

Characterization of a Novel Long Noncoding RNA, SCAL1, Induced by Cigarette Smoke and Elevated in Lung Cancer Cell Lines

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The incidence of lung diseases and cancer caused by cigarette smoke is increasing. The molecular mechanisms of gene regulation induced by cigarette smoke that ultimately lead to cancer remain unclear. This report describes a novel long noncoding RNA (lncRNA) that is induced by cigarette smoke extract (CSE) both *in vitro* and *in vivo* and is elevated in numerous lung cancer cell lines. We have termed this lncRNA the smoke and cancer-associated lncRNA-1 (SCAL1). This lncRNA is located in chromosome 5, and initial sequencing analysis reveals a transcript with four exons and three introns. The expression of SCAL1 is regulated transcriptionally by nuclear factor erythroid 2-related factor (NRF2), as determined by the small, interfering RNA (siRNA) knockdown of NRF2 and kelch-like ECH-associated protein 1 (KEAP1). A nuclear factor erythroid-derived 2 (NF-E2) motif was identified in the promoter region that shows binding to NRF2 after its activation. Functionally, the siRNA knockdown of SCAL1 in human bronchial epithelial cells shows a significant potentiation of cytotoxicity induced by CSE *in vitro*. Altogether, these results identify a novel and intriguing new noncoding RNA that may act downstream of NRF2 to regulate gene expression and mediate oxidative stress protection in airway epithelial cells.

Keywords: lincRNA; cancer; smoke; NRF2; epigenetics

Recent advances in sequencing technology have revealed that significant numbers of noncoding RNAs are expressed and have significant functions in regulating gene expression. Noncoding RNAs have been classified according to size as either micro-RNAs (less than 200 base pairs) to long noncoding RNAs (greater than 200 base pairs; lncRNAs). lncRNAs have been further subdivided, based on their location relative to coding genes. Native antisense transcripts overlap with coding genes, intronic lncRNAs are expressed from the introns of coding genes, and long intergenic noncoding RNAs (lincRNAs) are found in the genome between coding genes (1). Unlike micro-RNAs, lncRNAs are much less well characterized, and how they function in biology and gene regulation remains an active area of investigation.

Using gain and loss of function approaches, the function of individual lncRNAs are gradually being elucidated. X-inactive specific transcript (XIST), one of the earliest identified lncRNAs, plays an essential role in sex-specific imprinting by epigenetically silencing one of the two X chromosomes in female zygotes

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CLINICAL RELEVANCE

The molecular mechanisms of gene regulation induced by cigarette smoke that ultimately lead to cancer remain unclear. We describe a novel long noncoding RNA (lncRNA) that is induced by cigarette smoke both *in vitro* and *in vivo*, and is elevated in numerous lung cancer cell lines. This is the first demonstration of the smoke and cancer-associated lncRNA-1 (SCAL1), the new noncoding RNA that may act downstream of nuclear factor erythroid 2-related factor to regulate gene expression and mediate oxidative stress protection in airway epithelial cells.

through the recruitment of polycomb repressor complexes (PRC2) (2). Other lncRNAs, such as HOX antisense intergenic RNA (HOTAIR) and P21-associated ncRNA DNA damage activated (PANDA), have also been found to regulate the chromatin state through their effects on the PRC2 complex (3, 4). Beyond its novelty in regulating important biological functions through epigenetic mechanisms, emerging evidence suggests that lncRNAs are associated with and may cause diseases such as cancer. Abnormal expressions of lncRNAs have been associated with cancers of the lung and breast (5, 6). Specific lncRNAs may be transcribed by tumor suppressors such as p53, and regulate cellular responses to DNA damage (3, 7). The mechanisms by which lncRNAs regulate transcription have been reviewed recently, and are broadly classified as signals, decoys, guides, and scaffolds (8). Recently, however, an lncRNA has been found in the cytosol, and it regulates its target genes by posttranscriptional mechanisms (9). Further developments in forthcoming years will likely reveal additional levels of gene regulatory mechanisms in addition to the predominantly transcriptional mechanisms characterized thus far.

Lung cancer is the leading cause of all cancer deaths worldwide. The mechanism of smoke-induced injury that ultimately leads to lung cancer remains unclear. Chemical and oxidative stress from smoke can activate the transcription factor nuclear factor erythroid 2-related factor (NRF2) in cells (10, 11). The loss of function of NRF2 sensitizes lung cells both *in vitro* and *in vivo* to damage and acute lung injury, as well as chronic obstructive pulmonary disease-like changes (12, 13). NRF2 is normally produced in abundance, but its cytosolic concentrations are kept low by its binding to kelch-like ECH-associated protein 1 (KEAP1), which leads to its degradation by ubiquitination and proteasomal digestion. The activation of NRF2 occurs when its cytosolic inhibitor KEAP1 undergoes reactions with oxidants or electrophiles in specific cysteine residues, leading to its dissociation from NRF2. Free NRF2 can then translocate into the nucleus and alter the expression of protective antioxidant genes (14).

Although many coding genes that are targets of NRF2-mediated transcription have been identified, less is known about whether (or how many) noncoding RNAs can also be transcriptionally activated

by NRF2. Although other lncRNAs have been identified that are transcriptionally activated by p53 after genotoxic stress (3, 7) or induced by purified tobacco carcinogens (15), to our knowledge, no specific lncRNA has been identified thus far that is regulated by NRF2. In this report, we show that a novel lncRNA, the smoke and cancer-associated lncRNA-1 (SCAL1), is induced by smoke and up-regulated in smokers' airways and various cancer cell lines. We further demonstrate that its expression is NRF2-dependent, and short, interfering RNA (siRNA) experiments reveal that it plays a protective role in suppressing smoke-induced toxicity in airway epithelial cells. This may suggest that SCAL1 is a novel, specific downstream mediator of NRF2's effects on the cytoprotection of lung cells.

MATERIALS AND METHODS

Cell Cultures

A549, CL1-0, CL1-5, H1975, HCC-827, NCI-H292, and PC9 cancer cells were cultured in RPMI + 10% FBS. Normal human primary bronchial epithelial (NHBE) cells were obtained from airway tissues provided by the National Disease Research Interchange (Philadelphia, PA) and the University of California at Davis Medical Hospital (Sacramento, CA), with patient consents. The protocol for human-tissue procurement was periodically reviewed and approved by the University Human Subject Research Review Committee of University of California at Davis. NHBE cells were grown in Clonetics BEGM medium (Cambrex Lonza, East Rutherford, NJ) with all hormones and growth factors included in the package, except for the retinoic acid. The human HBE1 cell line was a gift from J. R. Yankaskas at the University of North Carolina (16).

Analysis of lncRNAs from RNA-seq and Chromatin Immunoprecipitation-seq Datasets

RNA seq data were analyzed by converting the files to FASTA for alignment, using the NCBI Sequence Read Archive (SRA) Toolkit, version 2.0.0. These files were then aligned to the Hg19 transcriptome using the Burrows-Wheeler transform (17), followed by conversion to Sequence Alignment/Map (SAM) format files using SAMtools (18). Unaligned reads were removed by Picard (<http://picard.sourceforge.net>), and the files were converted back to FASTA format. These were compared with lncRNAs determined from (19), and the read counts were extracted with a Perl script (provided upon request) and imported into R using DESeq, version 1.6.1 (20). The smoker and nonsmoker datasets in the NCBI SRA included accession numbers SRP005411 (21) and GSE29006 (22). For the chromatin immunoprecipitation (ChIP)-seq dataset, accession number GSE37589 was used from the NCBI Gene Expression Omnibus (GEO).

Exposure of Cultured Cells to Cigarette Smoke Extract

Cultures of HBE1 and NHBE cells were exposed to cigarette smoke extract (CSE), using a protocol similar to that previously described (23). Briefly, research cigarettes (Kentucky Tobacco R&D Center, Lexington, KY) were lit, and mainstream smoke was suctioned with a 60-ml catheter tip syringe containing 5 ml of medium. The medium was then shaken vigorously for 20 seconds. This procedure was repeated four times. The resulting medium was sterilized through a 0.22- μ m filter and designated as 100% CSE. Dilutions were produced for the appropriate concentrations in treatments, as depicted in the figures. Control media were prepared similarly, except with filtered air instead of cigarette smoke.

siRNA Transfection Studies

The siRNA for human NRF2 (GTAAGAAGCCAGATGTAAAdtd) and KEAP1 (GGGCGTGGCTGTCTCAATdtd) was previously described (24). SCAL1-1 (CCCACAAAAGGAAGAAAAdtd) and SCAL1-2 (CAUUUCAGACUAAAUAAdtd) siRNAs were designed with the iScore designer (25), and a negative control siRNA was ordered

from Life Technologies (Grand Island, NY). All transfection was based on a reverse-transfection protocol, as described by Amarzguioui (26).

Western Blot Analysis

For nuclear and cytosol extracts, cells were extracted using the NE-PER Kit (Thermo Scientific, Waltham, MA), according to the manufacturer's protocol. After standard SDS-PAGE and transfer, Western blotting was performed as described previously (32) with anti-NRF2 (H300; Santa Cruz Biotechnology, Santa Cruz, CA) and anti- β -actin (Sigma-Aldrich, St. Louis, MO).

RT-PCR of Partial SCAL1 Transcript

After RNA isolation by Trizol (Invitrogen, Carlsbad, CA) and cDNA synthesis with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI), primers near the putative 5' and 3' ends of SCAL1 (forward, 5'-GTGTCAAGCTCGGATTGCCT-3'; reverse, 5'-GAGCCCACACTCAGGTTTC-3') were used in a PCR reaction with GoTaq (Promega), followed by standard agarose gel electrophoresis. The amplicon was then cloned into TA vectors (Invitrogen) and sequenced.

Quantitative RT-PCR

Quantitative RT-PCR was performed on an Applied Biosystems 5700 thermocycler (Applied Biosystems, Foster City, CA), as described previously. The primers included, for SCAL1, forward (5'-ACCAGCTGTCCCTCAGTGTCT-3') and reverse (5'-AGGCCTTTA TCCTCGGGTTGCCT-3'); for NRF2, forward (5'-ATTGAGCAAG TTTGGGAGGA-3') and reverse (5'-AAGACACTGTAACCTCAG GAATGGA-3'); and for β -actin (27), forward (5'-GCCGGAAA TCGTGCGTGACATT-3') and reverse (5'-GATGGAGTTGAAG GTAGTTTCGTG-3'). The relative abundance of message expression after normalization with β -actin was used as the measurement of gene expression.

Drug Treatment and Cell Viability Assays

Cells were treated with CSE at the previously described doses for 2 hours, followed by media change and 24-hour recovery. Viable cell numbers were checked by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) assay (Promega), according to the manufacturer's instructions.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed according to the method of Weinmann and colleagues (28). The primers for the PCR included SCAL1 ChIP forward (5'-GGCAGGAAGGAGTGTGT CAT-3') and reverse (5'-GCACCTGTCTAAGGCAATCC-3'). The antibody to NRF2 (H300) was obtained from Santa Cruz Biotechnology.

Statistical Analysis

Experiments were performed in triplicate, and repeated for at least three independent passages. The numbers of experiments are stated in the figure legends as *n*. Standard errors of the means were calculated for all treatment groups, and were used for the error bars shown in the figures. For the calculation of *P* values, two-tier Student *t* tests were used for experiments with two treatment groups only. For experiments with more than two treatment groups, ANOVA was used, followed by *post hoc* analysis with the Newman-Keuls multiple-comparisons test. Statistical significance is noted in the figures when *P* < 0.05.

RESULTS

A Novel lncRNA Is Elevated in the Airway Epithelium of Cigarette Smokers and Metastatic-Prone Cancer Cells

Previous reports have shown that aggressive malignant characteristics in lung cancer can be attributed to lncRNAs, such as metastasis

associated lung adenocarcinoma transcript 1 (MALAT1) (5, 29). Because continued cigarette smoke exposure can promote aggressive malignant phenotypes in lung cancer (30, 31), we hypothesized that additional novel lncRNAs may exist that are related to cigarette smoke exposure and malignant characteristics in cancer. To identify these novel lncRNAs, we first used a noninvasive lung cancer cell line CL1-0 and its more metastatic-prone subclone CL1-5, as previously characterized (32), and we then performed next-generation RNA sequencing. A top-ranked hit that was differentially expressed in CL1-5 versus CL1-0 cells was a specific lncRNA identified previously as XLOC_004924 (19) (Figure 1A). The increased expression of XLOC_004924 RNA in CL1-5 compared with CL1-0 was further confirmed in these cell lines via quantitative RT-PCR (Figure 1B). This lncRNA is located

on chromosome 5 at approximate locations (chr5:90598846–90610200) in an intergenic region between two coding genes, G protein-coupled receptor 98 (GPR98) and arrestin domain-containing 3 (ARRDC3) (Figure 1C). Using primers near the putative 5' and 3' ends identified from a putative RNA-seq fragment (TCONS_00009724) in the Human Body Map lincRNAs database, we amplified a 750-base pair partial fragment of XLOC_004924 from CL1-5 and HBE1 cells. Sequencing the amplicon in both cells gave an identical partial transcript that showed four putative exons and three introns (Figure 1C). This is different from the TCONS transcript in that ours lacked a third exon that appears to be spliced out in our cells, and may represent an alternative spliced variant. We next determined whether this lncRNA was elevated in cigarette smokers

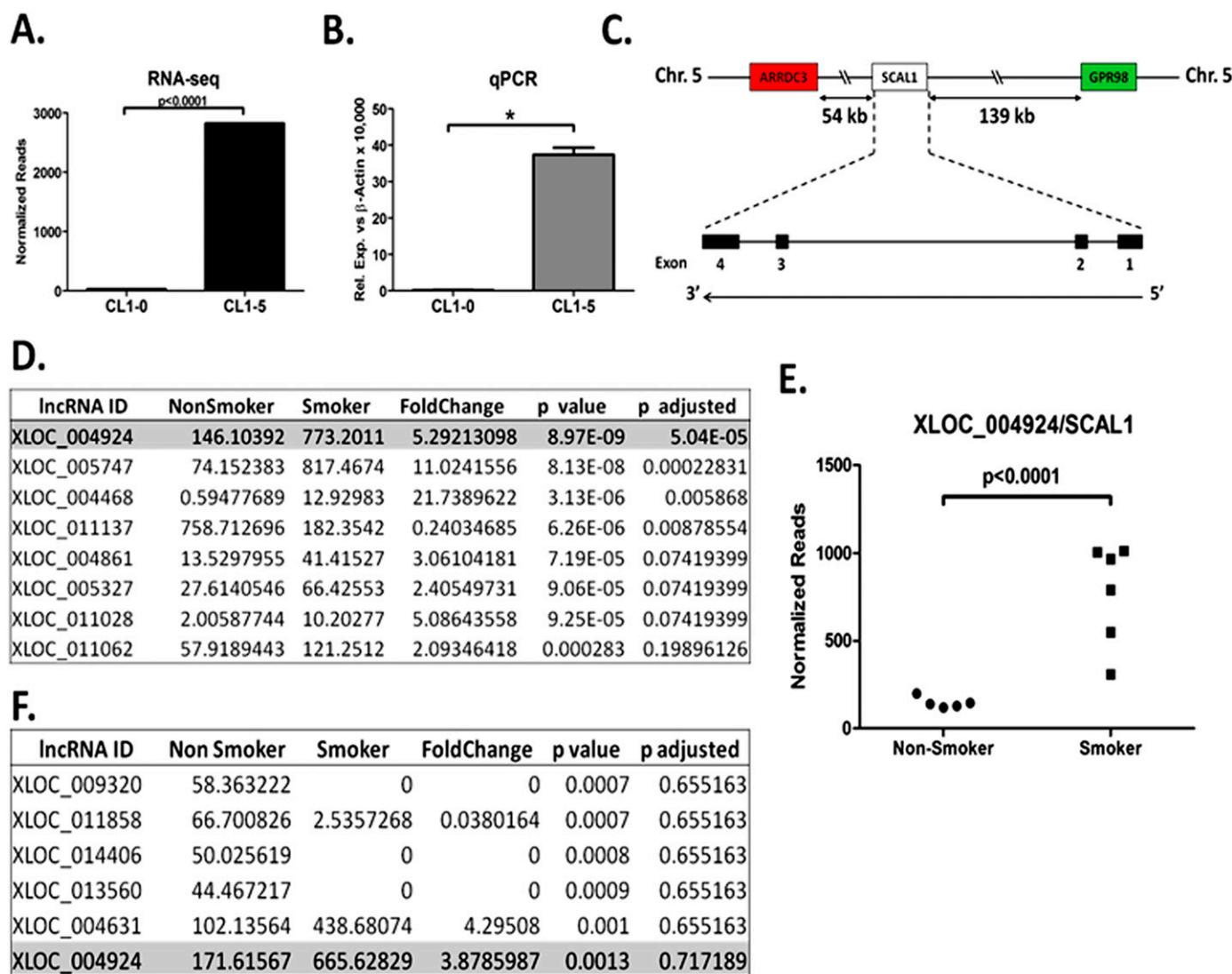


Figure 1. Smoke and cancer-associated long noncoding RNA-1 (SCAL1) expression in CL1-0/5 cells and in nonsmokers and smokers. (A) Expression of SCAL1 in CL1-5 shows approximate 15-fold increase compared with CL1-0 on RNA-seq based on normalized reads. (B) Quantitative PCR (qPCR) for cDNA samples of CL1-0 and CL1-5 shows an approximate 200-fold increase of SCAL1 in CL1-5 compared with CL1-0. (C) Chromosome 5 location of SCAL1 shows it is in an intergenic region between arrestin domain containing 3 (ARRDC3) and G protein-coupled receptor 98 (GPR78) with a putative splicing pattern of 4 exons and 3 introns. (D) Top long noncoding RNAs (lncRNAs) derived from NCBI Sequence Read Archive (SRA) accession number SRP005411 show differential expression between smokers and nonsmokers. The nomenclature by Cabili and colleagues (19) was used to denote the lncRNAs, and SCAL1 is noted as XLOC_004924. The ranking in this data analysis is based on the lowest adjusted *P* value, which was adjusted for false-discovery rate. (E) A scatterplot shows the expression of SCAL1 in the individual patients from D, as based on normalized reads. (F) Another experiment with NCBI SRA accession number GSE29006, using pooled sampling from smokers versus nonsmokers, showed a 3.8-fold increase in SCAL1 (shaded gray) compared with nonsmokers. Chr. 5, chromosome 5. Error bars represent SEMs. **P* < 0.05.

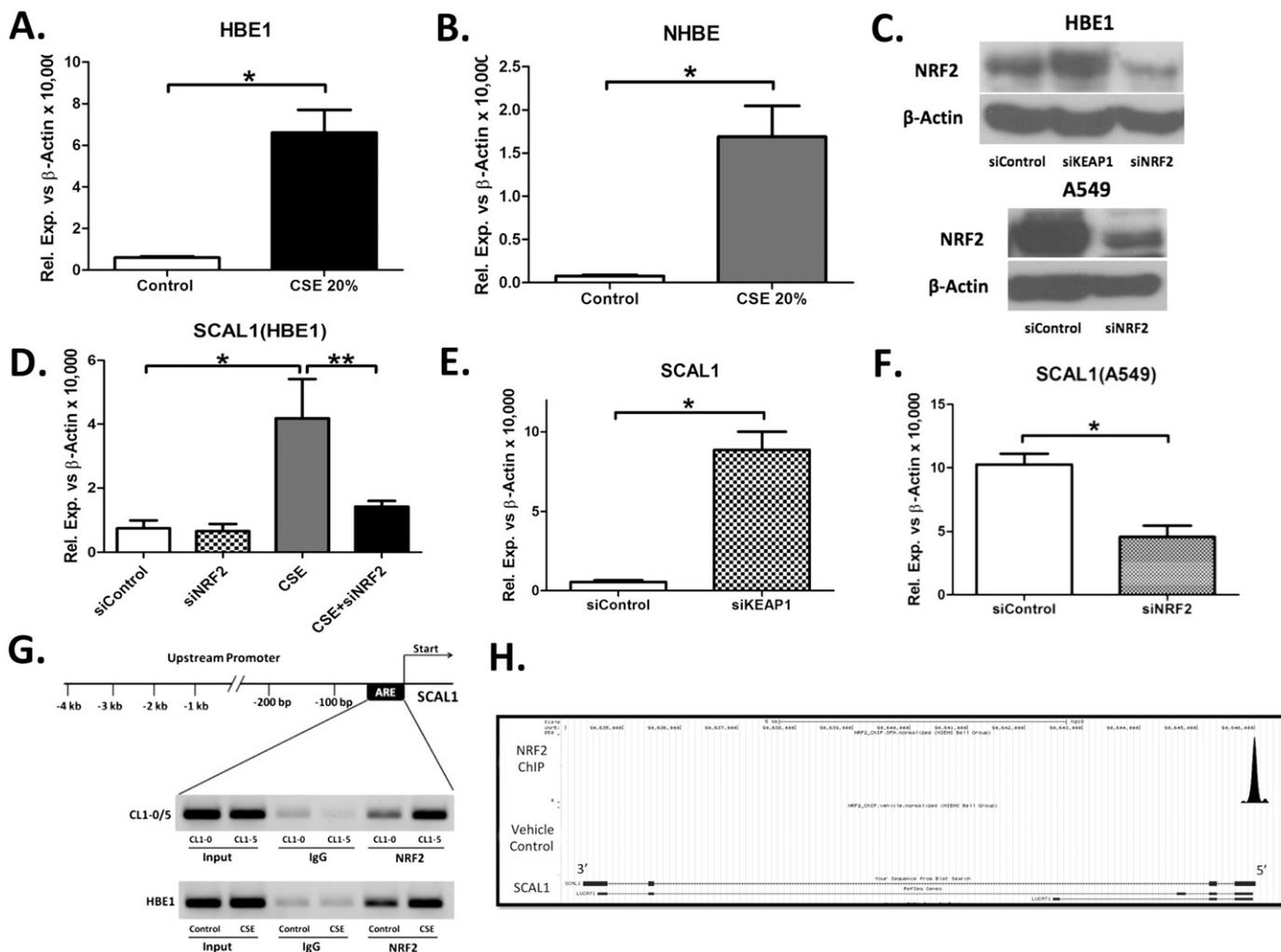


Figure 2. Induction of SCAL1 by cigarette smoke extract (CSE) exposure and dependence on nuclear factor erythroid 2-related factor (NRF2). (A) Human bronchial epithelial (HBE1) cells exposed to CSE at 20% for 2 hours, followed by a 16- to 20-hour recovery, show a robust stimulation of SCAL1 expression. (B) Similar exposure of primary normal human primary bronchial epithelial (NHBE) cells from five different patients showed a similar induction of SCAL1. (C) Western blot shows nuclear concentrations of NRF2 after the short, interfering RNA (siRNA) knockdown of NRF2 in A549 cells, and of kelch-like ECH-associated protein 1 (KEAP1) and NRF2 in HBE1 cells. (D) SCAL1 induction after CSE is significantly reduced by NRF2 siRNA knockdown. (E) Activation of NRF2 by KEAP1 siRNA knockdown leads to a significant induction of SCAL1 without smoke exposure. (F) The knockdown of NRF2 in A549 cells reduces SCAL1 expression. (G) Chromatin immunoprecipitation (ChIP) assay shows increased NRF2 binding to a putative NFE2 binding site of the SCAL1 promoter in CL1-5 versus CL1-0 cells, and in smoke-treated versus non-smoke-treated HBE1 cells. (H) Analysis of the ChIP-seq dataset accession number GSE37589 (35) shows increased NRF2 binding to the putative NF-E2 site in the SCAL1 promoter after sulforaphane treatment in human lymphoblastoid cells. * $P < 0.05$. ** $P < 0.05$.

from two publically available RNA-seq databases. These two datasets were available in the SRA at the NCBI under accession numbers SRP005411 (21) and GSE29006 (22). Several lncRNAs that were differentially expressed between smokers and non-smokers were identified and listed in tables in Figures 1D and 1F, ranked by P -adjusted values or P values. We found that XLOC_004924 was elevated in the airway epithelia of cigarette smokers versus nonsmokers in both of these datasets (Figures 1D and 1F). In the first dataset (21), five nonsmokers were compared with six smokers. The amount of XLOC_004924 was 5.3-fold higher in smokers versus nonsmokers, with an adjusted P value of 5.0×10^{-5} (Figures 1D and 1E). In the second study (22), the smokers' epithelia demonstrated a 3.9-fold increase in XLOC_004924, compared with nonsmokers (Figure 1F), which is consistent with the results of the first study. As will be shown, because this lncRNA appeared to have an association

with cancer as well, we have termed this lncRNA the smoke and cancer-associated lncRNA-1 (SCAL1).

SCAL1 Is Induced by Cigarette Smoke in Airway Epithelial Cells in an NRF2-Dependent Manner

Because SCAL1 appeared to be elevated in the airway epithelia of smokers, we tested whether CSE *in vitro* could induce its expression in airway epithelial cultures. Using the immortalized cell line HBE1 and primary NHBE cells, we exposed cell samples to CSE at a 20% concentration for 2 hours, followed by a recovery period of 16–20 hours. Quantitative PCR subsequently showed an increased expression of SCAL1 in both HBE1 and NHBE cells (Figures 2A and 2B). Thus the increased SCAL1 expression in the smokers' airways seen in Figures 1D–1F) is likely attributable to a direct effect of cigarette smoke on epithelial cells. These

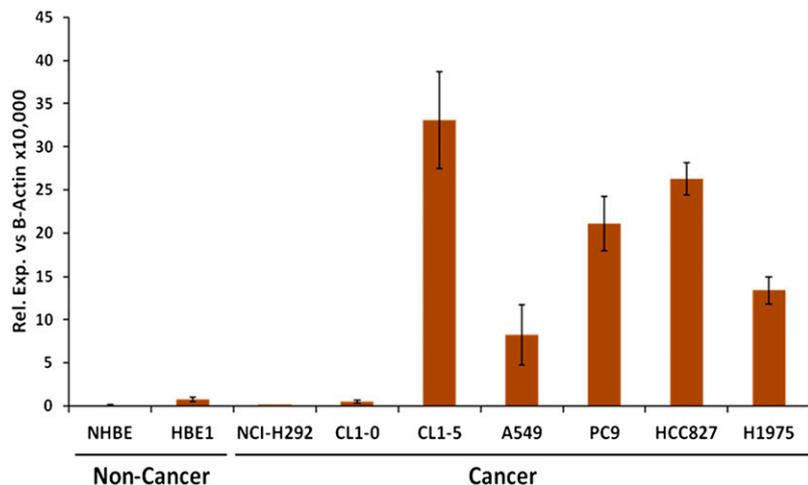


Figure 3. Expression of SCAL1 in normal, immortalized, and lung cancer cell lines. NHBE and HBE1 (immortalized) are noncancerous cells and show a low expression of SCAL1. Low expression is also seen in some lung cancer cell lines (CL1-0 and NCI-H292), but SCAL1 is markedly increased in several other cell lines (A549, PC9, CL1-5, HCC-827, and H1975). Gene expression was quantitated by quantitative PCR. Error bars represent the SEMs.

data, combined with the results of the SRA datasets looking at smokers versus nonsmokers, strongly suggest that SCAL1 is inducible by cigarette smoke both *in vitro* and *in vivo*.

Among the many signaling pathways activated by oxidative stress and smoke, NRF2-KEAP1 is one of the major examples and is critical in protecting lung epithelial cells from cellular damage (11–13, 33, 34). Thus we hypothesized that it may be a key regulator of SCAL1 expression. To test this hypothesis, we performed two siRNA knockdown approaches. One involved the siRNA knockdown of NRF2 in high SCAL1 expressing A549 cells and CSE-activated HBE1 cells, and the other involved the knockdown of a key regulator protein, KEAP1, that suppresses NRF2 activation before CSE/oxidative stress in non-smoke-treated HBE1 cells. As shown in Figures 2C, 2D, and 2F, the inhibition of NRF2 in A549 and CSE-exposed HBE1 cells suppressed SCAL1 expression. In contrast, inhibition of the upstream regulator, KEAP1, by siRNA could elevate SCAL1 expression in HBE1 cells without CSE exposure (Figure 2E). To delineate further the role of NRF2 in regulating SCAL1 expression, we checked its promoter region up to around 10,000 base pairs upstream of the RNA seq fragment TCONS_00009724. A putative NRF2 binding NF-E2 site was identified upstream of the TCONS_00009724 fragment. To determine whether NRF2 binds to this motif, ChIP was performed in CL1-0 versus CL1-5 cells, and in HBE1 cells with and without CSE treatment. As seen in Figure 2G, NRF2 showed increased binding to this region of the promoter in high SCAL1 expressing CL1-5 versus low expressing CL1-0 cells, as well as in HBE1 cells after CSE treatment. In further support of these data, we analyzed a publically available ChIP-seq dataset involving the sulforaphane (SFN, an activator of NRF2) treatment of lymphoblastoid cells (35). As seen in Figure 2H, NRF2 activation by SFN in these cells demonstrated the strong binding of NRF2 (Figure 2H, *large black peak*) to a location in chromosome 5 that is nearly identical to the putative NF-E2 motif of SCAL1. More importantly, analysis of this ChIP-seq dataset of 10,000 base pairs upstream and downstream of TCONS_00009724 showed no additional significant binding of NRF2 after SFN treatment, suggesting that this is the only NRF2 binding site for SCAL1. These results all strongly support a direct role of NRF2 in the induction of SCAL1 transcriptional expression in airway epithelial cells.

SCAL1 Is Elevated in Many Lung Cancer Cell Lines

Because SCAL1 was identified in a highly metastatic-prone cancer cell line (Figures 1A and 1B), we asked whether it was also

elevated in other lung cancer cell lines. We screened a small panel of lung cancer cells (A549, NCI-H292, PC9, CL1-0, CL1-5, NCI-H1925, and HCC-827) and compared the expression of SCAL1 to that of the immortalized HBE1 and NHBE cells under basal cell culture conditions. As seen in Figure 3, HBE1 and NHBE cells demonstrated a relatively low expression of SCAL1. In some of the lung cancer cell lines, such as CL1-0 and NCI-H292, the expression was also comparably low. However, the expression was markedly elevated in some cancer cell lines such as A549, PC9, CL1-5, NCI-H1975, and HCC-827. Of particular interest in these cell lines was the baseline activation of NRF2. Previously reports suggested that cells with KEAP1-inactivating mutations such as A549 (24) and HCC-827 (36) may have high levels of basal NRF2 activation (24, 37). Intriguingly, NRF2 can also be constitutively activated by excessive EGFR activation (38, 39), which is common in lung cancer and present in cell lines such as PC9, HCC-827, and NCI-1975 (38, 40). In contrast, NCI-H292 has been shown to have low levels of NRF2 activation (38). Although only correlative, these expression results are consistent with the siRNA knockdown results in Figure 2, suggesting that cancer cells may also up-regulate SCAL1 expression through an NRF2-based mechanism.

siRNA Knockdown of SCAL1 Potentiates Cigarette Smoke Toxicity

To elucidate the putative function of SCAL1, we designed two nonoverlapping siRNA sequences (siSCAL1-1 and siSCAL1-2) that could knock down SCAL1 expression efficiently. As shown in Figure 4A, these siRNAs had achieved 87% and 92% knock-downs, respectively, of CSE-induced SCAL1 expression in HBE1 cells after a 48-hour incubation. Using this approach, we tested whether SCAL1 knockdown could affect the cell survival of CSE-exposed HBE1 cells. We exposed siSCAL1-transfected HBE1 cells to increasing concentrations of CSE media from 10% to 30% for 2 hours, followed by a recovery period of 20 hours. As shown in Figure 4B, a significant increase in toxicity occurs after SCAL1 is knocked down by both siRNA sequences, as seen by the reduced viability of the cells versus the short, interfering control (a shift to the left of the viability curve). This increase in toxicity was notable for higher doses of smoke at 20%, 25%, and 30%. A trend toward a higher level of toxicity was evident in the second siRNA (siSCAL1-2) versus the first siRNA (siSCAL1-1) sequence, but the results were not statistically significant. This may have occurred because of the slightly higher knockdown efficacy of SCAL1 from the siSCAL1-

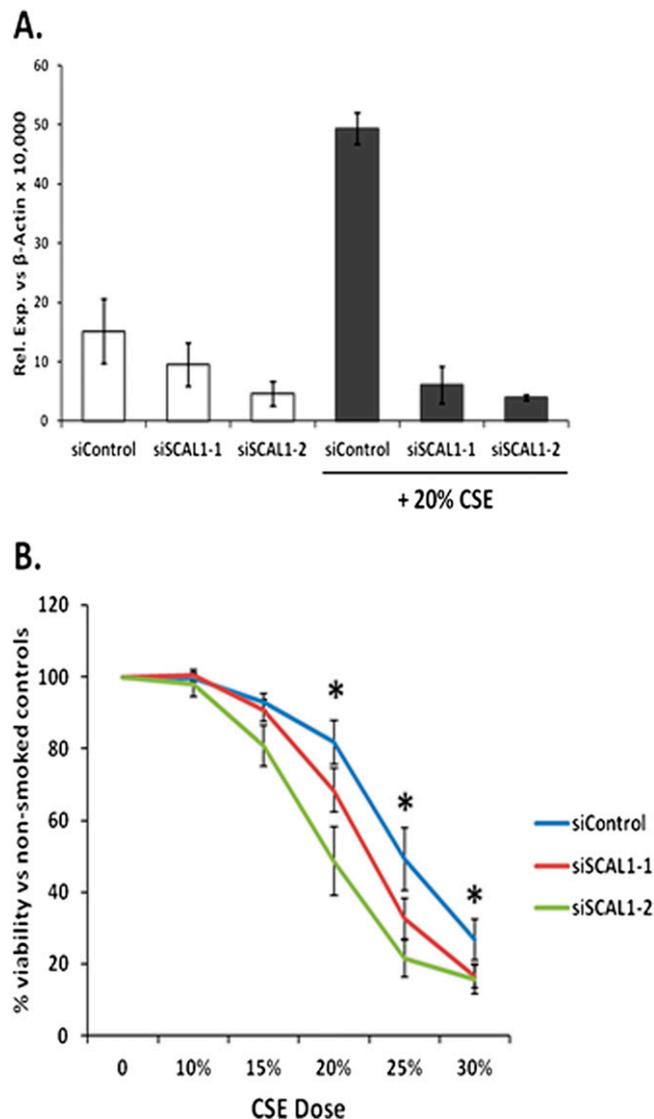


Figure 4. SCAL1 siRNA knockdown potentiates smoke-induced toxicity in HBE1 cells. (A) Two pairs of siRNA duplexes designed to SCAL1 produced 87% (siSCAL1-1) and 92% (siSCAL1-2) knockdown after CSE treatment in HBE1 cells. (B) Percent cell viability (y axis) of the HBE1 cells exposed to increasing concentration of CSE (x axis) is plotted. Percent cell viability represents (CSE-exposed cells/non-CSE-exposed cells) $\times 100$ for each siRNA species. A significant increase in toxicity of the smoke-treated HBE1 cells is evident after SCAL1 knockdown, as noted by the shift to the left of the viability curve. This increase in toxicity was significant for both siRNAs, and was statistically significant for the higher doses at 20%, 25%, and 30%. * $P < 0.05$ ($n = 5$). Error bars represent the SEMs. Rel. Exp., relative expression.

2 sequence versus the siSCAL1-1 sequence. Because the potentiation in toxicity to cigarette smoke occurs after the knockdown of SCAL1 by both siRNA species, these results are unlikely attributable to off-target effects. In summary, these results strongly suggest that SCAL1 plays a vital role in mediating some of the cytoprotective functions of NRF2 toward the oxidative and electrophilic stress triggered by cigarette smoke exposure.

DISCUSSION

In this report, we describe our initial characterization of a novel lncRNA, SCAL1, that is induced by cigarette smoke in airway

epithelial cells. This inducibility by cigarette smoke appears to be related to the transcription factor NRF2, and is seen both *in vitro* and *in vivo*. A putative NRF2 binding site was identified by ChIP assays, suggesting this lncRNA is transcriptionally regulated by NRF2. This lncRNA is also elevated in many lung cancer cell lines, and appears to play a functional role in cytoprotection against cigarette smoke-induced toxicity. Because NRF2 has been known to play a protective role in this process, our results here suggest that SCAL1 may be a novel key downstream mediator of NRF2 in regulating the genes responsible for oxidative stress protection.

Whereas micro-RNAs have long been known to play important roles in regulating gene expression at the posttranscriptional level, lncRNAs have been increasingly recognized as another class of regulator RNA molecules that function primarily at the transcriptional level. Despite rapidly accumulating data in this area, there have been no reports, to our knowledge, of a lncRNA that is regulated by the transcription factor NRF2 or that may mediate protection against cigarette smoke-induced toxicity. Although NRF2 has been recognized as a transcription factor activated in response to various environmental and chemical stressors, its cytoprotective function has largely been ascribed to its direct transcriptional regulation of numerous antioxidant coding genes, such as heme oxygenase 1, NAD(P)H:quinone reductase (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC), and thioredoxin. Its direct regulation of these genes has been well established, but whether NRF2 can regulate other vital coding genes indirectly through noncoding RNAs remains unclear. Recently, various micro-RNA species have been identified to regulate NRF2 concentrations. No lncRNAs have yet been identified that are regulated by NRF2, although several well-characterized lncRNAs such as PANDA and lincRNA-p21 have been found to be transcribed by p53 in response to DNA damage. Our analysis of several publically available databases suggests that cigarette smoke can alter the expression of numerous lncRNAs (Figures 1D and 1F) in airway epithelial cells. Our initial characterization of SCAL1, combined with these previous lncRNA studies, supports the idea that a functional role played by these novel RNA species may involve globally regulating large sets of genes that protect against environmental stresses. The exact mechanism of how SCAL1 mediates its protective effects remains unclear. Cigarette smoke can cause cellular damage from multiple mechanisms, including DNA damage, membrane damage from lipid peroxidation, autophagy-induced apoptosis, cellular detachment, and mitochondrial dysfunction (41–45). More specific studies in the future on SCAL1 and what possible genes it may regulate should be able to delineate which of these mechanisms it may protect against in cigarette smoke-induced cell injury. Although NRF2 plays an important role in protecting normal cells from oxidative stresses (12), it can also be hijacked by cancer cells to protect them from chemotherapeutic agents used in treatment. Because of NRF2's well recognized role in mediating chemoresistance in cancer cells, SCAL1's elevated expression in lung cancer cell lines could suggest it is one of the downstream mediators of this effect. Future studies of SCAL1 should be able to further reveal what types of genes it regulates and how it mediates some of its protection against environmental stresses such as cigarette smoke and its role in cancer.

In conclusion, our data identify a novel lncRNA, termed SCAL1, that is induced by cigarette smoke in the airways of smokers and is up-regulated in many lung cancer cell lines. Its expression is regulated by NRF2, and it plays a functional role in mediating protection against cigarette smoke-induced toxicity. Future studies will be able to delineate its precise mechanism of

action further in regulating gene expression and cytoprotection against smoke-induced toxicity.

Author disclosures are available with the text of this article at www.atsjournals.org.

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