forward-5'GAGTCAACGGATTTGGTCGT3' reverse-5'GAC AAGCTTCCCGTTCTCAG 3'; *TERT* forward-5' GGCAAGTCCTACGTCCAGTG 3', reverse-5' GGGCATAGCTGAGGAAGGTT 3'.

Induction of SSCs and HFSCs into melanocyte differentiation: For melanocyte differentiation, 70-80 per cent confluent cultures of SSCs and HFSCs were incubated in the medium with the following composition: 50 per cent molecular cellular and developmental biology (MCDB) 201 medium, 40 per cent Ham's F12, nutrient mix (Sigma, USA), supplemented with 10 per cent foetal calf serum, 2mM/ml L-glutamine (Gibco, USA), 10^{-4} mol/l L-ascorbic acid, 10 nM/ml phorbol 12-myristate 13-acetate (PMA), 10 ng/ml cholera toxin, 20 ng/ml fibroblast growth factor (PeproTech, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, USA). Geneticin (Sigma, USA) at a concentration of 100 µg/ml was used to remove the contaminating fibroblast.

Functional assessment of melanocytes by L-DOPA staining: The functionality of the differentiated melanocytes was checked *in vivo* by the ability of the melanocytes to reduce the L-DOPA (L-3,4-dihydroxyphenylalanine) into DOPA-chrome with the help of tyrosinase enzyme. Cultured melanocytes were fixed with 10 per cent formalin in phosphate buffer saline (PBS) for 3 h at 4°C. Cells were rinsed with PBS and incubated with 0.05 mg/ml L-DOPA (Sigma-Aldrich, USA) in PBS for 3 h at 37°C. Following incubation, cells were rinsed with PBS and fixed with 10 per cent buffered formalin for 1 h. Functional melanocytes were stained brown in the presence of L-DOPA.

Induction of SSCs and HFSCs into neuronal differentiation: For neuronal differentiation 70-80 per cent confluent cultures of stem cells were incubated in the neurobasal medium (Invitrogen, USA) containing 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, USA) supplemented with 20 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF) (PeroTech, USA), 10 ng/ml B-27 supplement (Invitrogen, USA) and 2 mM/ml L-glutamine (Gibco, USA). Geneticin (100 µg/ml) was added to remove the contaminating fibroblast. Medium was changed every third day, the cells started to change their morphology after 4 to 5 days of culture. This protocol was standardized in our laboratory (unpublished data).

Immunofluorescence staining for stem cells, melanocytes and neuronal cells: Cells grown over the cover slips or cytospin preparations were taken for immunofluorescence staining. Cells were fixed in 4 per cent paraformaldehyde for 10 min at room temperature, blocked with 2 per cent bovine serum albumin (BSA), and stained with primary antibodies. For HFSCs cells; CK15, CK19, and β1-integrin, (Millipore, USA) antibodies were used. HMB45 (Human Melanoma Black 45) and S100 (S100 because of their solubility in a 100%-saturated solution with ammonium sulphate at neutral *p*H.) (Millipore, USA) antibodies were used to stain differentiated melanocytes. NF (neurofilament) and TH (tyrosine hydroxylase) (eBiosciences, USA) antibodies were used to stain differentiated neurons. Negative control was used by omitting the primary antibody. Fluorescein isothiocyanate (FITC) and Texas red (TR) (BD, USA) conjugated secondary antibodies were used to develop fluorescence signal. Propidium iodide (PI) and 4;6-diamidino-2-phenylindole (DAPI) (Sigma, USA) were used for nuclear staining.

qRT-PCR analysis for the gene expression pattern in melanocytes and neuronal cells derived from SSCs and HFSCs[19]: The relative expressions of *MITF* (microphthalmia-associated transcription factor) and *TYR* (tyrosinase) genes in melanocytes and *NF* and *TH* genes in neuronal cells were compared with their expression in native skin tissue using SYBR green chemistry as described earlier. The primers used for the study were as follows- *MITF* forward 5'ACCTCGGAACTGGGACTGAG 3', reverse 5'GGGGACACTGAGGAAAGGAG 3'; *TYR* forward 5'ACGTCTTCCTGAACCACAGG 3', reverse 5'CGTGGGGTCACTGTAACCTT 3'; *NF* forward 5'TGGGAAATGGCTCGTCATTT 3' reverse 5'CTTCATGGAAGCGGCCACTT 3' and TH

forward 5'GGTCGCGCTGCCTGTACT 3', reverse 5'TCATCACCTGGTCACCAAGTT 3'.

Statistical analysis: Student t test was used for statistical calculations between two groups and One Way-ANOVA for multiple groups.

Results

Initiation of cell growth from the skin explant took a little more time *i.e.* 6-7 days in comparison to the hair follicle explant, which took 4-5 days. The cell sheet obtained from both the tissue explants showed a typical honey comb morphology, and growth pattern of keratinocyte stem cells. Hair follicle stem cells could be expanded for 10 passages as compared to skin stem cells which could be taken for up to eight passages. The doubling time was 3.7±0.8 and 4.6±0.4 days for skin stem cells and hair follicle stem cells, respectively.

Characterization of in vitro expanded cells: The cells expanded from both the tissue were positive for keratinocyte stem cell markers K19, β1-integrin and CK15 as revealed by immunofluorescence (Fig.1). The cells were negative for the expression of the markers in the negative control slides.

Cell proliferation assay by MTT: The MTT assay was performed for the determination of SSCs and HFSCs proliferation rate for upto 15 days in culture (Fig. 2A). The cell proliferation was evident from the increase in the absorbance of the purple formazan formed. There was a difference in the absorbance of the formazan formed from 3rd day onwards and the difference in the absorbance was maintained till 15th day. SSCs showed higher absorbance in comparison to the HFSCs, indicating a higher rate of cell proliferation for SSCs.

Expression of TERT gene in SSC and HFSC: The fold expression of *TERT* gene in HFSCs and the SSCs was 5.433 ± 0.616 and 2.583 ± 0.518, respectively (Fig. 2B), indicating HFSCs to have a significantly (P<0.001) higher level transcription of *TERT* gene compared to SSCs.

Differentiation into melanocytes: The protocol to differentiate the stem cells into melanocytes was of 21 days. The melanocytes differentiation initiated after 8-10 days in the differentiation medium for stem cells of both skin and hair origin. The culture dish became homogenously confluent with melanocytes

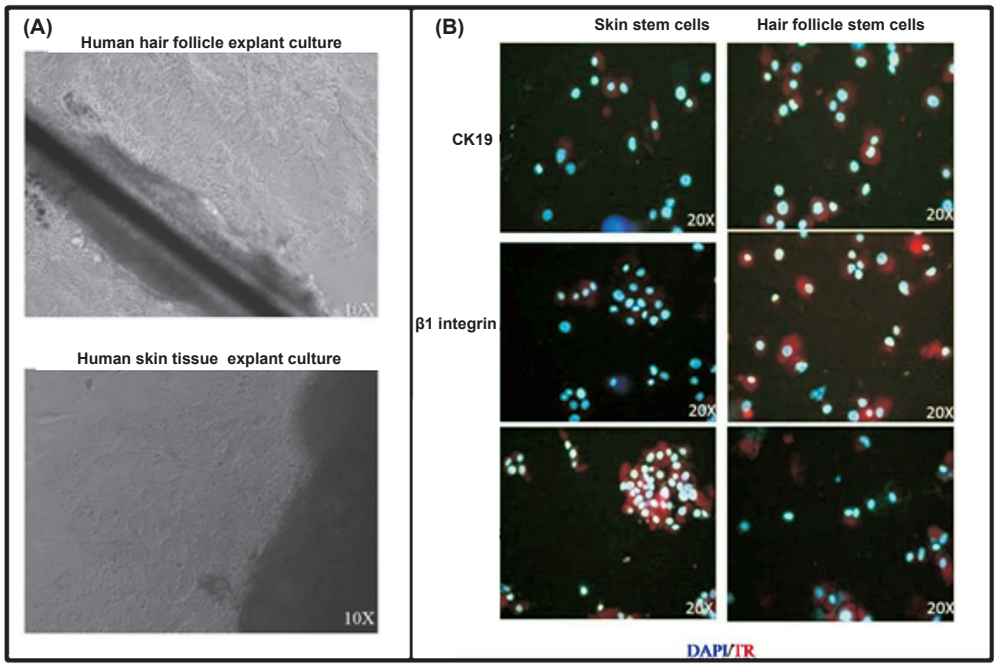

Fig. 1. Expansion and charcaterization of skin and hair follicle stem cells. **(A).** Photomicrograph showing the explant culture of hair and skin tissue over fibronectin coated culture dish. The cells started coming out from the explant periphery after a week in culture media. **(B).** The cells were positive for the expression of CK-19, β1-integrin and CK-15 as revealed by immunofluorescence. DAPI (blue colour) was used as a nuclear stain. The secondary antibody was conjugated with Texas red (red colour).

in almost 25-30 days. The morphological appearance of the melanocytes obtained from the two sources appeared to be similar. There was no visible difference in the melanocytes stained with HMB-45 (recognizes melanosomal protein gp100) and S-100 (recognizes calcium binding protein within melanocytes) antibodies, for immunofluorescence, differentiated from HFSCs and SSCs (Fig. 3A,B)

Determination of in vitro functional aspect of melanocytes differentiated from SSCs and HFSCs by L-DOPA staining: The melanocytes derived from skin and hair follicle were functionally active as demonstrated by L-DOPA assay. The percentage of cells found to be functionally active were 55.06 ± 5.24 and 61.27 ± 3.64 for melanocytes derived from HFSCs and SSCs, respectively (Fig. 3C).

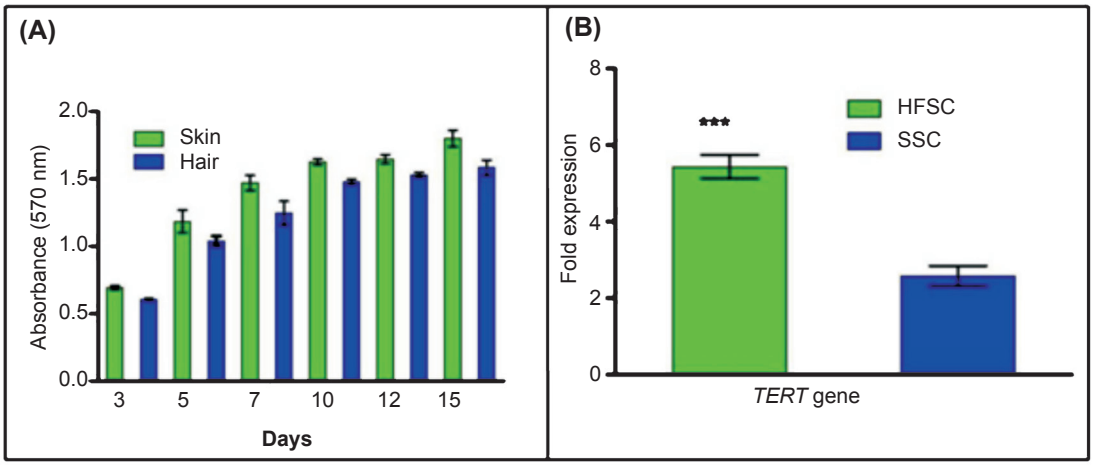

Fig. 2. Analysis of skin and hair follicle stem cell proliferation. **(A).** The cell proliferation for SSCs and HFSCs at day 3, 5, 7, 10, 12 and 15. The data represent the mean ± SD of five independent experiments. **(B).** The expression of *TERT* gene was significantly (P<0.001) higher in hair follicle stem cells as compared to the skin stem cells. The data represent mean ± SD of five independent experiments.

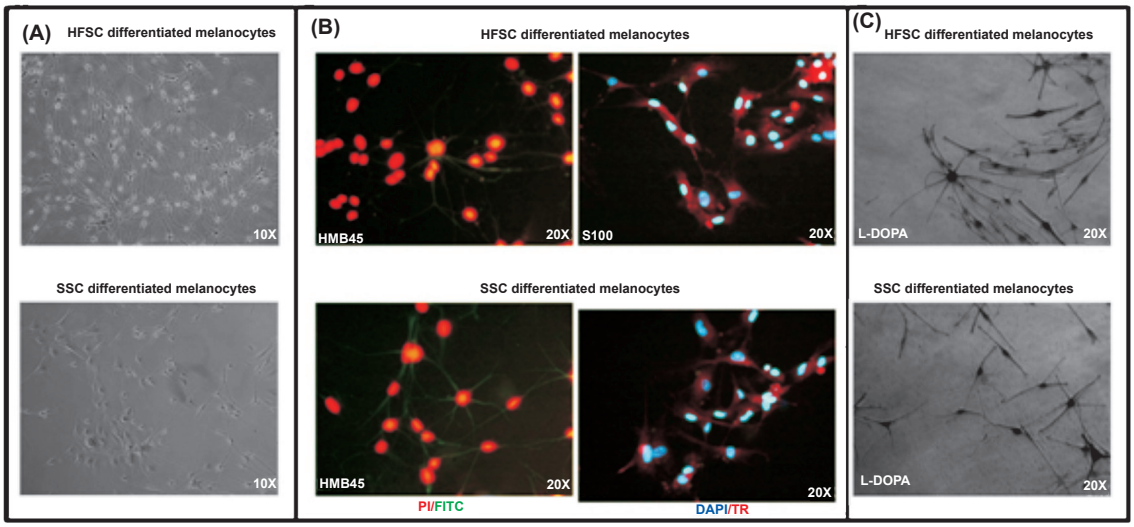

Fig. 3. Differentiation of SSCs and HFSCs into melanocytes and their characterization. **(A).** Photomicrographs depicting the differentiation of SSCs and HFSCs in melanocytes. **(B).** SSCs and HFSCs differentiated melanocytes were positive for the melanocyte markers HMB-45 and S-100 markers as revealed by immunofluorescence. PI (red colour) or DAPI (blue) has been used as nuclear stain. The secondary antibody was conjugated with Texas red (red colour) or FITC (green colour). **(C).** Functional assay of the differentiated melanocytes was done by L-DOPA staining.

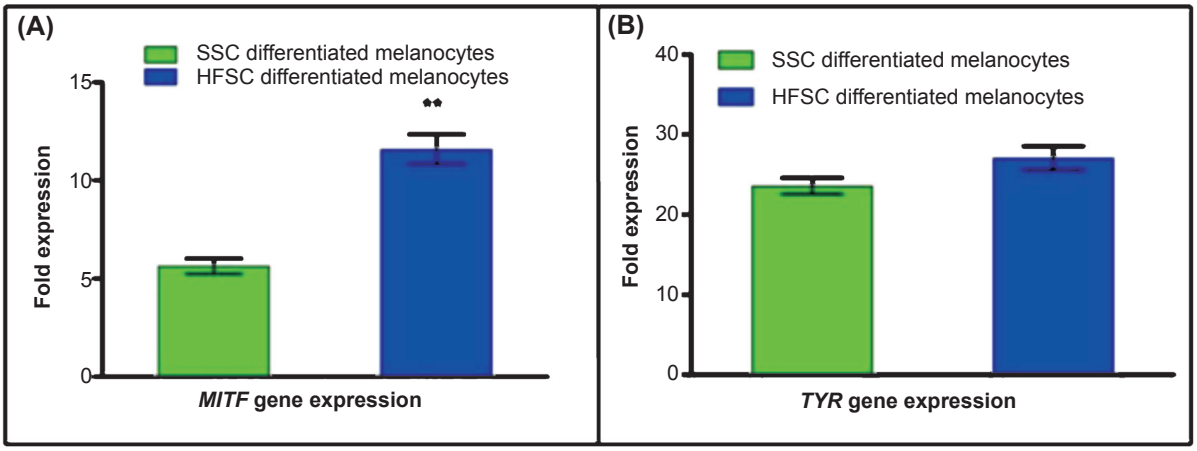

Fig. 4. Characterization of differentiated melanocytes for specific transcripts by qRT-PCR. **(A).** Expression of *MITF* gene was significantly (P<0.01) higher in melanocytes derived from HFSCs as compared to the SSCs derived melanocytes. **(B).** There was no significant difference in the expression of tyrosinase (TYR) in melanocytes differentiated from either SSCs or HFSCs. The data represent mean ± SD fold expression of five independent experiments.

Quantitative RT-PCR for the relative expression of MITF and TYR gene in melanocytes derived from HFSCs and SSCs: The fold expression of *MITF* gene was 5.63 ± 0.66 folds for SSCs derived melanocytes and 11.58±1.32 folds for HFSCs derived melanocytes which was significantly (P<0.01) higher. The fold expression of *TYR* in HFSCs derived melanocytes and SSCs derived melanocytes was 27.09 ± 2.60 and 23.56 ± 1.75 folds, respectively (Fig. 4).

Differentiation into neuronal cells: Protocol for neuronal differentiation was of 14 days. The stem cells started to change their morphology in the differentiation medium by 4-5 days. The differentiated cells were positive for neuronal markers NF and TH by immunofluorescence (Fig. 5).

Quantitative RT-PCR for the relative expression of TH and NF genes in neuronal cells differentiated from HFSCs and SSCs: The fold expression of *TH* gene was 27.56 ±3.44 folds for SSCs derived neuronal cells and 6.2±1.158 folds for HFSCs derived neuronal cells. The fold expression of *NF* gene in SSCs derived neuronal cells and HFSCs derived neuronal cells was 48.03 ± 6.07 folds and 4.89 ± 1.03 folds, respectively (Fig. 6). The fold expression of both the genes was significantly (P<0.001) higher in SSCs derived neuronal cells.

Discussion

Epidermal tissues of hair and skin are rich source of multipotent stem cells. Hair follicle and skin tissue, apart from the bone marrow, are perhaps the only tissues, which have got the niche for diverse kind of stem cells- melanocyte stem cells, keratinocyte stem cells, and mesenchymal stem cells[21,22]. The skin largely contains the keratinocyte stem cells (KSCs), in the basal layer. Lately, two distict population of stem cells have been identified in skin dermis known as skin derived progenitors (SKPs) and dermal sheath cells (DSCs)[8-11,13,23,24].

The cell expanded in culture contained a mixed population of cell types. The stem cells can be maintained in culture for longer period of time compared to other cell types. The culture expanded cells from skin and hair were positive for the keratinocyte stem markers. There was no significant difference in the positivity for the expression of stem cell markers. There was no significant difference in the proliferation rate of SSCs versus HFSCs, however, the telomerase activity was significantly higher in HFSCs. This indicates that with almost same proliferation rate in both groups of the cells, HFSCs had the advantage of being passaged for longer period of time as indicated by higher telomerase gene expression.

The melanocytes showed multidendritic morphology, similar to that expected *in situ*. We used PMA, as inducer of melanocyte, in the induction medium which promoted cell proliferation and helped multiple dendrite formation[25,26]. Contaminating fibroblasts in cultures were eliminated by the addition of geneticin, which resulted in homogenous population of differentiated cells[25,26]. The mode of action of geneticin is by blocking the protein synthesis which ultimately kills the cells. The differentiated cells from

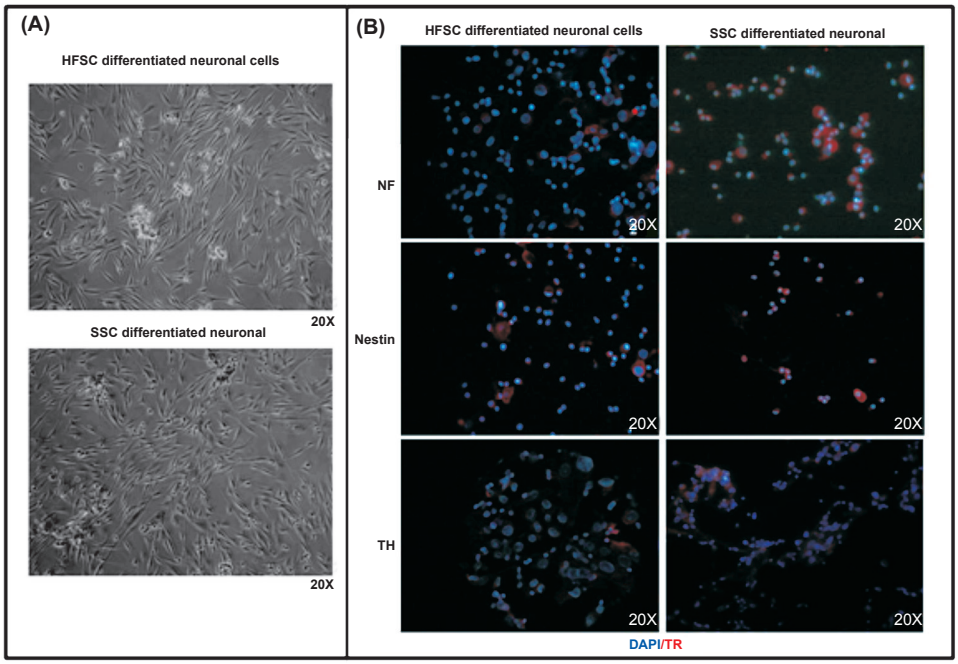

Fig. 5. Differentiation of SSCs and HFSCs cells into neuronal cells and their characterization. **(A).** Photomicrographs depicting differentiation of SSCs and HFSCs into neuronal cells. **(B).** Immunofluorescence image of SSCs and HFSCs derived neuronal cells stained neuronal cell specific markers NF, nestin and TH antibody. DAPI (blue colour) was used as a nuclear stain. The secondary antibody was conjugated with Texas red (red colour).

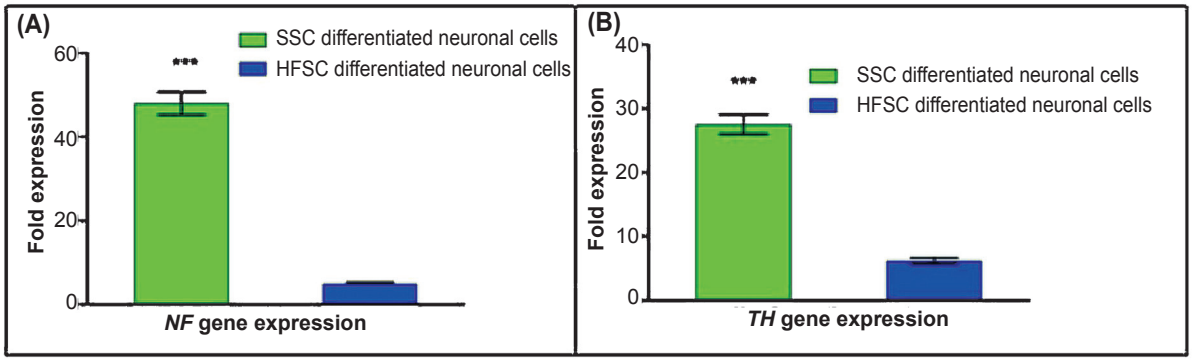

Fig. 6. Characterization of differentiated neuronal cells for specific transcripts by qRT-PCR. **(A).** The expression of *NF* gene was significantly (P<0.001) higher in SSCs derived neuronal cells in comparison to HFSCs derived neuronal cells. **(B).** The expression of *TH* gene was significantly (P<0.001) higher in SSCs derived neuronal cells in comparison to HFSCs derived neuronal cells. Values are mean ± SD of five independent experiments.

both the origins were positive for the melanocytes markers, *MITF* and tyrosinase (*TYR*) by qRT-PCR and HMB-45, and S-100 by immunofluorescence. The melanocytes were also positive for L-DOPA stain indicating their functionality, however, the efficiency of generation of functional melanocytes was higher in SSCs derived melanocytes. There was a population of cells which acquired the dentritic phenotype but lacked L-DOPA staining. These are the amelanotic melanocytes (AMMC)[27,28]. These AMMC are considered to be melanocyte stem cells population[29,30]. The importance of SSC and HFSC is not just limited

to the cells of the epidermal lineage. Nestin positive cells have been isolated and differentiated from skin and hair follicle tissue into neuronal cells[30,31]. Neuronal cells differentiated from SSCs showed significantly higher expression of the neuronal cell specific genes (*TH* and *NF*)[32,33] in comparison to the HFSCs derived neuronal cells. The observation may be explained in view of skin tissue harbouring a special niche of stem cells, which are known as SKPs[20,25]. The SKPs are known to have close relationship with neuronal cells. The SKPs tend to have spontaneous differentiation tendency towards neuronal lineage. However, there is no report on comparative study of the neuronal cells differentiated from SSCs and HFSCs.

This was a preliminary study which investigated the candidate cells appropriate for neuronal and melanocyte lineage differentiation. The differentiation studies indicated hair to be a better source for melanocyte differentiation and skin to be more inclined for neuronal differentiation. Future studies involving more number of samples and exploring the functional aspects of differentiated melanocytes and neuronal cells need to be initiated.

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***Conflicts of Interest*:** None.

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