



Title	Possible role of epiregulin from dermal fibroblasts in the keratinocyte hyperproliferation of psoriasis
Author(s)	Iwata, Hiroaki; Haga, Naoya; Ujiie, Hideyuki
Citation	Journal of dermatology, 48(9), 1433-1438 https://doi.org/10.1111/1346-8138.16003
Issue Date	2021-09
Doc URL	http://hdl.handle.net/2115/86680
Rights	This is the peer reviewed version of the following article: Iwata, H, Haga, N, Ujiie, H. Possible role of epiregulin from dermal fibroblasts in the keratinocyte hyperproliferation of psoriasis. J Dermatol. 2021; 48: 1433–1438, which has been published in final form at https://doi.org/10.1111/1346-8138.16003 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	article (author version)
File Information	J Dermatol 1346-8138.16003.pdf



[Instructions for use](#)

1 **The possible role of epiregulin from dermal fibroblasts in the keratinocyte hyperproliferation of**
2 **psoriasis**

3 Hiroaki Iwata*, Naoya Haga, Hideyuki Ujiiie

4

5 Running head: Fibroblast-derived epiregulin in psoriasis

6

7 Department of Dermatology, Faculty of Medicine and Graduate School of Medicine, Hokkaido
8 University

9

10 North 15 West 7, Kita-ku, Sapporo 060-8638, Japan

11 Tel: +81-11-706-7387

12 Fax: +81-11-706-7820

13

14 **Key words:** psoriasis, acanthosis, growth factor, fibroblast, epiregulin

15

16 *Correspondence: Hiroaki Iwata

17 Department of Dermatology, Hokkaido University Graduate School of Medicine,

18 North 15 West 7, Kita-ku, Sapporo 060-8638, Japan

19 E-mail: hiroaki.iwata@med.hokudai.ac.jp

20

21 Word count: 1,500words

22 Figures: 3

23

24 Financial disclosure: The work was supported by Eli Lilly and Company and AbbVie GK.

25 Publishable disclosure: The authors have no conflicts of interest to declare.

26

27

1 **Abstract**

2 Psoriasis, an immune-mediated inflammatory disease, is characterized by keratinocyte
3 hyperproliferation. Tumor necrosis factor alpha (TNF α), interleukin (IL)-23, and IL-17A play critical
4 roles in the pathogenesis of psoriasis. IL-17A secreted by Th17 acts more directly against keratinocytes
5 than TNF α or IL-23 do. Regarding the receptors of cytokine, fibroblasts also express receptors against
6 IL-17A and TNF α , and induce the production of growth factors. Epiregulin (EREG), an epidermal
7 growth factor receptor (EGFR) ligand, is produced by both keratinocytes and fibroblasts. EREG
8 enhances keratinocyte proliferation and differentiation. We hypothesized that fibroblasts stimulated
9 with IL-17A and/or TNF α may play a role in epidermal hyperproliferation through the production of
10 epidermal growth factors in psoriasis. The mRNA expression of EREG was found to be significantly
11 upregulated by co-stimulation with IL-17A and TNF α (mean 49.2-fold). Furthermore, the stimulation
12 with TNF α alone, but not IL-17A alone, induced significant increases. Immunofluorescent staining
13 demonstrated that the protein expression level of EREG was also increased in fibroblasts stimulated
14 with these cytokines. Stimulation with EREG significantly enhanced keratinocyte proliferation in vitro.
15 In human psoriatic patients' skin, immunofluorescence staining of EREG showed significantly high
16 intensity in the dermis of lesional skin. In conclusion, cytokine stimulations with TNF α and IL-17A
17 induce the overexpression of EREG from dermal fibroblasts in the lesional skin of psoriasis, and plays
18 a role in epidermal hyperproliferation.

19

20

1 **Introduction**

2 Psoriasis is an immune-mediated inflammatory disease characterized by keratinocyte
3 hyperproliferation. Among the pathomechanisms of psoriasis, it is well known that tumor necrosis
4 factor alpha (TNF α), interleukin (IL)-23, and IL-17 play critical roles, and biologics block these
5 cytokines or their receptors. Of these cytokines, IL-17A, which is mainly secreted by Th17 cells, is
6 thought to act more directly against keratinocytes than TNF α or IL-23 does, and to induce keratinocyte
7 proliferation(1). In general, growth factors also contribute to cell proliferation. A previous report
8 showed that epiregulin (EREG), an epidermal growth factor receptor (EGFR) ligand, is overexpressed
9 in psoriatic lesions(2). This overexpression is thought to be involved in epidermal hyperproliferation
10 via Erk/JNK, MAP kinase, and stat3 signaling(2,3).

11 The IL-17 receptor family consists of five members: IL-17RA, RB, RC, RD, and RE. These receptors
12 are composed of a heterodimer, such as IL-17RA/IL-17RC for IL-17A, IL-17F and IL-17A/IL-
13 17F(4,5). IL-17RA is expressed in almost all cell types, and fibroblasts produce inflammatory
14 cytokines and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF),
15 and EREG via IL-17A signaling(4,5). Of these growth factors, EREG is produced by both fibroblasts
16 and keratinocytes(6,7). EREG enhances the proliferation and differentiation of keratinocytes(7) and
17 of follicular keratinocytes, leading to hair growth(8). In addition, keratinocytes secrete EREG and
18 stimulate keratinocyte growth in an autocrine manner(7). Dermal–epithelial interactions, both direct
19 and indirect, play essential roles in skin biology, such as in skin homeostasis, cancer progression, and
20 wound healing(9,10). Fibroblast-derived EREG is known to promote epidermal tumors(11,12), cancer
21 invasion(13), and wound healing(14,15).

22 Based on this evidence, we hypothesized that fibroblasts activated by IL-17A may play an important
23 role in epidermal hyperproliferation through the production of epidermal growth factors, especially
24 EREG.

1 **Materials and Methods**

2 **Patients**

3 Cryo-skin samples were obtained from 5 patients with psoriasis vulgaris whose diagnosis was
4 confirmed by histological examination. This study was approved by the Hokkaido University Certified
5 Review Board and was performed in accordance with the Declaration of Helsinki.

6

7 **Cell cultures and cytokine stimulation**

8 Normal human fibroblasts obtained from uninvolved skin of surgical specimens were cultured for 24
9 hours in Dulbecco's modified Eagle's medium (Life Technologies, Tokyo, Japan) containing 1.8 mM
10 of calcium. An antibiotic-antimycotic solution (Sigma Aldrich, St. Luis, MO) and 10% fetal calf serum
11 were supplied to the medium. Immortalized human keratinocytes (Ker-CT) purchased from ATCC
12 (Manassas, VA) were cultured in keratinocyte growth medium (KGM-Gold, Lonza, Basel,
13 Switzerland).

14 The recombinant human TNF α , IL-17A and IL-23 were purchased from PeproTech (Cranbury, NJ,
15 10602HNAE5), from Thermo Fisher Scientific (Waltham, MA, PHC9174) and from abcam
16 (Cambridge, UK, ab106889), respectively.

17

18 **Primer and quantitative polymerase chain reaction (qPCR)**

19 Fibroblasts were stimulated with PBS or cytokines for 48 hours. mRNA was extracted from fibroblasts.
20 Single-stranded cDNA was synthesized using the SuperScript IV (Thermo Fisher Scientific, Waltham,
21 MA). qPCR assays were performed using RT² SYBR GREEN/ROX PCR Master Mix (Qiagen,
22 Valencia, CA) and Step-OnePlus (Applied Biosystems). Relative expression ratios were normalized
23 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each primer sequence is listed below.

24 *EGF* F: GTTAGTCTCTCAGCACCGCA R: GTGCAGGACCCACACAAGTA

1 *FGF1* F: AAAGCGTGGGGGAGGTGTAT R: ATTTGGTGTCTGTGAGCCGT
 2 *FGF2* F: GCGACCCTCACATCAAGCTA R: AGCCAGGTAACGGTTAGCAC
 3 *FGF7* F: GAAAGGCTCAAGTTGCACCAG R: GCTATTTGACTTTTGTGTTTGTGCT
 4 *FGF10* F: AGATGTCCGCTGGAGAAAGC R: TCTCCAGGATGCTGTACGGG
 5 *EREG* F: CTCTGCCTGGGTTTCCATCTT R: ACTGGACTCTCCTGGGATACAT
 6 *GAPDH* F: ACCATCTTCCAGGAGCGAGA R: GACTCCACGACGTACTCAGC

7

8 **Immunofluorescence staining**

9 For the immunofluorescence staining, the cells were cultured on cover glass and incubated for 48
 10 hours with cytokines (Matsunami Glass, Kishiwada, Japan). The cells were washed with PBS and
 11 fixed with 4% paraformaldehyde at room temperature for 15 minutes. After permeabilization with
 12 0.25% TritonX-100 for 10 minutes, the cells were incubated with goat anti-epiregulin (R&D Systems,
 13 Minneapolis, MN, final concentration: 10 µg/ml) at 4°C overnight. To stain the human cryo-skin
 14 samples, 5 µm-thick sections were incubated with goat anti-epiregulin (10 µg/ml), rabbit anti-CD3
 15 (Roche diagnostics, Rotkreuz, Switzerland) and mouse anti-CD68 (Thermo Fisher Scientific, Waltham,
 16 MA, 1:100 dilution). After washing with PBS, cells or sections were stained with FITC-conjugated
 17 anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:100 dilution), FITC-
 18 conjugated anti-rabbit IgG (SouthernBiotech, Birmingham,AL, 1:100 dilution) and FITC-conjugated
 19 anti-mouse IgG (SouthernBiotech, 1:100 dilution). The fluorescent intensity was measured by ImageJ
 20 (min-max, 0-256).

21

22 **Keratinocyte proliferation assay**

23 On day 0, 1.0×10^4 cells/well were cultured in 96-well plates and then treated with growth factor or
 24 cytokine (concentration of 100 ng/ml); recombinant human EREG (PeproTech, Cranbury, NJ, 100-

04), TNF α , IL-17A, and EGF (PeproTech, Cranbury, NJ, AF-100-15). For 72 hours of incubation, cell proliferation was measured by the Premix WST-1 Cell Proliferation Assay System according to the manufacturer's instruction (Takara Bio, Kusatsu, Japan, MK400). The indicated optical density (OD) was the value after the OD value of the control well was subtracted (KGM-gold and Premix WST-1).

Statistical analysis

Statistical calculations were performed using SigmaPlot (Version 14.5, Systat Software, Chicago, IL). To compare relative gene expression levels and cell proliferation, one-way ANOVA was used. A p-value of <0.05 was considered statistically significant.

Results

Co-stimulation with IL-17A and TNF α induced the EREG overexpression of mRNA and proteins in fibroblasts

We investigated the relative gene expressions of six growth factors under cytokine stimulation (final concentration: 100 ng/ml, N=4). EGF, FGF1, FGF7, or FGF10 did not change under any stimulation condition (Figure 1a). FGF2 was significantly upregulated by co-stimulation with IL-17A and TNF α (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation with IL-17A and TNF α (Figure 1a, EREG, mean 49.2-fold). In addition, TNF α alone, but not IL-17A alone, induced significant increases of EREG (mean 18.2-fold). This indicates that co-stimulation with IL-17A and TNF α has a synergistic effect and not just an additive effect. Based on this result, we focused on EREG expressed by fibroblasts under cytokine stimulation. We next investigated protein expression level. Immunofluorescence staining of EREG clearly showed strong fluorescence intensity under co-stimulation with IL-17A and TNF α (Figure 1b, TNF α +IL-17A). Stimulation with TNF α alone also demonstrated slight intensity compared to those with PBS or IL-17A alone.

1

2 **EREG enhanced keratinocyte proliferation**

3 Next, we investigated keratinocyte proliferation under cytokine or growth factor stimulation (N=8).

4 The 100 ng/ml concentration significantly enhanced cell proliferation with EREG stimulation, but not
5 with TNF α or IL-17A (Figure 1c).

6

7 **EREG was highly expressed in the dermis of lesional skins**

8 Finally, we studied EREG staining in psoriasis patients. Representative images are shown in Figure 2.

9 EREG was highly stained in the dermis of psoriasis lesions (Figure 2a, red square, Psoriasis 1 and 2),
10 but not in the dermis of healthy controls. The average fluorescent intensity of lesional dermis was

11 significantly higher than that of normal control (Figure 2b, dermis). The fluorescent intensity of
12 epidermis was not significantly different (Figure 2b, epidermis). Psoriatic lesional skin from three

13 patients (Patients 1, 2 and 3) out of five patients showed positive staining of EREG in the basal layer,
14 with one patient showing a particularly high expression of EREG in the lower epidermis (Psoriasis 2).

15 Interestingly, we found that several round cells in the papillary dermis and in the epidermis were highly
16 expressing EREG (white arrow). To confirm which inflammatory cells express EREG, we performed

17 immunofluorescent staining of CD3 and CD68. Most of infiltrating cells in the papillary dermis and
18 in the epidermis were CD3-positive T cells, but rarely CD68 macrophages (Figure 3c).

19

20

1 Discussion

2 Fibroblasts express receptors of IL-17A, IL-17C, and TNF α , but not of IL-23 (Human Protein Atlas).
3 Then, we focused on the function of the growth factors produced by fibroblasts stimulated with IL-
4 17A and TNF α . Interestingly, the combination of TNF α and IL-17A induced a synergistic effect on the
5 expression of EREG from fibroblasts. We found no significant effects on EREG expression level by
6 IL-23 stimulation (data not shown). Immunofluorescence staining of EREG demonstrated high-
7 intensity staining in the upper dermis of psoriatic skin. EREG from fibroblasts that are stimulated by
8 TNF α and IL-17A might induce the epidermal hyperproliferation seen in psoriasis. It has been known
9 that the proliferation of keratinocytes and epidermal tumor cells is enhanced by stimulation with
10 EREG(2,8,11,15). In this study, EREG demonstrated significantly high proliferation.
11 We found EREG expression to be high not only in the dermis but also in the epidermis, especially in
12 the basal layer (Figure 2). Shirakata et al. investigated *EREG* mRNA expression in the epidermis of
13 normal humans and psoriasis patients(2). They found faint expression in the basal layer of normal skin,
14 and found overexpression in the spinous layer, but not in the basal layer, of lesional psoriatic skin.
15 Immunofluorescence staining may not be sensitive enough, compared to *in-situ* hybridization.
16 However, protein expression is generally more directly associated with the phenotype than mRNA
17 expression is. In addition, we found several round EREG-positive cells in the papillary dermis and in
18 the epidermis by immunofluorescence staining. A previous report showed that peripheral blood cells,
19 particularly macrophages, express EREG(16). However, the present study revealed most of the
20 infiltrating cells to be CD3-positive T cells. This suggests that the EREG-positive round cells might
21 be T cells, rather than macrophages.
22 In conclusion, TNF α and IL-17A, which are essential cytokines in psoriasis pathogenesis, induce the
23 overexpression of EREG from fibroblasts and other cells (Figure 3). Although the systemic blocking
24 of growth factors can have serious side effects, topical application may be a potential therapeutic

1 option for psoriasis.

2

3

4

5

6 **Acknowledgments**

7 We wish to sincerely thank Ms. Mika Tanabe for her technical assistance. The work was supported by
8 research grants from Eli Lilly and AbbVie.

9

10 **Authorship contributions**

11 HI performed the experiments. HI and NH performed medical examinations on the patients. HI and
12 HU designed the study. HI wrote the manuscript, and all the coauthors had final approval of the
13 submission.

14

15 **Abbreviations**

16 epiregulin (EREG), epidermal growth factor receptor (EGFR), interleukin (IL), epidermal growth
17 factor (EGF), fibroblast growth factor (FGF), tumor necrosis factor alpha (TNF α), quantitative
18 polymerase chain reaction (qPCR)

19

1 **References**

- 2 **1** Wu L, Chen X, Zhao J *et al.* A novel IL-17 signaling pathway controlling keratinocyte
3 proliferation and tumorigenesis via the TRAF4-ERK5 axis. *J Exp Med* 2015; **212**: 1571–1587.
- 4 **2** Shirakata Y, Kishimoto J, Tokumaru S *et al.* Epiregulin, a member of the EGF family, is over-
5 expressed in psoriatic epidermis. *J Dermatol Sci* 2007; **45**: 69–72.
- 6 **3** Sano S, Chan K S, Carbajal S *et al.* Stat3 links activated keratinocytes and immunocytes required
7 for development of psoriasis in a novel transgenic mouse model. *Nat Med* 2005; **11**: 43–49.
- 8 **4** Miossec P, Kolls J K. Targeting IL-17 and T H 17 cells in chronic inflammation. *Nat Rev Drug*
9 *Discov* 2012; **11**: 763–776.
- 10 **5** Blauvelt A, Chiricozzi A. The Immunologic Role of IL-17 in Psoriasis and Psoriatic Arthritis
11 Pathogenesis. *Clin Rev Allergy Immunol* 2018; **55**: 379–390.
- 12 **6** Toyoda H, Komurasaki T, Uchida D *et al.* Distribution of mRNA for human epiregulin, a
13 differentially expressed member of the epidermal growth factor family. *Biochem J* 1997; **326**:
14 69–75.
- 15 **7** Toyoda H, Komurasaki T, Uchida D *et al.* Epiregulin. A novel epidermal growth factor with
16 mitogenic activity for rat primary hepatocytes. *J Biol Chem* 1995; **270**: 7495–7500.
- 17 **8** Jeong H S, Kwack M H, Joo H W *et al.* Platelet-derived growth factor-AA-inducible epiregulin
18 promotes elongation of human hair shafts by enhancing proliferation and differentiation of
19 follicular keratinocytes. *J Dermatol Sci* 2020; **97**: 168–170.
- 20 **9** Werner S, Krieg T, Smola H. Keratinocyte-fibroblast interactions in wound healing. *J Invest*
21 *Dermatol* 2007; **127**: 998–1008.
- 22 **10** Russo B, Brembilla N C, Chizzolini C. Interplay between keratinocytes and fibroblasts: A
23 systematic review providing a new angle for understanding skin fibrotic disorders. *Front Immunol*
24 2020; **11**

- 1 **11** Yoshikawa M, Kojima H, Yaguchi Y *et al.* Cholesteatoma Fibroblasts Promote Epithelial Cell
2 Proliferation through Overexpression of Epiregulin. PLoS One 2013: **8**
- 3 **12** Sasaki E, Arakawa T, Fujiwara Y *et al.* Epiregulin stimulates proliferation of rabbit gastric cells
4 in primary culture through autophosphorylation of the epidermal growth factor receptor. Eur J
5 Pharmacol 1997: **338**: 253–258.
- 6 **13** Wang Y, Jing Y, Ding L *et al.* Epiregulin reprograms cancer-associated fibroblasts and facilitates
7 oral squamous cell carcinoma invasion via JAK2-STAT3 pathway. J Exp Clin Cancer Res 2019:
8 **38**
- 9 **14** Roy S, Khanna S, Rink C *et al.* Characterization of the acute temporal changes in excisional
10 murine cutaneous wound inflammation by screening of the wound-edge transcriptome. Physiol
11 Genomics 2008: **34**: 162–184.
- 12 **15** Neufert C, Becker C, Türeci Ö *et al.* Tumor fibroblast-derived epiregulin promotes growth of
13 colitis-associated neoplasms through ERK. J Clin Invest 2013: **123**: 1428–1443.
- 14 **16** Riese D J, Cullum R L. Epiregulin: Roles in normal physiology and cancer. Semin Cell Dev Biol
15 2014: **28**: 49–56.
- 16
- 17

1 Figure legends

2

3 Figure 1 Overexpression of EREG from fibroblasts by co-stimulation with TNF α and IL-17A

4 Fibroblasts were stimulated with cytokines for 48 hours. a) qPCR analysis of growth factors under
5 cytokine stimulation. The Y axis is the gene expression level relative to that of GAPDH b)

6 Representative immunofluorescence staining of EREG. Scale bar: 50 μ m. c) Keratinocytes were
7 stimulated with growth factors or cytokines (100 ng/ml). Asterisk indicate a significant P-value (P <
8 0.05).

9

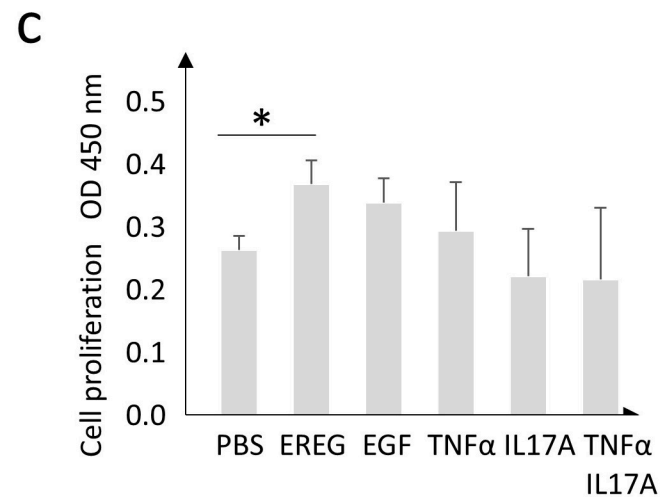
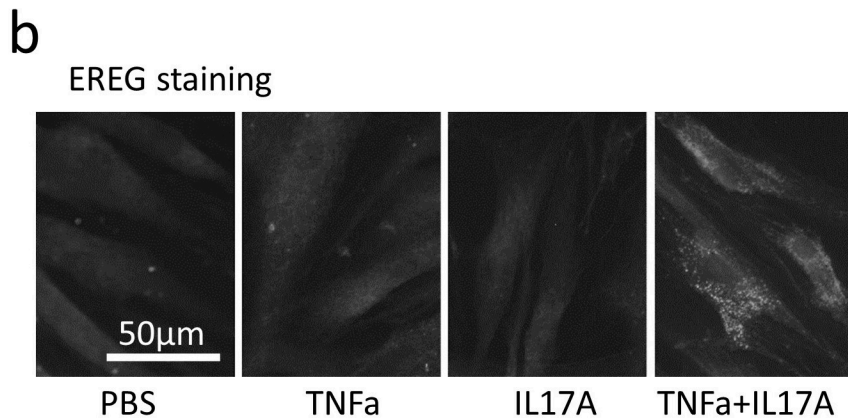
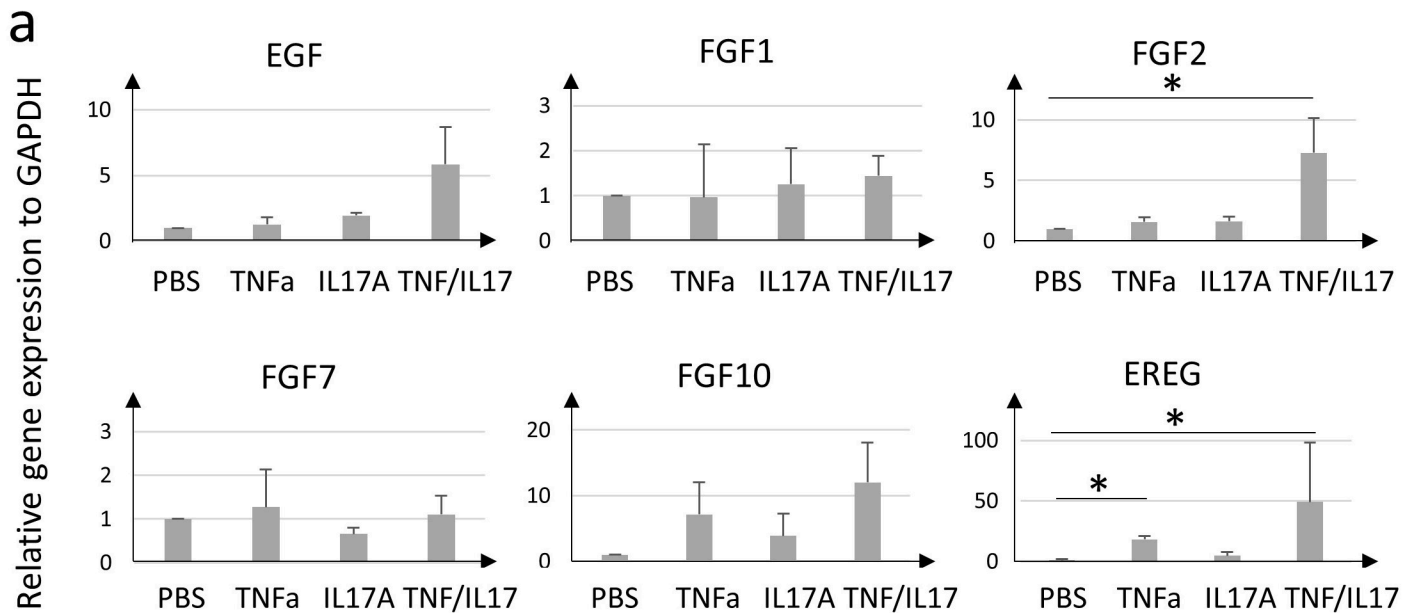
10 Figure 2 Lesional psoriatic skin overexpress EREG in the dermis

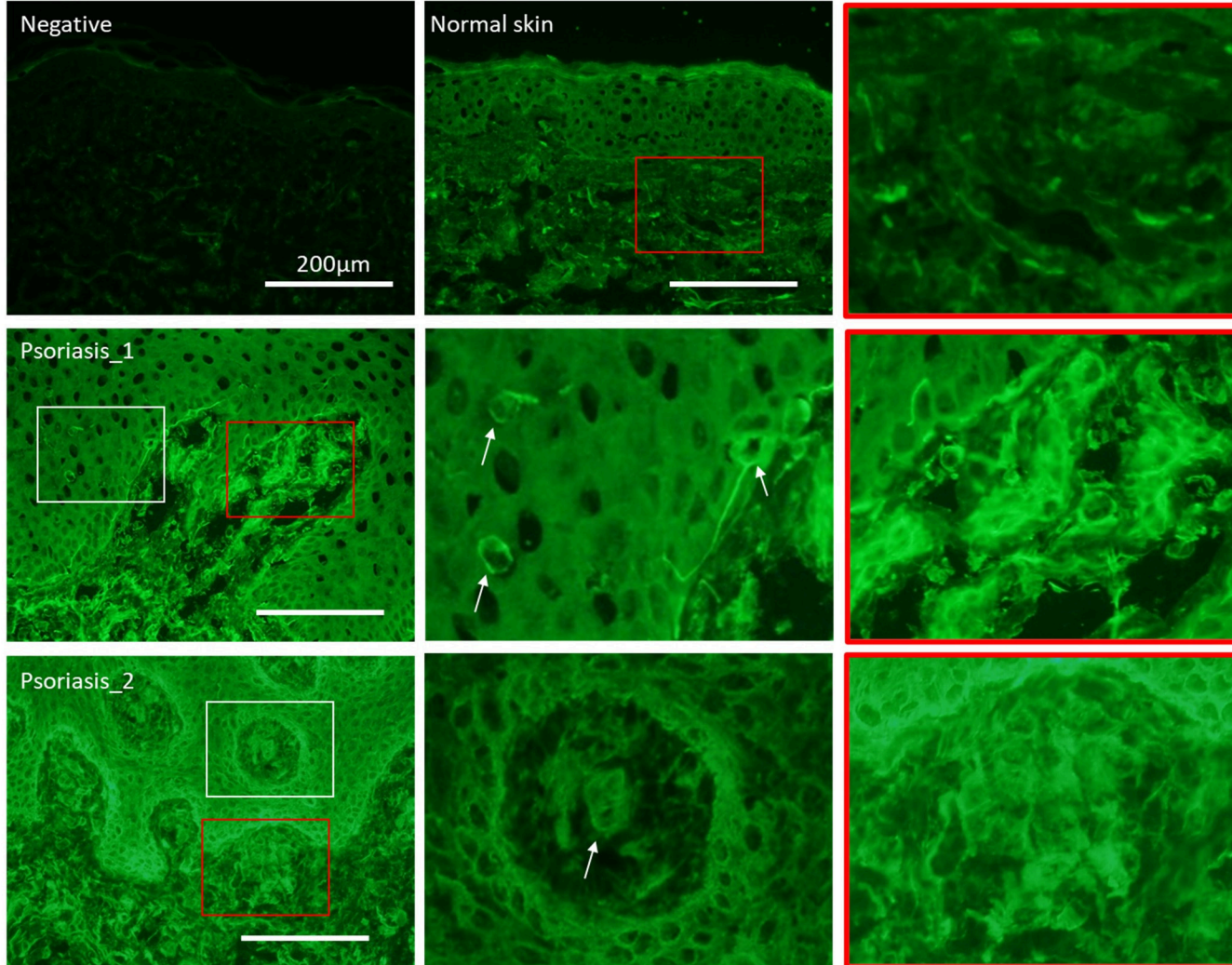
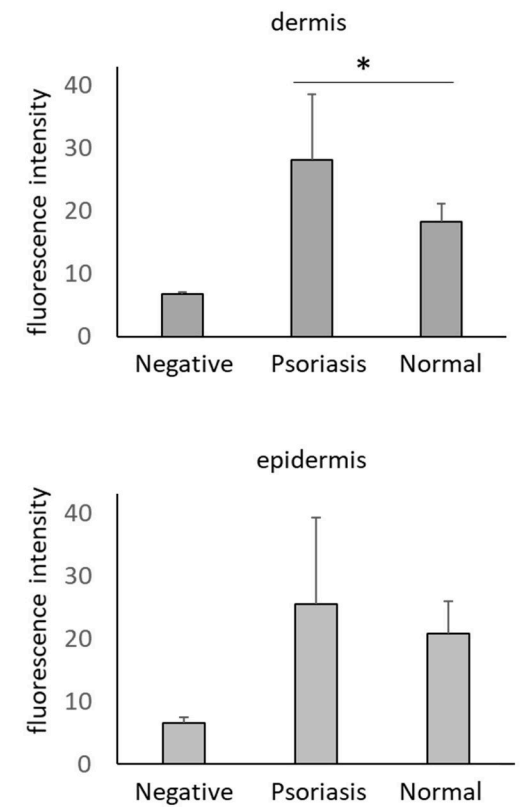
11 a) Immunofluorescence staining demonstrates high intensity in the dermis, especially in the papillary
12 dermis of psoriatic lesions (Psoriasis 1 and 2). High magnification images show that some round cells
13 are highly stained and are found in the papillary dermis and in the epidermis of psoriatic lesions
14 (squared area from psoriatic lesions, white arrow). Negative used PBS instead of primary antibody in
15 normal human skin. Scale bar: 200 μ m. b) The average of fluorescent intensity in the dermis and
16 epidermis. Asterisk indicate a significant P-value (P < 0.05). c) Immunofluorescence staining show
17 CD3-positive cells in the papillary dermis (left) and epidermis (right). Scale bar: 50 μ m.

18

19 Figure 3 Schematic of the hypothesis

20 Dermal dendritic cells produce TNF α and IL-23. The IL-23 induces IL-17A production from Th17
21 cells. Both the TNF α and the IL-17A stimulate fibroblasts and generate EREG. The EREG induces the
22 epidermal hyperplasia seen in psoriasis. TNF α and the IL-17A may also enhance epidermal
23 hyperplasia. Black arrows demonstrate known signals, and red arrows indicate the hypothesis based
24 on this study.



a**b****c**