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***Nicastrin* haploinsufficiency alters expression of type I interferon-stimulated genes: the relationship to familial hidradenitis suppurativa**

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Summary

Background.—Hidradenitis suppurativa (HS), also called acne inversa, is a chronic skin disease. The symptoms can be severe, and include intensely painful nodules and abscesses in apocrine-gland rich inverse skin, such as the buttocks, under the arms and in the groin. Autosomal dominant forms of HS exist, but are rare. Some of these kindred have heterozygous loss-of-function rare variants in the γ -secretase complex component nicastrin (*NCSTN*).

Aim.—To investigate the effect of *NCSTN* haploinsufficiency on human keratinocytes and assess potential mechanisms for lesion development.

Methods.—*Nicastrin* was knocked down using a small hairpin RNA construct in both a keratinocyte cell line (HEK001) and an embryonic kidney cell line (HEK293), and differential gene expression was assessed using RNA microarray. Using the HEK293 line, a heterozygous deletion of *NCSTN* was created with CRISPR/Cas9 genome editing, and nuclear factor kappa B (NF κ B) activity was assessed using a luciferase reporter.

Results.—Compared with controls, the keratinocyte *NCSTN* knockdown cell line demonstrated significantly increased expression of genes related to the type I interferon response pathway. Both HEK001 and HEK293 knockdowns demonstrated evidence of altered growth. There was a small but significant increase in NF κ B signalling in response to tumour necrosis factor treatment in HEK293 cells genome-edited for reduced *NCSTN*.

Conclusions.—Our data suggest a role for increased keratinocyte inflammatory responsiveness in familial HS. Confirming this phenotype and characterizing additional effects in different cell types will require study beyond cell lines, such as in primary cells and tissues.

Introduction

Hidradenitis suppurativa (HS) is a chronic, severe and disfiguring skin disease. The estimated prevalence in white populations ranges from 0.1 to 1%.^{1,2} In the USA, the highest prevalence is in postpubertal African American girls.² Lesions primarily develop in the

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axillae, groin, pubic area, perianal region, submammary/inframammary folds and inner thighs.³ Mild HS consists of inflamed and painful nodules, while severe lesions progress to clusters of interconnected subcutaneous abscesses that drain a purulent, malodorous fluid. Even mild HS is intensely painful and significantly reduces a person's quality of life.⁴⁻⁶

In 1985, Fitzsimmons and Guilbert showed evidence for rare autosomal dominant inheritance of HS.⁷ Rare genetic variants in γ -secretase complex components, particularly nicastrin, have since been discovered in some kindred with familial (f)HS and confirmed in different populations.⁸ Active γ -secretase requires nicastrin (NCSTN), anterior pharynx-defective (APH)1 (A or B), presenilin enhancer γ -secretase subunit (PEN)2 and presenilin (PSEN)1 or PSEN2. The γ -secretase complex cleaves type-1 single-pass transmembrane proteins to release intracellular (cytoplasmic) and extracellular (ectodomain) fragments. One suggested fHS mechanism is disrupted NOTCH signalling, which is a key regulator in hair follicle development and maintenance, and HS wounds are driven more by follicle occlusion than by apocrine inflammation. Direct evidence for disease pathogenesis by impaired NOTCH signalling has been lacking. This is further complicated by the large number of potential γ -secretase substrates with diverse molecular functions, which makes predicting the effect of *NCSTN* haploinsufficiency difficult.

The rare *NCSTN* variants identified in fHS to date are all loss-of-function, suggesting haploinsufficiency. To assess the biological impact of *NCSTN* haploinsufficiency we used small hairpin (sh)RNA to knock down *NCSTN* in human epidermal keratinocyte (HEK001) and human embryonic kidney (HEK293) cell lines, and profiled the transcriptomes via microarray. We later generated a heterozygous *NCSTN* deletion in HEK293 cells. Our overall approach is shown in Fig. S1.

Methods

General cell culture conditions

HEK001, HEK293 and HEK293T cell lines (American Type Culture collection) were cultured at 37 °C with 5% CO₂. HEK001 cells were cultured in serum-free medium (Keratinocyte-SFM) supplemented with human recombinant Epidermal Growth Factor and Bovine Pituitary Extract (Life Technology catalogue no. 17005042), while HEK293 and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, glutamine 2 mmol/L, penicillin 100 /mL and streptomycin 100 µg/mL.

Nuclear factor κ B luciferase assay

HEK293 cells were plated at 100 000 cells/well in a 24-well plate 24 h prior to transfection using transit-LT1. NF κ B-luc (firefly luciferase containing proximal NF κ B binding sites) and Renilla luciferase (pGL4 as a transfection control) were used at a 24 : 1 ratio for transfection, and 4 h after transfection, some cultures with were treated with 20 ng/mL tumour necrosis factor (TNF) (catalogue no. T0157; Millipore-Sigma, St Louis, MO, USA). At 24 h post-transfection, cells were harvested and luciferase activity assayed (Dual-Luciferase Reporter Assay System E1910; Promega, Madison, WI, USA).

Growth curves

Cells were plated at 3000 cells/well and proliferation was measured (CellTitre 96 Non-Radioactive Cell Proliferation Assay G4000; Promega, Madison, WI, USA) according to the manufacturer's instructions, starting on Day 2 and continuing for up to 7 consecutive days. Colour change was detected by absorbance at 570 nm in a 96-well plate reader (UQuant; BioTek, Winooski, VT, USA).

Genome editing

NCSTN in HEK293 cells was targeted using CRISPR/Cas9. The gRNA target and single-guide RNA were ordered as IDT gBlocks and cloned using the pCR-Blunt TOPO vector (Table S2). HEK293 cells were transfected with the plasmids for single-guide RNA¹⁰ and humanized Cas9 (JDS246; cat no. 43861; AddGene, Watertown, MA, USA; kind gift of K. Joung), then single-cell clones were screened for deletion.

Statistical analysis

For microarray differential expression analysis, Illumina microarray data (GEO GSE57949) were analysed using *R* software (beadarray and limma packages). Genes with a false-discovery rate corrected *P* value of < 0.05 were considered to be differentially expressed. Known pathway enrichment was determined using the gProfileR g:COSt tool.⁹ For the NF κ B luciferase assays, significance of NF κ B induction (firefly/Renilla) was calculated with *R* software using Tukey honest simple differences in an ANOVA model. For growth curves, significance within day and cell type was calculated by two-sided *t*-test after subtracting background.

Results

***Nicastrin* mRNA can be reduced to less than 50% of wildtype levels using small hairpin RNA**

Five shRNA constructs were tested in triplicate (A06-A10; Table S4) for their ability to knock down *NCSTN* mRNA in the HEK001 and HEK293 cell lines using real-time quantitative (RT-q) PCR (Fig. 1). While both A08 and A09 had knockdown activity in both cells, A08 demonstrated the strongest knockdown and was used for further study. Using 2-week puromycin selection, HEK001 and HEK293 stable knockdowns were generated for A08, as was a control (shLuc; targeting luciferase), all of which were in triplicate. Knockdown was confirmed prior to array profiling (Fig. S2), and later a stable knockdown for an shRNA targeting green fluorescent protein (shGFP) was generated as an additional control.

HEK001 keratinocyte *Nicastrin* knockdown lines have increased type I interferon activation and disruption of cell cycle related genes

HEK001 knockdown of *NCSTN* resulted in the differential expression of 1548 probes for 1393 unique genes (Fig. 2A; Table S8), with some confirmed by RT-qPCR (Fig. S3). The focus was on genes with adjusted $P < 0.05$ and an absolute fold change (FC) of at least 1.50 ($n = 364$ increased, $n = 324$ decreased).

Genes with at least 1.5-fold decrease were enriched for cell cycle pathways (Fig. S4A; Table S9), including histone proteins [H2A histone family member Z (*H2AFZ*): -2.69 FC], DNA topoisomerase (DNA topoisomerase II alpha (*TOP2A*): -1.99 FC] and centromere proteins [centromere protein V (*CENPV*): -1.98 FC], while genes with at least 1.5-fold increased expression were enriched for type I interferon (IFN) pathways (Fig. S4B; Table S10). Among the genes with the highest increase were several associated with inflammation, including chemokines [C-X-C motif ligand 14 (*CXCL14*) 4.91 FC], cytokines [interleukin 33 (IL33): 2.69 FC] and IFN inducible proteins [IFN α -inducible protein 6 (*IFI6*): 3.52 FC; IFN-induced GTP-binding protein Mx1 (*MX1*): 2.29 FC].

HEK293 NCSTN knockdowns have decreased NOTCH and interferon signalling

The HEK293 knockdowns had differential expression for 3089 probes representing 2813 genes (Fig. 2B; Table S11). More than 1300 genes exhibited at least an absolute 1.5 FC ($n = 607$ increased, $n = 784$ decreased). Again, a subset was confirmed by RT-qPCR (Fig. S5).

Decreased expression genes were enriched for small molecule and steroid biosynthesis (Fig. S6A; Table S12). There were also enrichments for NOTCH-related signalling, including categories for general NOTCH signalling, NOTCH receptor processing and NOTCH3 signalling. The genes linked to these pathways included *NCSTN* (-3.82 FC), *Jagged-2* (*JAG2*; -2.51 FC), *NOTCH3* (-3.06 FC) and *MYC* (-1.26 FC). However, no differences were detected in *HEY1* (adjusted $P = 0.83$) or *HES1* (adjusted $P = 0.79$), which are canonical immediate downstream genes regulated by NOTCH signalling. Thus, while *NCSTN* depletion resulted in decreased NOTCH pathway components, there was no evidence for differences in downstream NOTCH signalling. In contrast to the HEK001 genes, the HEK293 genes with decreased expression were enriched for IFN signalling, including *MX1* (-1.63 FC), *IFI6* (-2.25 FC), *IFIT1* (-3.84 FC), IFN-induced transmembrane protein 1 (*IFITM1*) (-6.36 FC), *IFITM2* (-3.20 FC) and *IFITM3* (-1.84 FC). Genes with increased expression were primarily enriched for categories related to senescence, HDAC activity and methylation (Fig. S6B; Table S13).

Nicestrin knockdown alters cell metabolism after exponential growth

Because of enrichments in growth-related categories seen in both cell lines, cell proliferation was assayed in the stable knockdown lines. There was no difference for knockdowns in either cell line during the exponential growth phase (Fig. 3); however, *NCSTN* knockdowns in both lines demonstrated significantly reduced signal in the postexponential phase (Table S5). As this is a colourimetric conversion assay, the reduced conversion could be driven by increased cell death, increased rates of senescence, or both.

HEK293 cell lines have altered nuclear factor κ B activation with decreased Nicestrin

Treating patients with sporadic HS with anti-TNF monoclonal antibodies can be an effective treatment, thus this study investigated whether NF κ B responses, a pathway downstream of TNF, were activated in the knockdown cells. Three stable knockdowns (A08, shLuc, shGFP) were generated; some cells received no treatment, while others were stimulated with TNF for NF κ B-responsive luciferase induction (Fig. 4A; Table S6). The unstimulated A08 line demonstrated significantly increased NF κ B activity compared with the shLuc and shGFP

unstimulated lines (1.7-fold to 1.9-fold increase). The TNF-stimulated lines had significantly increased NF κ B activity compared with unstimulated lines; however, there was no difference between the different TNF-stimulated samples.

Wildtype HEK293 cells were compared with HEK293 cells having a heterozygous nicastrin deletion (~25% of wildtype expression; Fig. S7). There was no difference at baseline (Fig. 4B; Table S7), and again, all TNF-stimulated cells had increased NF κ B activity compared with unstimulated cells. However, the deletion line had a significant (approximately 1.75-fold) increase ($P = 0.02$) in NF κ B activity in response to TNF stimulation.

These data suggest that reduced *NCSTN* expression in HEK293 cells results in dysregulated NF κ B activity. In this study, there was a discrepancy with regard to whether the dysregulation was observed at baseline or after TNF stimulation, which may be a result of the intrinsic differences of the two models.

Discussion

The term ‘hidradenitis’ suggests a disease driven by glandular inflammation. However, it has been shown that an HS-like phenotype can be produced by applying occlusive tape, suggesting that follicular occlusion is the initiating step.¹¹ Histological studies confirm that follicular occlusion is the most common feature of affected skin^{12,13} and that lesional follicles may have weak basement membranes that are prone to rupture.¹⁴ Ruptured follicles could then initiate the inflammatory response. The increased type I IFN signature in keratinocyte transcriptomes and the increased NF κ B signalling we saw in HEK293 cells by luciferase assay suggests that individuals haploinsufficient for *NCSTN* could have increased inflammatory responsiveness. We previously helped describe keratinocyte hyperinflammatory responses in particular rare variants in *CARD14*, supporting this possibility.¹⁵ However, it is unclear whether individuals with decreased *NCSTN* have increased frequency of follicular occlusion compared with controls or increased reaction to follicular occlusion. In the current study, an unexpected finding was that reduced nicastrin caused disrupted IFN signalling in both HEK001 and HEK293 cell lines, suggesting a role for γ -secretase in regulating inflammatory function in keratinocytes and raising the question of what additional systemic effects exist for individuals with familial HS. The decreased IFN signalling in the HEK293 knockdowns is discordant with the increased HEK001 response, but helps support the role for γ -secretase in regulating IFN responses in different cell types.

Our results also bring into question whether disrupted NOTCH signalling is a driver of familial HS. There is little direct evidence that individuals with *NCSTN* haploinsufficiency have reduced NOTCH signalling. *NCSTN* is not essential *in vivo* for processing all γ -secretase substrates.^{16,17} Another study failed to find a reduced number of γ -secretase complexes in primary cells from haploinsufficient individuals,¹⁸ and several *NCSTN* missense mutations linked to fHS have been shown to facilitate NOTCH signalling.¹⁹ Direct profiling of human lesional ($n = 17$) and nonlesional skin ($n = 13$) revealed substantial inflammation without a strong signal for disrupted NOTCH signalling.²⁰ However, a study with small interfering RNA knockdown of *NCSTN* in HACAT keratinocytes did support disrupted NOTCH signalling and also found reduced NOTCH1–3 staining in a patient

biopsy.²¹ Overall, the available evidence does not refute a role for NOTCH signalling, but also does not support NOTCH signalling as the sole mechanism. Regulation of inflammatory responses is a strong secondary candidate mechanism and is supported by other evidence. The prevalence of HS in patients with inflammatory bowel disease is higher than that in the general population.²² A TNF promoter single nucleotide polymorphism (rs361525) was enriched in a small cohort of patients with HS in a previous study²³ and is independently associated with psoriasis.²⁴ TNF levels are also higher in lesional skin than control skin.²⁵ In the current study, we do not know whether the cell growth phenotype is a primary effect of haploinsufficiency or a secondary effect of our system. If it is a primary effect of haploinsufficiency in keratinocytes, it could contribute to the lesions behaving as nonhealing chronic wounds.

Conclusion

This study helps to highlight the potential for diverse and cell-type specific responses to decreased levels of *NCSTN*. If there are pleiotropic effects for NCSTN loss, they are likely to be observed only with the correct environmental stimulus in the right cell type. NOTCH signalling may be involved in pathogenesis, but NOTCH-independent inflammation may be a critical therapeutic target. It will be important to distinguish whether fHS shares pathological mechanisms with sporadic HS, or whether it is a severe phenocopy with unique mechanisms. Further study in patient tissue and in primary cell lines will be required to help tease apart these possibilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What's already known about this topic?

- *NCSTN* haploinsufficiency has been identified as a high-penetrance risk factor for developing HS, based on genetic studies in families with autosomal dominant HS.
- The mechanism by which *NCSTN* haploinsufficiency causes disease in the skin is not well understood.

What does this study add?

- This study suggests that keratinocytes may help drive inflammatory responses in the skin of individuals with nicastrin insufficiency that has familial HS.
- It also suggests that *NCSTN* haploinsufficiency may have other systemic effects that are both cell type-specific and environmentally responsive.

Figure 1.

Relative expression results for the 5 shRNA constructs (A06-A10). The *x* axis is the small hairpin (sh)RNA construct used and the *y* axis is the relative expression to nontransduced controls. Points indicate mean relative expression and error bars represent standard deviation. Grey boxes indicate the standard deviation of the control cells. Two different Nicastrin (NCSTN) amplicons (circle and triangle) were tested, with each having three technical replicates. A08 had the most consistent knockdown effect.

Figure 2.

Volcano plots for (a) HEK001 and (b) HEK293 knockdowns. Effect size is shown on the x axis (log₂ fold change) and the adjusted significance on the y axis. Some top genes are individually labelled. The horizontal line represents adjusted $P < 0.05$ and the vertical lines represent changes of 1.5-fold.

Figure 3.

The x axis shows the ligand treatment and the y axis shows the log₂-transformed relative fluorescence units (RFU). (a) Data from shRNA knockdowns, (b) data from genome-edited cells. Each point represents in individual replicate. Point shape and colour denote the small hairpin (sh)RNA knockdown or genome-edited status. Each experiment was performed in triplicate. Significant differences are shown as asterisk-labelled lines. shRNA The Nicastrin (*NCSTN*) knockdown had increased baseline nuclear factor (NF) κ B activity, but the genome-edited line had increased activity only after stimulation.

Figure 4.

The *x* axis shows the ligand treatment and the *y* axis shows the log₂-transformed relative fluorescence units (RFU). (a) Data from small hairpin (sh)RNA knockdowns; (b) data from genome-edited cells. Each point represents in individual replicate. Point shape and colour denote the shRNA knockdown or genome-edited status. Each experiment was performed in triplicate. Significant differences are shown as asterisk-labelled lines. shRNA The Nicastrin (*NCSTN*) knockdown had increased baseline NFκB activity, but the genome-edited line had increased activity only after stimulation.