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Masaru Honma, Masako Minami-Hori, Hidetoshi Takahashi, Hajime Iizuka

(Original article)

Podoplanin Expression in Wound and Hyperproliferative Psoriatic Epidermis: Regulation by TGF-β and STAT-3 Activating Cytokines, IFN-γ, IL-6, and IL-22

Masaru Honma, Masako Minami-Hori, Hidetoshi Takahashi and Hajime Iizuka

Department of Dermatology, Asahikawa Medical University, Midorigaoka-Higashi 2-1-1-1, Asahikawa, JAPAN

Correspondence:

Masaru Honma MD PhD, Department of Dermatology, Asahikawa Medical University, Midorigaoka-Higashi 2-1-1-1, Asahikawa 078-8510, JAPAN

E-mail: wanwan@asahikawa-med.ac.jp

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Abstract

Background Podoplanin (PDPN)/T1α/aggrus/PA2.26 antigen, a transmembranous glycoprotein, is a well-known lymphatic endothelial marker. Recent evidence indicates that PDPN is also expressed in keratinocytes especially of sebaceous glands.

Objective To verify expression-pattern and the regulatory mechanism of PDPN in human epidermal keratinocytes

Methods PDPN-expression pattern was analyzed in normal and psoriatic epidermis by immunostaining. The regulatory mechanism of PDPN-expression of keratinocytes by cytokines was analyzed using specific inhibitors, siRNA, and adenoviral shRNA of signaling pathways.

Results In normal skin, PDPN was expressed on the basal cell layer of sebaceous glands and on the outer root sheath of hair follicles. While no expression was detected in the normal interfollicular epidermis, PDPN was detected in the basal cell layer of wound and hyperproliferative psoriatic epidermis, where the granular layer is lacking. TGF-β1 and IFN-γ independently upregulated PDPN-expression of keratinocytes via TGF-β receptor-Smad pathway and JAK-STAT pathway, respectively. IL-6 and IL-22 also stimulated PDPN-expression of keratinocytes accompanied by STAT-3

phosphorylation. siRNA of STAT-1, inhibitors of STAT-3 signaling, AG490, STAT-3 inhibitor VI, and si/shRNA of STAT-3 inhibited the PDPN-expression of keratinocytes induced by IFN-γ, IL-6 and IL-22 but not by TGF-β1.

Conclusion These results indicate that TGF-β1, IFN-γ, IL-6, and IL-22 induce PDPN-expression of keratinocytes, which might be significantly involved in the wound healing process as well as in the pathomechanism of hyperproliferative psoriatic epidermis.

1. **Introduction**

Podoplanin (PDPN)/T1α/aggrus/PA2.26 antigen is a 38-40 kD, mucin-type transmembranous glycoprotein originally identified as a molecule expressed in lung type I alveolar cells, and is broadly expressed in various tissues, such as glomerular podocytes, chondroid plexus of central nervus system, the ciliary epithelium of eye, intestine, and malignant tumors [1-4]. PDPN has been known as a lymphatic endothelial marker and the expression surrounding malignant tumors is one of the prognostic factors associated with lymphangiogenesis and distant metastasis [5-7]. PDPN also plays a role in epithelial-mesenchymal transition (EMT), which is significantly involved in tumor invasion and metastasis [8]. Thus, the PDPN-expression is frequently detected in the leading edge of malignant epithelial tumors, such as breast cancer and esophageal carcinoma [9]. The expression of PDPN is also recognized in epidermis, such as TPA-treated hyperproliferative mouse epidermis [9], psoriatic lesional epidermis [10], and the basal cell layer of sebaceous glands [11]. Although exogenous overexpression of PDPN increases cell motility in immortalized mouse keratinocytes [12], precise function of PDPN in human epidermal keratinocytes remains unclear.

In the present study, we analyzed the expression of PDPN in epidermal keratinocytes. PDPN was upregulated in wound and in psoriatic hyperproliferative

epidermis. Since TGF-β and STAT-3 activation is involved in both skin conditions [14], we analyzed the expression of PDPN by TGF-β1 and various STAT-3 activating cytokines in keratinocytes. TGF-β1 stimulated PDPN-expression of keratinocytes in a Smad- but not STAT-3-dependent manner, and IFN-γ, IL-6, and IL-22 stimulated the expression in both STAT-1 and STAT-3-dependent manner. These stimulatory effects on PDPN-expression might be significantly involved in the wound healing process as well as in the pathomechanism of hyperproliferative psoriatic epidermis.

2. Materials and methods

2.1. Cell culture

Neonatal human epidermal keratinocytes (HEK) (Cambrex, Charles City, IA) were cultured in KBM-2 serum-free medium (Cambrex).

2.2. Tissue sections and immunostaining

Formalin-fixed and paraffin-embedded tissue sections were obtained from wounded skin and from 20 cases of psoriasis with informed consent and the approval of the Asahikawa Medical University ethical committee. Paraffin-embedded sections were dewaxed and subjected to antigen retrieval by incubation in 10 mM sodium citrate buffer (pH 6.0) at 100°C for 10 minutes. Sections were then blocked with 10% BSA in phosphate-buffered saline (PBS) for 1 hr and incubated with primary antibodies diluted in 1% BSA in PBS overnight at 4°C. Alexa fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA), or DAKO Envision system (DAKO, Glostrup, Denmark) was employed for visualization. Nuclei were stained with Hoechst 33342 dyes (Invitrogen). Immunofluorescent-stained sections were digitally recorded using a fluorescent microscope system (Olympus, Tokyo, JAPAN). This study was conducted according to the Declaration of Helsinki Principles.

2.3. Antibodies and reagents

Mouse monoclonal antibody, D2-40 (DAKO), and rat monoclonal antibody, NZ-1 (AngioBio, Del Mar, CA), were used for the detection of PDPN. Mouse monoclonal anti-β-tubulin antibody (Sigma, St. Louis, MO), anti CD-34 antibody (DAKO), rabbit anti-E-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-STAT-1 antibody, anti-phospho-STAT-1 antibody, anti-STAT-3 antibody, anti-phospho-STAT-3 antibody, anti-Smad-2 antibody, anti-phospho-Smad-2/3 antibody and anti-Smad-4 antibody (Cell Signaling Technology, Danvers, MA) were employed for the detection of each molecule. JAK inhibitor I, TGF-β type I receptor kinase inhibitor IV, AG490, STAT-3 inhibitor VI (Calbiochem, La Jolla, CA), TGF-β1, IFN-γ, IL-6 (PeproTech, Rocky Hill, NJ), and IL-22 (WAKO, Osaka, JAPAN) were used at the indicated concentrations.

2.4. Western Blotting

Cells were rinsed twice with ice-cold PBS and lysed in ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN) as previously described [15]. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL). Cell lysate (20–50 μg per a lane) was separated by SDS-PAGE and transferred to Hybond-P Nitrocellulose membranes (Amersham Bioscience, Piscataway,

NJ). Blotted membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk and then incubated with primary antibodies overnight at 4°C. Proteins were visualized with anti-mouse or anti-rabbit IgG horseradish-peroxidase–linked antibodies (Amersham Bioscience) for 1 hr, followed by chemiluminescence detection (ECL plus, Amersham Bioscience). Intensity of each data from triplicated experiments was measured using ImageJ image analyzer. Then the data were analyzed for statistics.

2.5. RT-PCR

Total RNA (5 μg) was extracted from primary human keratinocytes using RNeasy mini kit (Qiagen, Hilden, Germany). The RNA was reverse transcribed by oligo dT primer using ImProm-II™ Reverse Transcription System (Promega, Madison, WI). Subsequently, the same amount of first strand cDNA was amplified using ExTaq DNA polymerase (TAKARA, Otsu, JAPAN) and indicated primers' pairs. Primers' sets of 5'-GCCATCCTAAAAGCCACCCC-3' and 5'-CACCCACTCCCAGGGAGACC-3', 5'-TCCAGGAACCAGCGAAGACC-3' and 5'-ACGATGATTGCACCAATGAAGC-3' were used for the amplification of human β-actin and PDPN, respectively.

2.6. Transfection of siRNA

Fifty to 100μM of control scramble (TAKARA) and STAT-1, STAT-3, Smad-2/3, or Smad-4 specific siRNA (Cell Signaling Technology) were transfected into normal human keratinocyte and HaCaT cells using Transit-TKO transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's protocol.

2.7. Adenovirus vector and its infection to HEK

A pair of DNA oligomers for STAT-3-specific shRNA (5'-GATCCAACATCTGCCTAGATCGGCTACTGTGAAGCCACAGATGGGTAGC CGATCTAGGCAGATGTTTTTTTTA-3' and 5'-AGCTTAAAAAAAACATCTGCCTAGATCGGCTACCCATCTGTGGCTTCACA GTAGCCGATCTAGGCAGATGTTG-3') was subcloned into BamHI and HindIII sites of pBAsi-hU6. The shRNA construct with human U6 RNA polymerase promoter was subcloned into ClaI site of pAxcwit cosmid. Then, adenovirus vector was generated using the cosmid by COS-TPC method (TAKARA, Otsu, JAPAN) [16]. Adenovirus generated using pAxcwit was used as a control vector. Adenoviral vectors were amplified in HEK293 cells and each multiplicity of infection (MOI) was titered by limiting dilution method according to the manufacturer's protocol (TAKARA). For the infection of adenovirus vector, HEK was incubated with the same MOI of each

adenovirus vector in culture medium for 1 hr, and then the medium was exchanged to fresh one.

2.8. Three-dimensional cultured epidermis

LabCyte EPI-MODEL 12, a three-dimensional epidermal culture model, was purchased from J-TEC (Gamagori, JAPAN). Each reconstituted epidermis was treated with IL-22 (50 ng/ml) for 4 days, then fixed with 4% formaldehyde after the cultivation, paraffin-embedded, and sectioned for each staining.

3. Results

3.1. PDPN-expression in normal human skin

Immunostaining using mouse monoclonal antibody, D2-40, revealed that PDPN is highly expressed in lymphatic endothelial cells and the basal cell layer of sebaceous glands but not in normal human interfollicular epidermis (Fig. 1a and b). Marked PDPN-expression was detected in the outer root sheath (ORS) of hair follicles from the mid portion down to the hair bulb excluding the bulge area (Fig. 1c). The expression-pattern correlated with the expression of CD-34, a marker of ORS inferior to the bulge area in human hair follicles [17] (Fig. 1d). NZ-1, a rat monoclonal antibody against human PDPN, showed similar expression pattern (data not shown).

3.2. PDPN-expression in wounded skin and in psoriasis

While PDPN-expression was not detected in normal human interfollicular epidermis (Fig. 1a), marked expression was detected in wound and psoriatic epidermis. PDPN-expression was localized on the basal cells of the leading edge of the wound (Fig. 2b) and those of the psoriatic epidermis (Fig. 2d). Not all the psoriatic epidermis showed the PDPN-expression. It was detected in 7 out of 20 lesional psoriatic epidermis samples. PDPN-positive psoriasis was mostly manifested as hyperproliferative expanding lesion without the granular layer [18]; stable and/or regressing psoriasis with

granular layer was negative for PDPN. In PDPN-positive psoriatic epidermis, the expression was higher in the basal cells of rete ridges than those on the dermal papillae. The PDPN-expression was never detected in the non-lesional psoriatic epidermis (data not shown). Similar findings were observed by using another PDPN antibody, NZ-1 (data not shown).

3.3. TGF-β1 and IFN-γ upregulate PDPN-expression of HEK via TGF-β receptor-activation and JAK-STAT signaling pathway, respectively.

Wound and psoriatic epidermis share several inflammatory cytokines including TGF-β and IFN-γ [19–26]. The effect of these cytokines on PDPN-expression of HEK cells was analyzed. Both TGF-β1 and IFN-γ induced PDPN-expression of HEK cells at 24 hr following the stimulation (Fig. 3a). RT-PCR analysis revealed that PDPN mRNA was upregulated by these cytokines, which was detected at 6 hr following the stimulation (Fig. 3b). The PDPN-upregulation required relatively high concentrations of these cytokines. TGF-β1 required 2 ng/ml and IFN-γ required 50 ng/ml for the induction of PDPN (Fig. 3c). The upregulation of PDPN by IFN-γ was also observed in HaCaT cells, an immortalized human keratinocyte, but not in A431 or HSQ-89, human squamous cell carcinoma cell-lines (Fig. S1a). As expected, TGF-β1-dependent Smad-2 phosphorylation was inhibited by TGF-β type I receptor kinase inhibitor IV, and

IFN-γ-dependent STAT-1 and STAT-3 phosphorylation was inhibited by JAK inhibitor. The PDPN-expression induced by TGF-β1 was inhibited by TGF-β type I receptor kinase inhibitor IV, and that by IFN-γ was inhibited by JAK inhibitor (Fig. 4a).

3.4. PDPN-expression of keratinocytes induced by IFN-γ depends on STAT-1 and STAT-3 signaling pathways

Inhibitors for STAT-3 signaling, AG490, STAT-3 inhibitor VI, and STAT-3 inhibitory peptides, suppressed the upregulation of PDPN induced by IFN-γ (Fig. 4b and data not shown). While the phosphorylation of STAT-3 was more markedly suppressed by AG490, STAT-1-phosphorylation was also suppressed by AG490 in a dose-dependent manner (Fig. 4b). Both STAT-3 shRNA and STAT-1 siRNA inhibited the IFN-γ-dependent upregulation of PDPN, indicating that both STAT-1 and STAT-3 pathways are involved in the PDPN-induction (Fig. 4c and d).

3.5. PDPN-expression of keratinocytes induced by TGF-β1 depends on Smad2/3 and Smad-4 signaling pathways

To verify whether TGF-β1-induced PDPN-expression depends on Smad signaling pathway, experiments using siRNA targeting Smad-2/3 and Smad-4 were performed (Fig. 4e). PDPN-induction by TGF-β1 was markably suppressed by both siRNA for Smad-2/3 and Smad-4, indicating Smad-2/3 and -4 are essential for the

On the other hand, the TGF-β1-induced PDPN-expression was not affected by STAT-3-specific gene silencing (Fig. S2) or by the STAT-3 inhibitors, suggesting an independent PDPN-induction mechanism of TGF-β1 from STAT-3 signaling pathway.

3.6. IL-22 and IL-6 stimulate PDPN-expression of keratinocytes through the activation of STAT-3.

Recent studies have shown that IL-22 and IL-6 are significantly involved in the pathomechanism of psoriasis [19-21, 27]. PDPN-expression was induced by these cytokines, which was accompanied by STAT-3-phosphorylation (Fig. 5a). AG490, STAT-3 inhibitor VI and shRNA of STAT-3 inhibited both PDPN-expression and STAT-3-phosphorylation (Fig. 5b and data not shown). The IL-22-dependent PDPN-induction was also inhibited by STAT-3 siRNA in HaCaT cells (Fig. S1b). Thus, IL-22 and IL-6 stimulated PDPN-expression of keratinocytes through the STAT-3 signaling pathway. Using reconstituted three-dimensional epidermis, PDPN-expression was also analyzed. Following IL-22-stimulation the induction of PDPN was detected in the basal cell layer of the reconstituted epidermis (Fig. 5c), which was not detected in the reconstituted epidermis without the IL-22-treatment. The IL-22-treated reconstituted

epidermis showed several features mimicking psoriatic epidermis, such as epidermal acanthosis without the granular layer, and parakeratotic horny layer.

4. Discussion

Our results indicate the role of PDPN in the wound and psoriatic hyperproliferative epidermis. While no PDPN-expression was detected in the normal interfollicular epidermis, marked expression was detected in the outer root sheath of the hair follicles as well as in the basal cell layer of sebaceous glands. Interestingly, the outer root sheath of the hair follicle is known to show a similar phenotype to wound and psoriatic epidermis [28, 29].

Regulation of PDPN-expression has been described. These include cytokines/growth factors [29-33], transcriptional factors [9, 34, 35], Src [36], and cell contact [36]. In a fibrosarcoma cell-line (HT1080 cells), TGF-β induces PDPN-expression via TGF-β receptor-Smad-4 pathway, which was not confirmed in HaCaT cells [29] (Fig. S1). Our results indicate that PDPN-induction by TGF-β1 in HEK is dependent on both Smad-2/3 and -4 pathways [Fig. 4e].

IFN-γ is known to activate both STAT-1 and STAT-3 [38] and our study demonstrated that the IFN-γ-dependent PDPN-expression of keratinocytes depends on both STAT-1 and STAT-3-activation, because siRNA, shRNA and inhibitors of STAT-1 and STAT-3 signaling inhibited the PDPN expression of keratinocytes. Other inhibitors of the STAT-3 signaling pathway, AG490 and STAT-3 inhibitory peptide, also abolished

the effect of IFN-γ on PDPN-induction of keratinocytes (Fig. 4b and data not shown). As expected, the STAT-3 phosphorylation induced by IFN-γ was inhibited by AG490. However, the STAT-1 phosphorylation, albeit to a lesser extent, was also inhibited by AG490 in our system (Fig. 4b), the significance of which remains to be determined. Recent evidence indicates that STAT-3 activation is significantly involved in wound healing as well as in psoriatic epidermis [14, 27]. IFN-γ and IL-27 induce various chemokines upregulated in psoriatic epidermis, such as CXCL9/10/11 in a STAT-1-dependent manner [39-41]. Psoriatic epidermis shows a similar phenotype to wounded epidermis and the activation of IFN-γ and TGF-β signaling has been described in both skin conditions [25, 26]. Involvement of IL-6 and IL-22 in the pathomechanism of psoriasis is interesting in this context [19-21, 27], because both IL-6 and IL-22 stimulate STAT-3 signaling of keratinocytes [27]. Our results indicate that IL-6 and IL-22 induce PDPN-expression of keratinocytes again through the activation of STAT-3. A similar regulatory mechanism has been described in other molecules, such as S100A7/psoriasin [42]. Thus, the induction of PDPN by various cytokines reconfirms the essential role of STAT-3 signaling in keratinocyte biology specifically in the hyperproliferative condition. The reconstituted three dimensional epidermis clearly demonstrated that IL-22 induces PDPN-expression acompanied by STAT-3

phosphorylation. The PDPN-expression of CD34-expressing ORS inferior to the bulge area and the presence of undifferentiated basal cells of sebaceous glands suggest that these keratinocytes are also under the control of the STAT-3-activation. Because the proliferation of keratinocytes is more marked in the basal cells of rete ridges than in those on the dermal papillae [43], more marked expression of PDPN on the basal cells of rete ridges is consistent with the observation that STAT-3-dependent signaling is significantly involved in the hyperproliferative keratinocytes. This is also consistent with the finding that PDPN-positive psoriatic epidermis was mostly manifested as hyperproliferative expanding epidermis, which lacks the granular layer [18]. Our results indicate the essential role of Smad- and STAT-signaling pathways in the cytokine-induced PDPN-expression. However, further study would be required to verify whether these signaling pathways is directly associated with the induction of PDPN, because these cytokines except for TGF-β1 require 24 hr or more for the detection of the effect.

The precise function of PDPN in keratinocytes remains to be determined. While PDPN does not increase cell proliferation in immortalized keratinocytes [13], the PDPN-expression on the basal cells of hyperproliferative and/or migrating epidermis, such as subbulge area of anagen hair follicles, sebaceous glands, psoriasis, and wounded

which may contribute to the epidermal remodeling [42, 44] as well as to EMT [8, 37, 45]. Activated matrix metalloproteinase and destabilization of adherens junctions of PDPN-expressing cells might also be interpreted as the epidermal remodeling process [9, 46]. Further study would be required to determine the role of PDPN in terms of proliferation/migration of epidermal keratinocytes.

Our results indicate that TGF-β1, IFN-γ, IL-6, and IL-22 induce PDPN-expression of keratinocytes, which might be significantly involved in the wound healing process as well as in the pathomechanism of psoriatic hyperproliferative epidermis. TGF-β1 seems to upregulate the PDPN expression through Smad-2/3 and -4-activation but in a STAT-3-independent manner, while IFN-γ, IL-6, and IL-22 upregulate the expression in both STAT-1- and STAT-3-dependent manner. Because PDPN is also detected in the leading edge of various epithelial tumors, the PDPN-expression might also be significantly involved in other biological processes such as EMT.

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References

[1] Dobbs LG, Williams MC, Gonzalez R. Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. Biochim Biophys Acta 1988; 970: 146-156.

[2] Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D. Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol 1997; 151: 1141-52.

[3] Williams MC, Cao Y, Hinds A, Rishi AK, Wetterwald A. T1 a protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus and ciliary epithelia of adult rats. Am J Respir Cell Mol Biol 1996; 14: 577-585.

[4] Watanabe M, Sugimoto Y, Tsuruo T. Expression of a Mr 41,000 glycoprotein associated with thrombin-independent platelet aggregation in high metastatic variants of murine B16 melanoma. Cancer Res 1990; 50: 6657-62.

[5] Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G, Detmar M. T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J 2003; 22: 3546-56.

[6] Renyi-Vamos F, Tovari J, Fillinger J, Timar J, Paku S, Kenessey I, Ostoros G, Agocs L, Soltesz I, Dome B. Lymphangiogenesis correlates with lymph node metastasis, prognosis, and angiogenic phenotype in human non-small cell lung cancer. Clin Cancer Res 2005; 11: 7344-53.

[7] Straume O, Jackson DG, Akslen LA. Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. Clin Cancer Res 2003; 9: 250-6.

[8] Martín-Villar E, Megías D, Castel S, Yurrita MM, Vilaró S, Quintanilla M. Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition. J Cell Sci 2006; 119: 4541-53.

[9] Wicki A, Christofori G. The potential role of podoplanin in tumour invasion. Br J Cancer 2007; 96: 1-5.

[10] Gandarillas A, Scholl FG, Benito N, Gamallo C, Quintanilla M. Induction of PA2.26, a Cell-Surface Antigen Expressed by Active Fibroblasts, in Mouse Epidermal Keratinocytes During Carcinogenesis. Mol Carcinogen 1997; 20: 10–18.

[11] Henno A, Blacher S, Lambert CA, Deroanne C, Noël A, Lapière C, de la Brassinne M, Nusgens BV, Colige A Histological and transcriptional study of

angiogenesis and lymphangiogenesis in uninvolved skin, acute pinpoint lesions and established psoriasis plaques: an approach of vascular development chronology in psoriasis. J Dermatol Sci 2010; 57: 162-9.

[12] Yang HM, Cabral E, Dadras SS, Cassarino DS. Immunohistochemical expression of D2-40 in benign and malignant sebaceous tumors and comparison to basal and squamous cell carcinomas. Am J Dermatopathol 2008; 30: 549-54.

[13] Scholl FG, Gamallo C, Vilaró S, Quintanilla M. Identification of PA2.26 antigen as a novel cell-surface mucin-type glycoprotein that induces plasma membrane extensions and increased motility in keratinocytes. J Cell Sci. 1999; 112: 4601-13.

[14] Sano S, Chan KS, Carbajal S, Clifford J, Peavey M, Kiguchi K, Itami S, Nickoloff BJ, DiGiovanni J. Stat3 links activated keratinocytes and immunocytes required for development of psoriasis in a novel transgenic mouse model. Nat Med 2005; 11: 43-9.

[15] Honma M, Benitah SA, Watt FM. Role of LIM kinases in normal and psoriatic human epidermis. Mol Biol Cell 2006; 17: 1888-96.

[16] Honma M, Namikawa K, Mansur K, Iwata T, Mori N, Iizuka H, Kiyama H. Developmental alteration of nerve injury induced glial cell line-derived

neurotrophic factor (GDNF) receptor expression is crucial for the determination of injured motoneuron fate. J Neurochem 2002; 82: 961-75.

[17] Ohyama M, Terunuma A, Tock CL, Radonovich MF, Pise-Masison CA, Hopping SB, Brady JN, Udey MC, Vogel JC. Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest 2006; 116: 249-60.

[18] Iizuka H, Honda H, Ishida-Yamamoto A. Epidermal remodeling in psoriasis (II): a quantitative analysis of the epidermal architecture. J Invest Dermatol 1997; 109: 806-10.

[19] Lowes MA, Bowcock AM, Krueger JG. Pathogenesis and therapy of psoriasis. Nature 2007; 445: 866-73.

[20] Sabat R, Philipp S, Höflich C, Kreutzer S, Wallace E, Asadullah K, Volk HD, Sterry W, Wolk K. Immunopathogenesis of psoriasis. Exp Dermatol 2007; 16: 779-98.

[21] Griffiths CE, Barker JN. Pathogenesis and clinical features of psoriasis. Lancet 2007; 370: 263-71.

[22] Shirakata Y, Kimura R, Nanba D, Iwamoto R, Tokumaru S, Morimoto C, Yokota K, Nakamura M, Sayama K, Mekada E, Higashiyama S, Hashimoto K. Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. J Cell Sci 2005; 118: 2363-70.

[23] Romanowska M, Reilly L, Palmer CN, Gustafsson MC, Foerster J. Activation of PPARbeta/delta causes a psoriasis-like skin disease in vivo. PLoS One 2010; 5: e9701.

[24] Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H, Durham SR, Schmidt-Weber CB, Cavani A. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest 2009; 119: 3573-85.

[25] Flisiak I, Chodynicka B, Porebski P, Flisiak R. Association between psoriasis severity and transforming growth factor beta1 and beta2 in plasma and scales from psoriatic lesions. Cytokine 2002; 18: 121-5.

[26] Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, Abello MV, Novitskaya I, Pierson KC, Gonzalez J, Krueger JG, Lowes MA. Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. J Invest Dermatol 2009; 129: 79-88.

[27] Zheng Y, Danilenko DM, Valdez P. Interleukin-22, a Th17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 2007; 445: 648-51.

[28] Mansbridge JN, Knapp AM. Changes in keratinocyte maturation during wound healing. J Invest Dermatol 1987; 89: 253-63.

[29] Bernot KM, Coumombe PM, McGowan KM. Keratin 16 expression defines a subset of epithelial cells during skin morphogenesis and the hair cycle. J Invest Dermatol 2002; 119: 1137-49.

[30] Suzuki H, Kato Y, Kaneko MK. Induction of podoplanin by transforming growth factor-beta in human fibrosarcoma. FEBS Lett 2008; 582: 341-5.

[31] Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, Christofori G. Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. Cancer Cell 2006; 9: 261-72.

[32] Gröger M, Loewe R, Holnthoner W, Embacher R, Pillinger M, Herron GS, Wolff K, Petzelbauer P. IL-3 induces expression of lymphatic markers Prox-1 and podoplanin in human endothelial cells. J Immunol 2004; 173: 7161-9.

[33] Al-Rawi MA, Watkins G, Mansel RE, Jiang WG. The effects of interleukin-7 on the lymphangiogenic properties of human endothelial cells. Int J Oncol 2005; 27: 721-30.

[34] Jiang WG, Davies G, Martin TA, Parr C, Watkins G, Mansel RE, Mason MD. The potential lymphangiogenic effects of hepatocyte growth factor/scatter factor in vitro and in vivo. Int J Mol Med 2005; 16: 723-8.

[35] Durchdewald M, Guinea-Viniegra J, Haag D, Riehl A, Lichter P, Hahn M, Wagner EF, Angel P, Hess J. Podoplanin is a novel fos target gene in skin carcinogenesis. Cancer Res 2008; 68: 6877-83.

[36] Petrova TV, Mäkinen T, Mäkelä TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Ylä-Herttuala S, Alitalo K. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. EMBO J 2002; 21: 4593-9.

[37] Shen Y, Chen CS, Ichikawa H, Goldberg GS. SRC induces podoplanin expression to promote cell migration. J Biol Chem 2010; 285: 9649-56.

[38] Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002; 3: 651-62.

[39] Giustizieri ML, Mascia F, Frezzolini A, De Pità O, Chinni LM, Giannetti A, Girolomoni G, Pastore S. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. J Allergy Clin Immun 2001; 107: 871–7.

[40] Kanda N, Shimizu T, Tada Y, Watanabe S. IL-18 enhances IFN-gamma-induced production of CXCL9, CXCL10, and CXCL11 in human keratinocytes. Eur J Immunol 2007; 37: 338-50.

[41] Shibata S, Tada Y, Kanda N, Nashiro K, Kamata M, Karakawa M, Miyagaki T, Kai H, Saeki H, Shirakata Y, Watanabe S, Tamaki K, Sato S. Possible roles of IL-27 in the pathogenesis of psoriasis. J Invest Dermatol 2010; 130: 1034-9.

[42] Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J Immunol 2005; 174: 3695-702.

[43] Iizuka H, Ishida-Yamamoto A, Honda H. Epidermal remodelling in psoriasis. Br J Dermatol 1996; 135: 433-8.

[44] Meng F, Cheng X, Yang L, Hou N, Yang X, Meng A. Accelerated re-epithelialization in Dpr2-deficient mice is associated with enhanced response to TGFbeta signaling. J Cell Sci 2008; 121: 2904-12.

[45] Zavadil J, Cermak L, Soto-Nieves N, Böttinger EP. Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J 2004; 23: 1155-65.

[46] Scholl FG, Gamallo C, Quintanilla M. Ectopic expression of PA2.26 antigen in epidermal keratinocytes leads to destabilization of adherens junctions and malignant progression. Lab Invest 2000; 80: 1749-59.

Figure legends

Figure 1 PDPN-expression in normal human skin.

Anti-PDPN monoclonal antibody, D2-40 (green), and anti-E-cadherin rabbit polyclonal antibody (red) were applied to the skin (a-c). A: normal human skin. b: sebaceous gland. c: hair follicle. In d, D2-40 (red) and anti-CD34 antibody (green) were applied on the hair follicle. Scale bars indicate 100 μm.

Figure 2 PDPN-expression in diseased epidermis

PDPN-expression was detected on the basal cells of re-epithelizing wounded epidermis (a, b) and in psoriasis (c, d); a & c: HE staining; b & d: immunofluorescence staining of PDPN (green). Red staining of b shows E-cadherin-expression. Scale bars indicate 100 μm.

Figure 3 Regulation of PDPN-expression in HEK by TGF-β1 and IFN-γ

The time course of western blot analysis is represented by a. Up-regulation of PDPN-expression in HEK cells by TGF-β1 (5 ng/ml) and IFN-γ (50 ng/ml). TGF-β1 stimulation was accompanied by p-Smad-2/3 and IFN-γ stimulation was

accompanied by p-STAT-1, respectively. In b, the time course of RT-PCR analysis is shown. In c, the dose response analysis by western blotting is shown. Up-regulation of PDPN was analyzed at 24hr following the stimulation with various doses of TGF-β1 and IFN-γ.

Figure 4 Analyses of TGF-β1 and IFN-γ signaling on keratinocytes

PDPN-induction by IFN-γ and TGF-β1 was inhibited by JAK inhibitor I and TGF-β type I receptor kinase inhibitor IV, respectively (a). TGF-β type I receptor kinase inhibitor IV (10 nM) and JAK inhibitor I (10 μM) were added 1 hr before the TGF-β1 and IFN-γ stimulation. Cell lysates were collected 24hr following the cytokine stimulation. The PDPN-induction by IFN-γ was also inhibited by AG490, a STAT-3 specific inhibitor (b). Cell lysates were collected 24hr following the cytokine stimulation. AG490 (20 μM and 100 μM) was administered 1 hr before IFN-γ stimulation. STAT-3 phosphorylation was more markedly suppressed by AG490 than STAT-1 phosphorylation (*P<0.01 compared with DMSO-control, respectively). STAT-1 phosphorylation was also suppressed by AG490 in a dose-dependent manner. Both STAT-1 siRNA (siSTAT-1) and STAT-3 shRNA (shSTAT-3) inhibited the PDPN induction by IFN-γ (c and d). Control scramble (ct) and STAT-1 or STAT-3-specific

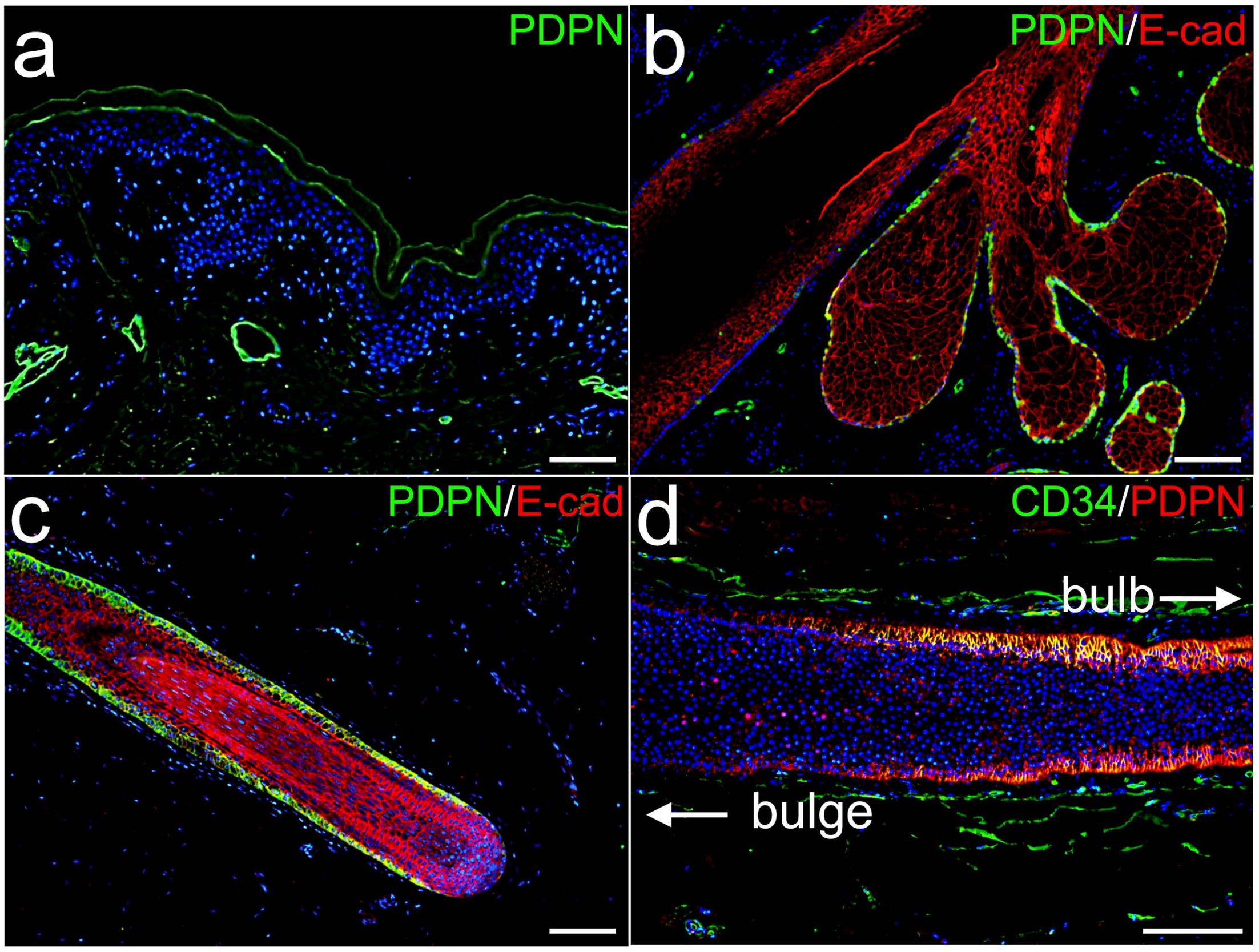
siRNA were transfected 48 hr prior to TGF-β1 or IFN-γ-stimulation. Adenovirus vector was also infected 48 hr prior to IFN-γ-stimulation. The TGF-β1-dependent PDPN-induction was remarkably reduced by siRNA of Smad-2/3 (siSmad-2/3) and Smad-4 (siSmad-4) (e). PDPN-expression was analyzed by western blotting using cell lysates 24 hr following the stimulation (5 ng/ml of TGF-β).

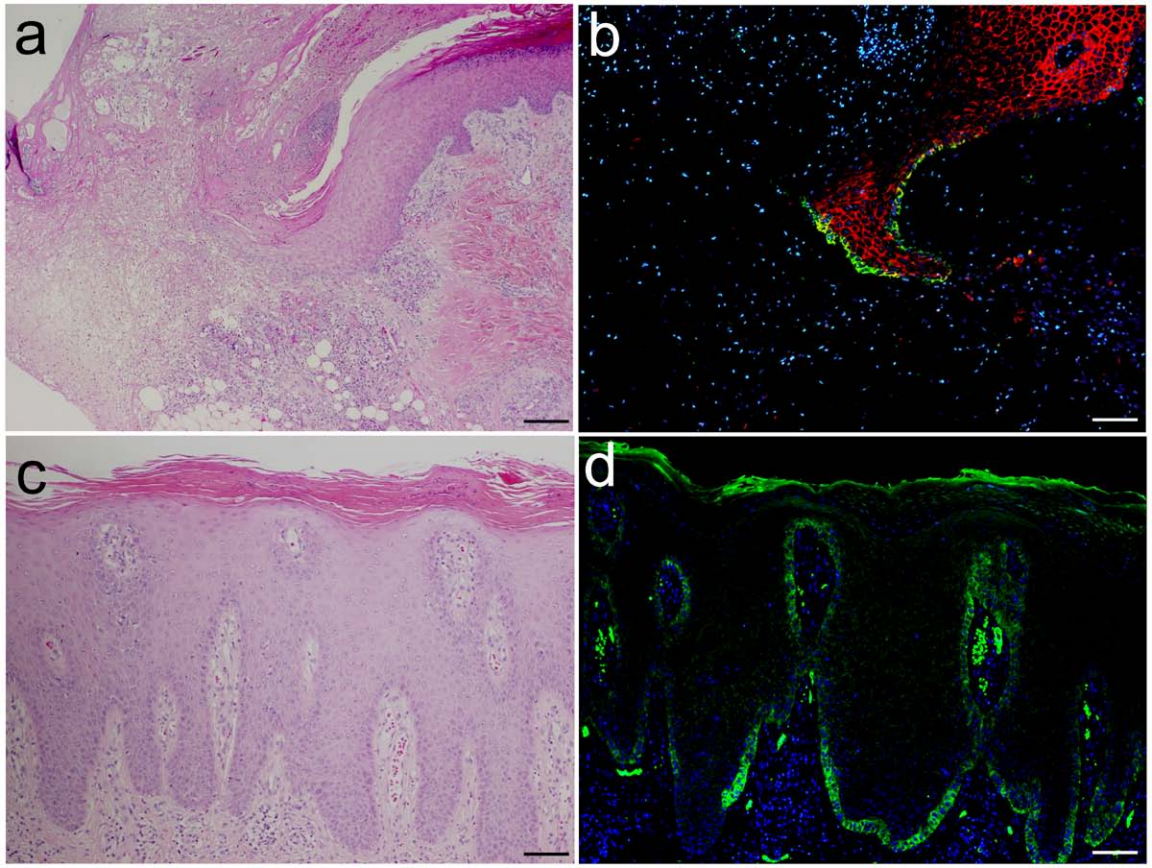
Figure 5 Effects of IL-6 and IL-22 on PDPN-expression of HEK and reconstituted epidermis.

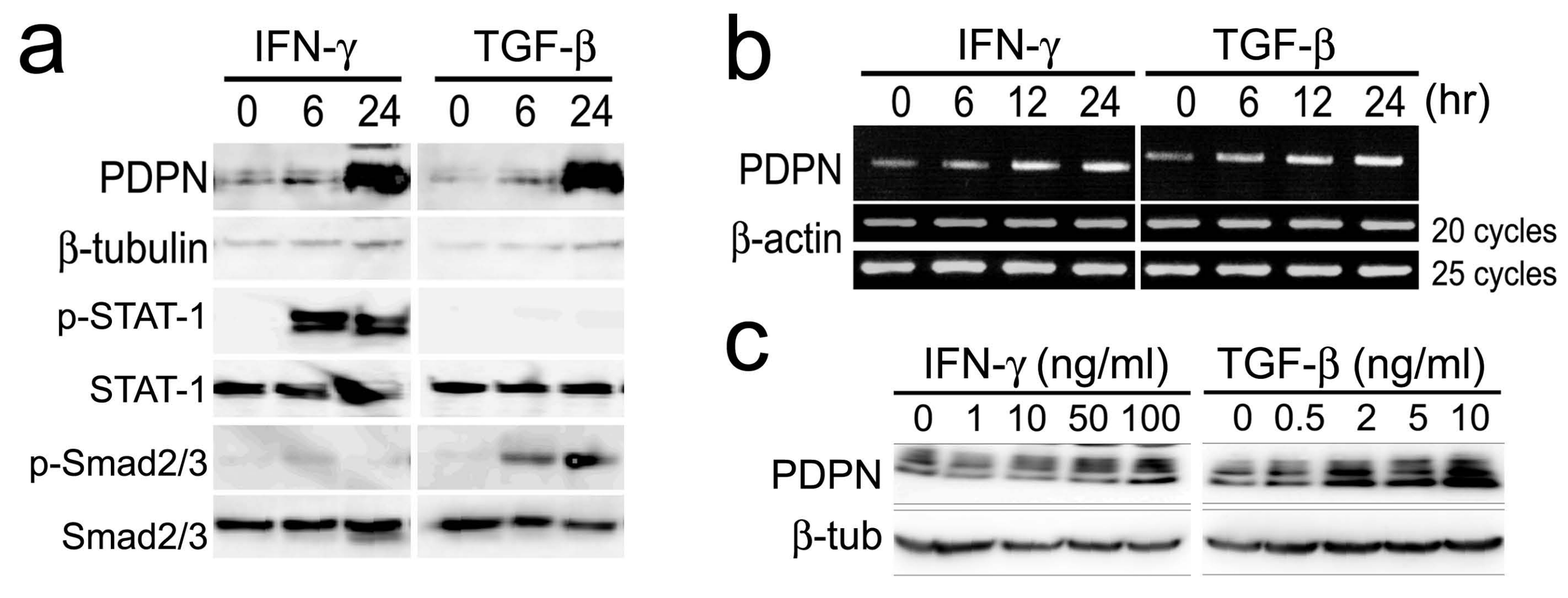
a. IL-6 (10 ng/ml) and IL-22 (50 ng/ml) induced STAT-3 phosphorylation.

b. IL-6 (10 ng/ml) and IL-22 (50 ng/ml) induced PDPN-expression. This was inhibited by STAT-3 shRNA (shSTAT-3). The cells were infected with control (cont) and shSTAT-3 adenovirus vectors 36 hr prior to the cytokine treatment. Cell lysate was collected and analyzed 30 hr following the IL-6 and IL-22 treatment.

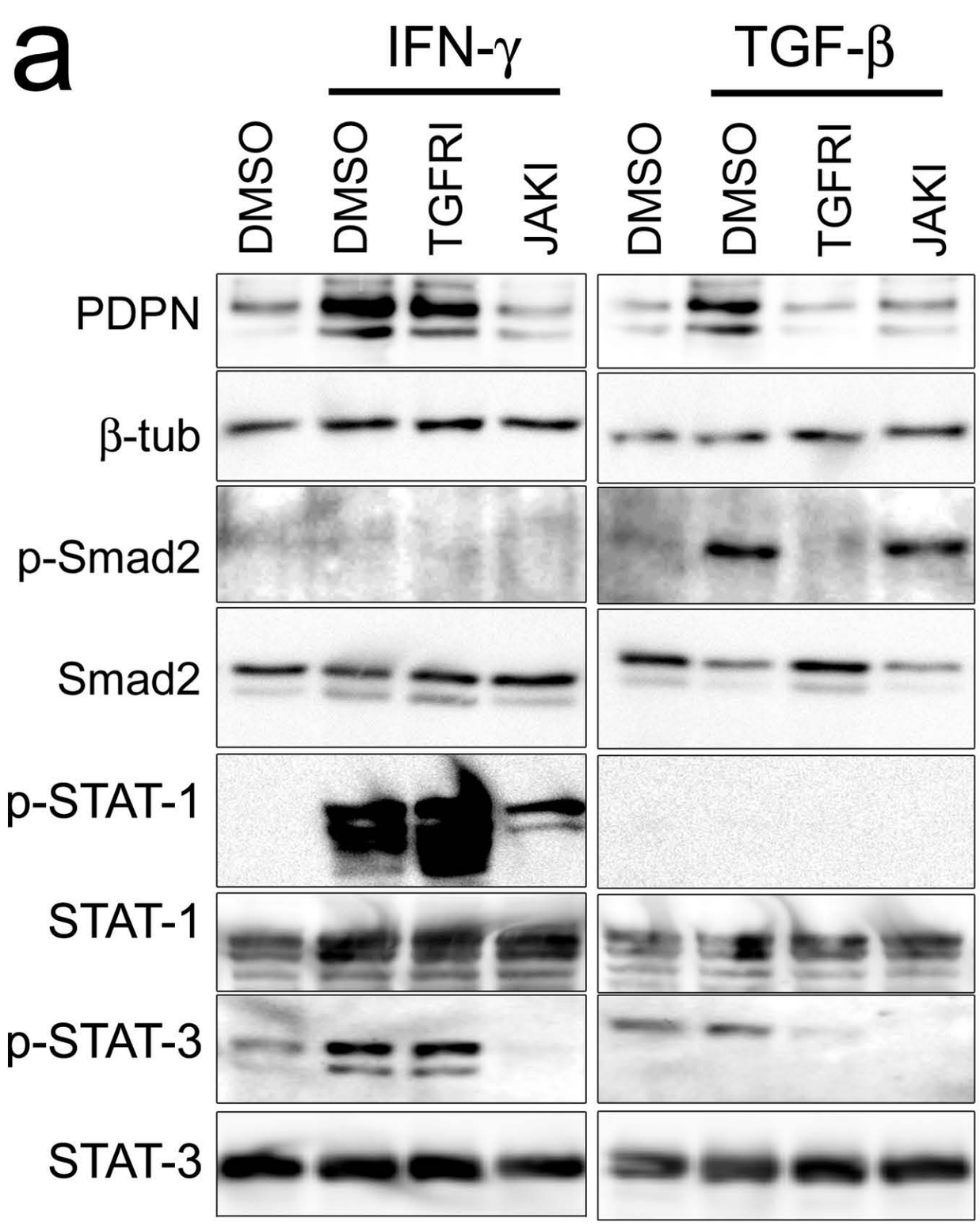
c. IL-22 induced PDPN-expression in reconstituted epidermis. Note acanthosis and parakeratosis in IL-22-treated epidermis.


a
PDPN
b
PDPN/E-cad
c
PDPN/E-cad
d
CD34/PDPN
bulb
bulge

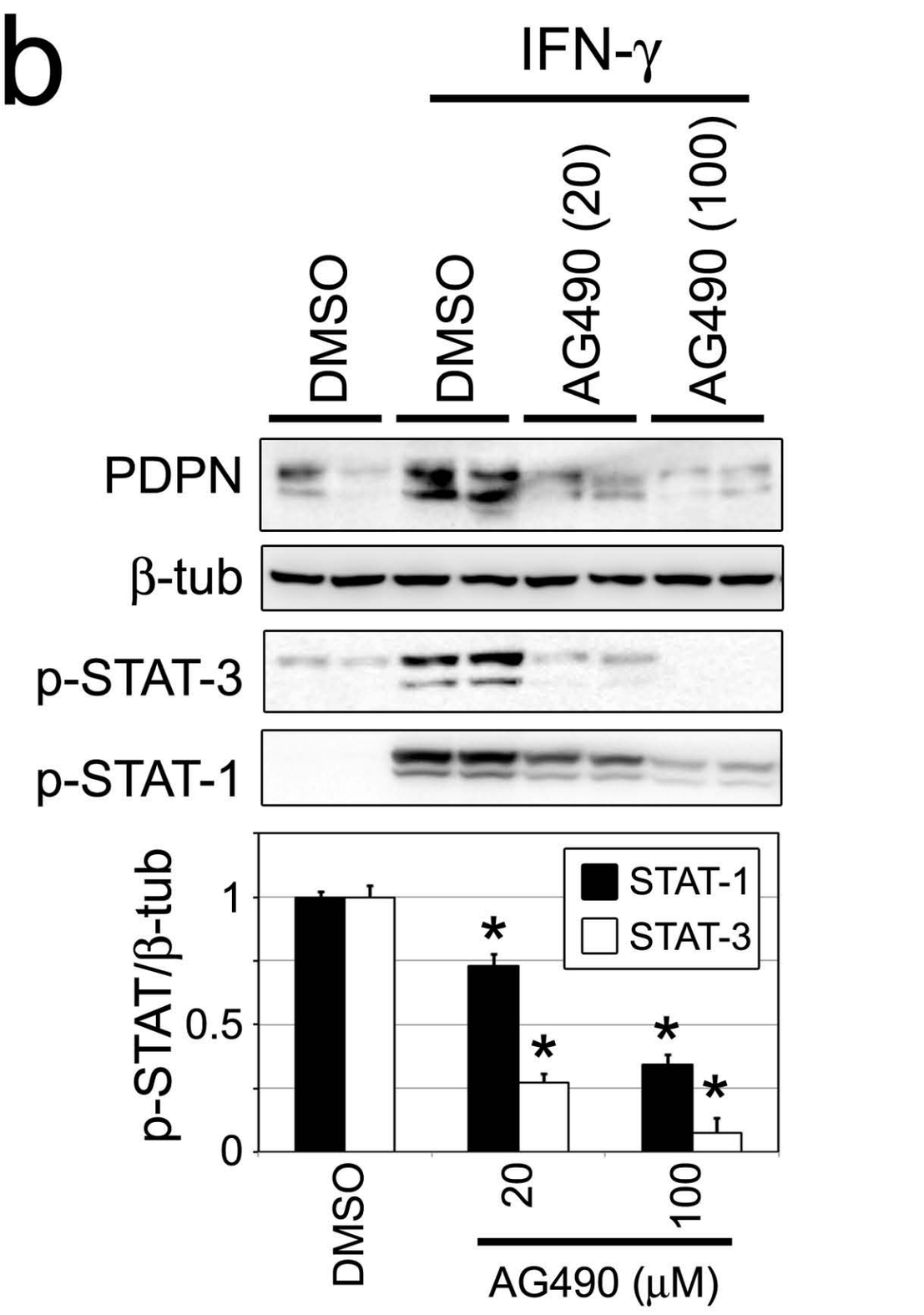

a
b
c
d


a
IFN-γ
TGF-β
0 6 24 0 6 24
PDPN
β-tubulin
p-STAT-1
STAT-1
p-Smad2/3
Smad2/3
b
IFN-γ
TGF-β
0 6 12 24 0 6 12 24 (hr)
PDPN
β-actin
20 cycles
25 cycles
c
IFN-γ (ng/ml)
TGF-β (ng/ml)
0 1 10 50 100
0 0.5 2 5 10
PDPN
β-tub

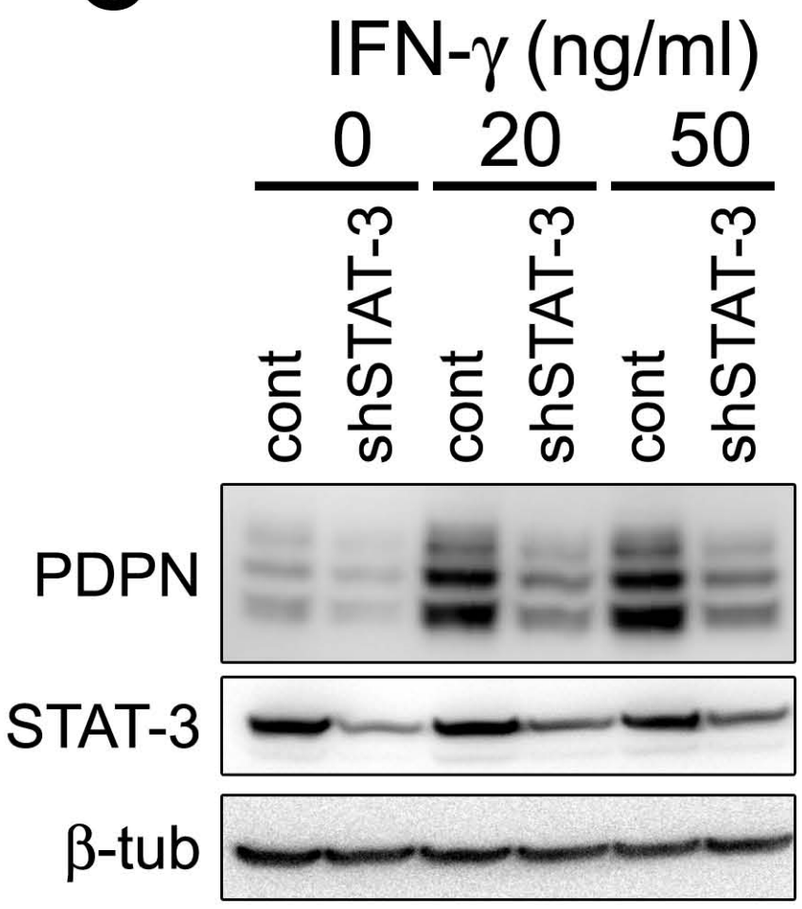
a


IFN-γ
TGF-β
DMSO
DMSO
TGFRI
JAKI
DMSO
DMSO
TGFRI
JAKI
PDPN
β-tub
p-Smad2
Smad2
p-STAT-1
STAT-1
p-STAT-3
STAT-3

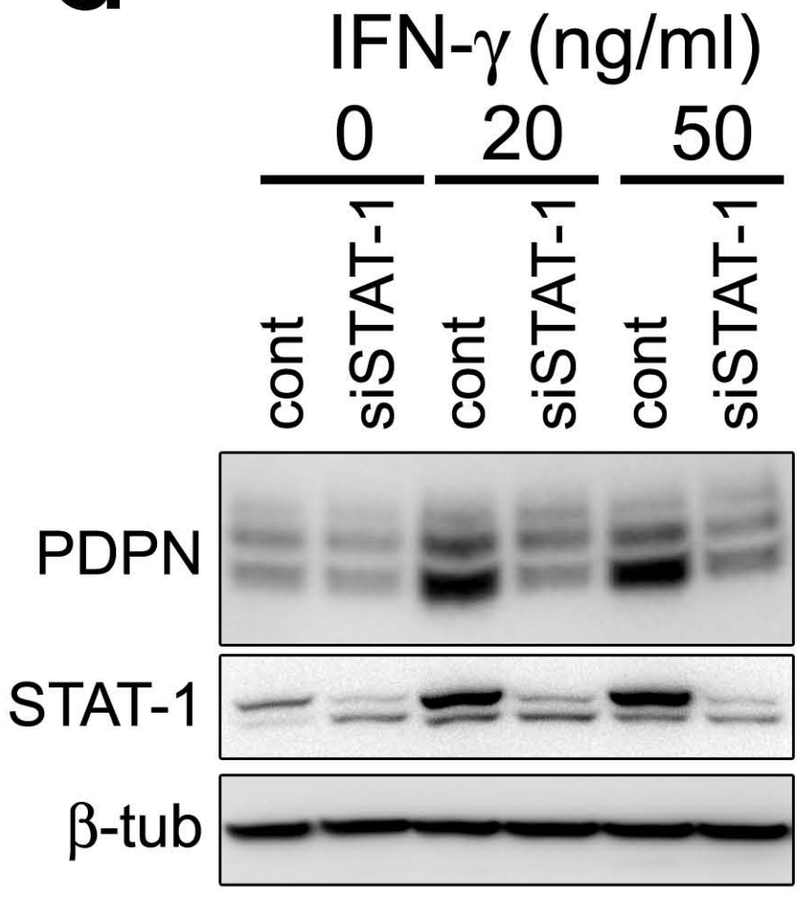
b


IFN-γ
DMSO
DMSO
AG490 (20)
AG490 (100)
PDPN
β-tub
p-STAT-3
p-STAT-1
p-STAT/β-tub
STAT-1
STAT-3
1
0.5
0
*
*
*
*
DMSO
20
100
AG490 (μM)

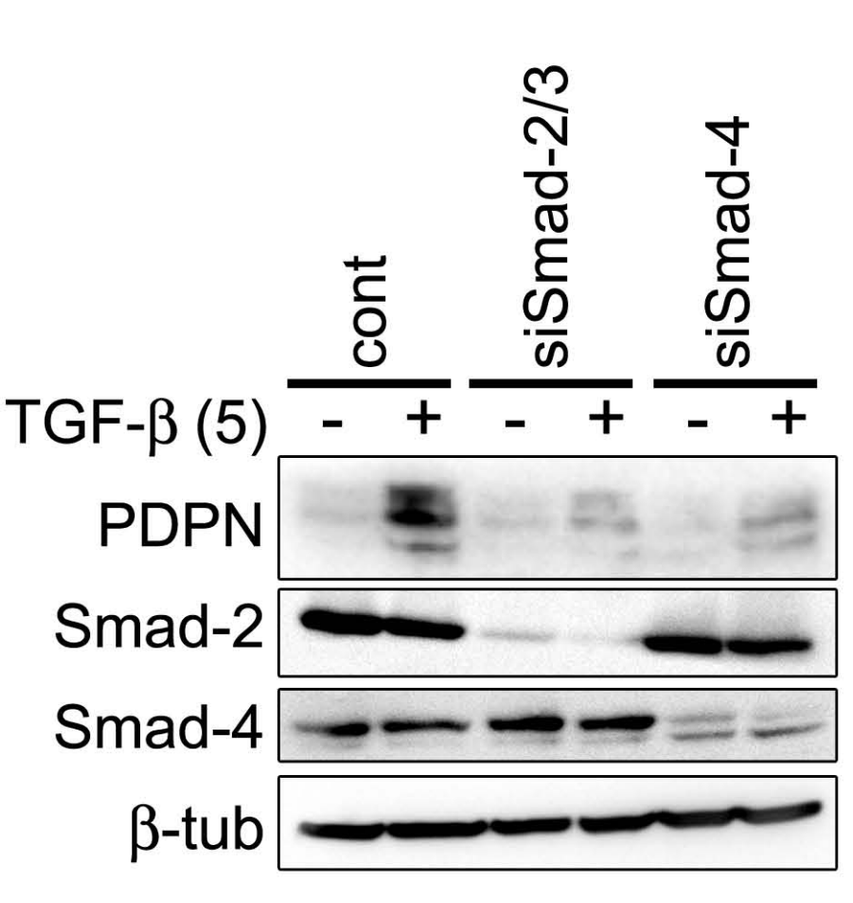
c

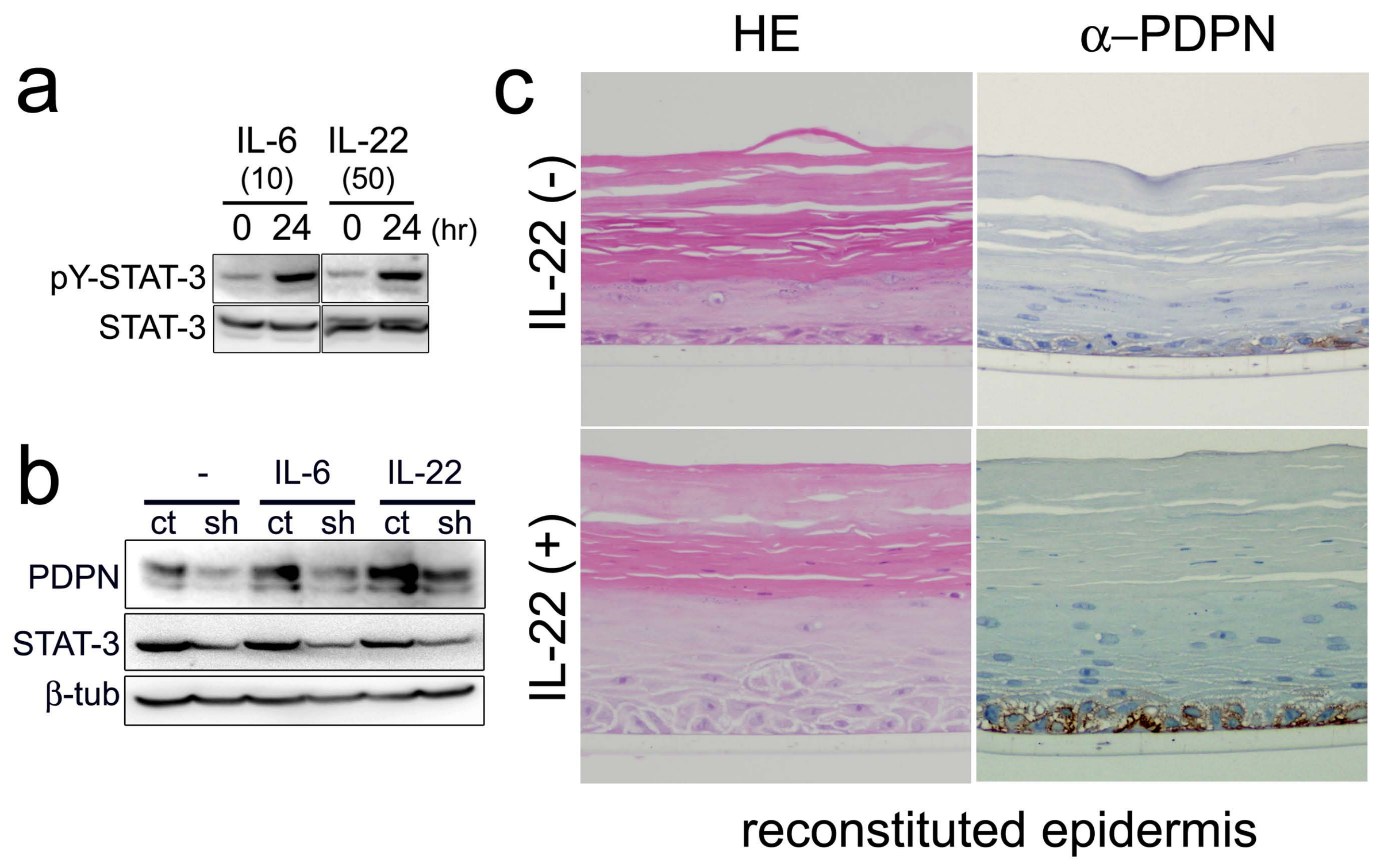

IFN-γ (ng/ml)
0
20
50
cont
shSTAT-3
cont
shSTAT-3
cont
shSTAT-3
PDPN
STAT-3
β-tub

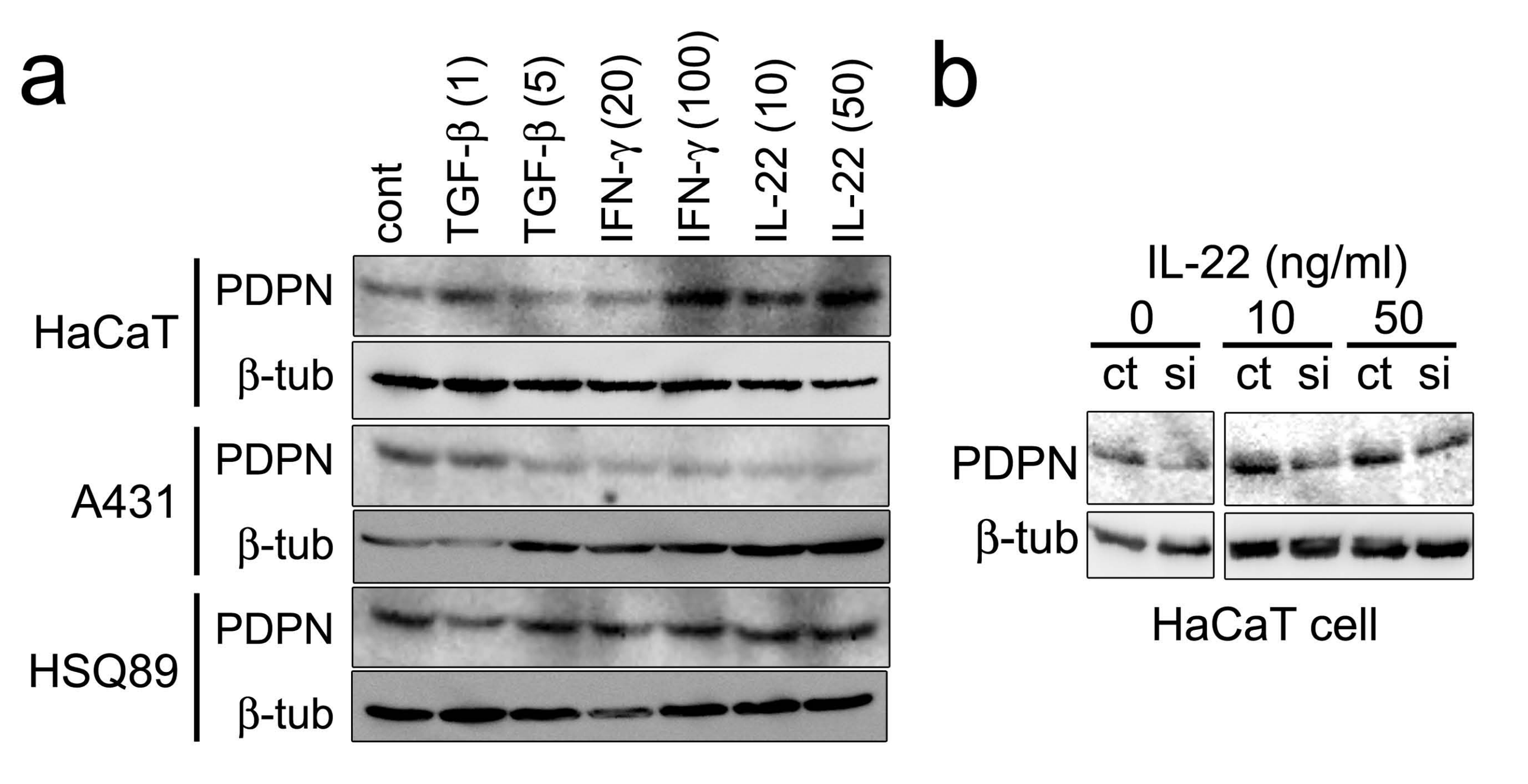
d

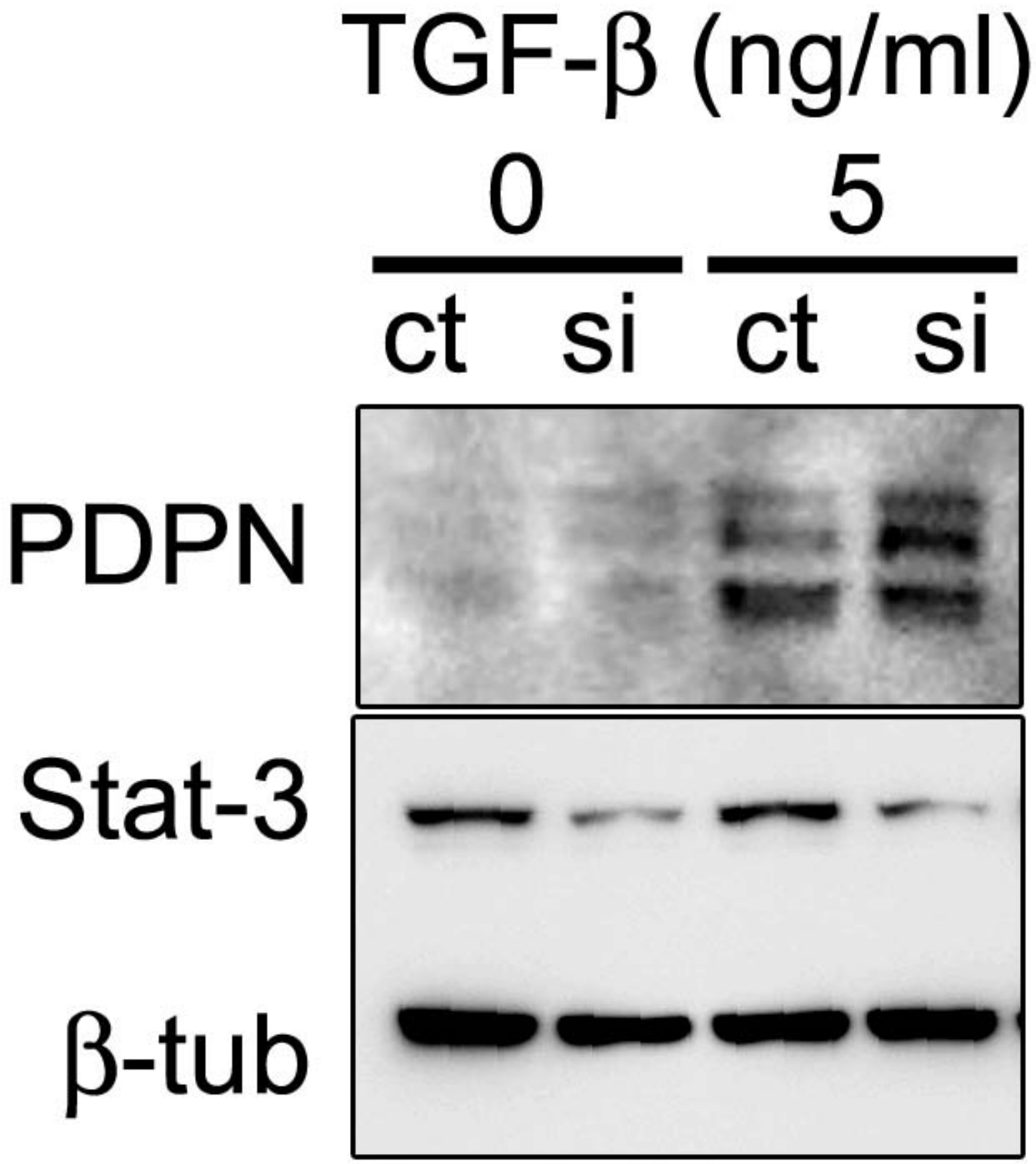

IFN-γ (ng/ml)
0
20
50
cont
siSTAT-1
cont
siSTAT-1
cont
siSTAT-1
PDPN
STAT-1
β-tub

e


cont
siSmad-2/3
siSmad-4
TGF-β (5)
-
+
-
+
-
+
PDPN
Smad-2
Smad-4
β-tub


a
IL-6
(10)
IL-22
(50)
0 24 0 24 (hr)
pY-STAT-3
STAT-3
b
-
IL-6
IL-22
ct sh ct sh ct sh
PDPN
STAT-3
β-tub
c
HE
α–PDPN
IL-22 (-)
IL-22 (+)
reconstituted epidermis


a
cont
TGF-β (1)
TGF-β (5)
IFN-γ (20)
IFN-γ (100)
IL-22 (10)
IL-22 (50)
HaCaT
PDPN
β-tub
A431
PDPN
β-tub
HSQ89
PDPN
β-tub
b
IL-22 (ng/ml)
0
10
50
ct si ct si ct si
PDPN
β-tub
HaCaT cell


TGF-β (ng/ml)
0
5
ct
si
ct
si
PDPN
Stat-3
β-tub