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Role of Dermatopontin in re-epithelialization: Implications on keratinocyte migration and proliferation

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Re-epithelialization is a key event in wound healing and any impairment in that process is associated with various pathological conditions. Epidermal keratinocyte migration and proliferation during re-epithelialization is largely regulated by the cytokines and growth factors from the provisional matrix and dermis. Extracellular matrix consists of numerous growth factors which mediate cell migration via cell membrane receptors. Dermatopontin (DPT), a non-collagenous matrix protein highly expressed in dermis is known for its striking ability to promote cell adhesion. DPT also enhances the biological activity of transforming growth factor beta 1 which plays a central role in the process of wound healing. This study was designed to envisage the role of DPT in keratinocyte migration and proliferation along with its mRNA and protein expression pattern in epidermis. The results showed that DPT promotes keratinocyte migration in a dose dependant fashion but fail to induce proliferation. Further, PCR and immunodetection studies revealed that the mRNA and protein expression of DPT is considerably negligible in the epidermis in contrast to the dermis. To conclude, DPT has a profound role in wound healing specifically during re-epithelialization by promoting keratinocyte migration via paracrine action from the underlying dermis.

Wound healing is a complex biological process involving a cascade of cellular, biochemical and molecular events which proceed in an orderly fashion to restore the structural and functional integrity of the impaired tissue[1]. Essentially the process of tissue repair is broadly considered to occur in three distinctive yet overlapping phases *viz.* inflammatory, proliferative and remodeling[2]. During these phases various inflammatory cytokines, mitogenic growth factors, cell adhesion molecules, proteases and enzyme inhibitors present in the extracellular matrix (ECM), interact in a well-orchestrated manner to restore the tissue function[3].

Re-epithelialization of wounded tissue involves the formation of new epithelium and skin appendages by activating keratinocytes which proliferate, migrate and differentiate, reestablishing the protection of the underlying tissue[4,5]. This process is primarily governed by the ECM components through signal transduction via cell membrane receptors[6,7]. Cell migration is a dynamic interplay between cell adhesion and ECM through organization of cytoskeletal actin to form focal adhesion points which are mostly regulated by a class of membrane receptors called Integrins[8–10]. The influence of growth factors like PDGF (Platelet Derived Growth Factor), EGF (Epidermal Growth factor), KGF (Keratinocyte Growth Factor) and ECM molecules such as collagen, fibronectin, laminin and MMPs on re-epithelialization are studied extensively[7,11–15].

Dermatopontin (DPT), a 22 kDa Tyrosine Rich Acidic Matrix Protein (TRAMP) first purified in bovine dermis is known to interact with transforming growth factor beta 1 (TGFβ1), fibronectin and decorin thereby assuming an important role in the process of wound healing[16–19]. DPT regulates the architecture of ECM through acceleration of collagen and fibronectin fibrillogenesis[18,20]. DPT was also identified as proteoglycan binding protein, and is known to enhance adhesion of fibroblasts and keratinocytes via $\alpha 3\beta 1$ integrin[21–23]. Owing to its abundant expression in the skin and multiple roles during wound healing and ECM reassembly, an attempt is made in this study to elucidate the role of DPT in keratinocyte proliferation and migration. Additionally, the status quo of its expression in the epidermis has also been investigated.

Results

DPT influences keratinocytes migration. The migratory potential of DPT on adhered keratinocytes was assessed using standard scratch wound assay. The wounded keratinocyte monolayer when treated with various concentrations (50–500 pg/mL) of DPT showed a dose dependant increase in the migration (fig. 1 and fig. 2). The percentage of wound area recovered after 8 hours in DPT treated and untreated cells were

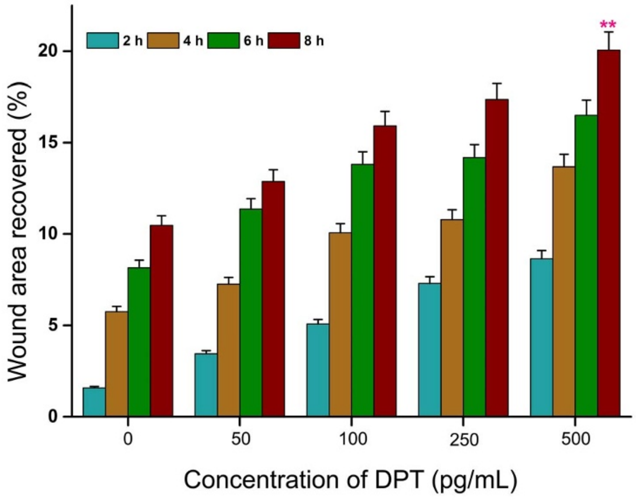

Figure 1 | Effect of DPT on keratinocytes migration. Graphical illustration of the percentage of wound area recovered with various concentrations of DPT treatment. ** P-Value (0.0258) was calculated using student's t-test for the indicated concentration by comparing with the untreated cells at the same time point.

20.05 and 10.47 respectively. The recovery of wound as a measure of cell migration was twice in treated cells when compared to the untreated cells indicating that DPT significantly influences keratinocyte cell migration.

DPT enhances lamellipodia formation in keratinocytes. The extension of lamellipodia, an indicator of cell migration was assessed by staining the actin fibers. Phalloidin staining of F- actin showed the formation of thick fiber assembly and focal adhesion points in the DPT treated cells (fig. 3) confirming the involvement of DPT in keratinocyte migration. The lamellipodia formation captured after scratch assay is shown in the fig. 4. The untreated

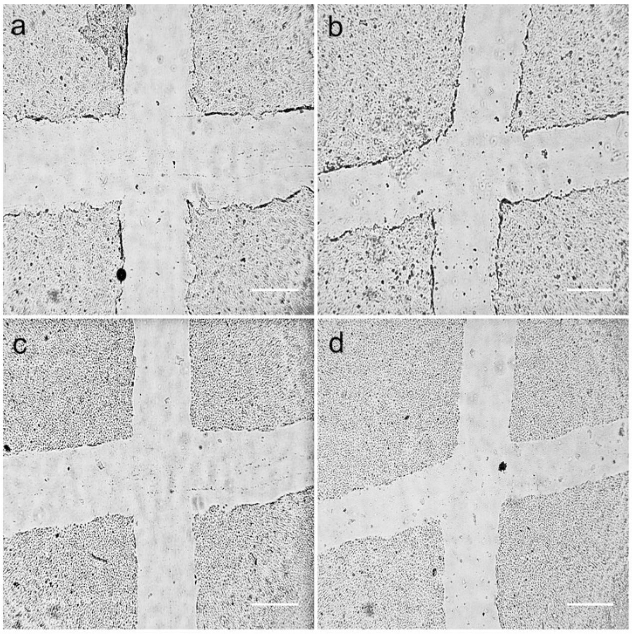

Figure 2 | Effect of DPT on wounded keratinocyte monolayer. Representative microscopic images showing the migratory pattern in (a & c) untreated and (b & d) treated cells at 0 (a & b) and 8 (c & d) hours. Scale bar – 100 μm.

cells show very few lamellipodia formations when compared to the treated cells which displayed multiple lamellipodial extensions indicating the enhancement in the migratory rate of HaCaT cells by DPT.

Proliferative potential of DPT on keratinocytes. The results of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay is illustrated in fig. 5. The intensity of the color measured in control and treated cells was notably unaltered indicating that DPT has no effect on the proliferation of HaCaT cells. In spite of the increase in the concentration (up to 100 ng/mL) and the treatment period (up to 72 hours) no substantial change was observed confirming that DPT has no potential role in keratinocyte proliferation.

DPT mRNA transcripts expression in epidermis. Semi quantitative PCR analysis revealed that DPT gene is not expressed in epidermis and HaCaT cells (fig. 6a). On the other hand, normal fibroblasts and dermis showed a prominent expression of DPT. The expression of internal control gene, RPL32, was observed in all the samples imparting that the mRNA expression of DPT is restricted to dermis. The absence of non-specific amplicons clearly indicated the specificity of the primers used.

DPT protein expression in epidermis. DPT protein expression was analyzed using western blotting and immunohistological studies. The results of the western blotting shown in the fig. 6b clearly depicted that DPT is not expressed in epidermis and HaCaT cells, in contrast to the conspicuous expression in the dermis and normal fibroblasts. In histological analysis, the sections treated with anti – human DPT and subsequent HRP or FITC conjugated secondary antibody revealed that DPT protein levels are markedly low in epidermis than in dermis (fig. 7 and fig. 8). The brown color developed due to HRP-DAB reaction was present throughout the dermis localizing on the collagen bundles. The epidermis showed a very feeble or no color development indicating the absence of antigen (DPT protein). The bright signals in the immunofluorescence studies also corroborated that DPT is absent and abundant in the epidermis and dermis respectively.

Discussion

Adhesion-mediated migration plays a central role in many physiological processes like angiogenesis, fetal development, wound healing, etc.[24] and among other functions, DPT is known for its striking ability to promote adhesion in a variety of cell types including keratinocytes[23]. Keratinocytes when treated with DPT showed an enhanced migration suggesting that DPT promotes keratinocyte motility independently. Lamellipodia based motility is one of the prominent strategies involved in the cell migration[25]. The lamellipodial projections formed by the polymerization of actin undergoes cycles of protrusion and retraction leading to cell migration[26,27]. Here in our study, we have showed that DPT induces multiple lamellipodial protrusions and focal adhesion contacts (subset in fig. 3) in the direction of cell migration. Thus, hitherto an undetermined role of DPT has been deciphered in this study. DPT is known to interact with $\alpha 3\beta 1$integrin efficiently, which are in turn reported to be one of the prime receptors expressed by the keratinocytes during re-epithelialization[23,28,29]. But, recent *in vivo* studies on $\alpha 3\beta 1$ integrin's precise role divulged that the surface receptor is not essential for re-epithelialization and further inhibits the directional migration of keratinocytes during wound healing[30,31]. Taken together, based on these findings we presume that DPT might interact with other receptors also to mediate its migratory function in keratinocytes. However, studies on DPT's mechanism of action and *in vivo* functional validations are required to understand its role completely during cutaneous wound healing.

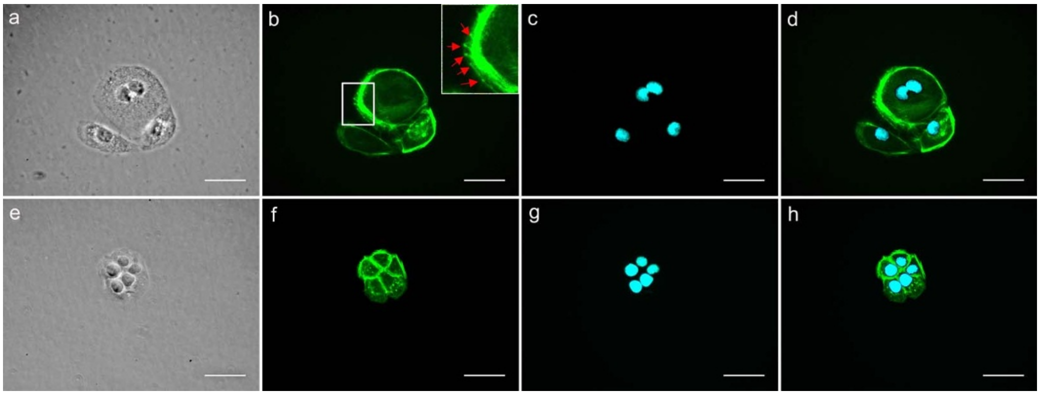

Figure 3 | Phalloidin staining of cells treated with and without DPT. The number of thick and extended lamellipodia forming cells was higher in DPT treated cells (a - d) than in control cells (e - h). A typical representation of images are (a & e) Phase contrast image showing the cytoplasmic protrusions formed. (b & f) F-Actin filaments stained with Phalloidin (green) showing the thick actin network formed at the migrating edge, a typical phenotype of the migratory cells. (c & g) Nucleus stained with DAPI (blue). (d & h) Fused image showing the stained nucleus and F-actin. The focal adhesion points formed in the direction of migration are indicated by red arrows in the subset of image b. Scale bar – 50 μm.

Cell proliferation is a key phenomenon in the re-epithelialization process during wound healing. Following tissue insult, under the influence of various growth factors and cytokines, keratinocytes at the rear of wound margins multiply rapidly forming a dense hyper proliferative epithelium[5,7]. These cells then migrate forward on the wound bed restoring the barrier function of the epidermis protecting the underlying tissue. DPT enhances the biological activity of TGFβ1 which is known to inhibit keratinocyte proliferation through c-Myc signaling pathway[32]. Moreover, DPT is previously shown to induce quiescence in BALB/c 3T3 cells[33] making a notion that it might be involved in growth suppression of cells. Nonetheless, our results showed that DPT has no effect on the growth of keratinocytes. These results suggest that DPT might have explicit functions on different types of cells.

The expression profile of DPT in different organs of porcine and human are well documented with skin being the richest source (~15 mg/Kg of wet weight)[19,34,35]. Hitherto, macrophages and mesenchymal cells, specifically fibroblasts and myofibroblasts are attributed for the production of DPT[36,37]. However, the expression of DPT by other cell types is poorly understood. Our PCR analysis apparently revealed that DPT gene is expressed neither in epidermis nor by HaCaT keratinocytes in contrast to the high expression in dermis and fibroblasts. The protein expression and localization studies on normal skin further confirmed the absence of DPT in the epidermis. The strong signals owing to DPT expression elicited in the dermis suggest that DPT was produced predominantly by the dermal cells and not by the epidermal cells.

Epithelial-mesenchymal interactions play a crucial role in the regulation of tissue development, homeostasis, repair, etc. and in skin it is mostly facilitated by paracrine-acting factors[38]. Few growth factors and cytokines are previously identified to work in a paracrine mode in modulating keratinocyte functions[39]. The present study unraveled that DPT might also act in a paracrine fashion on epidermal keratinocytes promoting its migration. The altered expression of DPT in various pathological conditions including carcinomas and fibrosis are studied earlier[35,36,40–43]. Recently we have reported that DPT expression is altered in chronic cutaneous wounds due to specific protease degradation aiding the defective wound healing[44]. Additionally, we have observed that prior incubation of DPT with specific proteases modified its ability to promote keratinocyte migration (data not shown, unpublished). These data along with its newly recognized paracrine function suggest that the expression and functions of DPT are vital and are tightly regulated in various compartments of skin.

Thus, this study concludes that DPT has an important role in re-epithelialization by promoting keratinocyte migration. Further, the expression of the protein is negligible in epidermis and hence mediates its action in a paracrine manner. Further studies on the expression of this protein and understanding its other functions in different phases of wound healing may aid in establishing therapeutic targets for cutaneous tissue regeneration.

Methods

Cell culture. Immortalized human epidermal keratinocytes, HaCaT, was obtained from National Centre for Cell Science, Pune, India and normal skin fibroblasts (NSF) were isolated from circumcision samples using regular explant culture techniques[45]. The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 units/mL), gentamicin (30 μg/mL) and amphotericin B (2.5 μg/mL). The cells were maintained at 37°C in a humidified 5% CO_2 incubator (Binder, Germany) in 25 cm^2 culture flasks. All the chemicals were procured from Sigma- Aldrich (USA) and are cell

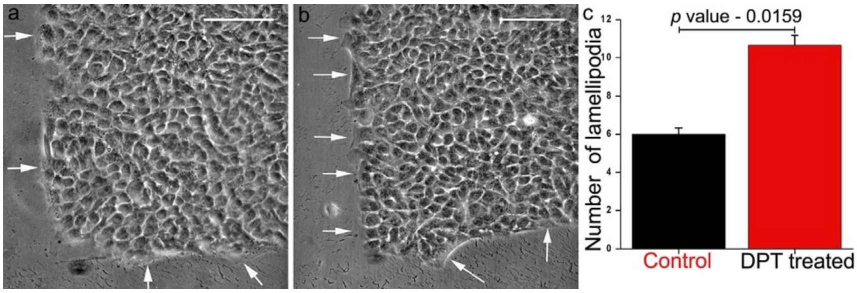

Figure 4 | Phase contrast images showing lamellipodia formation after scratch assay experiment. (a) Untreated cells and (b) DPT (500 pg/mL) treated cells. (c) Graphical representation of number of lamellipodia formed in cells with and without DPT treatment. The images were captured after fixing the cells. The lamellipodia observed in cells are indicated with arrows. Scale bar – 100 μm.

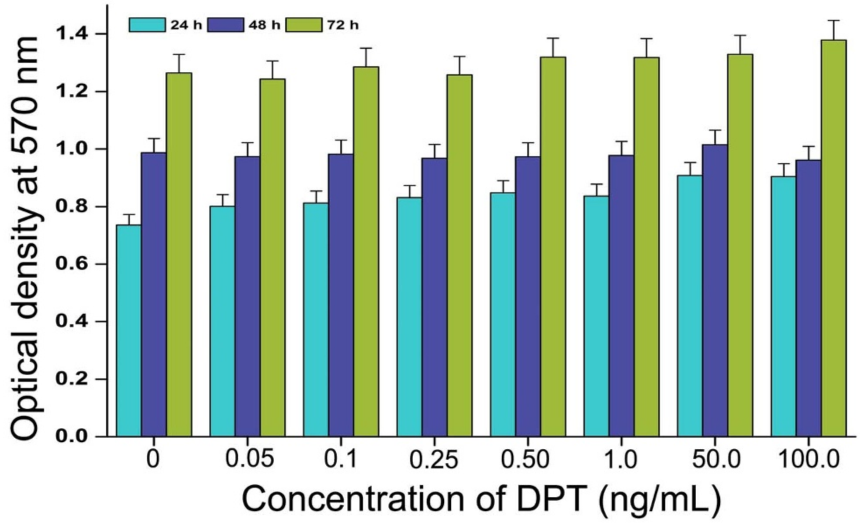

Figure 5 | Effect of DPT on Keratinocyte growth. Graphical depiction of the proliferative potential of DPT on keratinocytes assessed through MTT assay. The values represent the mean of three repeated experiments with triplicates for each concentration.

culture tested. The tissue collection and the experimental protocols performed, in accordance with the proper guidelines and regulations were approved by the Institutional Ethical Committee of Central Leather Research Institute and prior informed consent for donation of clinical specimens was obtained from all the participants.

Scratch wound assay. Equal density of 2×10^5 cells (HaCaT) per well was seeded in a 24 well tissue culture plate and allowed to become confluent overnight in a CO_2 incubator. A scratch wound was created on the monolayer of cells using a 200 µL pipette tip. The cells were then washed twice with warm PBS and fresh medium without serum containing different concentrations of human recombinant DPT (rDPT) (4629-DP, R&D systems, USA) were added in triplicates. The migration of the cells was captured using a phase contrast microscope (Leica Microsystems, Germany) at a time interval of 2 hours and the wound area was measured using image J[46] software. The percentage of wound recovered was calculated using the formula given below and its significance was computed using student's t-test. After the study period cells were fixed and observed for lamellipodia formation in a phase contrast microscope (Leica Microsystems, Germany). The protrusions formed in the treated and untreated cells were counted manually and analyzed for significance using student's t-test. For both migration and lamellipodia formation (n = 3) two-tailed test was performed.

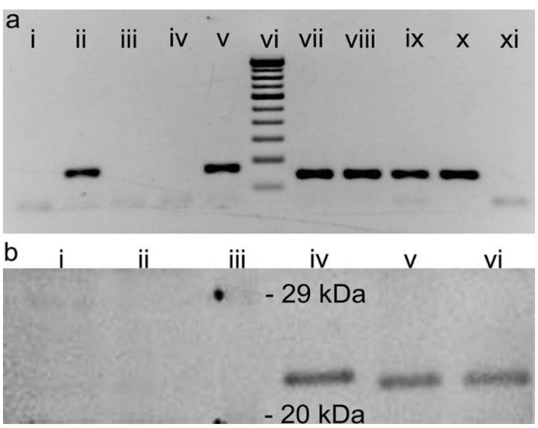

Figure 6 | Expression of DPT in epidermis and dermis. (a) Agarose gel images showing DPT PCR products, 168 bp. (i–v) and RPL32 PCR products, 147 bp. (vii–xi). Lanes: i & xi) No template control for DPT and RPL32 respectively, ii & x) NSF, iii & ix) HaCaT cells, iv & viii) Epidermis, v & vii) Dermis, vi) 100 bp DNA ladder. (b) Western blotting analysis of protein homogenates from i) HaCaT cells and ii) epidermis revealing the absence of DPT protein in contrary to the prominent expression in v) dermal and vi) NSF protein extracts. The bands were compared with iv) rDPT and iii) protein molecular weight marker to determine the band size.

$$\text{Percentage of wound area recovered} = \frac{(\text{Initial wound area} - \text{Final wound area})}{\text{Initial wound area}} \times 100$$

Phalloidin staining. The characteristic lamellipodia formation of the migrating cells was identified by staining the cytoskeletal actin filaments. After treating the HaCaT cells with rDPT (500 pg/mL) overnight, cells were washed twice with PBS and fixed with 3.7% formaldehyde. The fixed cells were then washed, extracted with 0.1% triton X 100 (Sigma-Aldrich, USA) and blocked with 1% BSA. The cells were later washed twice with PBS and stained with Oregon Green® 488 Phalloidin (Invitrogen, USA) for 30 min in a dark humidified chamber. For control cells same procedure was performed excluding rDPT treatment. The excess stain was removed by washing with PBS and images were captured using a fluorescence microscope (Leica Microsystems, Germany).

Cell proliferation assay. The proliferative potential of DPT on HaCaT cells was assessed by MTT assay[47]. Equal density of 12×10^3 cells/well was seeded in a 24 well tissue culture plate. Following overnight incubation, fresh medium without serum containing different concentrations of rDPT were added in triplicates. After 24, 48 and 72 hours the cells were treated with MTT (0.5 mg/mL in PBS) for 3 hours at 37°C. The formazan complex formed by the live cells was dissolved and measured colorimetrically at 570/630 nm using a micro plate reader (Bio-Rad, USA).

RNA isolation and polymerase chain reaction. Total RNA was extracted from 90% confluent HaCaT and NSF cells using TRIzol® (Invitrogen, USA) following the manufacturer's instructions. Epidermis was separated from the dermis as described elsewhere[48], and RNA was isolated from epidermis and dermis following the same procedure mentioned above. The extracted RNA was quantified using Nanodrop 2000 (Thermofischer, USA), and 2 µg of total RNA was converted to cDNA using ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England BioLabs, UK) according to the manufacturer's protocol. Polymerase chain reaction was then performed at pre optimized conditions using the synthesized cDNA in a gradient thermal cycler (Eppendorf, Germany) using Red dye master mix (Bangalore Genei, India) and primers (DPT: Forward - 5′ TATTCCTGCTGGCTAACAACA 3′, Reverse - 5′ ACAGTCGTATTCAGTCATCCG 3′; RPL32: Forward - 5′ GCCCTCAGACCCCTTGTG 3′, Reverse - 5′ CCTTGAATCTTCTACGAACCCT 3′). NSF and dermis were used as positive controls for the experiment and RPL32 gene served as an internal control. After amplification the products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and photographed using GelDoc XR documentation system (Bio-Rad Laboratories Inc, USA).

Western blotting. Proteins samples isolated from the phenol-ethanol supernatant obtained after DNA precipitation during RNA isolation procedure were quantified using BCA (Bicinchoninic acid, Sigma-Aldrich, USA) assay. Protein homogenates were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions followed by electro-transfer on to PVDF membrane (Millipore Inc., USA) using a wet transfer system (Bio-Rad Laboratories Inc., USA). The membranes were then blocked with 5% skim milk, washed and probed with anti-human DPT antibody (1 : 1000 dilutions; sc-376863) for 1 hour at 37°C. Appropriate secondary antibody conjugated with alkaline phosphatase (sc-2037) was added to the membrane and incubated for 1 hour at 37°C. All antibodies were purchased from

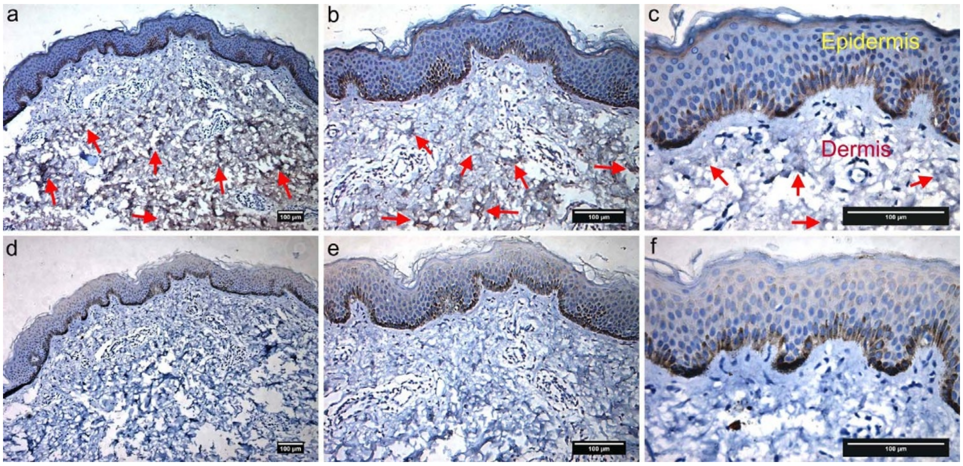

Figure 7 | Immunohistochemical analysis of normal skin for expression and localization of DPT protein. (a, b & c) Sections treated with anti-human DPT primary antibody at different magnifications. (d, e & f) Position-matched control sections without primary antibody treatment at different magnifications. The brown color developed due to HRP-DAB reaction is indicated by arrows. Scale bar – 100 μm.

Santa Cruz Biotechnology Inc. (USA). Bands were visualized using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) solution (Sigma-Aldrich, USA) and imaged with GelDoc XR documentation system (Bio-Rad Laboratories Inc, USA).

Immunohistochemical analysis. Paraffin embedded normal human skin tissue, cut into 5 μm sections were de-waxed with xylene, rehydrated through a series of alcohols to deionized water. Antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6.0) using the microwave method. Sections were then blocked and subsequently treated with anti-human DPT antibody (sc-376863, Santa Cruz Inc, USA) at 1 : 100 dilutions for 1 hour at room temperature in a humidified container. One set of slides were then treated with appropriate secondary antibody (1 : 400 dilution; sc – 2010, Santa Cruz Biotechnology Inc., USA) conjugated with FITC (Fluorescein isothiocyanate). The other set was developed using Novolink™ polymer detection kit (Leica Biosystems, Germany) with DAB (3, 3′-Diaminobenzidine) as a chromogenic substrate. The sections were further washed with TBS (pH 7.4) and counterstained with hematoxylin. For control sections, the same procedure was performed skipping the primary antibody treatment. All the sections were then aqueous mounted and images were captured using phase contrast or fluorescence microscope (Leica Microsystems, Germany).

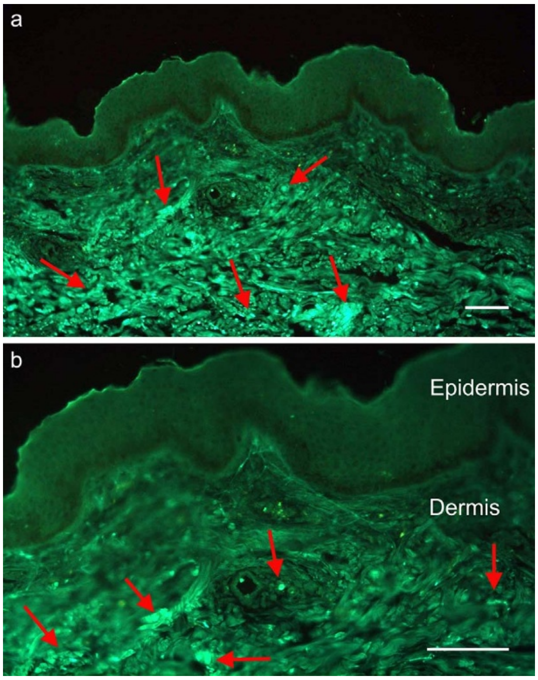

Figure 8 | Immunofluorescent detection of DPT protein in normal skin sections. (a) Sections treated with FITC conjugated secondary antibody showing strong signals in the dermis (indicated by arrows). ii) Magnified image of the same section revealing the absence of DPT protein in the epidermis. Scale bar – 100 μm.

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Author contributions

V.R.K. designed and performed the experiments and prepared the manuscript. P.S.K. approved the experiments and corrected the manuscript.

Additional information

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