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1	The possible role of epiregulin from dermal fibroblasts in the keratinocyte hyperproliferation of
2	psoriasis
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<b>5</b>	Running head: Fibroblast-derived epiregulin in psoriasis
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27	

### 1 Abstract

 $\mathbf{2}$ Psoriasis, an immune-mediated inflammatory disease, is characterized by keratinocyte hyperproliferation. Tumor necrosis factor alpha (TNFα), interleukin (IL)-23, and IL-17A play critical 3 roles in the pathogenesis of psoriasis. IL-17A secreted by Th17 acts more directly against keratinocytes 4  $\mathbf{5}$ than TNF $\alpha$  or IL-23 do. Regarding the receptors of cytokine, fibroblasts also express receptors against 6 IL-17A and TNFα, and induce the production of growth factors. Epiregulin (EREG), an epidermal 7growth 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 $\alpha$  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 $\alpha$  (mean 49.2-fold). Furthermore, the stimulation 12with TNFα alone, but not IL-17A alone, induced significant increases. Immunofluorescent staining 13demonstrated that the protein expression level of EREG was also increased in fibroblasts stimulated 14with these cytokines. Stimulation with EREG significantly enhanced keratinocyte proliferation in vitro. 15In human psoriatic patients' skin, immunofluorescence staining of EREG showed significantly high 16intensity in the dermis of lesional skin. In conclusion, cytokine stimulations with TNF $\alpha$  and IL-17A 17induce the overexpression of EREG from dermal fibroblasts in the lesional skin of psoriasis, and plays 18a role in epidermal hyperproliferation.

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#### 1 Introduction

 $\mathbf{2}$ Psoriasis is an immune-mediated inflammatory disease characterized by keratinocyte hyperproliferation. Among the pathomechanisms of psoriasis, it is well known that tumor necrosis 3 4 factor alpha (TNF $\alpha$ ), interleukin (IL)-23, and IL-17 play critical roles, and biologics block these  $\mathbf{5}$ 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  $TNF\alpha$  or IL-23 does, and to induce keratinocyte 7proliferation(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 12are composed of a heterodimer, such as IL-17RA/IL-17RC for IL-17A, IL-17F and IL-17A/IL-1317F(4,5). IL-17RA is expressed in almost all cell types, and fibroblasts produce inflammatory 14cytokines and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), 15and EREG via IL-17A signaling(4,5). Of these growth factors, EREG is produced by both fibroblasts 16and keratinocytes(6,7). EREG enhances the proliferation and differentiation of keratinocytes(7) and 17of follicular keratinocytes, leading to hair growth(8). In addition, keratinocytes secrete EREG and 18stimulate 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 20wound healing(9,10). Fibroblast-derived EREG is known to promote epidermal tumors(11,12), cancer 21invasion(13), and wound healing(14,15). 22Based on this evidence, we hypothesized that fibroblasts activated by IL-17A may play an important

role in epidermal hyperproliferation through the production of epidermal growth factors, especially
EREG.

3

#### **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

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

The recombinant human TNFα, IL-17A and IL-23 were purchased from PeproTech (Cranbury, NJ,
10602HNAE5), from Thermo Fisher Scientific (Waltham, MA, PHC9174) and from abcam
(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 RT<sup>2</sup> 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: AAAGCGTGGGGGGGGGGGGTGTAT	R: ATTTGGTGTCTGTGAGCCGT
2	FGF2	F: GCGACCCTCACATCAAGCTA	R: AGCCAGGTAACGGTTAGCAC
3	FGF7	F: GAAAGGCTCAAGTTGCACCAG	R: GCTATTTGACTTTTGTTTGTTGCT
4	FGF10	F: AGATGTCCGCTGGAGAAAGC	R: TCTCCAGGATGCTGTACGGG
5	EREG	F: CTCTGCCTGGGTTTCCATCTT	R: ACTGGACTCTCCTGGGATACAT
6	GAPDH	F: ACCATCTTCCAGGAGCGAGA	R: GACTCCACGACGTACTCAGC

 $\mathbf{7}$ 

# 8 Immunofluorescence staining

9 For the immunofluorescence staining, the cells were cultured on cover glass and incubated for 48 10hours 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 120.25% TritonX-100 for 10 minutes, the cells were incubated with goat anti-epiregulin (R&D Systems, 13Minneapolis, MN, final concentration: 10 µg/ml) at 4°C overnight. To stain the human cryo-skin 14samples, 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, 16MA, 1:100 dilution). After washing with PBS, cells or sections were stained with FITC-conjugated 17anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:100 dilution), FITC-18conjugated anti-rabbit IgG (SouthernBiotech, Birmingham, AL, 1:100 dilution) and FITC-conjugated 19anti-mouse IgG (SouthernBiotech, 1:100 dilution). The fluorescent intensity was measured by ImageJ 20(min-max, 0-256).

21

### 22 Keratinocyte proliferation assay

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

1	04), TNFα, IL-17A, and EGF (PeproTech, Cranbury, NJ, AF-100-15). For 72 hours of incubation, cell
2	proliferation was measured by the Premix WST-1 Cell Proliferation Assay System according to the
3	manufacturer's instruction (Takara Bio, Kusatsu, Japan, MK400). The indicated optical density (OD)
4	was the value after the OD value of the control well was subtracted (KGM-gold and Premix WST-1).
5	
6	Statistical analysis
7	Statistical calculations were performed using SigmaPlot (Version 14.5, Systat Software, Chicago, IL).
8	To compare relative gene expression levels and cell proliferation, one-way ANOVA was used. A p-
9	value of <0.05 was considered statistically significant.
10	
11	Results
12	Co-stimulation with IL-17A and TNFa induced the EREG overexpression of mRNA and
13	proteins in fibroblasts
$13\\14$	proteins in fibroblasts We investigated the relative gene expressions of six growth factors under cytokine stimulation (final
13 14 15	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
13 14 15 16	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 TNFα
13 14 15 16 17	proteins in fibroblastsWe 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 TNFα (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation
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<ol> <li>13</li> <li>14</li> <li>15</li> <li>16</li> <li>17</li> <li>18</li> <li>19</li> <li>20</li> </ol>	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 TNFα (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, EREG, mean 49.2-fold). In addition, TNFα alone, but not IL-17A alone, induced significant increases of EREG (mean 18.2-fold). This indicates that co-stimulation with IL-17A and TNFα has a synergistic effect and not just an additive effect. Based on this result, we
<ol> <li>13</li> <li>14</li> <li>15</li> <li>16</li> <li>17</li> <li>18</li> <li>19</li> <li>20</li> <li>21</li> </ol>	<b>proteins in fibroblasts</b> 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 (figure 1a). FGF2 was significantly upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, EREG, mean 49.2-fold). In addition, TNFα alone, but not IL-17A alone, induced significant increases of EREG (mean 18.2-fold). This indicates that co-stimulation with IL-17A and TNFα 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
<ol> <li>13</li> <li>14</li> <li>15</li> <li>16</li> <li>17</li> <li>18</li> <li>19</li> <li>20</li> <li>21</li> <li>22</li> </ol>	<b>proteins in fibroblasts</b> 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 TNFα (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, EREG, mean 49.2-fold). In addition, TNFα alone, but not IL-17A alone, induced significant increases of EREG (mean 18.2-fold). This indicates that co-stimulation with IL-17A and TNFα 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
<ol> <li>13</li> <li>14</li> <li>15</li> <li>16</li> <li>17</li> <li>18</li> <li>19</li> <li>20</li> <li>21</li> <li>22</li> <li>23</li> </ol>	<b>proteins in fibroblasts</b> 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 TNFα (Figure 1a, FGF2, mean 7.28-fold). Interestingly, EREG was extremely upregulated by co-stimulation with IL-17A and TNFα (Figure 1a, EREG, mean 49.2-fold). In addition, TNFα alone, but not IL-17A alone, induced significant increases of EREG (mean 18.2-fold). This indicates that co-stimulation with IL-17A and TNFα 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 TNFα (Figure 1b, TNFα+IL-17A). Stimulation with TNFα

1

## 2 EREG enhanced keratinocyte proliferation

Next, we investigated keratinocyte proliferation under cytokine or growth factor stimulation (N=8).
The 100 ng/ml concentration significantly enhanced cell proliferation with EREG stimulation, but not
with TNFα 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 12epidermis was not significantly different (Figure 2b, epidermis). Psoriatic lesional skin from three 13patients (Patients 1, 2 and 3) out of five patients showed positive staining of EREG in the basal layer, 14with one patient showing a particularly high expression of EREG in the lower epidermis (Psoriasis 2). 15Interestingly, we found that several round cells in the papillary dermis and in the epidermis were highly 16expressing EREG (white arrow). To confirm which inflammatory cells express EREG, we performed 17immunofluorescent staining of CD3 and CD68. Most of infiltrating cells in the papillary dermis and 18in the epidermis were CD3-positive T cells, but rarely CD68 macrophages (Figure 3c).

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#### 1 Discussion

 $\mathbf{2}$ Fibroblasts express receptors of IL-17A, IL-17C, and  $TNF\alpha$ , 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 $\alpha$ . Interestingly, the combination of TNF $\alpha$  and IL-17A induced a synergistic effect on the  $\mathbf{5}$ 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-7intensity staining in the upper dermis of psoriatic skin. EREG from fibroblasts that are stimulated by 8 TNF $\alpha$  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 12the basal layer (Figure 2). Shirakata et al. investigated EREG mRNA expression in the epidermis of 13normal humans and psoriasis patients(2). They found faint expression in the basal layer of normal skin, 14and found overexpression in the spinous layer, but not in the basal layer, of lesional psoriatic skin. 15Immunofluorescence staining may not be sensitive enough, compared to *in-situ* hybridization. 16However, protein expression is generally more directly associated with the phenotype than mRNA 17expression is. In addition, we found several round EREG-positive cells in the papillary dermis and in 18the 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 20infiltrating cells to be CD3-positive T cells. This suggests that the EREG-positive round cells might

21 be T cells, rather than macrophages.

In conclusion,  $TNF\alpha$  and IL-17A, which are essential cytokines in psoriasis pathogenesis, induce the overexpression of EREG from fibroblasts and other cells (Figure 3). Although the systemic blocking of growth factors can have serious side effects, topical application may be a potential therapeutic

1	option for psoriasis.
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5	
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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 $\alpha$ ), quantitative
18	polymerase chain reaction (qPCR)
19	

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16		
17		

# 1 Figure legends

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3	Figure 1 Overexpression of EREG from fibroblasts by co-stimulation with $TNF\alpha$ 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: 50µm. c) Keratinocytes were
7	stimulated with growth factors or cytokines (100 ng/ml). Asterisk indicate a significant P-value (P $\leq$
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: 200µm. 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: 50µm.
18	
19	Figure 3 Schematic of the hypothesis
20	Dermal dendritic cells produce TNF $\alpha$ and IL-23. The IL-23 induces IL-17A production from Th17
21	cells. Both the TNF $\alpha$ and the IL-17A stimulate fibroblasts and generate EREG. The EREG induces the

epidermal hyperplasia seen in psoriasis. TNFα and the IL-17A may also enhance epidermal
hyperplasia. Black arrows demonstrate known signals, and red arrows indicate the hypothesis based

24 on this study.











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