

Inhibition of Transcription Factor Specificity Protein 1 Alters the Gene Expression Profile of Keratinocytes Leading to Upregulation of Kallikrein-Related Peptidases and Thymic Stromal Lymphopoietin

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Transcription factor specificity protein 1 (Sp1) is involved in diverse cellular functions. We recently found that Sp1 was significantly decreased in skin biopsy samples obtained from patients with atopic dermatitis (AD) and had an even greater reduction in AD patients with a history of eczema herpeticum. In the current study, we sought to better understand the role of Sp1 in skin biological processes by using a small-interfering RNA (siRNA) technique to knock down Sp1 gene expression in normal human keratinocytes (NHKs) and investigated the genome-wide gene expression profiling of Sp1-silenced NHKs. The gene arrays revealed that 53 genes had greater than 3-fold changes in the expression in Sp1-silenced NHKs as compared with scrambled siRNA-silenced cells. Strikingly, six kallikrein (KLK)-related peptidase genes, namely *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK10*, and *KLK12*, were upregulated in NHKs following Sp1 silencing. Functionally, protease activity was significantly enhanced in Sp1-silenced keratinocytes as compared with scrambled siRNA-silenced keratinocytes. Moreover, thymic stromal lymphopoietin (TSLP), an epithelial-derived T_H2-promoting cytokine, was induced in Sp1-silenced keratinocytes because of elevated KLK activity. These results indicate that Sp1 expression deficiency leads to abnormally increased KLK protease activity in keratinocytes and may contribute to T_H2 immune responses in the skin by inducing TSLP.

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INTRODUCTION

Sp1 is a transcription factor that belongs to the SP/XKLF (specificity protein/Kruppel-like factor) family. Its DNA-binding domain possesses three C2H2-type zinc fingers that have higher binding affinities to GC boxes and lower binding affinities to CT and GT boxes (Wierstra, 2008). Aside from regulating gene expression by binding to its own binding sites, Sp1 appears as a versatile partner for many other transcription factors in activating or repressing its responsive genes. Sp1 was originally characterized as a transcription factor

for constitutive activation of housekeeping genes. More recently, it has become clear that Sp1 also possesses regulatory function and is actively involved in tissue-specific gene expression and responses to induced signals (Hu *et al.*, 2007; Wu *et al.*, 2009). Although Sp1 gene expression is ubiquitous, several studies have shown that its expression varies substantially in different cell types or in the same cell type at different stages of development (Saffer *et al.*, 1991).

The human kallikrein (KLK)-related peptidase family comprises 15 secreted serine proteases (KLK1–KLK15) that possess trypsin- or chymotrypsin-like enzyme activities (Yousef and Diamandis, 2001). To date, eight KLK proteins, namely KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, and KLK14, have been found in stratum corneum tissues and skin appendages (Komatsu *et al.*, 2005, 2006). The KLK function has been proposed as desquamatory enzymes by causing degradation of corneodesmosomal adhesion proteins in the stratum corneum and leading to shedding of the outmost layer of the skin (Borgono *et al.*, 2007; Eissa and Diamandis, 2008; Eissa *et al.*, 2011). KLKs' protease activity and their corresponding inhibitors are strictly regulated under physiological conditions (Cork *et al.*, 2009). Elevated KLK gene expressions have been found in skin diseases with skin

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Abbreviations: AD, atopic dermatitis; ADEH, atopic dermatitis with a history of eczema herpeticum; AEBF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; CRNN, cornulin; FLG, filaggrin; KLK, kallikrein; KRT, keratin; NHK, normal human keratinocyte; NS, Netherton's syndrome; siRNA, small-interfering RNA; Sp1, specificity protein 1; SPINK5, serine protease inhibitor Kazal-type 5; TSLP, thymic stromal lymphopoietin; VNN3, vanin 3

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barrier disorders, such as psoriasis (Komatsu *et al.*, 2007a), acne rosacea (Yamasaki *et al.*, 2007), and atopic dermatitis (AD) (Komatsu *et al.*, 2007b).

Human thymic stromal lymphopoietin (TSLP) is a cytokine mainly expressed by atopic epithelial cells such as keratinocytes (Reche *et al.*, 2001). Human dendritic cells, stimulated by TSLP, produce chemokines that attract T_H2-type CD4⁺ T cells; and TSLP-treated dendritic cells drive CD4⁺ T cells to differentiate into a T_H2 phenotype producing IL4, IL5, and IL13 (Reche *et al.*, 2001; Soumelis *et al.*, 2002). The discovery that TSLP promotes T_H2 immune responses inspired new insights into the important role of the epithelium in initiating and controlling immune responses. Indeed, TSLP expression has been significantly elevated in the epidermis from patients with AD and Netherton's syndrome (NS), a disease with severe AD-like skin manifestations (Soumelis *et al.*, 2002; Briot *et al.*, 2009). Interestingly, Briot *et al.* (2009) found that unstrained KLK5 in NS patients was responsible for elevated TSLP expression.

Recently, we found that inhibition of Sp1 gene expression enhanced vaccinia virus and herpes simplex virus replication in keratinocytes by attenuating the anti-viral innate immune response (Bin *et al.*, 2011). Furthermore, we found that Sp1 gene expression was significantly decreased in skin biopsy samples obtained from patients of AD. Interestingly, AD patients with a history of eczema herpeticum (ADEH) had significantly lower levels of Sp1 than did those without a history of viral infection. AD is a chronic inflammatory skin disease, which is characterized by elevated T_H2 infiltration and loss of skin barrier function (Cork *et al.*, 2009; Boguniewicz and Leung, 2010). ADEH subjects have more severe eczema and increased T_H2 responses than do regular AD (Beck *et al.*, 2009). The findings of Sp1 deficiency in AD and ADEH prompted us to further investigate the role of Sp1 in keratinocytes' biological processes. In this study, we reported that Sp1 silencing changed the expression profile of keratinocytes and led to the upregulation of six KLK family members. The elevated gene expression of KLKs resulted in significantly enhanced protease activity and augmentation of TSLP levels in Sp1-silenced cells. The current study indicates that epidermal Sp1 deficiency may contribute to T_H2 immune responses in the skin by enhancing the expression of TSLP in keratinocytes.

RESULTS

Inhibition of Sp1 alters gene expression profiling of keratinocytes

To gain better insights into the molecular perturbations induced by reduced Sp1 expression in keratinocytes, we used a gene microarray approach to identify genes that were affected by the downregulation of Sp1. Normal human keratinocytes (NHKs) were transfected with small-interfering RNA (siRNA) duplexes specifically targeting Sp1 or control-scrambled siRNA. Sp1 mRNA levels and proteins levels in NHKs were monitored up to 4 days after transfection of siRNA duplexes. As shown in Supplementary Figure S1 online, both Sp1 mRNA and protein were decreased in NHKs following transfection of Sp1 siRNA duplexes at day 1 and this difference persisted for 4 days. Transfection with control-

scrambled siRNA did not affect Sp1 expression. RNA extracted from NHKs after 72 hours of transfection with siRNA duplexes was used for gene profiling studies. Three independent experiments were performed and analyzed using Affymetrix GeneChip Human Genome U133 plus 2.0 arrays. Sp1 downregulation was associated with a substantial modification of gene transcription profiles. Using scrambled siRNA-transfected cells as the reference, we found 1,268 genes with unique gene symbol identifiers to be differentially expressed with >1.5-fold change. The expression of 576 genes was increased, whereas that of 692 genes was decreased. With a 3-fold change of expression, 53 genes with unique gene symbol identifiers were found differentially expressed with 27 upregulated and 26 downregulated genes. Table 1 shows the most upregulated and downregulated genes. Sp1 inhibition had an effect on the expression of many genes involved in multiple cellular functions, including metabolism, inflammation, proliferation, and apoptosis, etc.

Among the top four most upregulated genes associated with Sp1 silencing, *S100A12* is known to be a proinflammatory protein that possesses chemotactic activity for monocytes and mast cells (Yan *et al.*, 2008); KLK-related peptidase 6 (KLK6) is a serine protease involved in the process of desquamation (Borgono *et al.*, 2007); VNN3 (vanin 3) is a protein belonging to the pantetheinase enzyme family that catalyzes the conversion of vitamin B5 into the antioxidant cysteamine (Martin *et al.*, 2001, 2004). Expressions of these three genes have been previously reported to be increased in the skin of AD patients and other allergic diseases (Komatsu *et al.*, 2007b; Yang *et al.*, 2007; Jansen *et al.*, 2009). Using real-time PCR in NHK cells from three different donors and three different Sp1 siRNA duplexes, we confirmed that the gene expression of these three genes are indeed upregulated in Sp1-silenced NHK cells as compared with cells transfected with scrambled siRNA duplexes (Figure 1).

Keratin 13 (KRT13) and keratin 19 (KRT19) are the top two most downregulated genes in Sp1-silenced NHKs, and keratin 15 (KRT15) was also downregulated 3.8-fold following Sp1 silencing. Both KRT19 and KRT15 are biomarkers for skin stem cells (Pontiggia *et al.*, 2009), whereas mutations in KRT13 result in white sponge naevus (OMIM 193900) (Shibuya *et al.*, 2003). We confirmed that these three genes were downregulated in Sp1-silenced NHK cells by real-time PCR in NHKs from three different donors (Figure 1).

KLK family members are upregulated in Sp1-silenced keratinocytes

Several lines of evidence have implicated KLK family members in the pathogenesis of T_H2-mediated skin diseases including AD and NS, a rare genetic skin disease with AD-like skin manifestations (Bitoun *et al.*, 2002; Komatsu *et al.*, 2006, 2007b). Therefore, we searched further for KLK family members with expression changes following Sp1 silencing in NHK cells. We were struck by the finding that 10 probes representing 6 KLK family members were upregulated in Sp1-silenced NHK cells analyzed by gene profiling. As shown in Table 2, KLK6, KLK10, KLK12, KLK8, KLK5, and KLK7 were upregulated to different degrees from 1.45- to 5.27-fold.

Table 1. Genes affected by Sp1 silencing

Affymetrix ID	Symbol	Full name	Relative intensity (mean ± SEM)		Fold change	P-value
			Scrambled siRNA	Sp1 siRNA		
Metabolism						
212816_s_at	CBS	Cystathionine-β-synthase	76.97 ± 17.94	463.1 ± 169.8	6.02	NS
206643_at	HAL	Histidine ammonia-lysase	539.4 ± 20.38	2521 ± 140.2	4.67	**
206177_s_at	ARG1	Arginase, liver	646.5 ± 55.23	2667 ± 348.5	4.12	*
217127_at	CTH	Cystathionase (cystathionine γ-lyase)	286.7 ± 82.59	1056 ± 172.6	3.68	NS
211788_s_at	TREX	Three prime repair exonuclease 2	155.4 ± 11.99	564.2 ± 31.81	3.63	***
231202_at	ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	21.67 ± 4.486	85.3 ± 28.12	3.94	NS
203438_at	STC2	Stanniocalcin 2	214.9 ± 67.02	670.8 ± 124.8	3.12	*
205047_s_at	ASNS	Asparagine synthetase	873.8 ± 224.7	2999 ± 825.5	3.43	NS
238029_s_at	SLC16A14	Solute carrier family 16, member 14 (monocarboxylic acid transporter 14)	10.53 ± 1.364	34.97 ± 3.597	3.32	**
208126_s_at	CYP2C18	Cytochrome P450, family 2, subfamily C, polypeptide 18	71.33 ± 2.483	218.3 ± 15.59	3.06	***
224009_x_at	DHRS9	Dehydrogenase/reductase (SDR family) member 9	390.6 ± 16.85	1213 ± 197.8	3.10	*
208998_at	UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)	955.4 ± 2.776	196.9 ± 17.97	−4.85	***
223044_at	SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	232.0 ± 7.365	54.43 ± 9.879	−4.26	***
210519_s_at	NQO1	NAD(P)H dehydrogenase, quinine 1	1724 ± 32.77	501.5 ± 44.00	−3.43	**
206561_s_at	AKR1B10	Aldo-keto reductase family 1, member B10 (aldose reductase)	563.5 ± 35.33	171.3 ± 18.33	−3.29	***
201042_at	TGM2	Transglutaminase 2(C polypeptide, protein-glutamine-γ-glutamyltransferase)	296.4 ± 21.87	92.30 ± 13.51	−3.21	***
Cell growth and apoptosis						
215785_s_at	CYFIP2	Cytoplasmic FMR1-interacting protein 2	71.40 ± 3.544	275.6 ± 22.37	3.85	***
226492_at	SEMA6D	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	49.83 ± 5.912	13.20 ± 1.464	−3.77	**
202409_at	IGF2///INS-IGF2	Insulin-like growth factor 2 (somatomedin A)///INS-IGF2 readthrough transcript	2082 ± 37.40	597.7 ± 37.49	−3.48	***
211959_at	IGFBP5	Insulin-like growth factor-binding protein 5	98.93 ± 15.52	29.27 ± 4.53	−3.38	*
1552701_a_at	CARD16	Caspase recruitment domain family, member 16	320.9 ± 26.74	89.83 ± 8.07	−3.57	**
205081_at	CRIP1	Cysteine-rich protein 1 (intestinal)	164.0 ± 9.40	47.57 ± 0.38	−3.44	***
Transcription factor						
236265_at	SP4	Sp4 transcription factor	135.3 ± 9.734	517.3 ± 57.95	3.82	**
Inflammation						
205863_at	S100A12	S100 calcium-binding protein A12	101.8 ± 5.4	690.6 ± 9.04	6.78	***
220528_at	VNN3	Vanin 3	27.00 ± 0.51	152.3 ± 15.09	5.64	**
Receptor and transmembrane protein						
204007_at	FCGR3B	Fc fragment of IgG, low-affinity IIIb, receptor (CD16b)	36.37 ± 4.70	242.0 ± 42.53	6.65	**
221107_at	CHRNA9	Cholinergic receptor, nicotinic, α-9	44.60 ± 0.70	190.7 ± 33.45	4.27	
228176_at	SIPR3	Sphingosine-1-phosphate receptor 3	101.8 ± 9.18	24.30 ± 3.24	−4.19	**
Mucus secretion						
209173_at	AGR2	Anterior gradient homolog 2 (<i>Xenopus laevis</i>)	321.4 ± 11.59	106.7 ± 8.23	−3.01	***

Table 1 continued on the following page

Table 1. Continued

Affymetrix ID	Symbol	Full name	Relative intensity (mean ± SEM)		Fold change	P-value
			Scrambled siRNA	Sp1 siRNA		
Membrane protein						
219313_at	GRAMDIC	GRAM domain containing 1C	43.00 ± 0.15	146.1 ± 20.22	3.40	**
229927_at	LEMD1	LEM domain containing 1	129.2 ± 10.31	26.07 ± 1.24	−4.96	***
232176_at	SLITRK6	SLIT and NTRK-like family, member 6	134.8 ± 5.48	35.00 ± 8.96	−3.85	***
228080_at	LAYN	Layilin	184.9 ± 3.89	53.13 ± 4.30	−3.48	***
214297_at	CSPG4	Chondroitin sulfate proteoglycan 4	182.0 ± 6.53	55.67 ± 12.32	−3.27	***
220090_at	CRNN	Cornulin	98.43 ± 9.19	31.30 ± 2.89	−3.14	**
Epidermis development/intermediate filament						
207935_s_at	KRT13	Keratin 13	4027 ± 154.0	515.7 ± 108.7	−7.81	***
201650_at	KRT19	Keratin 19	2149 ± 163.7	309.4 ± 88.33	−6.95	***
204734_at	KRT15	Keratin 15	3911 ± 155.2	1067 ± 230.4	−3.66	***
Extracellular matrix						
206101_at	ECM2	Extracellular matrix protein 2, female organ and adipocyte specific	56.73 ± 12.23	253.9 ± 71.26	4.48	NS
208978_at	CRIP2	Cysteine-rich protein 2	1431 ± 52.16	455.8 ± 65.88	−3.14	***
211964_at	COL4A2	Collagen, type IV, α2	360.7 ± 18.67	111.2 ± 7.16	−3.24	***
Wnt signaling pathway						
202036_s_at	SFRP1	Secreted frizzled-related protein 1	178.8 ± 44.04	55.97 ± 6.56	−3.20	NS
Serine protease						
204733_at	KLK6	Kallikrein-related peptidase 6	80.87 ± 2.30	463.1 ± 39.02	5.72	***
Protease inhibitor						
202833_s_at	SERPINA1	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1	68.03 ± 1.62	18.37 ± 0.86	−3.70	***
Unknown function						
1558212_at	FLI35024	Hypothetical LOC401491	27.97 ± 2.97	118.4 ± 31.6	4.23	*
227410_at	FAM43A	Family with sequence similarity 43, member A	172.0 ± 10.24	650.7 ± 70.41	3.78	**
230765_at	KIAA1239	KIAA1239	32.63 ± 2.85	117.5 ± 16.24	3.60	NS
226905_at	FAM101B	Family with sequence similarity 101, member B	187.2 ± 17.27	638.0 ± 65.02	3.41	*
232689_at	LOC284561	Hypothetical protein LOC284561	31.10 ± 4.82	112.7 ± 31.41	3.62	***
1558195_at	LOC283404	Hypothetical protein LOC283404	121.2 ± 9.83	402.8 ± 65.64	3.32	**
226723_at	CCDC23	Coiled-coil domain containing 23	299.3 ± 9.36	908.3 ± 69.25	3.03	**
218723_s_at	C13orf15	Chromosome 13 open reading frame 15	187.6 ± 19.75	32.73 ± 5.55	−5.73	**
236984_at	C4orf26	Chromosome 4 open reading frame 26	32.20 ± 1.79	10.63 ± 1.22	−3.03	**

Abbreviations: ANOVA, analysis of variance; NHK, normal human keratinocyte; siRNA, small-interfering RNA; Sp1, specificity protein 1.

Genes identified by microarray analysis (Affymetrix GeneChip Human Genome U133 plus 2.0) have greater than 3-fold of upregulation or downregulation following Sp1 silencing in NHK (three independent experiments). Differential gene expression following Sp1 silencing relative to control was determined by GeneSpring GX 7.3 statistical analysis module ANOVA and *t*-test.

P* < 0.05; *P* < 0.01; ****P* < 0.001.

We investigated the dynamic gene expression of KLK family members up to 4 days following Sp1 silencing. As shown in Figure 2, KLK5, KLK6, KLK7, and KLK12 were increased in expression 2 days after transfection of Sp1

siRNA, whereas KLK8 and KLK10 were upregulated 1 day after transfection of Sp1 siRNA. However, the greatest increase of all six genes as compared with controls was at 3 days after transfection. We also harvested protein lysates

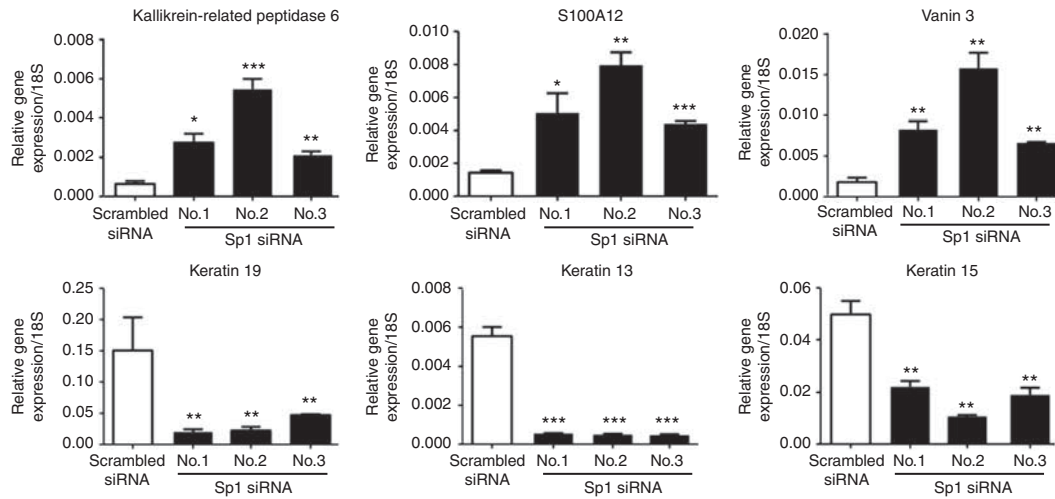


Figure 1. Confirmation of differentially expressed genes revealed by gene profiling. NHK cells were transfected with scrambled siRNA duplexes and three different Sp1 siRNA duplexes for 3 days. mRNA levels of KLK6, S100A12, VNN3, KRT19, KRT13, and KRT15 were measured by quantitative real-time PCR. Data are shown as mean $\pm$ SEM. Results are representative of three experiments. * P <0.05; ** P <0.01; *** P <0.001. KLK, kallikrein; KRT, keratin; NHK, normal human keratinocyte; siRNA, small-interfering RNA; Sp1, specificity protein 1; VNN3, vanin 3.

Table 2. KLK family members are upregulated in Sp1-silenced keratinocytes

Probe set	Gene name	Relative intensity (mean $\pm$ SEM)		Ratio (Sp1 siRNA/scrambled siRNA)	P-value
		Scrambled siRNA	Sp1 siRNA		
204733_at	KLK6	88.88 $\pm$ 4.02	425.95 $\pm$ 67.56	5.27	***
215808_at	KLK10	53.46 $\pm$ 6.04	187.17 $\pm$ 69.40	3.5	*
220782_x_at	KLK12	85.39 $\pm$ 7.17	233.29 $\pm$ 13.00	2.73	***
233687_s_at	KLK8//KLK9	47.97 $\pm$ 8.30	112.87 $\pm$ 6.54	2.35	***
234316_x_at	KLK12	96.42 $\pm$ 7.28	219.07 $\pm$ 32.08	2.27	**
209792_s_at	KLK10	3541.23 $\pm$ 320.10	6723.03 $\pm$ 415.80	1.9	***
1552319_a_at	KLK8	219.66 $\pm$ 5.91	363.16 $\pm$ 22.27	1.65	***
222242_s_at	KLK5	4633.18 $\pm$ 599.77	7657.66 $\pm$ 160.40	1.65	**
205778_at	KLK7	3077.18 $\pm$ 306.69	4459.33 $\pm$ 759.10	1.45	*
239381_at	KLK7	2275.83 $\pm$ 72.40	3542.96 $\pm$ 102.97	1.56	***

Abbreviations: KLK, kallikrein; siRNA, small-interfering RNA; Sp1, specificity protein 1.

Genes identified by microarray analysis (Affymetrix GeneChip Human Genome U133 plus 2.0).

* P <0.05; ** P <0.01; *** P <0.001.

from cells after 3 days of silencing Sp1 and confirmed that six kinds of KLK protein levels were increased in Sp1-silenced NHK cells (Supplementary Figure S2 online).

Protease activity is enhanced in Sp1-silenced keratinocytes but does not affect skin barrier protein FLG protein level

The KLK proteins are secreted proteins and possess trypsin- and chymotrypsin-like activities (Yousef and Diamandis, 2001). Therefore, we investigated whether upregulation of KLKs in Sp1-silenced NHKs led to enhanced protease activity. As shown in Figure 3a, the functional protease activity in cell culture supernatants from Sp1-silenced NHK cells was significantly increased as compared with scrambled siRNA-silenced NHK cells.

Previous studies have reported that human KLK5 and KLK7 degraded corneodesmosome components, desmoglein 1, desmocollin 1, and corneodesmosin, by *in vitro* proteolysis assay (Simon *et al.*, 2001; Caubet *et al.*, 2004). However, we did not detect degradation of these proteins in Sp1-silenced NHK cells (data not shown). We then investigated whether skin barrier protein filaggrin (FLG) protein level was decreased in Sp1-silenced NHK cells after 5 days of Ca^{2+} -driven differentiation. As shown in Figure 3b, both pro-FLG and monomer FLG were not decreased in Sp1-silenced NHK cells as compared with cells transfected with scrambled siRNA. On the contrary, FLG protein level was increased in Sp1-silenced NHKs as compared with cells transfected with scrambled siRNA. However, if Sp1-silenced NHKs were

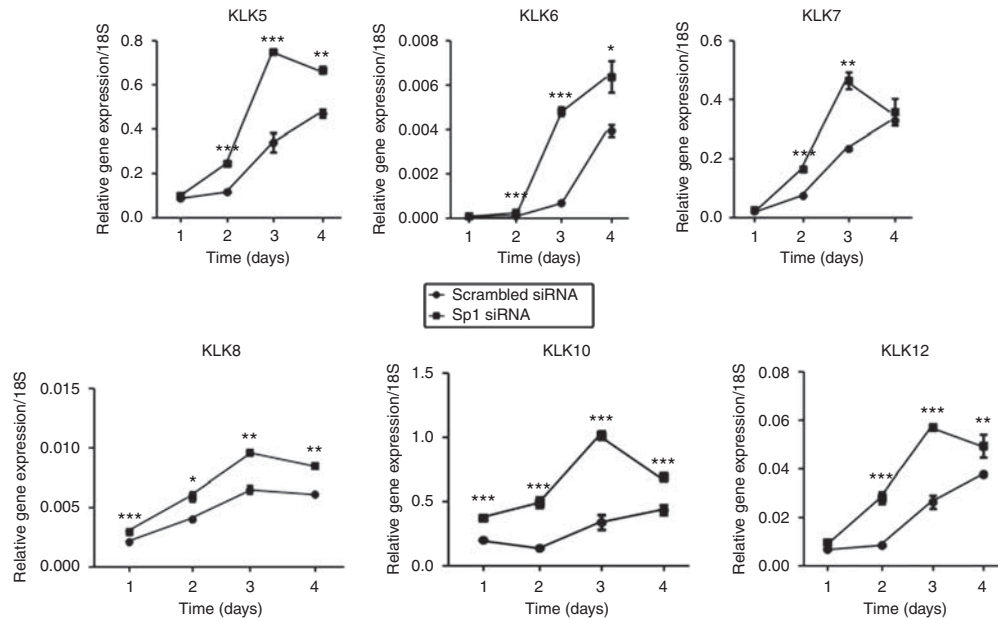


Figure 2. The dynamic changes of KLK gene expression following Sp1 silencing in NHK cells as compared with controls. mRNA levels of KLK5, KLK6, KLK7, KLK8, KLK10, and KLK12 were measured by quantitative real-time PCR from 1 day up to 4 days after transfection of Sp1 and scrambled siRNA duplexes. Data are presented as mean $\pm$ SEM. Results are representative of three experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. KLK, kallikrein; NHK, normal human keratinocyte; siRNA, small-interfering RNA; Sp1, specificity protein 1.

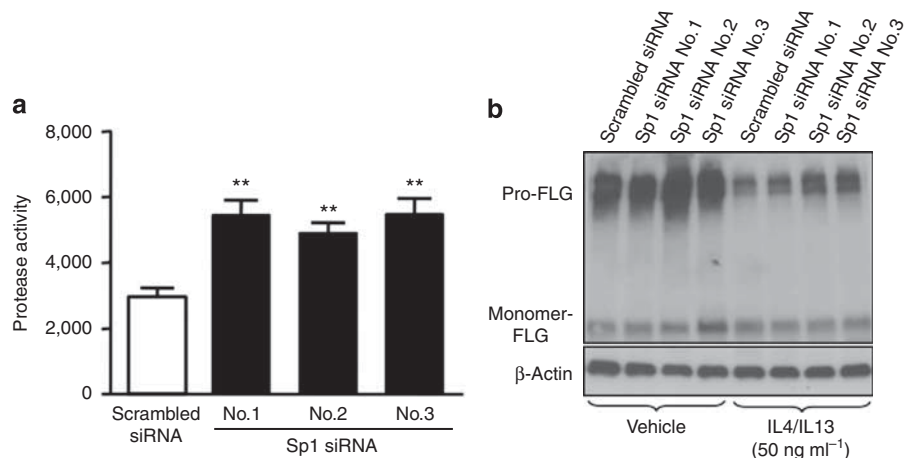


Figure 3. Sp1 silencing leads to enhanced protease activity but not degradation of FLG. (a) NHK cells were transfected with scrambled siRNA duplexes and three different Sp1 siRNAs for 3 days. Culture supernatants were incubated with fluorescence-conjugated casein substrate for 48 hours, and protease activity was determined based on the generation of fluorescent product from this substrate as described in the "Materials and Methods" section. Data are presented as mean $\pm$ SEM of triplicate experiments. ** $P < 0.01$. (b) FLG protein expression was detected by western blot. NHK cells were transfected with scrambled siRNA duplexes and three different Sp1 siRNAs and differentiated at 1.3 mM Ca^{2+} in the absence and presence of IL4/IL13 for 5 days. FLG, filaggrin; NHK, normal human keratinocyte; siRNA, small-interfering RNA; Sp1, specificity protein 1.

differentiated in the presence of $\text{T}_{\text{H}2}$ cytokines (IL4/IL13), FLG protein expression levels were suppressed.

TSLP gene expression is upregulated in Sp1-silenced NHKs depending on KLKs' protease activity

TSLP is believed to have a central role in atopic inflammatory diseases and it has been found to be induced by KLK5 (Soumelis *et al.*, 2002; Ebner *et al.*, 2007; Briot *et al.*, 2009). As multiple KLK proteins were upregulated and protease

activity was enhanced in Sp1-silenced keratinocytes, we were interested in whether TSLP was upregulated in Sp1-silenced keratinocytes. We first searched for TSLP expression in our gene array data, and found that its expression was increased in Sp1-silenced NHKs by 2.7-fold as compared with controls. We further confirmed TSLP gene expression at both the mRNA and the protein levels. As shown in Figure 4a, TSLP mRNA was significantly increased at days 3 and 4 following Sp1 silencing as compared with scrambled

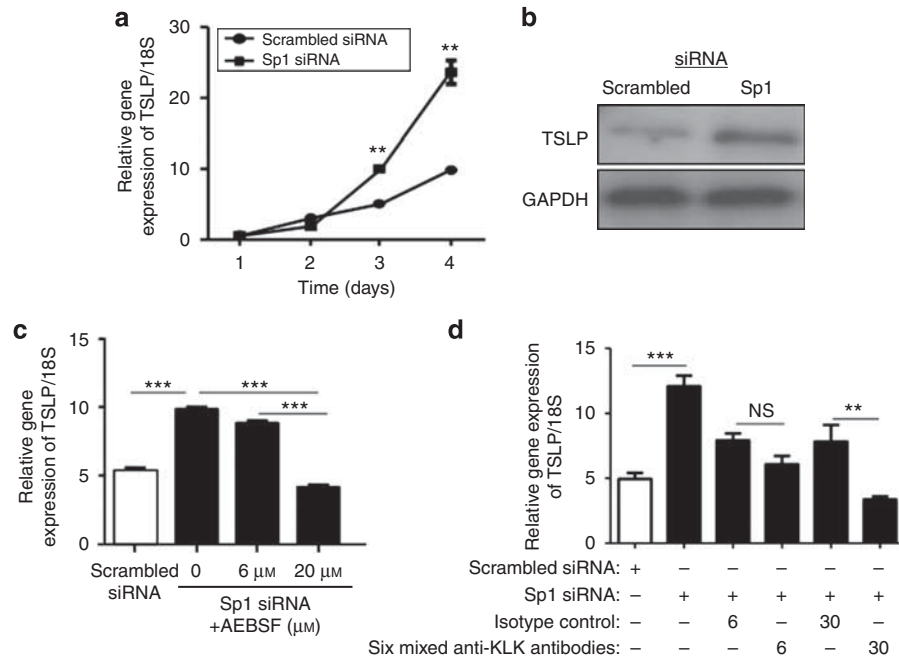


Figure 4. TSLP gene expression is upregulated in Sp1-silenced NHKs depending on enhanced protease activity. (a) TSLP mRNA is significantly increased at days 3 and 4 following Sp1 silencing as compared with NHKs transfected with scrambled siRNA. (b) TSLP protein was detected by western blot in NHKs 4 days after transfection of siRNA duplexes. (c) NHK cells were transfected with scrambled siRNA and Sp1 siRNA duplexes in the absence and presence of protease inhibitor AEBF for 3 days. mRNA levels of TSLP were measured by quantitative real-time PCR. (d) NHK cells were transfected with scrambled siRNA and Sp1 siRNA duplexes in the absence and presence of six anti-KLK-neutralizing antibodies and isotype control IgG for 3 days (see the "Materials and Methods" section for details). mRNA levels of TSLP were measured by quantitative real-time PCR. Data are presented as mean $\pm$ SEM of triplicate experiments. ** $P < 0.01$; *** $P < 0.001$. AEBF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; NHK, normal human keratinocyte; siRNA, small-interfering RNA; Sp1, specificity protein 1; TSLP, thymic stromal lymphopoietin.

siRNA-transfected keratinocytes. We harvested protein from cells after 4 days of transfection with Sp1 siRNA duplexes and found that the TSLP protein was increased in Sp1-silenced keratinocytes detected by western blot (Figure 4b).

As upregulation of TSLP followed KLKs' upregulation as indicated by our time course study, we determined whether TSLP upregulation in Sp1-silenced NHK cells was a response to increased KLK activity. To test this hypothesis, we used a serine protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBF), to inhibit protease activity in Sp1-silenced NHKs. As shown in Figure 4c, TSLP upregulation was significantly inhibited in Sp1-silenced NHK cells along with increased concentration of AEBF. To further define whether TSLP upregulation is a secondary response to increased KLK production, we used neutralizing antibodies to block the effects of the six upregulated KLKs in Sp1-silenced NHKs. As shown in Figure 4d, TSLP was not upregulated in Sp1-silenced NHK cells in the presence of KLK-neutralizing antibodies. Thus, TSLP upregulation in Sp1-silenced NHKs was dependent on augmented KLK protease activity.

DISCUSSION

The goal of the current study was to advance our understanding of the biological consequence of keratinocyte deficiency of Sp1 expression. In this paper, we demonstrated that six KLK family members, namely KLK5, KLK6, KLK7, KLK8, KLK10, and KLK12, were significantly increased in

Sp1-silenced keratinocytes. The functional significance of our gene expression data was supported by the observation of enhanced functional protease activity. TSLP gene expression was also significantly augmented in Sp1-silenced NHKs. As growing evidence has shown that enhanced protease activity in the epidermis is detrimental to skin barrier function and TSLP orchestrates allergic inflammation (Descargues *et al.*, 2005; Hachem *et al.*, 2006; Elias *et al.*, 2008; Ziegler, 2010), our findings suggest that Sp1 epidermal deficiency may have a role in allergic skin diseases, such as AD and ADEH.

Among the KLKs expressed in the skin, only KLK5 (a tryptic enzyme) and KLK7 (a chymotryptic enzyme) have been studied in great detail in terms of their enzymatic activities and substrates (Cork *et al.*, 2009). *In vitro* experiments have shown that KLK5 and KLK7 can degrade desmosomal adhesion proteins including desmoglein 1, corneodesmosin, and desmocollin 1 (Caubet *et al.*, 2004). Their enzymatic activities can be affected by pH and the presence of serine protease inhibitors (Deraison *et al.*, 2007). Of note, seven KLKs proteins, including KLK5, KLK6, KLK7, KLK8, KLK10, KLK13, and KLK14, have been found to be elevated in the stratum corneum of patients with AD (Komatsu *et al.*, 2007b). However, the mechanism by which KLKs are upregulated in AD is incompletely understood. Mutations in the serine protease inhibitor Kazal-type 5 (*SPINK5*) gene has been reported in patients with NS (Chavanas *et al.*, 2000; Bitoun *et al.*, 2002). *SPINK5* encodes the protein of the

lymphoepithelial Kazal-type 5 serine protease inhibitor, which is a specific inhibitor of KLK5, KLK7, and KLK14. Briot *et al.* (2009) have recently demonstrated that hyperactivity of KLK5 in lymphoepithelial Kazal-type 5 serine protease inhibitor-deficient keratinocytes of patients with NS has a key role in causing atopic skin lesions, thus highlighting the clinical significance of enhanced epidermal serine protease activity in the pathogenesis of NS. The reports of *SPINK5* mutations in NS have attracted several groups to investigate the association of *SPINK5* gene variants in AD (Walley *et al.*, 2001; Kato *et al.*, 2003; Nishio *et al.*, 2003; Kabesch *et al.*, 2004; Moffatt, 2004; Folster-Holst *et al.*, 2005; Jongepier *et al.*, 2005; Hubiche *et al.*, 2007). However, their results were contradictory, suggesting that *SPINK5* genetic variants only partially account for genetic predisposition to AD. We propose that epidermal deficiency of Sp1 in AD and ADEH may be an additional mechanism leading to elevated KLKs in patients with AD.

Our data also support the finding of Briot *et al.* that increased serine protease activity significantly augments TSLP gene expression in keratinocytes. This is an important confirmatory finding as TSLP is a critical cytokine that promotes T_H2 cell development in the pathogenesis of AD (Ziegler, 2010). TSLP-treated dendritic cells induce naive CD4⁺ T cells to undergo proliferation and differentiation into T_H2 lymphocytes, leading to enhanced production of IL4, IL5, and IL13 (Soumelis *et al.*, 2002). Transgenic mice overexpressing TSLP specifically in keratinocytes develop spontaneous AD disease (Yoo *et al.*, 2005). TSLP gene expression is elevated in the epidermis of patients with AD and NS (Soumelis *et al.*, 2002; Briot *et al.*, 2009).

Aside from KLKs and TSLP, several other genes that are differentially expressed in Sp1-silenced NHKs have also been implicated in the pathogenesis of AD. In this study, it was found that *S100A12*, a chemoattractant for monocytes and mast cells (Yan *et al.*, 2008), is the most upregulated gene following Sp1 silencing. Of interest, a recent study demonstrated that *S100A12* induces degranulation of mast cells and amplifies IgE-mediated responses, implicating its involvement in allergic inflammatory response (Yang *et al.*, 2007). *VNN3*, another gene that we found is also greatly upregulated following Sp1 silencing, has been reported to be significantly increased in AD skin lesions (Jansen *et al.*, 2009). However, the biological function of *VNN3* in the skin is still not clear. Our gene array data in Table 1 also showed downregulation of *CRNN* (Cornulin) in Sp1-silenced HNK. As the *CRNN* gene is located in the epidermal differentiation complex gene cluster and its protein is found mainly in the granular layer of the epidermis (Contzler *et al.*, 2005), it is considered to be a marker of late epidermal differentiation. *CRNN* is significantly downregulated in the skin of patients with AD (Lieden *et al.*, 2009).

Taken together, reduced Sp1 leads to a unique pattern of altered gene expression, which mimics some of the dysregulatory features of the AD skin. Importantly, we have demonstrated that Sp1 deficiency leads to enhanced protease activity and overproduction of the major pro-T_H2 cytokine, TSLP, in keratinocytes; to our knowledge this is previously

unreported. Although we did not detect the degradation of desmoglein 1, desmocollin 1, corneodesmosin, and FLG in Sp1-silenced NHKs, we showed that T_H2 cytokines treatment can suppress FLG protein expression in Sp1-silenced NHKs. These results suggest that Sp1 deficiency in the skin of patients with AD and ADEH may contribute to the pathogenesis of these complex skin diseases by inducing TSLP, which in turn promotes the elevation of T_H2 cytokines.

MATERIALS AND METHODS

Keratinocyte culture

The NHKs were purchased from Cascade Biologics/Invitrogen (Portland, OR) and maintained in serum-free EpiLife medium containing 0.06 mM CaCl₂ and S7 supplemental reagent (Cascade Biologics/Invitrogen) under standard tissue culture conditions.

siRNAs silencing experiment and serine protease inhibitor treatment

Sp1 and silencer negative control 1 siRNA duplexes were purchased from Ambion (Austin, TX). Sequences for targeting Sp1 were as follows: Sp1 no. 1 siRNA: 5'-GCAACAUGGGAAUUAUGAA-3'; Sp1 no. 2 siRNA: 5'-GGCAGACCUUUACAACUCA-3'; Sp1 no. 3 siRNA: 5'-CCACAAGCCCAACAAUCA-3'. NHK cells were plated in 24-well plates at 1 × 10⁵ per well the day before transfection. Cells were transfected with siRNA duplexes at a final concentration of 10 nM using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Sp1 no. 1 siRNA was used if there was no indication. Serine protease inhibitor, AEBSF, was purchased from Sigma-Aldrich (St Louis, MO). AEBSF (6–20 μM) was added to NHK cells after overnight incubation with Sp1 siRNA duplexes, and then cells were incubated with AEBSF for 2 days. Mouse IgG_{2A} isotype control, mouse IgG_{2B} isotype control, monoclonal anti-human KLK5, KLK6, KLK7, KLK8, KLK10, and KLK12 were purchased from R&D Systems (Minneapolis, MN). In all, 1 μg ml⁻¹ of each KLK antibody or 5 μg ml⁻¹ of each KLK antibody was added to Sp1-silenced NHK cells for 3 days, 6 or 30 μg ml⁻¹ mixed mouse IgG_{2A} and IgG_{2B} as controls.

RNA isolation and real-time PCR

Total RNA was isolated from cells using RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's guidelines. RNA was reverse transcribed into cDNA using superScript III reverse transcriptase from Invitrogen and analyzed by real-time reverse transcriptase-PCR using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) as described previously (Nomura *et al.*, 2003). Primers and probes for human *Sp1*, *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK10*, *KLK12*, *KRT13*, *KRT15*, *KRT19*, *VNN3*, *S100A12*, *TSLP*, and *18S* were purchased from Applied Biosystems. Quantities of all target genes in test samples were normalized to the corresponding 18S levels.

Gene microarray analysis

The microarrays used for this study were Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA) containing probe sets of 54,000 transcripts. Total RNA (5 μg) isolated from Sp1-silenced NHK cells and scrambled siRNA-transfected NHK cells was converted to double-stranded cDNA and then to biotinylated cRNA. After fragmentation and quality confirmation, 15 μg of biotinylated cRNA

was hybridized to microarrays. After washing and staining with streptavidin-phycoerythrin, the arrays were scanned using a GeneArray scanner (Hewlett Packard, Palo Alto, CA). Data were analyzed using Affymetrix Microarray Suite 5.0 software and GeneSpring 10.5 software (Agilent Technologies, Santa Clara, CA).

Western blot

Whole-cell extracts were prepared in the presence of 1% (vol/vol) of protease inhibitor cocktail and 1% (vol/vol) of phosphatase inhibitor cocktail (Sigma-Aldrich). Protein was then separated using SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The blots were then blocked and incubated with primary and secondary antibodies. Rabbit anti-human Sp1, rabbit anti-human TSLP, and rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-FLG monoclonal antibody was purchased from Vector Laboratories (Burlingame, CA) and mouse anti- β -actin was from Sigma-Aldrich. Blots were developed with ECL Detection Reagents (GE Healthcare Bio-Sciences, Piscataway, NJ).

Protease activity assay

Protease activity in cell culture supernatants was measured using EnzChek Protease Assay Kit green fluorescence (Molecular Probes/Invitrogen) according to the manufacturer's instructions. In brief, cell supernatants from HNK cells with treatment of different siRNA duplexes were mixed with same volume of BODIPY FL casein substrate in 10 mM Tris-HCl, pH 7.8, and incubated at 37°C for 48 hours. Fluorescence intensity was measured at excitation/emission of ~505/513. Protease activity was determined as increased fluorescence intensity.

Statistical analysis

All statistical analyses were conducted using GraphPad prism, version 5.03 (GraphPad, San Diego, CA). Comparisons of expression levels were performed using analysis of variance techniques and independent sample *t*-tests as appropriate. Differences were considered significant at $P < 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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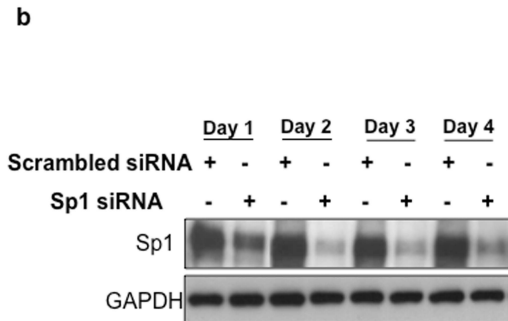
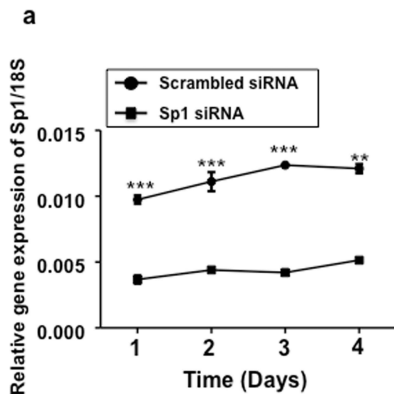
SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplemental Figure 1. Sp1 gene expression is inhibited following transfection of Sp1 siRNA duplexes. NHK cells were transfected with scrambled siRNA and Sp1 #1 siRNA duplexes. Sp1 mRNA (**a**), and protein expression (**b**), were monitored from one day up to four days following siRNA transfection.

siRNA

Scrambled Sp1

KLK5

KLK6

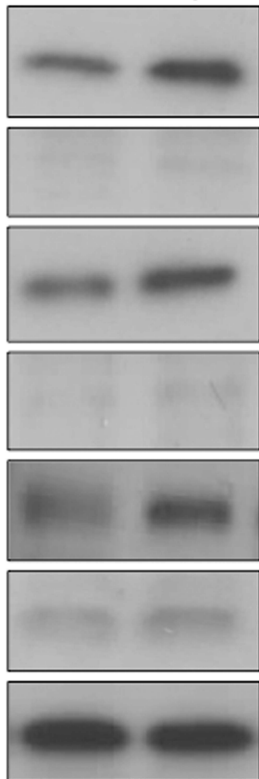
KLK7

KLK8

KLK10

KLK12

GAPDH



Supplemental Figure 2.

KLK5, KLK6, KLK7, KLK8, KLK10, KLK12 protein levels were increased in Sp1-silenced NHK cells. Cell lysates were harvested at day 3 after Sp1 silencing with final 4 hours' incubation with Golgi-stop.