TWEAK/Fn14 Activation Contributes to the Pathogenesis of Bullous Pemphigoid

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TWEAK participates in various cellular effects by engaging its receptor of Fn14. Increased levels of soluble TWEAK are associated with systemic autoimmunity in patients with lupus erythematosus, rheumatoid arthritis, or dermatomyositis. However, the role of TWEAK in bullous pemphigoid (BP) remains unknown. In this study, we found an elevated serum level of TWEAK and a positive correlation between serum TWEAK and anti-BP180 antibodies. Immunohistochemistry showed strong TWEAK and Fn14 expression and implied an opposite relationship between the TWEAK and BP180 expression in skin samples from BP patients. In vitro TWEAK stimuli reduced BP180 expression in HaCaT cells and inhibited the adhesion of cells to the culture dish. Consistently, the transfection of Fn14 small interfering RNA preserved BP180 and protected cells from losing adherence. Moreover, such effect of TWEAK correlated with activation of the extracellular signal-regulated kinase and NF-kB pathways. TWEAK may serve as a biomarker or therapeutic target of BP.

INTRODUCTION
Bullous pemphigoid (BP) is a common autoimmune disease characterized by the appearance of subepidermal blisters in skin and the presence of circulating autoantibodies against structural components (including BP180, also called collagen XVII) of hemidesmosomes and inclusions against structural components (including BP180, Nishie, 2014; Schmidt and Zillikens, 2013). BP mainly affects people 70 years or older, and the morbidity and mortality of BP increase with age because of disease-specific factors (Barrick et al., 2015; Di Zenzo et al., 2012). In addition, the patients with BP are more prone to malignancies, pulmonary diseases, and neurologic disorders than their counterparts (Cai et al., 2015; Di Zenzo et al., 2012; Kokkonen et al., 2017). Therefore, to understand BP mechanistically is urgent for both monitoring and treating BP and associated diseases.

TWEAK, a member of the tumor necrosis factor (TNF) superfamily, engages with its sole signaling receptor of Fn14 and is involved in multiple biological processes, including immune responses and tissue repair (Chen et al., 2017; Cheng et al., 2015; Rayego-Mateos et al., 2013; Xia et al., 2015). The major pathogenic effect of TWEAK is believed to rely on the induction of proinflammatory cytokines and chemokines, such as RANTES, MCP-1, IP-10, IL-6, and IL-8 (Liu et al., 2011). The expression of TWEAK and Fn14 is up-regulated in a number of human skin diseases (Cheng et al., 2015, 2016; Doerner et al., 2015; Zimmermann et al., 2011). In recent years, an increasing amount of evidence has implicated the participation of TWEAK in the pathogenesis of a wide variety of autoimmune diseases that include systemic lupus erythematosus, rheumatoid arthritis, polymyositis, and dermatomyositis (Burkly et al., 2011; Peng et al., 2014; Xu et al., 2016). Moreover, TWEAK/Fn14 blockade, using genetically modified mice or neutralizing antibodies, ameliorated experimental autoimmune diseases, such as cutaneous or neuropsychiatric lupus erythematosus and nephrotoxic serum nephritis (Doerner et al., 2015; Wen et al., 2013; Xia et al., 2012, 2015). However, to our knowledge, there are no data on the role of TWEAK/Fn14 activation in the development of BP. Because a TWEAK/Fn14-targeting pharmaceutical is undergoing clinical trials (Wajant, 2013), a further understanding of TWEAK actions in BP may guide new therapeutic approaches for this disease.

Actually, TWEAK/Fn14 activation affects the expression of junction proteins in podocytes, glomerular endothelial cells, and tubular epithelial cells (Benzal et al., 2015; Xia et al., 2015). The alteration of junction proteins may further damage the linkage between the extracellular and intracellular structural elements involved in cell adhesion, or even cause cell death and loss of structural integrity (Xia et al., 2015). Considering the fact that BP correlates with the abnormalities of junction proteins and the inflammatory response of keratinocytes, we speculated about the...
potential effect of TWEAK/Fn14 signals on the pathogenesis of this disease. The purpose of this study was to elucidate the expression of TWEAK and Fn14 in patients with BP and the role of their interaction in the development of BP lesions.

RESULTS
Serum levels of TWEAK and anti-BP180 IgG correlate positively in patients with BP
We measured the levels of TWEAK in the sera and blister fluid of patients with BP and the sera of normal control subjects. The serum TWEAK levels in patients were significantly higher than those in normal control subjects (Figure 1a). Moreover, a positive correlation was found between the serum levels of TWEAK and anti-BP180 IgG (Figure 1b). Furthermore, the downstream proteins of RANTES, MCP-1, and IP-10 were determined, showing significant increase in sera from patients compared with normal control subjects (Figure 1c).

TWEAK expression is up-regulated in skin lesions of BP
We also assessed the TWEAK expression in blister fluid and found higher levels in BP patients than in normal sera, though not differing from sera in BP patients (Figure 1a). Consistently, histopathological analysis showed stronger staining of both TWEAK and Fn14 in lesional skin, whereas normal or nonlesional skin displayed slight staining (Figure 2a). In contrast, the expression of BP180 was strong in normal and nonlesional tissues but weak in lesional tissues (Figure 2a). The stained epidermis was quantitated for staining intensities and showed a negative linear correlation between TWEAK (or Fn14) and BP180 expression (Figure 2b and c). The perilesional tissues showed high expression of TWEAK and Fn14 and low expression of BP180, which was similar to that of lesional samples (see Supplementary Figure S1 online).

TWEAK stimulation reduces BP180 expression in HaCaT cells
The effect of TWEAK on BP180 expression was examined by culturing keratinocytes in vitro. The results showed that the mRNA level of BP180 increased but its protein expression decreased with TWEAK stimulation (Figure 3a–c). By immunofluorescence, HaCaT cells expressed less BP180 upon TWEAK stimulation (Figure 3d). In addition, there was an elevated mRNA expression of RANTES, MCP-1, IP-10, IL-6, and IL-8 in cells after TWEAK stimulation (see Supplementary Figure S2 online).

By vibration detaching assay, the adherence of HaCaT cells to culture dish decreased significantly under TWEAK stimulation (Figure 3e). The appearance of cultures showed more detached cells in TWEAK-treated dishes (see Supplementary Figure S3 online).

TWEAK exhibits an effect on BP180 via binding to the receptor Fn14
Some HaCaT cells were transfected with small interfering RNA (siRNA) before TWEAK stimulation. By Western blotting, the transfection of Fn14 but not control siRNA restored BP180 expression in supernatants and lysates of cell cultures (Figure 4a and b). Similarly, immunofluorescent detection showed increased BP180 expression in the Fn14 siRNA-transfected cells when compared with controls (Figure 4c and d). Furthermore, the mRNA expression levels of RANTES, MCP-1, IP-10, IL-6, and IL-8 decreased in Fn14 siRNA-transfected cells (Figure 4e).

NF-κB and extracellular signal-regulated kinase (ERK) activation is involved in TWEAK-induced loss of BP180 in HaCaT cells
Previously, we and other groups showed that both NF-κB and ERK signals are linked to the TWEAK-introduced inflammatory effect on cutaneous cells and skin tissues (Cheng et al., 2016; Doerner et al., 2015). To understand the intermediate signals in TWEAK regulation of BP180 expression, the NF-κB− and ERK-related molecules were determined in HaCaT cells. The results showed that TWEAK up-regulated phosphorylated (p-)IκBα and IκBα and enhanced phosphorylation of ERK in a time-dependent manner (Figure 5a and b). Consistently, in Fn14-silenced TWEAK-treated cells, both p-ERK and p-IκBα levels were significantly lower than in control cells (see Supplementary Figure S4 online).

Specific inhibitors of NF-κB (Bay 11-7082) and mitogen-activated protein kinase/ERK (U0126) pathways were applied to TWEAK-stimulated cell cultures. We observed that Bay 11-7082 decreased the mRNA expression of BP180 (Figure 5c). Consistently, Western blotting showed an increase in BP180 protein upon the addition of Bay 11-7082 or plus U0126 (Figure 5d). Meanwhile, the expression of p-IκBα and p-ERK decreased in Bay 11-7082− and U0126-treated cells, respectively (Figure 5d, and see Supplementary Figure S5 online). Finally, the downstream cytokines (RANTES, MCP-1, IP-10, IL-6, and IL-8) were reduced after the administration of the two inhibitors (Figure 5e).

ADAMs participate in TWEAK-induced BP180 loss in HaCaT cells
Because the shedding of BP180 from the cell surface is directly associated with ADAM proteins (Jackow et al., 2016b), we investigated the effect of TWEAK on ADAM9, ADAM10, ADAM17, and MMP-9 expression in keratinocytes. By immunohistochemistry, ADAM9, ADAM10, ADAM17, and MMP-9 were weakly expressed in normal and nonlesional skin but highly expressed in lesional skin (Figure 6a, and see Supplementary Figure S6 online). Moreover, their mRNA and protein levels increased significantly in TWEAK-stimulated HaCaT cells (Figure 6b–d, and see Supplementary Figure S6 online). However, the transfection of ADAM17 siRNA enhanced the protein expression of BP180 in these cells (Figure 6e and f), although it did not affect its mRNA expression (see Supplementary Figure S7 online). Consistently, the transfection of Fn14 siRNA down-regulated the protein expression of ADAM17 (Figure 6g and h). Moreover, both mRNA and protein levels of ADAM9, ADAM10, ADAM17, and MMP-9 were inhibited by NF-κB (Bay 11-7082) or ERK (U0126) inhibitor (see Supplementary Figure S7).

DISCUSSION
In this study, we showed that both TWEAK and Fn14 are highly expressed in skin lesions of BP. Serum levels of TWEAK and anti-BP180 IgG correlate positively in patients with BP. Moreover, TWEAK stimulation reduces BP180...
expression via binding to Fn14 in cultured keratinocytes. The effect of TWEAK on BP180 expression in keratinocytes involves the activation of NF-κB and ERK pathways. Furthermore, some sheddases such as ADAM17 participate in the TWEAK-induced BP180 loss in these cells. Therefore, TWEAK may contribute to the pathogenesis of BP by affecting BP180 expression and cellular adherence, involving the activation of ERK and NF-κB signals.

Previous studies reported that TWEAK plays a role in disrupting cell junctions (Berzal et al., 2015; Wen et al., 2015; Xia et al., 2015). In MRL/lpr lupus-prone mice, the Fn14 knockout reduced the brain expression of VCAM-1, ICAM-1 and fibronectin, leading to attenuation of the integrity of blood-brain barrier (Wen et al., 2015). TWEAK/Fn14 activation can reduce the expression of nephrin, podocin, and tight junction protein-1 in podocytes and glomerular endothelial cells, preserving the integrity of renal filtration barrier (Xia et al., 2015). TWEAK also induces junctional instability in tubular epithelial cells (Berzal et al., 2015). In this study, we confirmed that TWEAK induces the loss of BP180 in keratinocytes and further leads to weaker cell adhesion. The silence of Fn14 expression in keratinocytes abrogates TWEAK-induced

Figure 1. Levels of TWEAK increase in both sera and blister fluid from patients with bullous pemphigoid (BP). (a) Serum and blister fluid levels of TWEAK were measured by ELISA. (b) In patients with BP, the relationship between serum TWEAK levels and anti-BP180 IgG titers was assessed by Spearman’s rank correlation. (c) The levels of proinflammatory cytokines were determined in serum and blister fluid samples. Number of BP patients = 16, number of healthy donors = 20. *P < 0.05, **P < 0.01, ***P < 0.001.
BP180 loss and proinflammatory cytokine production. Furthermore, both TWEAK and Fn14 expression is upregulated in BP lesions, whereas BP180 expression is reduced significantly. Hence, TWEAK/Fn14 activation is pivotal in the loss of BP180 expression and cell adhesion. However, TWEAK/Fn14 activation is not bound to BP180 loss in skin. In some skin inflammation such as psoriasis and human papillomavirus infection, TWEAK induces the proliferation of keratinocytes without blister formation (Cheng et al., 2015, 2016). This indicates that TWEAK regulates cell fate depending on local inflammatory microenvironments and that the in vitro findings need further verification through in vivo experiments.

Serum levels of TWEAK are elevated in various autoimmune disorders, such as rheumatoid arthritis (Park et al., 2008), systemic sclerosis (Yanaba et al., 2009), and systemic lupus erythematosus (Chen et al., 2017). Similarly, we confirmed the increase of TWEAK level in sera from patients with BP. Moreover, the TWEAK level correlates positively with the serum titer of anti-BP180 IgG, which reflects the disease activity of BP (Lee et al., 2012). Therefore, soluble TWEAK may be a useful marker for monitoring the disease.
activity of BP. Macrophages/monocytes are the main source of TWEAK in inflammatory tissues (Bird et al., 2013). Moreover, the infiltration of macrophages/monocytes is prominent in the skin lesions of patients with BP (Furudate et al., 2014). Therefore, local macrophages/monocytes ought to be the source of TWEAK in BP lesions.

BP180 deficiency can reduce keratinocyte-basement membrane adhesion and the size of hemidesmosome plaques (Qiao et al., 2009). The ectodomain of BP180 can shed constitutively from the cell surface in an ADAM-independent manner (Hofmann et al., 2009). A series of ADAMs (ADAM8, 9, 10, 15, and 17) and MMPs (MMP-2, -9, and -13) have been suggested to participate in BP180 cleavage or BP development (Franzke et al., 2009; Laval et al., 2014; Niimi et al., 2006; Zebrowska et al., 2009). We found that ADAM9, ADAM10, ADAM17, and MMP-9 are highly expressed in BP lesions and increase in keratinocytes upon TWEAK/Fn14 activation. ADAM17 is the major sheddase in keratinocytes, whereas other sheddases also contribute to shedding of BP180 (Franzke et al., 2002, 2009). ADAM17 expression is increased in lesional BP skin (Zebrowska et al., 2009). Moreover, TWEAK/Fn14 interaction directly induces ADAM17 activation in cultured cells (Rayego-Mateos et al., 2013). In this study, we found that the transfection of Fn14 siRNA reduced ADAM17 expression and that ADAM17 siRNA treatment increased...
the BP180 protein level in TWEAK-stimulated keratinocytes. Hence, ADAM17 and other sheddases participate in the TWEAK modulation of BP180 loss in keratinocytes.

TWEAK increases the mRNA level but decreases the protein level of BP180 in keratinocytes. Also, ADAM17 inhibition enhances protein expression but does not affect mRNA expression of BP180. This discrepancy suggested that ADAM17 regulates BP180 expression through potential posttranscriptional pathways. ADAM17 not only exerts proteolytic activity but also regulates signaling pathways. ADAM17 and other sheddases may act on the coiled coils in the NC16A domain, leading to the hydrolysis and cleavage of BP180, which produces a 120-kDa polypeptide (Nishie et al., 2012). ADAM17 is dispensable for Notch1 activity (Groot et al., 2013; Murthy et al., 2012). Moreover, TWEAK via ADAM17 can release the membrane-bound ligands of EGFR, which further triggers downstream responses, including mitogen-activated protein kinase/ERK activation and proinflammatory gene overexpression (Rayego-Mateos et al., 2013). Actually, shedding of BP180 is strongly responsive to epidermal growth factor stimulation (Jacko´w et al., 2016a). Hence, we suppose that the downstream signaling pathways may participate in the ADAM17 regulation of BP180 loss. Our results also showed that the inhibitors of NF-κB and ERK signaling reduce ADAM17 expression in keratinocytes under TWEAK stimulation. Methylprednisolone exerts therapeutic effects through inhibiting mitogen-activated protein kinase/ERK phosphorylation (Hellberg et al., 2013). Therefore, corticosteroids may be used to interfere with the TWEAK-mediated BP development. This speculated mechanism underlying the TWEAK-ADAMs axis in BP180 loss is illustrated in Supplementary Figure S8 online and needs to be thoroughly investigated in future studies.

TWEAK/Fn14 activation induces the production of proinflammatory cytokines (e.g., RANTES, MCP-1, IP-10, IL-6, and IL-8) in various types of cells including keratinocytes, renal resident cells, and vascular endothelial cells (Cheng et al., 2015; Wen et al., 2015; Xia et al., 2015). In fact, the elevation of these cytokines is prominent in sera from patients with BP (Nakashima et al., 2007). In both human and
 experimental murine BP, cytokines have been implicated to be essential for blister formation in this disease (Hammers and Stanley, 2016; Plée et al., 2015; Riani et al., 2017). We found that these proinflammatory cytokines have high levels in both sera and blister fluid from patients with BP. Moreover, TWEAK stimulation enhances the expression of proinflammatory cytokines in keratinocytes. Our results not only provided more evidence that cytokines are important in the pathogenesis of BP but also strongly suggested that TWEAK/Fn14 activation contributes to blister formation in BP by enhancing the inflammatory responses in keratinocytes.

The interaction between TWEAK signals and anti-BP180 antibodies remains unclear. In the murine BP model of IgG passive transfer, anti-BP180 IgG can trigger the formation of blisters in skin by inducing BP180 degradation (Lin et al., 2012). Additionally, some studies supported that the BP180 autoantibodies are the major cause of cytokine release in BP (Nishie, 2014; Van den Bergh et al., 2012). The activation of complements and inflammatory pathways may be responsible for the pathogenicity of anti-BP180 IgG (Nishie, 2014). Theoretically, the pathogenic anti-BP180 antibodies may enhance Fn14 expression and trigger
TWEAK/Fn14 signals. However, this study showed that TWEAK activation directly induces both BP180 loss and cytokine production of keratinocytes in anti-BP180 IgG-free media. Therefore, TWEAK/Fn14 activation plays an important role in the pathogenesis of BP as an additional contributor. TWEAK signals and anti-BP180 antibodies may act together in inflammatory cascades to aggravate the dermal-epidermal separation.

In conclusion, the serum level of TWEAK is elevated in patients with BP, and TWEAK/Fn14 signals are activated in the skin lesions. TWEAK/Fn14 activation can reduce BP180 expression and cell adhesion of keratinocytes. The role of TWEAK in the development of BP involves the activation of NF-κB and ERK pathways and the up-regulation of ADAM17 and other sheddases in keratinocytes. TWEAK may serve as a biomarker or therapeutic target for patients with BP.
MATERIALS AND METHODS

Sample collection
Skin tissues (lesional, nonlesional, and perilesional) and blister fluid were collected from patients with BP (n = 23), who had not received medication or physical therapy in the past 4 weeks. Nonlesional tissues were collected from locations far from BP lesions. Normal skin tissues and sera were collected from healthy donors (n = 20). There were no statistical differences in sex or age between patients and normal control subjects (P > 0.05). Participants’ demographic characteristics are detailed in Supplementary Table S1 online. This study was performed under the supervision of the Hospital Research Ethics Committee, and written informed consent was obtained from all subjects.

Immunohistochemistry
As described previously (Zou et al., 2015), paraffin sections were deparaffinized and rehydrated, followed by addition of Dual Endogenous Enzyme Block (DAKO, Glostrup, Denmark). Rabbit anti-Fn14 (or anti-TWEAK, anti-ADAM17, or anti-BP180) IgG (2 μg/ml; Abcam, Cambridge, MA) was used as the primary antibody. Rabbit anti-ADAM9 (or anti-ADAM10 or anti-MMP-9) IgG (2 μg/ml; Cell Signaling, Danvers, MA) was also the primary antibody. Sections were incubated with polymer-horseradish peroxidase–labeled goat anti-rabbit IgG and 3,3’-diaminobenzine-chromogen substrate (i.e., DAKO) in order before counterstaining with hematoxylin and eosin solution.

The positively stained areas were quantified by ImageJ software (National Institutes of Health, Bethesda, MD) according to a previously described method (Choudhry et al., 2016). Briefly, six to eight epidermal viewing fields were examined within each skin sample. Positively stained areas were expressed as a percentage of the whole field area (% positivity/mm²). Quantitative analyses were performed in a blinded fashion by two pathologists.

Cell culture
Human keratinocytes (HaCaT cell line) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA). Before stimulation assays, keratinocytes were starved in non–fetal bovine serum-supplemented medium for 24 hours. Then, TWEAK (100 ng/ml; R&D Systems, Minneapolis, MN) was added. In some experiments, the solutions of Bay 11-7082 (20 μmol/L, Sigma-Aldrich, St Louis, MO) and U0126 (10 μmol/L, Cell Signaling) were added and pre-incubated for 2 hours before TWEAK stimulation.

Immunofluorescence
As reported previously (Zhang et al., 2012), cultured cells growing on a glass-bottomed culture dish (MatTek, Ashland, MA) were incubated with primary antibody targeting BP180 (2 μg/ml; Abcam) after cold acetone fixation. Then, Alexa-488 conjugated anti-rabbit IgG (2 μg/ml; Jackson ImmunoResearch, West Grove, PA) was applied. After adding 4’,6-diamidino-2-phenylindole solution, the dishes were covered with slides and observed under a digital confocal microscope (Leica, Wetzlar, Germany).

ELISA
Serum levels of TWEAK, RANTES, MCP-1, IP-10, IL-6, and IL-8 were measured using commercial immunoassay kits (R&D Systems). MESACUP BP180-ELISA kit (MBL, Nagoya, Japan) was used for the serum titers of anti-BP180 IgG. In this kit, the NC16a recombinant proteins captured BP180 IgG in sera or fluid, and the second reaction was developed using peroxidase-conjugated goat anti-human IgG antibodies.

siRNA transfection
The siRNA oligonucleotides of Fn14 and ADAM17 (or control siRNAs) were purchased from Life Technologies. siRNA transfection was performed according to the recommended procedures of Lipofectamine 2000 transfection reagent (Life Technologies). Briefly, subconfluent cells in six-well plates were incubated with 75 pmol siRNA (in 7.5 μl of transfection reagent) for 24 hours. Then, the cells were incubated in medium for 24 hours before the next experiments. The efficiency of transfection was determined by quantitative real-time reverse transcriptase–PCR and Western blotting.

Quantitative real-time reverse transcriptase–PCR
Total RNA was extracted from cell lysates by using the Trizol reagent (Life Technologies). A commercial cDNA kit (Takara Bio, Kyoto, Japan) was used for reverse transcription. Next, quantitative real-time reverse transcriptase–PCR was performed on the One-Step PCR System (Applied Biosystems, Waltham, MA), with SYBR Green Master Mixes (Takara Bio) used as fluorescent dye. The PCR primers were synthesized by AuGCT DNA-SYN Biotech (Beijing, China) (see Supplementary Table S2 online).

Western blotting
Cell protein extracts were separated on electrophoresis gels and then transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). Rabbit antibodies to BP180 (recognizing amino acid residues 1300–1400, with two bands at 120 kDa and 180 kDa) or ADAM17 were purchased from Abcam. Rabbit antibodies to p-ERK, ERK, ADAM9, ADAM10, and MMP-9 were purchased from Cell Signaling. These antibodies were diluted to 0.2 μg/ml at use. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1 μg/ml, Abcam) was used as the secondary antibody. Signal was developed using an ECL chemiluminescence kit (Millipore). The intensities of bands were quantitated using ImageJ software.

Vibration detachment assay
This assay was performed according to Iwata et al. (2009). Briefly, HaCaT cells (8 × 10⁵) were seeded in 3.5-cm–diameter dishes and cultured for 48 hours. Then, cells were stimulated with TWEAK (100 ng/ml) for 6 or 48 hours. The adhesion of cells to the bottom of plates was assayed by determining the number of adherent cells after vibration with a vortex at grade 4.5 for 30 minutes. Cells remaining on the plate bottom were treated with 0.25% trypsin. The released cells were then counted.

Statistical analysis
Data were expressed as means ± standard error. GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Analysis of variance was used for the comparison of more than two groups. In comparing two groups, a two-tailed Student t test was used for statistical differences. Correlations between two parameters were analyzed using Spearman’s correlation coefficient by rank test. Differences were considered statistically significant at P < 0.05.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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REFERENCES
Y Liu et al.
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