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Decreased expression of suprabasin induces aberrant differentiation and apoptosis of epidermal keratinocytes: Possible role for atopic dermatitis

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ABSTRACT

Background: Suprabasin (SBSN), a secreted protein, is expressed in various epithelial tissues. The role of SBSN in epidermal differentiation and atopic dermatitis (AD) pathology remains largely unknown.
Objective: To evaluate the effects of SBSN on epidermal keratinocytes and its role in AD.
Methods: We examined the SBSN expression levels in the *stratum corneum* and the epidermis by proteome analysis and immunohistochemistry, respectively. The serum SBSN concentration was measured by ELISA. These values were compared between AD and healthy control. Morphological changes in the epidermis were investigated in SBSN-knockdown three-dimensional human living skin equivalent (LSE) model with or without IL-4/IL-13.
Results: Epidermal SBSN expression was decreased in AD lesional skin compared to healthy skin, as assessed by the *stratum corneum* proteome analysis and immunohistochemistry. The SBSN serum levels were significantly lower in AD patients than in normal subjects ($P<0.05$). The SBSN-deficient LSE exhibited compact *stratum corneum*, immature *stratum granulosum*, and increased keratinocyte apoptosis. Th2 cytokines, IL-4 and IL-13, did not affect SBSN expression in LSE. There were no differentiation-associated makers that were affected by the SBSN knockdown. SBSN deficiency-induced apoptosis of keratinocytes was exaggerated by IL-4/IL-13, and accordingly, the addition of recombinant SBSN induced significant keratinocyte proliferation ($P<0.05$).
Conclusion: Our data demonstrated that SBSN regulates normal epidermal barrier. Th2 cytokines unaffect SBSN expression in keratinocytes, but promote SBSN deficiency-induced apoptosis. It is suggested that SBSN has an anti-apoptotic activity, and its deficiency is involved in the pathogenesis of AD.
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1. Introduction

Suprabasin (SBSN) is expressed in various epithelial tissues of humans and mice, such as the epidermis, tongue, esophagus, and forestomach [1]. Since SBSN is a substrate for transglutaminase 2 and 3 activities, it is possibly involved in the process of epidermal differentiation [1]. During embryonic mouse development, *SBSN* mRNA is detected in epidermal keratinocytes at day 15.5, coinciding with epidermal stratification [1]. In relation to its differentiation-associated role, SBSN was found to be significantly downregulated in primary cultured keratinocytes when they were exposed to diesel particulate extract or its vapor [2]. Meanwhile, SBSN is known to be a secreted protein, elaborated from spinous cells of the stratified epithelia [3–5], suggesting that it may serve as a biomarker or a bioactive substance.

On the other hand, several studies have shown that SBSN plays a role for tumorigenesis. SBSN is a novel oncoprotein in epithelial neoplasms [6,7] and non-epithelial tumors [8–10], and it may represent a biomarker of lung adenocarcinoma [11]. SBSN has modest effects on cell proliferation [12] and induces angiogenesis [6,13]. Overexpression of SBSN in human esophageal squamous cell carcinoma is associated with advanced clinical stage, and upregulation of SBSN activates the Wnt/β-catenin signaling pathway, leading to nuclear localization of β-catenin and upregulation of cyclin D1 and c-Myc [11].

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By proteome analysis of *stratum corneum* (SC) samples taken from the human skin, we identified and quantified about 440 proteins [14]. In a comparison between atopic dermatitis (AD) patients and healthy subjects, we tentatively found that the expression of SBSN was decreased in the SC of AD patients. The present study was conducted to explore the expression and role of SBSN in AD pathology. We investigated the histopathological expression of SBSN in the skin and its concentration in the blood. We also performed *in vitro* study to examine the morphological and gene expression changes resulting from SBSN downregulation by using a human living three-dimensional skin model.

2. Materials and methods

2.1. Measurement of SBSN amounts in SC

This study was approved by the Medical Ethical Committee of Hamamatsu University School of Medicine. The procedure for proteome analysis of SC by hybrid quadrupole-orbitrap mass spectrometer was reported previously [14]. Briefly, after obtaining informed consent, we collected SC by a stripping technique with a cellophane tape from the flexor surface of the forearm of the subjects. The tape was dipped in toluene. After removal of the insoluble tape backing, the precipitate of centrifuged samples was washed with toluene. SC proteins were extracted from the dried sample. Acetone-purified extracts were reconstructed, and the protein samples were denatured, digested and purified. The samples were solved by 0.1% formic acid solution and analyzed by Q Exactive™ hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific).

2.2. Immunohistochemistry (IHC) for SBSN in human skin specimens

Skin biopsy samples obtained from patients with AD and normal subjects were used for immunohistochemical staining. All the patients and normal subjects were seen at Department of Dermatology, Hamamatsu University School of Medicine. Experimental protocol for human skin sample collections was conducted in accordance with the Declaration of Helsinki Principals and was approved by the Ethical Committees. Written informed consent was obtained from the patients, and if not, the opt-outs for the protocol were also opened to the general public by online at Hamamatsu University School of Medicine. The specimens were stained with hematoxylin and eosin for standard histopathology. Deparaffinized samples were activated by using citrate to retrieve the antigens and preceded overnight incubation with SBSN polyclonal antibody (1:100; PA5-25531, Thermo Fisher Scientific) or Filaggrin monoclonal antibody (1:100; SC-66192, Santa Cruz Biotech) at 4 °C. Labeling was visualized using the avidin-biotin complex method according to manufacturer's instructions. Images were obtained using a NanoZoomer slide scanner and NDP viewer (Hamamatsu, Japan).

2.3. Serum concentration of SBSN

Blood samples were obtained from patients with AD and psoriasis vulgaris (PV), and normal subjects. Written informed consent was collected from the patients. AD was diagnosed and subdivided into extrinsic AD and intrinsic AD [15,16] based on total IgE values and specific IgE levels to *Dermatophagoides* (*D.*). Extrinsic AD was defined as IgE >400 kU/L or 200 < IgE ≤ 400 plus class 2 or more of IgE specific to *D. pteronyssinus* and *D. farinae*, and intrinsic AD was defined as serum IgE levels ≤200 kU/L or 200 < IgE≤400 plus class 0 or 1 of the specific IgE [16]. Enrolled in this study were 20 extrinsic AD (mean age ± SD, 35.7 ± 12.8 years; 12 males, 8 females; mean ± SD of serum IgE, 7127.9 ± 7767.4 kU/L), 5 intrinsic AD (40.8 ± 4.9 years; 3 males, 2 females; 95 ± 45.6 kU/L), 12 Psoriasis vulgaris (58.1 ± 13.7 years; 9 males, 3 females) and 9 normal healthy subjects (42.2 ± 12.3 years; 5 males, 4 females). Serum was separated from blood samples and collected and stored at −80 °C until use. The SBSN concentration was quantified using an ELISA kit for SBSN (Cloud-Clone, Katy, Tx) according to the manufacturer's protocol.

2.4. Human living skin equivalent (LSE) model

The methods for preparation of LSE and collagen gel were described previously [17]. Briefly, a collagen gel, which consist of porcine collagen type I solution, DMEM, 20% fetal calf serum (FCS), 1% Anti-Anti (ThermoFischer Scientific, Walham, MA) and 0.1 N NaOH, was added to each culture insert (membrane pore size 3 μm; BD Falcon, San Diego, CA) in a six-well culture plate (BD Falcon). Following polymerization of the gel in the inserts, a solution of fibroblast-containing collagen was applied to each insert. DMEM supplemented with 10% FCS, 1% PC/SM and ascorbic acid was added into the polymerized fibroblast-containing gel. The cultured medium was changed twice per week. The gel was collected and used for constructing LSE. The dermal component was prepared for five days, then 1×10^5 NHEKs in 50 μL of EpiLife (ThermoFischer Scientific) with supplement S7 (ThermoFischer Scientific) and Anti-Anti were seeded onto the concave surface of the contracted gel. The LSE was kept submerged for 2 days. When keratinocytes reached confluence, the LSE was lifted to the air-liquid interface and cornification medium (CM) was added. CM was changed every other day. Two weeks after airlift, The LSE was harvested and used for the experiments. The LSE samples were embedded in paraffin for IHC, and the fresh frozen tissue was stored at −80 °C for gene expression analysis. In some experiments, LSE was cultured with 10 ng/ml of IL-4/IL-13 for 2 days and processed to standard histopathology, IHC and qRT-PCR.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from samples using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. cDNA was reverse transcribed from total RNA using the TaqMan RT reagents (ThermoFisher Scientific). The mRNA expression was analyzed with SYBR®GreenERTM qPCR Reagent system (ThermoFischer Scientific) using the Thermal Cycler Dice Real Time System II (TaKaRa, Tokyo, Japan). The primers and probe were ordered from Applied Biosystems, the catalog numbers are Hs01078781_m1 for SBSN, Hs00846307_s1 for IVL, Hs00221623_m1 for CLDN, Hs00559804_m1 for CAPN1, and Hs99999905_m1 for G APDH. GADPH was used as the reference genes. The relative expression was calculated according to the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$).

2.6. Cell proliferation assay

Cell proliferation was determined by using an MTT cell proliferation assay (Cayman Chemical, Ann Arbor, MI). Normal human epidermal keratinocytes (NHEKs; Kurabo, Tokyo, Japan) were plated into 96-well plates at a density of 1.0×10^4 cells/100 μL. After 24 h of culture, when reached 50% confluence, 100 pg/ml rSBSN (RC219831; OriGene, Rockville, MD) was added to the culture and cells were incubated for 48 h. Then, they were incubated with 10 μL of MTT reagent (5 μg/mL) for 4 h. After further incubation in a crystal dissolving solution for 4 h, the absorbance at 570 nm was recorded using a microplate reader (iMark; Bio-Rad, Tokyo, Japan).

2.7. Transmission electron microscope (TEM)

Specimens were pre-fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH7.4) and post-fixed with 1% osmium tetroxide in phosphate buffer, then dehydrated through a graded ethanol series, and embedded in Epon-Araldite mixture. Ultrathin section (approximately 80 nm) were stained with uranyl acetate and lead citrate, and examined with a JEM-1400Plus transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

2.8. Small-hairpin (sh) RNA transfection by lentiviral particles

MISSON pLKO.1-puro vector-based lentiviral particles containing shRNA cassette under the U6 promoter and puromycin resistance gene were produced from Sigma-Aldrich (St Louis, MO). As negative control, MISSON TRC2-pLKO.5-puro vector-based lentiviral particles containing a non-target shRNA (shc) was also used (Sigma-Aldrich). The sequences of shRNA targeting SBSN (shSBSN) or non-targeting shRNA (shc) were as follows:

shSBSN; sense 5′-CCCATGAGATCAACCATGGTA-3′,
antisense 5′-TACCATGGTTGATCTCATGGGTT-3′
shc; sense 5′-CAACAAGATGAAGAGCACCAA-3′,
antisense 5′-TTGGTGCTCTTCATCTTGTTG-3′

NHEKs, 2×10^5 cells, were seeded in 1 mL of antibiotic free EpiLife with S7 and 20 μg/mL of polybrene. Lentiviral particles containing shc or shSBSN were added into the culture medium at a multiplicity of infection (MOI) of 1, and NHEKs were incubated by shaking every 15 min. After one hour, 2 mL of antibiotics free EpiLife with S7 were added and the culture was continued for 24 h. Transduced NHEKs were selected in 3 mL of antibiotic free EpiLife with 2.5 μg/mL of puromycin. After additional 48 h of culture, transduced NHEKs were used for generating the LSE.

2.9. Statistical analysis

All statistical analysis and graphs were carried out using PRISM, version 7 (GraphPad, La Jolla, CA). Mann-Whitney U test was used for testing independent data. $P < 0.05$ was considered as the level of significance.

3. Results

3.1. Low expression of SBSN in SC of AD patients

Our previous proteome analysis using hybrid quadrupole-orbitrap mass spectrometer allowed us to quantify about 440 proteins in the tape-stripped SC samples from the human skin [14]. When we analyzed the data on SBSN, we found that the expression of SBSN was significantly decreased in the SC of AD patients (n = 6) compared with that of normal subjects (n = 3) (Fig. 1A).

By IHC, SBSN was positively stained mainly in the *stratum granulosum* and SC of the epidermis in normal subjects as seen in a representative case (Fig. 1B, Normal, upper panel). Since SBSN is a secreted protein, its expression was observed in the intercellular spaces. In AD lesional skin, the staining pattern varied as shown in two representative cases (Fig. 1B, AD, upper panel). One typical pattern represented positive staining in the upper prickle to granular layers without staining in the compact cornified layer (Fig. 1B; AD, upper panel). However, there were cases exhibiting the pattern similar to the normal skin when the SC had a basket weave pattern. Although the total amount of SBSN produced in the epidermis was unclear, the majority of AD patients had a relatively weak SBSN staining especially in the SC. Along with SBSN staining, we also performed IHC for FLG, whose staining intensity in the *stratum granulosum* was lower in AD lesional skin than in normal skin (Fig. 1B, lower panel).

3.2. Low serum level of SBSN in AD patients

The above finding prompted us to conduct another approach to quantify SBSN in AD patients. We measured the serum levels of SBSN in patients with AD and PV, and normal subjects by ELISA. We found that the SBSN level was significantly lower in AD patients, but not in PV, than in normal subjects, further demonstrating the decreased SBSN expression in AD (Fig. 2A). AD can be divided into the extrinsic and intrinsic types [15]. While extrinsic AD shows high serum IgE levels with specific IgE to environmental allergens, intrinsic AD has low serum IgE levels without specific IgE [16]. In this classification, intrinsic AD patients had significantly lower serum SBSN levels than extrinsic AD patients (Fig. 2B).

3.3. Histopathological changes in SBSN-deficient LSE model

The aforementioned findings and a recently reported observation [18] raised the possibility that the reduction of SBSN expression alters epidermal barrier in AD. To address the role of SBSN in epidermal differentiation, we knocked down SBSN expression with shRNA in LSE model. A non-target shRNA (shc) was used as control. Compared with the shc-treated LSEs, the SBSN-knockdown LSE exhibited compact SC without basket weave pattern and immature *stratum granulosum* with poor keratohyalin granule formation (Fig. 3A, HE). Notably, some keratinocytes in the

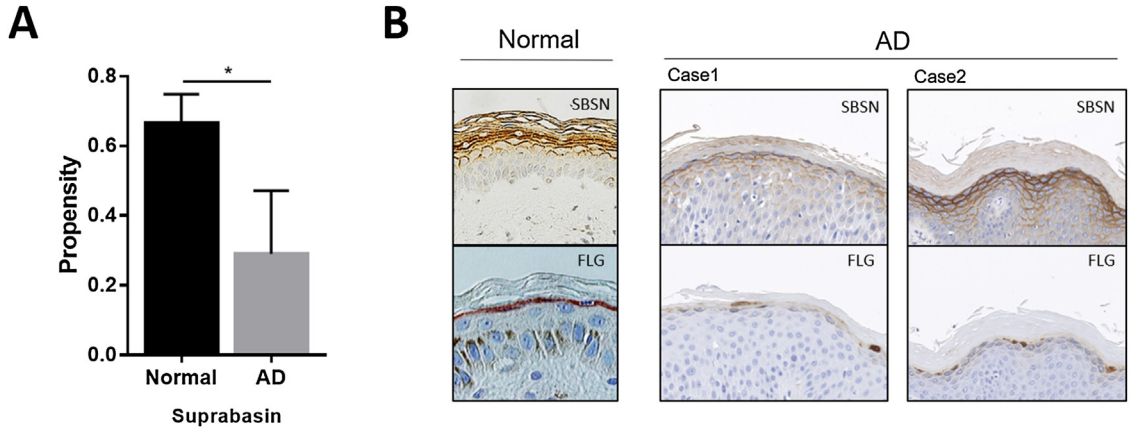

Fig. 1. Decreased levels of SBSN in *stratum corneum* of atopic dermatitis.
(A) Proteome analysis in *stratum corneum* of AD (n = 6) and healthy skin (n = 3), using hybrid quadrupole-orbitrap mass spectrometer. *$P < 0.05$. (B) Representative images of IHC of AD skin and healthy skin. SBSN, suprabasin; FLG, filaggrin.

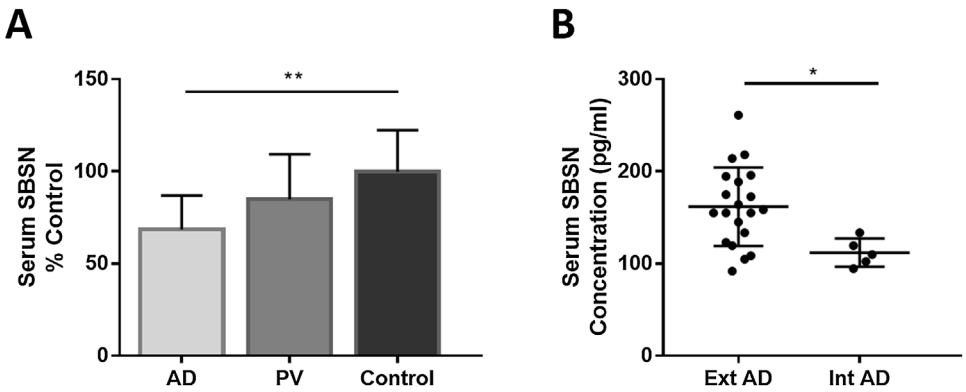

Fig. 2. Significantly lower SBSN serum levels in AD patients than in healthy individuals.
(A) SBSN serum level were measured by ELISA in AD patients (n = 13; SCORAD 39.5 ± 20.5), psoriasis vulgaris (PV) patients (n = 12), and healthy control (n = 9). $^{**}P < 0.01$.
(B) SBSN serum levels were measured by ELISA in intrinsic AD (Int AD; n = 5, SCORAD; 41 ± 17), extrinsic AD (Ext AD; n = 20, SCORAD; 51 ± 18). $^{*}P < 0.05$.

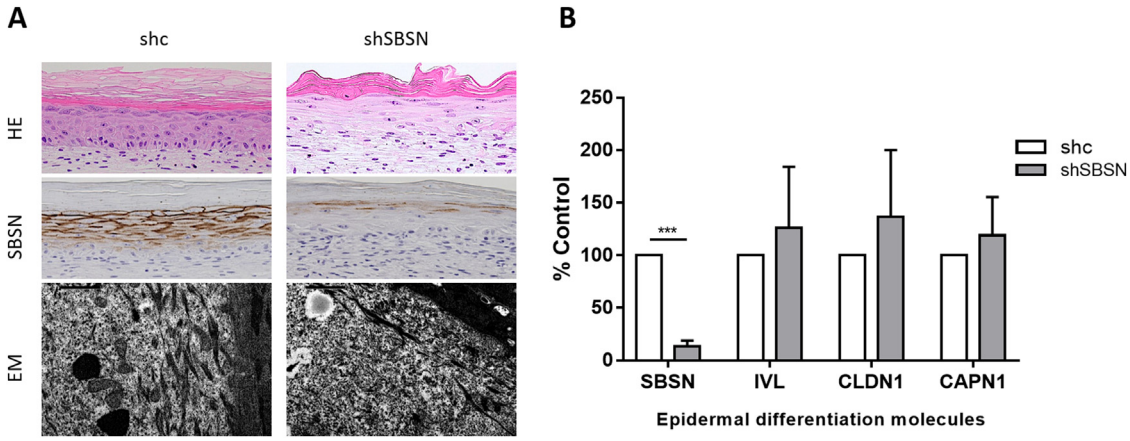

Fig. 3. Epidermal changes in SBSN-deficient LSE model.
LSE was treated with shSBSN or shc as control, and the histology, SBSN expression, and keratohyalin granule formation were examined. (A) Hematoxylin-eosin (HE) staining (upper panel), SBSN IHC (middle panel), and electron microscope (EM) (lower panel). (B) The expression of epidermal differentiation molecules in LSE treated with shSBSN (n = 3) or shc (n = 3) were analyzed by qRT-PCR. SBSN, suprabasin; ILV, involucrin; CLDN1, claudin-1; and CAPN1, calpain-1. $^{***}P < 0.001$.

SBSN-knockdown LSE showed aberrant cell shape and underwent apoptosis that obscured the dermoepidermal junction. IHC for SBSN disclosed marked reduction of SBSN expression in the shSBSN-treated epidermis compared to the shc (Fig. 3A, SBSN). The immature formation of keratohyalin granules in the shSBSN was confirmed by electron microscopy (Fig. 3A, EM).

The effects of shSBSN on the other differentiation-associated molecules were examined by qRT-PCR. While shSBSN significantly down-modulated the mRNA expression of SBSN, the mRNA expression for other molecules, including involucrin, claudin-1, and calpain-1, were unaffected. These findings suggest that the decreased SBSN expression leads to abnormal epidermal

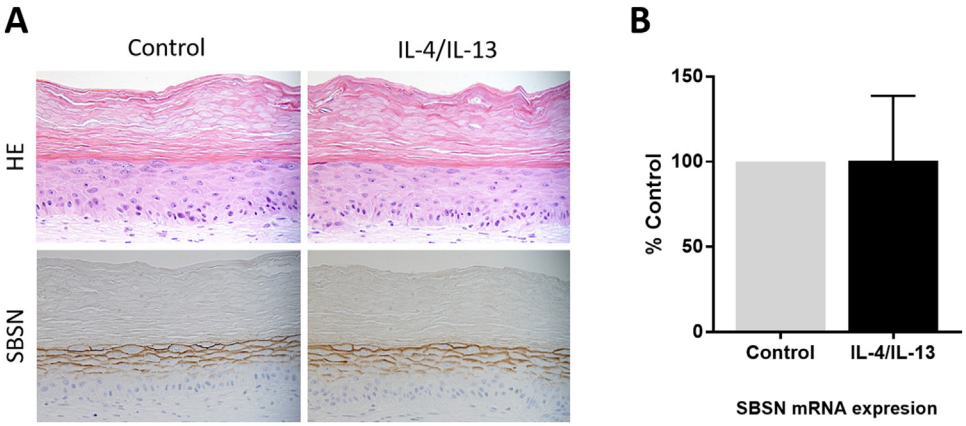

Fig. 4. No effect of IL-4/IL-13 on SBSN expression in LSE model.
LSE model was treated with 10 ng/mL of IL-4 and IL-13 (IL-4/IL-13) for 2 days or untreated (Control). (A) Hematoxylin-eosin staining (HE; upper panel) and SBSN IHC (lower panel). (B) The expression of *SBSN* mRNA in IL-4/IL-13-treated (n = 4) and untreated LSE (n = 4) by qRT-PCR.

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differentiation and keratinocyte apoptosis independently of the other differentiation markers.

3.4. No effect of Th2 cytokines on SBSN expression in LSE model

Th2 cells play a crucial role in AD [19], especially in extrinsic AD [15]. Th2 cytokines down-regulate the expression of differentiation-associated molecules, as represented by filaggrin [20]. The loss-of-function mutation of *filaggrin* gene is a typical cause of barrier impairment in AD, and its expression is further depressed by IL-4/IL-13 [20]. We therefore investigated whether IL-4/IL-13 modulates SBSN expression during keratinocyte differentiation. LSE was cultured in the presence of 10 ng/ml of IL-4/IL-13 for 2 days and processed to standard histopathology, IHC, and qRT-PCR. Morphologically, the IL-4/IL-13-treated-LSE was similar to the untreated LSE (Fig. 4A, HE). In addition, there was no difference in SBSN staining intensity (Fig. 4A, SBSN) or mRNA expression (Fig. 4B) between the IL-4/IL-13-treated and the untreated LSE models. These results indicate that Th2 cytokines do not affect the SBSN expression.

3.5. Promotion of keratinocyte apoptosis by IL-4/IL-13 in SBSN-knockdown LSE model

Although the IL-4/IL-13 did not alter the expression of SBSN in LSE model, we further investigated their effect on the SBS**N**-deficient keratinocytes. The SBSN knockdown LSE was cultured with 10 ng/ml of IL-4 and IL-13 for 2 days and subjected to the histopathology and qRT-PCR. Again, as compared with control (Fig. 5A, shc), SBSN knockdown induced apoptosis of keratinocytes (Fig. 5A, shSBSN). Further treatment of the LSE with simultaneously added IL-4/IL-13 exaggerated the apoptotic change of keratinocytes, as a large part of keratinocytes underwent apoptosis, giving rise to cleft formation between the epidermis and dermis (Fig. 5A, shSBSN + IL-4/IL-13).

It has been reported that SBSN induces modest proliferation of treated cells [12] and participates in the neoplastic proliferation of epithelial tumors [6,7]. We thus conducted cell proliferation assay using cultured keratinocytes and rSBSN. The addition of rSBSN to the culture induced moderate but significant proliferation of keratinocytes (Fig. 5B), suggesting that SBSN can prevents from apoptosis and results in cell proliferation.

4. Discussion

Our study showed that SBSN was expressed in the epidermis of human skin. We measured the SBSN amount by proteome analysis in SC samples and evaluated SBSN immunoreactivity by IHC in skin specimens. By using these methods, we found that epidermal SBSN expression was decreased in AD lesional skin compared to healthy skin. Because of the secreted property of SBSN, its presence was also detectable in the serum and its concentration was measurable by ELISA. Low concentration of SBSN was found in sera from patients with AD, especially intrinsic AD.

In another series of experiments, we conducted an *in vitro* study on SBSN expression using LSE model. In the SBS**N**-deficient epidermis induced by shSBSN, we found two types of morphological alterations. One is disordered epidermal differentiation, including compact SC and immature *stratum granulosum*, and the other one is keratinocyte apoptosis. Since differentiation and apoptosis are related to each other [21], these changes might stem from the same mechanism initiated by SBSN deficiency.

IL-4/IL-13 had no effect on the expression of SBSN in LSE model. This differs from the down-modulatory action of IL-4/IL-13 on filaggrin and other differentiation molecules [20]. In addition, there were no differentiation-associated makers that were affected by the SBSN knockdown. Therefore, SBSN may have independent kinetics from the other markers under the cytokine environment. While extrinsic AD is a common Th2-biased condition, intrinsic AD is a less frequent disorder where Th1 and Th17 activation occurs [15]. Provided that IL-4/IL-13 downmodulated SBSN expression, the serum SBSN concentration in extrinsic AD would be lower than that in intrinsic AD. In reality, however, intrinsic AD showed lower serum levels of SBSN. IL-4/IL-13 unaffected SBSN expression in our *in vitro* study, and accordingly, the serum level of SBSN was relatively kept in extrinsic AD. It is an issue whether SBSN deficiency might be causative for intrinsic AD. We are currently undergoing a study on the phenotypical and functional changes using SBS**N**-knockout mice.

Induction of apoptosis in shSBSN-treated LSE model and modest proliferation of keratinocytes by rSBSN suggest that SBSN

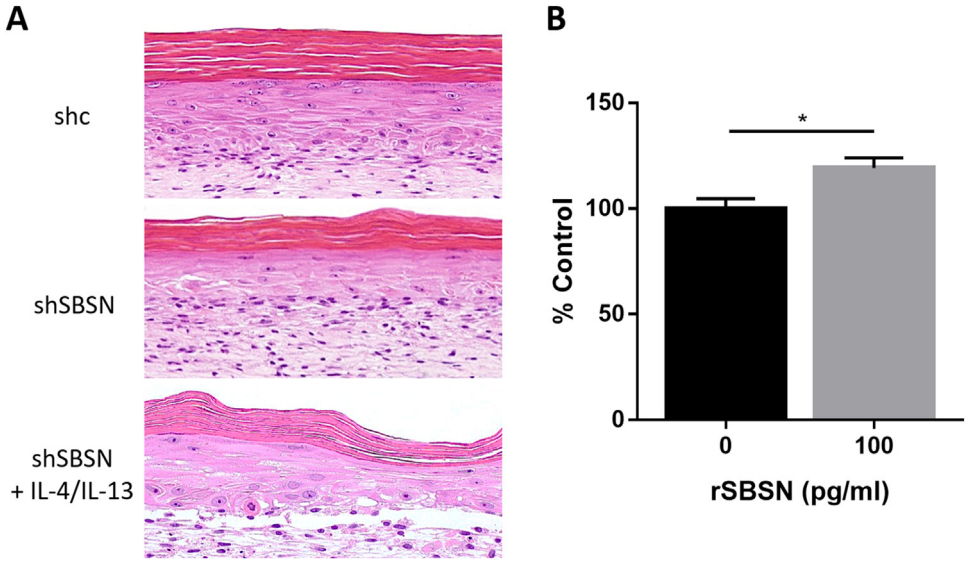

Fig. 5. Promotion of apoptosis by IL-4/IL-13 in SBSN-knockdown LSE model.
(A) Hematoxylin-eosin staining of shc-treated LSE (upper panel), shSBSN-treated LSE (middle panel), and hSBSN-and IL-4/IL-13-treated LSE (lower panel). (B) Keratinocyte proliferation of rSBSN-treated (n = 4) and untreated LSE (n = 4) were determined by MTT cell proliferation assay. $^{*}P < 0.05$.

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has an anti-apoptotic effect. Given that this secreted protein is less produced in AD, the lesional epidermis would easily undergo apoptosis upon stimulation. Our study demonstrated that IL-4/IL-13 exaggerated apoptotic damage in the shSBSN-treated epidermis. Once SBSN is downregulated, subsequent exposure to Th2 cytokines may deteriorate the condition. It is suggested that the deficiency of SBSN is related to allergy in the skin.

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None.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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