SnoN Is a Cell Type-specific Mediator of Transforming Growth Factor-β Responses*

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The transforming growth factor-β (TGF-β) family of secreted proteins have pleiotropic functions that are critical to normal development and homeostasis. However, the intracellular mechanisms by which the TGF-β proteins elicit cellular responses remain incompletely understood. The Smad proteins provide a major means for the propagation of the TGF-β signal from the cell surface to the nucleus, where the Smad proteins regulate gene expression leading to TGF-β-dependent cellular responses including the inhibition of cell proliferation. Recent studies have suggested that a nuclear Smad-interacting protein termed SnoN, when overexpressed in cells, suppresses TGF-β-induced Smad signaling and TGF-β inhibition of cell proliferation. However, the physiologic function of endogenous SnoN in TGF-β-mediated biological responses remained to be elucidated. Here, we determined the effect of genetic knockdown of SnoN by RNA interference on TGF-β responses in mammalian cells. Unexpectedly, we found that SnoN knock-down specifically inhibited TGF-β-induced transcription in the lung epithelial cell line Mv1Lu but not in HeLa or HaCaT cells. SnoN knock-down was also found to block TGF-β-dependent cell cycle arrest in Mv1Lu cells. Collectively, these data indicate that rather than suppressing TGF-β-induced responses, endogenous SnoN acts as a positive mediator of TGF-β-induced transcription and cell cycle arrest in lung epithelial cells. Our study also shows that SnoN couples the TGF-β signal to gene expression in a cell-specific manner.

The TGF-β1 superfamily of growth factors comprises a large family of cytokines that includes the TGF-β, activins, and the bone morphogenetic proteins (1, 2). Members of this family play critical and pleiotropic roles in development and homeostasis including the regulation of cell proliferation and extracellular matrix remodeling (3–6). Dysfunction of the TGF-β and TGF-β-regulated proteins contributes to disease including cancer (7–11). Members of the TGF-β superfamily of cytokines act on responsive cells via two types of cell surface receptors containing intrinsic serine/threonine kinase activity within their intracellular domains (2, 12). The TGF-β ligands form a heteromeric complex with specific TGF-β type I and II serine/threonine kinase receptors. Within this complex the type II kinase receptor phosphorylates and thereby activates the type I receptor (13, 14). Once stimulated, the type I receptor catalyzes the phosphorylation of the receptor-regulated Smad proteins, leading to their association with the common partner protein Smad4 (12, 15). The receptor-regulated Smad protein-Smad4 complex translocates to the nucleus, where it binds Smad binding elements within promoters of TGF-β-responsive genes and regulates their transcription (16, 17). The Smads contribute to either transcriptional activation or repression of distinct genes in TGF-β-treated cells (18, 19). These responses are believed to result from the type of transcriptional coregulators with which the Smads associate in the nucleus. Together with the transcriptional coactivators p300 and CBP, the Smad complex induces gene expression (20, 21). In contrast, the Smads are thought to recruit transcriptional corepressors including 5′-TG-3′-interacting factor to inhibit transcription (18, 22).

SnoN, a novel related gene product, belongs to the ski (gloan kettering virus gene product) family of nuclear proteins (23, 24). SnoN has been suggested to exert oncogenic behavior since SnoN overexpression triggers oncogenic transformation of chick and quail embryonic fibroblasts (25). Recent studies have demonstrated that the TGF-β-regulated Smads Smad2 and Smad3 as well as common partner protein Smad4 interact with and recruit SnoN to the Smad binding element within the promoters of TGF-β-responsive genes (26, 27). Overexpressed SnoN acts as a transcriptional repressor of TGF-β-induced genes (23, 24, 26). The ability of overexpressed SnoN to block TGF-β-induced gene expression and cell cycle arrest is thought to explain why SnoN promotes cell proliferation and transformation (28). A generally held view is that SnoN inhibits the basal promoter activity of TGF-β responsive genes (22, 23). Stimulation of cells with TGF-β leads to the ubiquitination of SnoN via the Smad2/3-dependent recruitment of the ubiquitin ligase Smurf2 (29, 30) and Cdh1-anaphase-promoting complex ubiquitin ligase complex (31, 32). SnoN ubiquitination and its consequent degradation are proposed to remove SnoN-inhibition of the TGF-β signaling pathway (23, 24, 26, 33).

Although the effects of overexpressed SnoN on TGF-β signaling have been characterized, the role of endogenous SnoN in TGF-β signaling remains to be elucidated. Mice in which the SnoN gene is disrupted were reported to be embryonic-lethal, thus precluding analysis of tumorigenesis in these mice (34). Mice that were heterozygous for the disrupted SnoN gene had an increased incidence of spontaneous and carcinogenic-induced lymphomas (34). In addition, SnoN+/− T cells and
mouse embryonic fibroblasts (MEFs) displayed increased rates of proliferation as compared with the wild type T cells and MEFs, respectively. Collectively, these data suggested that SnoN inhibits cell proliferation and appear to conflict with SnoN overexpression studies (34). In contrast, results of a second SnoN knock-out mouse, in which exon 1 was deleted, did not lead to embryonic lethality and suggested that proliferation of SnoN-deficient cells was impaired due to partial increased sensitivity to endogenous TGF-β (35). The contrasting data of the two SnoN knock-out studies might have occurred due to either distinct genetic backgrounds of the mice in the two studies or documented cleaved products in the second SnoN knock-out that might inhibit SnoN-related proteins in a dominant negative manner. Therefore, the specific question of the function of endogenous SnoN in the transcriptional and biological responses of epithelial cells to TGF-β remains to be clarified.

A powerful approach to study gene function involves the use of RNA interference (RNAi) to knock-down the products of genes of interest (36–38). This method is very efficient and specific in reducing target protein levels. In general, responses can be assessed within a relatively short time after knock-down of the target protein in cells, thus obviating the time needed for genetic compensatory mechanisms to appear. In this study we used a DNA-based approach of RNAi against SnoN to characterize SnoN protein functions in TGF-β-induced responses. We show that SnoN knock-down leads to specific blockade of TGF-β-induced transcription in the cell line mink lung epithelial cell (Mv1Lu) but not in HeLa or HaCaT cells. We also demonstrate that SnoN knock-down reduces the ability of TGF-β to induce cell cycle arrest in Mv1Lu cells. These data indicate that endogenous SnoN acts in a cell type-specific manner to mediate TGF-β transcriptional responses and thereby contributes to TGF-β-dependent inhibition of proliferation in epithelial cells. Our findings suggest that SnoN may, thus, exert tumor suppressive effects in specific epithelial cells.

EXPERIMENTAL PROCEDURES

Plasmids Construction—The U6/enhanced green fluorescent protein (EGFP) plasmid was constructed by subcloning a CMV-EGFP-PCR fragment amplified from a modified pCMV-EGFP vector into pBS/6 vector using convenient restriction sites. The SnoN RNAi plasmids U6/snoi-1/EGFP and U6/snoi-2/EGFP were constructed by ligating double-stranded DNA with each strand comprising 22-nucleotide-inverted SnoN sequences flanked by a 6-nucleotide sequence into digested U6/EGFP vector (see Fig. 1A and Refs. 39 and 40). The mouse SnoN 2 cDNA, which is identical to the mouse SnoN isoform except for a 138-nucleotide deletion in exon 3, was amplified from IMAGE clone 5387475 and subcloned into a CMV-driven mammalian expression vector. All constructs were confirmed by restriction digests and/or DNA sequence analyses (University of Calgary Core Sequencing Facility). The pBS/6 and U6/MEF2A RNAi plasmids were provided by A. Bonni, Harvard University, Boston, MA.

Cell Lines—Mink Lung epithelial cells (Mv1Lu) were cultured in minimum essential medium containing nonessential amino acids and 10% fetal bovine serum (FBS). HeLa, a human cervical carcinoma cell line, HaCaT, a human adult keratinocyte cell line, and 293T cells, a human kidney epithelial cell line, were cultured in Dulbecco’s modified essential medium containing 10% FBS. Cells were seeded for experiments as follows: Mv1Lu at 5 × 104, HaCaT at 8 × 104, HeLa at 6 × 104, and 293T at 1 × 105 cells/ml of media. 293T cells were transfected using the calcium phosphate method. HaCaT, HeLa, and Mv1Lu cells were transfected with the FuGENE 6.0 reagent according to the manufacturer’s instructions (Roche Applied Science).

Cell Extraction and Immunoblotting—Three days after transfection, cells were lysed as previously described (41, 42). Briefly, cells were incubated with TNTE (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) lysis buffer supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 100 μg/ml trypsin inhibitor, 100 μg/ml benzamidine chloride, 10 mg/ml antipain, and 50 μg/ml aprotinin) and phosphatase inhibitors (10 μM sodium pyrophosphate, 1 mM sodium orthovanadate, and 25 mM sodium fluoride) for 20 min at 4 °C. Lysates were then centrifuged at 15,000 × g for 10 min at 4 °C. SDS-protein sample buffer containing dithiothreitol was added to the supernatants, and the samples were boiled for 4 min. The protein content of each sample was resolved by SDS-PAGE and then transferred onto nitrocellulose membrane. The nitrocellulose membranes were immuno-blotted with antibodies against SnoN (H-317, Santa Cruz), GFP (B34, Covance, Cedarlane Laboratory Ltd), and actin (A2066, Sigma) and visualized by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham Biosciences) followed by detection using a Fluor-S Max Image (Bio-Rad).

Luciferase Reporter Assays—Cells seeded in a 24-well plate were cotransfected with 3TP-lux, ARE3-lux, or the c-Myc-lux luciferase reporter construct (0.1 μg/well), CMV-β-galactosidase vector (0.05 μg/well), and a SnoN RNAi (U6/snoi or U6/snoi/EGFP), a MEF2A RNAi (U6/MEF2A), or control (U6 or U6/EGFP) plasmid (0.05–0.1 μg/well) using the FuGENE 6.0 transfection reagent. A cDNA encoding the forkhead-containing transcription factor FoxH1 (FAST2) in a mammalian expression vector was included in all the ARE3-lux reporter transfections (43). One day post-transfection cells were incubated with 0.2% FBS-containing medium (for the 3TP-lux and ARE3-lux transfections) or 10% FBS containing medium (for the c-Myc-lux transfections) alone or together with 100 pM TGF-β (R&D Systems) for 20 h at 37 °C. 10-μl aliquots of cell lysates were assayed for luciferase activity using the beetle luciferin substrate (Promega) and measuring the light emitted using a Berthold Luminometer. β-Galactosidase expression in lysates was assayed using O-nitrophenyl-β-d-galactopyranoside as the substrate. Arbitrary luciferase activity (expressed in relative light units) was normalized to β-galactosidase activity to control for variations in transfection efficiency. Each experimental condition was carried out in triplicate, and the data are presented as the mean (±S.E.) except where indicated. Experiments were repeated at least three times.

Reverse Transcription-PCR—Mv1Lu cells were lysed using TRIzol lysis reagent (TRIZol; Invitrogen) followed by total RNA extraction and concentration using chloroform and isopropanol/ethanol, respectively. Poly(A)+cDNA was generated by subjecting the mink RNA to reverse transcription using the reverse transcriptase SuperScript II (Invitrogen) and digoxIDT-labeled (Amersham Biosciences) as the primer. Partial minus SnoN cDNA fragments corresponding to nucleotides 540–1000 of the mouse SnoN mRNA were PCR-amplified using the poly(A)+cDNA template and gene-specific SnoN oligonucleotides, selected for a high percentage of interspecies homology, as the primers. Forward primer 1 (5’TGAATTGGAAGAGAAAAGAAGCTC-3’), forward primer 2 (5’TGCGGAAAAGAAGGAC-3’), and the reverse primer (5’-GCTA-ACTCTCCTCACCTTTT-3’) corresponding to nucleotides 460–483, 524–544, and 1034–1056 of the mouse SnoN mRNA, respectively, were used for the amplification of the minus snoN cDNA.

Cell Cycle Analysis—Mv1Lu cells were seeded in a 100-mm dish and transfected with 10 μg/dish of the U6/EGFP control or U6/snoi-2/EGFP vector using the FuGENE 6.0 transfection reagent. Approximately 20 h later, cells from each transfection were trypsinized, diluted 2-fold, and subcultured equally into 2 × 100-mm dishes. The following day cells were preincubated for 4 h in 0.2% FBS containing medium and then were either left untreated or stimulated for 18 h with 100 pM TGF-β in 10% FBS-containing medium. Cells were harvested, fixed with 1% paraformaldehyde, permeabilized using 100% ethanol, and incubated with a propidium iodide (PI; Sigma)-RNase A (Roche Applied Science) solution to label total DNA content. Single cells were then analyzed for EGFP and PI-derived fluorescence by fluorescence-activated cell sorting (FACS) using a FACSCan (BD Biosciences) (The University of Calgary Flow Core Facility). Data acquisition was carried out using CellQuest Pro, and the relative distribution of cells in the different phases of the cell cycle was analyzed using Modfit LT (Verity Software House Inc.).

RESULTS

Knock-down of SnoN by RNA Interference—The finding that SnoN is ubiquitinated by the TGF-β-induced Smad2/3-Smurf2 or Smad2/3-Cdh1-anaphase-promoting complex ubiquitin ligase complexes suggests that SnoN is an important target of TGF-β-induced signaling. Because overexpressed SnoN displays oncogenic properties in cells, one interpretation of these signaling studies is that endogenous SnoN might impinge negatively on TGF-β responses, including importantly TGF-β-inhibition of cell cycle progression, and that TGF-β triggers the
ubiquitination and consequent degradation of SnoN to relieve SnoN-inhibition of TGF-β-induced signaling and cellular responses (23, 24). Although the results of overexpressed SnoN are consistent with this hypothesis, the physiological role of SnoN in TGF-β-induced responses remained to be elucidated.

To characterize SnoN function in TGF-β-induced responses, we used a plasmid DNA-based template method of RNAi because this method of gene silencing provides specific and efficient means of knocking-down target genes of interest (38, 44–46). We generated a construct containing a polymerase III RNA U6 promoter, driving the expression of a short hairpin (hp) RNA against SnoN (Fig. 1 A and B, and Refs. 39, 47, and 48). This snoi hpRNA transcript was designed to contain a 22-base pair siRNA stem with the two strands joined by a loop (Fig. 1, A and B). To aid in visualizing snoi hpRNAs-expressing cells, we inserted an EGFP cDNA driven by the CMV promoter into the pBS/U6 vector (“Experimental Procedures” and Ref. 46). Using this modified vector (U6/EGFP), we generated two SnoN RNAi plasmids, U6/snoi-1/EGFP and U6/snoi-2/EGFP (Fig. 1 B). The snoi-1 and snoi-2 hpRNAs were designed to target homologous sequences of the human and mouse SnoN mRNA, respectively, and are different from each other by three nucleotides.

To determine the effectiveness and the specificity of the SnoN RNAi plasmids, we carried out immunoblotting analysis of lysates of 293T cells that were transfected with the human or mouse SnoN expression vector alone or together with a SnoN RNAi plasmid or control U6 vector (Fig. 1 C). For mouse SnoN, we used an expression vector encoding the alternatively spliced isoform SnoN2 that is the most abundant isoform in mouse and that is identical to SnoN1 except for the absence of nucleotides 1280–1417 (49). In these experiments we found that the ex-
Fig. 3. Suppression of TGF-β-induced transcription by SnoN RNAi in Mv1Lu cells. A, lysates of Mv1Lu cells transfected with the 3TP-lux reporter and β-galactosidase constructs together with control (U6/EGFP) or a SnoN RNAi (U6/snoi/EGFP) plasmid and either left untreated or treated for 20 h with TGF-β were subjected to luciferase and β-galactosidase assays (see “Experimental Procedures”). i shows the basal (-TGF-β) and TGF-β-induced (+TGF-β) luciferase values normalized to the β-galactosidase values (relative light units (RLU)) from a single experiment. ii shows TGF-β-induced luciferase values relative to the basal luciferase values (TGF-β-dependent fold induction) averaged from five experiments. B, specificity of SnoN RNAi suppression of TGF-β-induced transcription. Mv1Lu cells were transfected with the 3TP-lux reporter and SnoN RNAi constructs together with plasmids encoding snoi hpRNAs (U6/snoi-2), MEF2A hpRNAs (U6/Mef2ai), or the control U6 vector and processed as described in A. C, rescue by SnoN-Res of SnoN RNAi-induced inhibition of TGF-β transcription. Mv1Lu cells were transfected with the 3TP-lux reporter and SnoN RNAi constructs together with the control (U6/EGFP) or an RNAi plasmid (U6/snoi-2/EGFP) along with a mammalian expression vector encoding an RNAi-resistant form of SnoN cDNA (SnoN-Res) or the empty control vector (pCMV6). The SnoN-Res construct and the corresponding control were transfected into cells at a final concentration of 0.8 μg/μl. Cells were either left untreated or treated
expression of snoi-1 hpRNAs ablated the expression of human SnoN but had little effect on the protein levels of mouse SnoN protein (Fig. 1C, upper panel). On the other hand, the expression of snoi-2 hpRNAs led to a greater than 90% reduction of the mouse SnoN gene product but only modestly reduced the level of human SnoN (Fig. 1C, upper panel). By contrast to the robust knock-down effects of snoi-1 and snoi-2 hpRNAs on human and mouse SnoN protein, respectively, SnoN RNAi had little effect on coexpressed EGFP or endogenous actin protein (Fig. 1C, middle and lower panels). Together, these results indicate that SnoN RNAi leads to the effective and specific knock-down of exogenous SnoN protein.

We next determined the effect of SnoN RNAi on the level of endogenous SnoN protein in the mink lung epithelial cell line Mv1Lu, which has been widely used to characterize TGF-β responses. First, we determined if mink SnoN might be the target of snoi-1 or snoi-2 hpRNAs. Because the mink SnoN gene sequence was not available, we carried out reverse transcription-PCR experiments using Mv1Lu-derived mRNA and appropriate SnoN primers (“Experimental Procedures”) to determine the homology between the designed hpRNAs and the target mink SnoN sequences. We isolated and sequenced three amplified fragments of SnoN cDNA containing the sequence of interest using a Mv1Lu poly(A)-enriched cDNA as the template. Through sequence alignment of the mink, mouse, and human SnoN we found that the 22 nucleotides of mink SnoN corresponding to human nucleotides 954–977 differ by only one nucleotide from mouse SnoN (Fig. 2A). Thus, we surmised that mink SnoN should be targeted more effectively by snoi-2 than snoi-1 hpRNAs. To test this idea, we transfected Mv1Lu cells with U6/GFP or the U6/snoi/EGFP plasmids. Transfected Mv1Lu cells were separated from non-transfected cells by fluorescence-activated cell sorting or FACS based on EGFP expression. Lysates of sorted EGFP-expressing cells were then analyzed for endogenous SnoN protein steady-state levels by SnoN-immunoblotting. Based on the results of two separate experiments, snoi-2 hpRNA expression reduced the level of endogenous SnoN protein by ~40–60% as compared with that of EGFP control (Fig. 2B). On the other hand, snoi-1 hpRNAs expression appeared to reduce endogenous SnoN protein by only 10% (Fig. 2Bi). Therefore, these experiments indicate that SnoN RNAi triggered a significant knock-down of endogenous SnoN protein in Mv1Lu cells, with snoi-2 hpRNAs demonstrating greater efficacy than snoi-1 hpRNA in inducing knock-down of endogenous SnoN in these cells.

SnoN Knock-down Blocks TGF-β Transcriptional Responses in Mv1Lu Cells—Having characterized the means to acutely knock-down SnoN in cells, we next asked if SnoN knock-down alters TGF-β responses in cells. We first determined the effect of SnoN RNAi on TGF-β transcriptional responses in Mv1Lu cells. Based on previous studies in which the overexpression of SnoN was found to inhibit the Smad signaling pathway and TGF-β-inhibition of cell proliferation (26, 31), we performed these experiments with the intention of testing the hypothesis that knock-down of SnoN might enhance Smad-dependent transcription.

One of the most widely used reporters to measure TGF-β responses in cells is the 3TP-lux reporter gene that contains TGF-β-responsive elements from the promoter of the plasminogen activator inhibitor-1 gene driving the expression of the firefly luciferase gene (50). We transfected Mv1Lu cells with the 3TP-lux reporter gene together with a SnoN RNAi plasmid or the control U6 vector and with a β-galactosidase expression plasmid to serve as an internal control for transfection efficiency. After transfection, cells were left untreated or stimulated with TGFβ (100 pM for 20 h). We measured the effect of SnoN knock-down on both basal (− TGF-β) and TGF-β-induced (+ TGF-β) 3TP-Lux reporter gene activity as shown in Fig. 3Ai. In addition, the effect of SnoN RNAi on TGF-β-induced 3TP-Lux reporter gene activity relative to the basal luciferase activity (TGF-β-dependent fold induction) compiled from several experiments is shown in Fig. 3Aii. As expected, the EGFP-expressing control cells displayed low levels of basal luciferase activity that increased dramatically upon TGF-β treatment (Fig. 3A, i and ii). SnoN knock-down had little if any effect on the basal activity of the 3TP-luciferase reporter gene (Fig. 3Ai). Surprisingly, and contrary to what might have been anticipated based on SnoN overexpression studies, SnoN knock-down significantly reduced TGF-β-induction of 3TP-lux reporter gene (Fig. 3Ai). The ability of SnoN RNAi to inhibit TGF-β-induced 3TP-lux reporter gene expression correlated directly with the extent of SnoN knock-down. Thus, although snoi-1 hpRNAs expression led to a modest reduction of around 30% in TGF-β-fold induction of the 3TP-lux reporter gene expression, the expression of snoi-2 hpRNAs inhibited TGF-β-induced transcription by 75% (Fig. 3A, i and ii). Together, these results indicate that endogenous SnoN is required for the ability of TGF-β to induce transcription in Mv1Lu cells.

To demonstrate that suppression of the TGF-β-induced transcription by SnoN hpRNAs is not due to potential nonspecific effects of double-stranded RNA, we compared the effect of expression of snoi hpRNAs with hpRNAs that target the unrelated gene encoding MEF2A protein (40) on TGF-β-dependent transcription (Fig. 3B). In contrast to the strong reduction of TGF-β-induced reporter activity by snoi hpRNAs, the MEF2A hpRNAs failed to suppress the TGF-β-induced 3TP-lux activity (Fig. 3B). These data suggest that inhibition of TGF-β tran...
scription by the SnoN RNAi is not due to the nonspecific activation of the RNAi machinery.

To establish that the SnoN RNAi inhibition of TGF-β-induced transcription is not due to off-target effects of the snoi hpRNAs expression, we performed a rescue experiment in which we tested the ability of a SnoN protein that is encoded by an RNAi-resistant form of SnoN cDNA (SnoN-Res) to reverse the effect of SnoN knock-down on TGF-β/H9252-induced transcription (Fig. 3C). In these experiments we found that SnoN-Res significantly reduced SnoN RNAi-triggered suppression of TGF-β-dependent 3TP-lux reporter gene expression (Fig. 3C). These data strongly support the conclusion that SnoN RNAi inhibits TGF-β induced transcription via the specific knock-down of endogenous SnoN rather than any potential off target effects of the snoi hpRNAs. Thus, we conclude that endogenous SnoN mediates TGF-β-induced 3TP-lux reporter gene expression in Mv1Lu cells.

SnoN Exerts Dual Effects on TGF-β-induced Transcription Depending on the Level of SnoN—Our finding that endogenous SnoN mediates TGF-β-induced 3TP-lux reporter gene expression in Mv1Lu cells appears to be paradoxical to the reported suppressive effect of overexpressed SnoN on the TGF-β-induced transcriptional response. We reasoned that this paradoxical effect might reflect a dependence on the concentration of SnoN in cells. Thus, at low levels including those that are found at physiologic concentrations, SnoN might contribute to the TGF-β response, whereas upon overexpression of large amounts, SnoN might squelch transcriptional cofactors that are required for TGF-β-induced transcription and, thus, trigger the inhibition of the TGF-β response. Consistent with this hypothesis, we found that overexpression of SnoN, achieved by transfecting Mv1Lu cells with large amounts of SnoN cDNA, led to a significant inhibition of TGF-β-induction of the 3TP-lux reporter gene in Mv1Lu cells (Fig. 4). By contrast, expression of low amounts of SnoN, by transfecting Mv1Lu cells with low amount of SnoN cDNA, remarkably potentiated the ability of TGF-β to induce the 3TP-lux reporter gene (Fig. 4). Together,
these results suggest that SnoN exerts opposing effects on TGF-β-induced transcription depending on the amount of SnoN in the cell. These results reconcile our SnoN RNAi findings and studies of SnoN overexpression.

**Endogenous SnoN Is a General Mediator of TGF-β-induced Transcription in Mv1Lu Cells**—We next tested the role of SnoN in the response of a second TGF-β-responsive promoter. In the ARE3-lux reporter gene the luciferase reporter gene is controlled by three tandem repeats of activin-responsive elements, found in the promoter of an activin-immediate early response gene in *Xenopus* (43, 51). Because activins and TGF-βs share the Smad signaling pathway to induce gene expression, this reporter gene is also widely used in studies of TGF-β transcriptional responses (27, 42, 51–54). We compared the effect of SnoN RNAi on the 3TP-lux and ARE3-lux promoter activity in the absence and presence of TGF-β stimulation (Fig. 5). Just as in the case of 3TP-luciferase activity, snoi-2 hpRNAs expression strongly suppressed TGF-β induction of the ARE3-luciferase activity (60 to 70% reduction) with little or no effect on its basal promoter activity (Fig. 5A). These results suggest that in Mv1Lu cells endogenous SnoN is a general positive mediator of TGF-β-induced transcription.

Having identified a positive role for endogenous SnoN in TGF-β-induced transcription in Mv1Lu cells, we next determined if endogenous SnoN also participates in the ability of TGF-β to repress transcription in these cells. TGF-β has been found to repress the expression of the c-Myc gene in cells via the TGF-β inhibitory elements of the c-Myc promoter (55, 56). In our experiments we confirmed that TGF-β treatment of Mv1Lu cells led to a significant suppression of a reporter gene that is controlled by the c-Myc TGF-β inhibitory element. The expression of snoi-2 hpRNAs had little effect on the percent of TGF-β repression of the c-Myc promoter even though in the same experiments snoi-2 hpRNAs robustly reduced TGF-β induction of the 3TP-lux reporter gene (Fig. 5B). Taken together, these results suggest that in Mv1Lu cells endogenous SnoN specifically mediates the ability of TGF-β to stimulate transcription.

To determine whether SnoN contributes to TGF-β-mediated transcription specifically in Mv1Lu cells or whether SnoN also contributes to TGF-β-induced transcription in other cells, we tested the effect of SnoN knockdown on TGF-β induction of the 3TP-lux reporter gene in Mv1Lu cells and in other TGF-β-responsive cells including HaCaT and HeLa cells. We found
that although SnoN knock-down inhibited TGF-β-induction of the 3TP-lux reporter gene in Mv1Lu cells (Fig. 5), SnoN knockdown failed to inhibit TGF-β-induction of the 3TP-lux reporter gene in HaCaT or HeLa cells (Fig. 6). This was not due to species differences of the cells, since neither snoi-1 nor snoi-2 reduced TGF-β-induction of the 3TP-lux reporter gene in the latter two cell lines that are of human origin. Together, these results show that SnoN knock-down inhibits TGF-β-transcriptional responses in a cell type-dependent manner.

**SnoN Knock-down Blocks TGF-β Cell Cycle Arrest of Mv1Lu Cells**—The dramatic decrease in the TGF-β-dependent transcriptional responses induced by SnoN RNAi raised the question of whether endogenous SnoN might mediate the ability of TGF-β to inhibit the proliferation of these cells.

We measured TGF-β-inhibition of Mv1Lu cell proliferation by carrying out cell cycle analysis using flow cytometry on transiently transfected cells that were either left untreated or treated for 18 h with TGF-β then fixed and labeled with PI. A first gate was set to determine the area versus width relationship of cells for the purpose of doublet discrimination (data not shown). A second gate was calculated using non-transfected cells to set the EGFP window (Fig. 7A). The data obtained from the PI-derived fluorescence (FL2-A, Fig. 7B) of EGFP gated cells (FL1-H, Fig. 7A) were then modeled to obtain the percentage of cells in the G1, G2/M phases of the cell cycle. As expected, we found that treatment of control EGFP-expressing cells with TGF-β (+ TGF-β) increased the percentage of cells accumulating in G2/M phase with a concomitant decrease in cells in S-phase as compared with the untreated control counterpart (−TGF-β) (Fig. 7B, upper two panels).
To induce SnoN knock-down, we used the RNAi plasmid snoi-2 hpRNAs since these hpRNAs triggered the effective knock-down of SnoN and inhibited TGF-β-induced transcription in Mv1Lu cells (Figs. 1–3). Remarkably, SnoN knock-down reduced the TGF-β-dependent increase in the percentage of cells accumulating in G_{1}/G_{0} (Fig. 7B, lower two panels and Fig. 7Cii). This was also reflected by a significantly lower percentage decrease in the number of cells in S-phase for the SnoN-silenced cells versus the control group (Fig. 7Cii). No evidence of cell death was found with SnoN RNAi as determined by microscopic analysis of EGFP-expressing cells and by the lack of an increase in sub-G_{1} DNA content in transfected cells by FACS analysis. Together, these results revealed that SnoN knock-down reduced the ability of TGF-β to inhibit cell cycle progression in Mv1Lu cells.

In summary, the data presented in this study show that endogenous SnoN acts in a cell type-specific manner to mediate TGF-β-induced transcription and TGF-β inhibition of epithelial cell proliferation. This function of endogenous SnoN occurs in a cell type-dependent fashion. These data, thus, point to an unexpected positive role of endogenous SnoN in TGF-β-induced biological responses.

**DISCUSSION**

In this study we have characterized a novel function for endogenous SnoN in TGF-β-induced responses. Genetic knock-down of SnoN in Mv1Lu cells revealed that SnoN plays a key role in linking the TGF-β signal to transcription. Endogenous SnoN was found to participate in TGF-β-induction of gene expression but not in TGF-β repression of transcription. In addition, SnoN mediated TGF-β-induced transcription in a cell-specific manner, contributing to the TGF-β transcriptional response in Mv1Lu cells but not in HeLa or HaCaT cells. Finally, we found that endogenous SnoN mediated the ability of TGF-β to inhibit cell cycle progression. Our findings provide support for the view that SnoN positively mediates TGF-β responses in lung epithelial cells and may, thus, exert tumor suppressor properties in these cells.

An important conclusion of our study is that endogenous SnoN impacts on TGF-β signaling in a distinct manner depending on the cell context. Our experiments reveal that endogenous SnoN plays a key positive role in TGF-β-induced transcription and cell cycle arrest in lung epithelial cells. However, in immune cells endogenous SnoN appears to counteract TGF-β-mediated responses (35). It will be interesting to determine how widespread each of these distinct functions of SnoN in TGF-β signaling is in different tissues of the body.

The finding that SnoN mediates TGF-β-induced transcription raises the important question of the mechanism by which SnoN mediates the TGF-β response. Because transcriptional coregulators typically act in concert with other proteins to activate transcription, it will be important in future studies to identify the protein complex that incorporates SnoN and that mediates the ability of TGF-β to stimulate transcription and to determine whether SnoN association with such a complex occurs in a cell-specific manner. Similarly, in future studies it will be important to determine whether the ubiquitination of SnoN, which under certain circumstances relieves SnoN inhibition of TGF-β signaling, regulates the positive role of SnoN in TGF-β-induced responses in lung epithelial cells.

Our findings indicating a key positive role for endogenous SnoN in TGF-β-induced responses contrast with results of SnoN overexpression studies, suggesting that SnoN suppresses TGF-β-induced Smad signaling and TGF-β-inhibition of cell proliferation (23, 24). The apparently paradoxical results of SnoN overexpression studies and of SnoN RNAi here can be reconciled by two possible underlying mechanisms. SnoN might interact with distinct transcriptional complexes in different cells, and some differences in endogenous and overexpressed SnoN function might result from the context of the cell as alluded to above. However, this explanation does not account for differences in the effects of endogenous and overexpressed SnoN in the same cells. Because SnoN is a transcriptional coregulator, the dose-dependent dichotomous effect of endogenous and overexpressed SnoN on transcription suggests the possibility that whereas at physiologic levels SnoN might be part of a transcriptional coactivator complex that mediates TGF-β-induced transcription, overexpressed SnoN might squelch these transcriptional coactivators and thereby inhibit the TGF-β transcriptional response. Consistent with this idea, whereas at higher concentrations overexpressed SnoN inhibits TGF-β-induced transcription, at endogenous levels or lower concentrations of expressed protein, SnoN enhances TGF-β-induced transcription. Together, these observations suggest the interesting possibility that physiologic levels of SnoN exert tumor-suppressive effects in specific cells, but upon overexpression, SnoN becomes oncogenic. Consistent with this idea, studies of heterozygous SnoN mice suggest that SnoN may act as a tumor suppressor (34), whereas in a number of studies overexpressed SnoN displays oncogenic behavior (25, 26, 28).

To sum up, our study reveals a novel role for endogenous SnoN protein as a positive mediator of TGF-β transcriptional responses and TGF-β inhibition of cell proliferation in mink lung epithelial cells. In addition, our work indicates that SnoN links TGF-β signaling to gene expression in a cell type-specific manner. A positive role of endogenous SnoN in mediating TGF-β inhibition of cell proliferation points to a possible tumor suppressor role for SnoN in certain cell types.

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