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14. ABSTRACT
Although the growth inhibitory effects of TGFβ play an important role in suppressing tumor cell proliferation early in tumorigenesis, TGFβ signaling also promotes malignant progression. SnoN and Ski negatively regulate TGFβ signaling by repressing the activity of the Smad proteins that act as effectors of the TGFβ receptor kinase complex to regulate expression of most TGFβ target genes. SnoN and Ski possess pro-oncogenic activity that promotes anchorage-independent growth of avian embryo fibroblasts. Here we confirm that expression of SnoN is elevated in human cancer cells and demonstrate that up-regulation of SnoN is necessary for the loss of anti-proliferative responses to TGFβ in two cancer cell lines. In addition, SnoN-deficient cancer cells are unable to undergo anchorage-independent growth or form tumors in nude mice. In contrast to these tumor-promoting effects of SnoN, we also show that SnoN can exert negative effects on cancer cell progression by inhibiting epithelial-to-mesenchymal transition (EMT). Cancer cells with reduced expression of SnoN exhibit increased motility, loss of cell adhesions, elevated stress fiber formation, and increased protease activity, and enhanced metastatic potential. Thus, SnoN possesses a dual role in epithelial cell tumorigenesis by both promoting the growth of tumor cells and suppressing cancer cell progression to a more invasive phenotype.

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TGF-beta signaling, oncogene, tumor suppressor, epithelial-to-mesenchymal transition (EMT)

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Introduction

TGFβ regulates a wide array of cellular activities through the Smad proteins. Upon phosphorylation by the active TGFβ receptor kinases, Smad2 and Smad3 oligomerize with Smad4, translocate into the nucleus, and regulate expression of TGFβ-responsive genes. The activity of the Smad complexes can be negatively regulated by two structurally related proteins, SnoN and Ski, which are involved in regulation of cellular transformation and differentiation. This research examines the role of SnoN and Ski in mammary epithelial cell tumorigenesis, focusing more in depth on SnoN. The work described here demonstrates that SnoN possesses both oncogenic and tumor suppressive activities in human breast cancer cells by stably reducing SnoN expression. On the one hand, SnoN can repress antiproliferative responses to TGFβ, which are frequently perturbed during early stages of tumorigenesis. Consistent with its pro-oncogenic role, SnoN expression was shown to be required for anchorage-independent growth of breast cancer cells and the formation of tumors in nude mice. In contrast to these tumor-promoting activities, SnoN can also repress epithelial-to-mesenchymal transition (EMT), thereby limiting tumor cell invasiveness and metastatic potential. SnoN-deficient cancer cells exhibit increased stress fiber formation, cell motility, and extracellular matrix production, and matrix metalloproteinase activity, contributing to a more malignant phenotype. Thus, I have identified a dual role for SnoN in the regulation of epithelial cell transformation.
Task 1. To generate breast cancer cells stably expressing SnoN and Ski small interfering RNA constructs to reduce expression of SnoN and Ski.

In order to generate breast cancer cells with reduced expression of SnoN and Ski, a small hairpin RNA (shRNA) vector targeting human SnoN was generated using the pSUPER vector system as described (Brummelkamp et al, 2002). Oligonucleotide pairs encoding shRNA against human SnoN and Ski were designed according to established guidelines using Oligoengine software. Forward and reverse primers were synthesized containing this sequence in sense and antisense orientation with an intervening linker. Primer pairs were designed to generate single-strand overhangs upon annealing that would allow the annealed duplex oligonucleotide to be cloned into BglIII and HindIII sites in the pSUPER retro puro vector. Forward and reverse primers were annealed and ligated into pSUPER retro puro vector that had been digested with BglIII and HindIII.

To introduce the shRNA vectors into breast cancer cells, transfection conditions were optimized for two breast cancer cell lines, MDA-MB-231 and MDA-MB-435 cells. Subsequently, each cell line was transfected with vectors encoding shRNA against SnoN (shSnoN) or Ski (shSki), together with the pBABE puro vector to permit selection using puromycin resistance. For each cell line, stable clones were generated in which expression of either SnoN alone, Ski alone, or both SnoN and Ski is reduced (Figure 1).

![Figure 1. Endogenous SnoN and Ski expression in breast cancer stable cell lines expressing shRNA constructs targeting SnoN, Ski, or both SnoN and Ski.](image)
Task 2. To examine the cellular effects of reducing SnoN and Ski levels on breast cancer cells.

Enhanced responsiveness to TGFβ.

I generated MDA-MB-231 and MDA-MB-435 cell lines in which expression of either SnoN alone, Ski alone, or both SnoN and Ski is reduced, and examined the effect of this reduction on TGFβ signaling responses. SnoN and Ski have been shown previously to repress TGFβ signaling responses (Stroschein et al 1999, Luo et al, 1999). Therefore, reducing the level of SnoN and Ski expression is likely to relieve this repression and allow TGFβ signaling to proceed. We first examined whether the shSnoN, shSki, and/or shSnoN+Ski cells were able to undergo TGFβ-elicited growth inhibition. Wild type MDA-MB-231 cells have completely lost any growth arrest response to TGFβ, and instead proliferate in the presence of TGFβ (Fig. 2a).

Reducing SnoN and Ski expression permits a growth arrest response to TGFβ in these cells. In MDA-MB-435 cells, which exhibit a moderate growth arrest response to TGFβ, downregulation of both SnoN and Ski expression resulted in a significant
increase in the ability to respond to TGFβ-elicited cell cycle arrest (Fig. 2b). In both breast cancer cell lines, reduction in Ski expression alone (shSki) had little, if any, effect on restoring TGFβ-induced growth inhibitory responses (Fig. 2a,b). In contrast, reducing SnoN expression alone (shSnoN) partially enhanced TGFβ-elicited growth arrest in MDA-MB-435 cells. Reduction of SnoN expression alone also had some effect in MDA-MB-231 cells, preventing their proliferation in response to TGFβ.

**Suppression of the transformed phenotype of breast cancer cells in vitro and in vivo.**

TGFβ signaling suppresses tumor growth at early stages of tumorigenesis through its ability to elicit growth arrest (Derynck et al, 2001; Siegel & Massague, 2003). Since SnoN and Ski repress TGFβ signaling, we reasoned that reducing their expression in cancer cells might diminish or reverse the transformed phenotype of these cells. To test this, we first examined the ability MDA-MB-231 breast cancer cells deficient in SnoN, Ski, or both SnoN and Ski expression to undergo anchorage-independent growth in a soft agar assay. Cells were embedded in soft agar and allowed to form colonies for approximately 3 weeks. Under these conditions, parental MDA-MB-231 cell lines formed colonies readily, whereas cells with reduced expression of SnoN and Ski were severely impaired in their growth in soft agar (Fig. 3). Interestingly, SnoN, but not Ski, appears to play a major role in anchorage-independent growth since downregulation of SnoN expression alone is sufficient to prevent anchorage-independent growth of MDA-MB-231 cells (Fig. 3). In contrast, reducing Ski expression had little impact on the ability of these breast cancer cells to form colonies in soft agar (Fig. 3). A similar pattern was observed in MDA-MB-435 cells with reduced expression of SnoN, Ski, or
both SnoN and Ski (data not shown). These results suggest that SnoN functions to promote oncogenic transformation.

MDA-MB-231 cells have been shown previously to undergo stellate growth when plated in a three-dimensional reconstituted basement membrane culture system, and this growth has been thought to reflect aspects of cellular transformation. We tested whether expression of SnoN and Ski affects the ability of MDA-MB-231 breast cancer cells to undergo stellate growth when cultured in this system. Reduction of both SnoN and Ski expression resulted in the loss of stellate projections (Fig. 4). Breast cancer cells expressing shSki exhibited partial inhibition of stellate growth, while shSnoN-expressing cells formed projections similarly to parental MDA-MB-231 cells (Fig. 4).

We next examined whether the decrease in the transforming activity of SnoN and Ski-deficient cells in culture resulted in reduced tumorigenicity in vivo. Nude mice were injected with parental cancer cell lines or those lacking SnoN and Ski expression, and tumor number and volume were measured after 8 weeks. Reduction of SnoN and Ski expression in breast cancer cells led to a significant decrease in average tumor volume, from 2 cm$^3$ in parental MDA-MB-231 cells to 0.07 cm$^3$ in cells lacking SnoN and Ski expression (Fig. 5). Downregulation of SnoN expression alone also significantly diminished the ability of breast cancer cells to form tumors in nude mice, with the average tumor volume of shSnoN-expressing cells was 0.6 cm$^3$. 

Figure 4. Three-dimensional growth of breast cancer cells with reduced expression of SnoN, Ski, or both SnoN and Ski in reconstituted basement membrane.

Figure 5. Tumor formation in athymic mice from breast cancer cells with reduced expression of SnoN, or both SnoN and Ski.
As with anchorage-independent growth, downregulation of Ski expression alone had little effect on tumorigenicity in vivo (data not shown).

From these preliminary studies in breast cancer cells with reduced expression of SnoN and Ski, it became apparent that the role of Ski in tumorigenesis was more complex than that of SnoN. SnoN expression is low in untransformed mammary epithelial cells and becomes increasingly elevated in breast cancer cells with malignant progression (Fig. 6). In contrast, Ski expression can be readily detected in untransformed mammary epithelial cells. Its expression was barely detectable in the noninvasive HMT-3522 T4 breast cancer cell line (T4), though Ski expression appeared significantly upregulated in metastatic MDA-MB-231 breast cancer cells (Fig. 6). Thus, the expression of SnoN and Ski appear to be regulated differently during malignant progression. Given these observations, and the significant role of SnoN in regulating mammary epithelial cell transformation, I chose to focus on the ability of SnoN to regulate various aspects of tumor cell progression in this study.

Enhancement of TGFβ-induced EMT.

Epithelial-to-mesenchymal transition (EMT) is a process by which tumor cells acquire the ability to invade surrounding tissues and blood vessels and undergo metastasis and is thought to be important for malignant progression in vivo. EMT is characterized by a number of morphological and biochemical changes, including increased cell motility and stress fiber formation, downregulation of adherens junctions and their affiliated proteins including E-cadherin, induction of extracellular matrix (ECM) proteins, and increased matrix metalloprotease (MMP) activity (Zavadil and Bottinger, 2005). Since SnoN potentiates oncogenic transformation and tumor
growth, we next asked whether and how SnoN affects EMT using the MDA-MB-231 cells expressing shSnoN.

We first examined whether expression of SnoN affects the motility of MDA-MB-231 cells using a wound-healing assay. A wound was created by scratching a confluent monolayer of cells with a pipet tip, and relative rates of cell motility were assessed by measuring percent closure of the wound after 48 hours of cell migration. Since the MDA-MB-231 cells already exhibit a high rate of cell migration, reducing SnoN expression only resulted in a moderate, but reproducible increase in cell motility (Fig. 7). These data, together with parallel data in lung cancer cells with reduced expression of SnoN (data not shown) suggest that SnoN functions to repress cell motility.

During EMT, many cells exhibit morphological changes that are thought to occur in part as a result of increased actin stress fiber formation (Savagner, 2001; Grunert et al, 2003). We therefore examined whether the reduction of SnoN expression affected stress fiber formation by staining cells with fluorescently labeled phalloidin. In parental MDA-MB-231 breast cancer cells, cellular actin was arranged cortically, with little or no stress fibers present (Fig. 8). As has been demonstrated previously in several cell types (Boland et al, 1996; Bhowmick et al, 2001; Shen et al, 2001), TGFβ treatment resulted in increased stress
fiber formation (Fig. 8). In shSnoN-expressing lung and breast cancer cells, actin stress fibers were observed even in the absence of TGFβ, and stress fiber formation was further enhanced upon stimulation with TGFβ (Fig. 8). Thus, downregulation of SnoN in breast cancer cells augmented actin stress fiber formation. The Rho family of small GTPases plays important roles in the regulation of cell growth, motility and actin stress fiber formation (Etienne-Manneville and Hall, 2002). Since reducing SnoN expression markedly enhanced actin stress fiber formation, we hypothesized that the enhanced stress fiber formation observed in SnoN-deficient cells may require RhoA activity. To test this, a dominant negative RhoA (RhoA T19N) was introduced into the shSnoN cells. In untransfected shSnoN MDA-MB-231 cells, actin was arranged in elongated stress fibers as observed previously (Fig. 8, 9). In contrast, cells expressing dominant negative RhoA exhibited diffuse cytoplasmic actin staining with no detectable stress fibers (Fig. 9), suggesting that RhoA activity is required for the increased stress fiber formation observed in SnoN-deficient cells.

Cofilin is an actin-severing protein that is inactivated upon phosphorylation by Lim kinase (LIMK) in a pathway that proceeds downstream of RhoA activation. Phosphorylation and inactivation of cofilin results in stabilization of actin filaments and concomitant stress fiber formation (Bamburg, 1999). Therefore, increased cofilin phosphorylation is a biochemical marker indicative of increased actin stress fiber formation. In parental breast cancer cells, TGFβ stimulation resulted in phosphorylation of cofilin (Fig. 10). In cells expressing shSnoN, the basal level of cofilin phosphorylation in untreated cells was heightened relative to parental cells, and TGFβ stimulation further increased the level of cofilin phosphorylation above that
observed in TGFβ-treated parental cells (Fig. 10). This is consistent with the observed increase in stress fiber formation in these cells.

Taken together, the RhoA pathway appears to function downstream of SnoN to mediate its effect on EMT.

Cells undergoing EMT also display increased ECM deposition as well as elevated activity of matrix metalloproteases (MMPs), for which ECM acts as a substrate. This dynamic production and degradation of ECM is thought to facilitate the movement of tumor cells during metastasis (Thiery, 2002; Grunert et al, 2003). In order to test whether SnoN expression affects the induction of ECM proteins, we examined the expression of PAI-1 in parental and shSnoN-expressing MDA-MB-231 cells in the absence and presence of TGFβ. TGFβ treatment induced a modest increase in PAI-1 expression in parental breast cancer cells (Fig. 11). This increase was significantly enhanced in shSnoN cells, suggesting that SnoN normally inhibits induction of PAI-1 by TGFβ (Fig. 11).

In situ zymography was employed to examine MMP activity in parental and shSnoN-expressing cells. Cells are plated on fluorescently labeled gelatin, which is a substrate for proteases such as MMP-2 and –9. Protease activity was assessed by quantifying the degradation of the fluorescently labeled substrate (Fig. 12). The proportion of cells exhibiting protease activity was significantly increased in shSnoN-expressing cells relative to parental cells (72% versus 18%, respectively), and this activity was suppressed by
the addition of MMP inhibitor GM6001, suggesting that the protease activity observed was specific to MMPs (data not shown). Treatment with TGFβ stimulated protease activity, as has been reported previously (Agarwal et al, 1994; Shimizu et al, 1996; Sehgal et al, 1999), and this TGFβ-induced protease activity was also markedly enhanced in SnoN-deficient tumor cells (data not shown).

![Image of MDA-MB-231 and shSnoN cells](image)

**Figure 12.** In situ zymography in MDA-MB-231 cells and their shSnoN-expressing derivatives. Cells were cultured on coverslips coated with gelatin-Alexa 488 for 20-24 h before processing for immunofluorescence microscopy. Representative in vitro zymography micrographs (left panel), and the quantified levels of protease activity (right panel) are shown for each assay.

**Task 3.** Overexpress SnoN or Ski in nontumorigenic mammary epithelial cells and examine the potentially transformed cellular phenotype.

Repeated efforts to stably overexpress either SnoN or Ski in nontumorigenic cells have proven unsuccessful. I have generated numerous SnoN and Ski vectors for mammalian expression, including constitutive as well as conditional promoter-driven expression and retroviral expression systems. Expression of SnoN or Ski appears to be toxic to untransformed cells. Twenty-four hours following transfection or infection, less than 5% of cells are positive for SnoN or Ski expression, and these cells fail to survive by forty-eight hours after transfection or infection (data not shown). Future students in the lab may continue this effort by attempting to express SnoN and Ski using an adenoviral expression system.

While carrying out these experiments in nontumorigenic mammary epithelial cells, I began investigating the intracellular localization of SnoN in untransformed versus malignant cells, which led to a separate avenue of research not delineated in the
original proposed statement of work. The findings from this study are nonetheless quite relevant to the topic of understanding the role of SnoN in mammary epithelial cell transformation. As such, I will briefly summarize the results of this work, and append the resultant published manuscript to this report (Krakowski et al, 2005). In the appended manuscript, “Cytoplasmic SnoN in normal tissues and nonmalignant cells antagonizes TGFβ signaling by sequestration of the Smad proteins,” I examined the intracellular localization of endogenous SnoN in normal and malignant tissues and found that, whereas SnoN is localized exclusively in the nucleus in cancer tissues or cells, in normal tissues and non-tumorigenic or primary epithelial cells, SnoN is predominantly cytoplasmic. Upon morphological differentiation or cell cycle arrest, SnoN translocates into the nucleus. In contrast to nuclear SnoN that represses the transcriptional activity of the Smad complexes, cytoplasmic SnoN antagonizes TGFβ signaling by sequestering the Smad proteins in the cytoplasm. Interestingly, cytoplasmic SnoN is resistant to TGFβ-induced degradation and therefore is more potent than nuclear SnoN in repressing TGFβ signaling. Thus, in this study I have identified a mechanism of regulation of TGFβ signaling via differential subcellular localization of SnoN that is likely to produce different patterns of downstream TGFβ responses and may influence the proliferation or differentiation states of epithelial cells.
Key Research Accomplishments

• Generated breast cancer cells in which endogenous SnoN, Ski, or both SnoN and Ski expression is stably reduced.

• Demonstrated that SnoN expression is required for antiproliferative responses elicited by TGFβ both *in vitro* and *in vivo*.

• Examined the level of endogenous SnoN and Ski expression in untransformed versus malignant mammary cell lines.

• Showed that SnoN-deficient breast cancer cells exhibit enhanced epithelial-to-mesenchymal transition (EMT), including increased stress fiber formation, cell motility, extracellular matrix deposition, and matrix metalloprotease activity.

• Demonstrated that the intracellular localization of SnoN is predominantly cytoplasmic in normal cells and tissues, whereas it is exclusively nuclear in breast cancer cells and tissues.

• Showed that cytoplasmic SnoN is not degraded upon TGFβ signaling and acts to repress the Smad proteins by sequestering them in the cytoplasm.
Reportable Outcomes


• Manuscript in preparation describing the dual role of SnoN in tumorigenesis, to be submitted later in 2006.


• Ph.D. degree in Molecular Cell Biology to be awarded in May, 2006.

• Cell lines developed:
  - MDA-MB-231 breast cancer cells with reduced expression of SnoN (shSnoN)
  - MDA-MB-231 breast cancer cells with reduced expression of Ski (shSki)
  - MDA-MB-231 breast cancer cells with reduced expression of SnoN and Ski (shSnoN+Ski)
  - MDA-MB-435 breast cancer cells with reduced expression of SnoN (shSnoN)
  - MDA-MB-435 breast cancer cells with reduced expression of Ski (shSki)
  - MDA-MB-435 breast cancer cells with reduced expression of SnoN and Ski (shSnoN+Ski)
Conclusion

This study examined the role of the TGFβ pathway co-repressors SnoN and Ski in tumorigenesis. Here, I confirm that expression of SnoN is elevated in human cancer cells and demonstrate that upregulation of SnoN is necessary for the loss of antiproliferative responses to TGFβ in two breast cancer cell lines. In addition, SnoN-deficient breast cancer cells are unable to undergo anchorage-independent growth or form tumors in nude mice. In contrast to these tumor-promoting effects of SnoN, I also show that SnoN can exert negative effects on cancer cell progression by inhibiting epithelial-to-mesenchymal transition (EMT). Cancer cells with reduced expression of SnoN exhibit increased stress fiber formation, cell motility, extracellular matrix deposition, and matrix metalloprotease activity. Thus, SnoN possesses a dual role in epithelial cell tumorigenesis by both promoting the growth of tumor cells and suppressing cancer cell progression to a more invasive phenotype.

Although a dual role for TGFβ in the regulation of tumorigenesis has been established in recent years, the question of whether SnoN can repress the tumor-promoting effects of TGFβ has not been investigated. In this report, we show that SnoN possesses oncogenic as well as tumor suppressive activities in human breast cancer cells. This research will contribute to our understanding of the complex role of the TGFβ signaling pathway in mammary epithelial cell carcinogenesis. Future studies examining the signaling mechanism by which SnoN regulates different stages of tumor cell progression will aid in elucidating the contribution of this protein and the related Ski protein to breast cancer etiology.
References


Appendix

Cytoplasmic SnoN in normal tissues and nonmalignant cells antagonizes TGF-β signaling by sequestration of the Smad proteins

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TGF-β is a ubiquitously expressed cytokine that signals through the Smad proteins to regulate many diverse cellular processes. SnoN is an important negative regulator of Smad signaling. It has been described as a nuclear protein, based on studies of ectopically expressed SnoN and endogenous SnoN in cancer cell lines. In the nucleus, SnoN binds to Smad2, Smad3, and Smad4 and represses their ability to activate transcription of TGF-β target genes through multiple mechanisms. Here, we show that, whereas SnoN is localized exclusively in the nucleus in cancer tissues or cells, in normal tissues and nontumorigenic or primary epithelial cells, SnoN is predominantly cytoplasmic. Upon morphological differentiation or cell-cycle arrest, SnoN translocates into the nucleus. In contrast to nuclear SnoN that represses the transcriptional activity of the Smad complexes, cytoplasmic SnoN antagonizes TGF-β signaling by sequestering the Smad proteins in the cytoplasm. Interestingly, cytoplasmic SnoN is resistant to TGF-β-induced degradation and therefore is more potent than nuclear SnoN in repressing TGF-β signaling. Thus, we have identified a mechanism of regulation of TGF-β signaling via differential subcellular localization of SnoN that is likely to produce different patterns of downstream TGF-β responses and may influence the proliferation or differentiation states of epithelial cells.

intracellular localization | signal transduction | differentiation | mammary epithelial cells

TGF-β regulates a wide array of cellular activities through the Smad proteins (1, 2). Upon phosphorylation by the TGF-β receptor kinases, Smad2 and Smad3 oligomerize with Smad4, translocate into the nucleus, and regulate expression of TGF-β-responsive genes (3–5). The activity of the Smad complexes can be negatively regulated by SnoN, which is a member of the Ski family of protooncoproteins that are involved in regulation of cellular transformation and differentiation (6, 7). In the nucleus, SnoN binds to Smad2, Smad3, and Smad4 on TGF-β-responsive promoters; also, it represses their ability to activate expression of TGF-β target genes by disrupting active heteromeric complexes of Smad2 or Smad3 with Smad4, by recruiting a transcriptional repressor complex containing N-CoR/SMRT, Sin3A, and HDAC1 and by blocking the binding of transcriptional coactivators (8–11). The ability of SnoN and Ski to antagonize TGF-β-induced growth arrest is thought to be important for their transforming activity (12).

The expression level of SnoN is subject to regulation by TGF-β. Immediately after TGF-β stimulation, SnoN is polyubiquitinated and degraded in a Smad- and proteasome-dependent manner, allowing the activation of TGF-β target genes (10, 13–16). After 2 h, TGF-β elicits a marked increase in the levels of SnoN through transcriptional activation (10). In malignant cells, SnoN expression is often elevated because of increased transcription, gene amplification, and/or protein stability (17–19). This elevated SnoN expression may be responsible for the resistance of malignant cancer cells to TGF-β-induced growth arrest. Thus, cellular levels of SnoN are intrinsically regulated, which may have a critical role in the appropriate and accurate control of TGF-β signaling.

Regulation of SnoN activity by intracellular localization has not been studied. SnoN has always been considered a nuclear protein, based on examination of ectopically expressed proteins in chicken embryo fibroblasts and tissue culture cell lines, as well as endogenous SnoN in tumor cell lines (12, 20, 21) (data not shown). Only two studies (22, 23) have reported that the localization of SnoN and the related Ski can change during malignant progression of specific types of cancer cells. However, the localization of these proteins has not been characterized carefully in normal tissues and nontumorigenic cells. The physiological significance of intracellular localization with respect to the function of SnoN also is not clear.

In this study, we examined the intracellular localization of endogenous SnoN in normal and malignant tissues and carried out mechanistic studies on the function of cytoplasmic SnoN. We show that SnoN exhibits predominantly cytoplasmic localization in nontumorigenic tissues and cells but is exclusively nuclear in malignant tissues and cell lines. Also, cytoplasmic SnoN can repress TGF-β signaling more potently than nuclear SnoN. Because TGF-β signaling has important roles in both cellular differentiation and malignant transformation, understanding the role of SnoN intracellular localization in the regulation of TGF-β signaling may provide insight into the ability of TGF-β to influence these processes in vivo.

Methods

Cell Culture and Reagents. We maintained 293T and Phoenix-Eco cells in DMEM containing 10% FBS. The Hep3B human hepatoma cell line (American Type Culture Collection) was cultured in MEM with 5% FBS. Ba/F3 pro-B cells were grown in RPMI medium 1640 with 10% FBS and 10% WEHI-conditioned medium as a source of IL-3. HMT-3522 S1 human mammary epithelial cells were propagated in chemically defined medium, as described (24). For 3D cultures, HMT-3522 S1 cells were embedded in Matrigel, as described (25). Adult human epidermal keratinocytes (HEKs) were obtained from Cascade Biologies (Portland, OR) and cultured in EpiLife serum-free medium containing EpiLife defined-growth supplement (Cascade Biologies). MDA-MB-231 (American Type Culture Collection) breast cancer cells were maintained in DMEM containing 5% FBS. A375 (American Type Culture Collection) malignant melanoma cells were cultured in DMEM containing 10% FBS. Mammary and skin tissue sections were generously provided by the University of California (San Francisco) Tissue Core and the Department of Plastic Surgery at the H´pital Saint-Louis (Paris), respectively.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HA, hemagglutinin; ECM, extracellular matrix; HEKa, adult human epidermal keratinocytes.

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Antisera against Smad3 (FL-425) and Smad4 (H552) were purchased from Santa Cruz Biotechnology. Monoclonal anti-Smad2 antibodies were purchased from BD Transduction Laboratories. Anti-Flag and anti-hemagglutinin (HA) antibodies were purchased from Sigma. Anti-phospho-Smad2 and anti-phospho-Smad3 antisera were a generous gift from Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Polyclonal antisera against SnoN are described in ref. 10. Anti-Ki67 antibody was obtained from Sigma. Alexa-fluorophore-conjugated secondary antibodies were purchased from Molecular Probes.

SnoN deletions and point mutations were generated by PCR and cloned into pCMV5b, pRK, or pBABE vectors for mammalian expression.

Transfection and Retroviral Infections. Transient transfections were performed by using Lipofectamine Plus reagents (Invitrogen) according to the manufacturer’s protocol. Ba/F3 cells stably expressing WT and mutant SnoN proteins were generated by transfecting Phoenix-Eco packaging cells with SnoN in the pMX-IREs-GFP vector. At 48 h after transfection, the viral supernatant was harvested and used to infect Ba/F3 cells with centrifugation for 2 h. The infected cells were then cultured for 48 h in complete medium before sorting for GFP-positive cells.

Immunofluorescence. Frozen tissue sections and tissue culture cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked in PBS containing 10% newborn calf serum, 1% BSA, and 0.02% Triton X-100. Transfected Flag- and HA-tagged proteins were detected by using antisera against the Flag or HA tags. Endogenous SnoN and Smad proteins were visualized with the antisera described above. For competition with the peptide antigen, anti-SnoN antibody was preincubated with the C-terminal peptide for 2 h at room temperature before being used for immunofluorescent staining. Immunofluorescence was observed with an Axiohot epifluorescence microscope or a confocal LSM 510 microscope (Zeiss).

Luciferase Assay. Hep3B cells were cotransfected with WT or mutant SnoN K30,31N and 0.5 μg of p3TP-lux. At 24 h after transfection, cells were serum-starved for 8 h and stimulated with 50 pM for 16 h, as described (10).

Growth-Inhibition Assays. We cultured 5 × 10^5 Ba/F3 cells with various concentrations of TGF-β1 for 4 days. Relative cell growth was determined by counting cells and expressing the number of TGF-β1-treated cells relative to the number of unstimulated cells.

Pulse–Chase Assays. Transfected 293T cells were starved with methionine- and cysteine-free media for 30 min, pulse-labeled with [35S]-Express (0.25 mCi/ml; 1 Ci = 37 GBq) for 30 min, and then chased with cold medium for various times before lysis. SnoN was purified by immunoprecipitation and resolved on SDS/PAGE.

Immunoprecipitation and Immunoblotting. Flag-tagged SnoN and Smad proteins were isolated from transfected 293T cells and infected Ba/F3 cell lines by immunoprecipitation with anti-Flag antibody, followed by elution with Flag peptide, as described (10). Expression of Flag-tagged SnoN or Smad proteins was detected by Western blotting with anti-Flag antibody. HA-SnoN bound to Smad proteins was visualized by immunoblotting with anti-HA antibody. HA-tagged Smad proteins that associated with Flag-SnoN were detected by immunoblotting with anti-HA antibody. Phosphorylation of Smad2 was detected by immunoblotting with anti-phospho-Smad2 antibody.

Results

SnoN Is Predominantly Cytoplasmic in Normal Human Tissues and Nontumorigenic Cell Lines. To examine the expression pattern of endogenous SnoN in normal tissues, we carried out immunofluorescent staining of normal human mammary tissue sections by using antibodies raised against a C-terminal (Fig. 1a) or N-terminal (Fig. 1b) peptide of SnoN, as well as a third antibody recognizing the C-terminal half of SnoN (data not shown) (10). SnoN was expressed in the luminal epithelial cells lining the mammary duct and was, surprisingly, predominantly cytoplasmic. Of the cells expressing SnoN, 51% exhibited exclusively cytoplasmic localization, 34% expressed both cytoplasmic and nuclear SnoN, and only 15% of cells showed exclusively nuclear SnoN. When tissue sections were stained with the C-terminal SnoN antibody in the presence of a peptide competitor, the fluorescent signal was reduced dramatically.

Fig. 1. SnoN localization varies in nontumorigenic versus malignant tissues and cell lines. Tissue sections derived from normal human mammary tissue (a–c), normal skin tissue (d), and stage II invasive ductal carcinoma (e) were stained for SnoN by using an antibody against the C (a, c–i) or N (b) terminus of SnoN. (c) The SnoN antibody was preincubated with a blocking peptide, as described in Methods. Endogenous SnoN was visualized in the nontumorigenic HMT-3522-S1 cell line (f), the invasive mammary carcinoma MDA-MB-231 cell line (g), primary HEKα (h), and the malignant melanoma A375 cell line (i). In all images, the nuclei were visualized by staining with Hoechst fluorescent dye.
observed in breast cancer cell lines and melanoma cells (Fig. 1). In cancer tissues, exclusively nuclear localization of SnoN was observed in both the cytoplasm and nucleus. [HMT3522 S1 (24) and 184 (26)] that retain the ability to be localized in at least two nontumorigenic mammary epithelial cell lines, SnoN was exclusively nuclear in 93% of cells. Thus, SnoN is predominately cytoplasmic in normal tissues and becomes exclusively nuclear in cancer cells.

We next surveyed established human and mouse cell lines to discern whether a similar pattern of SnoN localization exists in tissue culture cells. In many untransformed cell lines that have been passaged for many generations in tissue culture and have lost their functional capacities in comparison with their in vivo counterpart (such as NIH 3T3 cells), SnoN is localized in the nucleus (data not shown). However, in primary HEKa, as well as in at least two nontumorigenic mammary epithelial cell lines [HMT3522 S1 (24) and 184 (26)] that retain the ability to differentiate morphologically into acinus-like structures, localization of SnoN was observed in both the cytoplasm and nucleus (Fig. 1 f and h, and data not shown). Similar to what was observed in cancer tissues, exclusively nuclear localization of SnoN was observed in breast cancer cell lines and melanoma cells (Fig. 1 g and i, and data not shown).

Mapping of Nuclear Translocation Sequence in SnoN. To examine the function of cytoplasmic SnoN with regard to its ability to repress TGF-β signaling, we needed to generate a form of SnoN that localized exclusively in the cytoplasm. Because the mechanism that retains SnoN in the cytoplasm in normal cells is not known, we identified the nuclear localization sequence in SnoN. Deletion mutants of SnoN were generated (Fig. 2 a) and introduced into 293T cells by transfection, and their intracellular localization was determined by immunofluorescence with an anti-Flag antibody. As described in refs. 12 and 20, ectopically expressed WT SnoN was nuclear (Fig. 2 b). Deletion of the first 96 aa of SnoN resulted in exclusively cytoplasmic localization, whereas SnoN lacking the first 11 aa remained nuclear (Fig. 2 d). Mutation of lysines 16 and 17 to glutamine had no effect on nuclear localization of SnoN, whereas mutation of lysines 30 and 31 to asparagines had no effect on nuclear localization of SnoN, whereas mutation of lysines 30 and 31 to asparagines had no effect on nuclear localization of SnoN, whereas mutation of lysines 30 and 31 to asparagines had no effect on nuclear localization of SnoN, whereas mutation of lysines 30 and 31 to asparagines had no effect on nuclear localization of SnoN, whereas mutation of lysines 30 and 31 to asparagines had no effect on nuclear localization of SnoN, whereas mutation of lysines 30 and 31 to asparagines had no effect on nuclear localization of SnoN. Therefore, lysines 30 and 31 are required for the nuclear localization of SnoN, and mutation of these residues (SnoN K30,31N) allows us to examine the activity of cytoplasmic SnoN.

Cytoplasmic SnoN Is Able to Repress TGF-β Signaling. We first examined the ability of cytoplasmic SnoN to affect the transcription of TGF-β-target genes by using a luciferase reporter assay. SnoN K30,31N readily repressed TGF-β-induced transactivation when cotransfected with the p3TP-lux reporter construct into TGF-β-responsive Hep3B cells (Fig. 3 a). In multiple experiments, this

Fig. 2. Lysines 30 and 31 are required for nuclear localization of SnoN. (a) Schematic representations of SnoN deletion and point mutations. nuc, nuclear; cyto, cytoplasmic. (b) Localization of WT SnoN and SnoN mutant proteins. 293T cells were transfected with WT SnoN or the indicated SnoN mutants and stained with anti-Flag to determine the localization of ectopically expressed SnoN proteins. Nuclei were visualized by staining with Hoechst dye.

Fig. 3. Activity and properties of cytoplasmic SnoN. (a) Cytoplasmic localization of SnoN potently represses TGF-β-elicited transcripational activation. Hep3B cells were cotransfected with p3TP-lux together with empty vector, Flag-tagged WT SnoN, or SnoN K30,31N, as described in Methods. At 24 h after transfection, cells were serum-starved for 8 h and then treated with 50 pM TGF-β for 16 h before luciferase activity was measured. (b) Cytoplasmic localization of SnoN results in increased repression of TGF-β-induced growth arrest. Parental Ba/F3 cells, or Ba/F3 cells stably expressing Flag-tagged WT SnoN, SnoN 1–366, or SnoN K30,31N were treated with increasing concentrations of TGF-β and cultured for 4 days. Cell growth was calculated by cell counting and expressing the cell number as a percentage of the number of cells in unstimulated samples. SnoN expression was assessed by immunoblotting with anti-Flag antiserum. (c) Cytoplasmic SnoN is resistant to Smad3-mediated degradation in a pulse–chase assay. 293T cells were transfected with HA-SnoN and Flag-Smad3 and subjected to pulse–chase assays, as described in Methods. Smad3-bound SnoN was isolated by immunoprecipitation with anti-Flag antisera. SnoN was directly immunoprecipitated from 293T cells singly transfected with Flag-tagged SnoN (F-SnoN) as a control. (d) Cytoplasmic SnoN is resistant to TGF-β-induced degradation. Ba/F3 cells stably expressing Flag-tagged WT SnoN or SnoN K30,31N were treated with TGF-β for 1 h. SnoN levels were determined by anti-Flag immunoprecipitation, followed by immunoblotting with anti-Flag antiserum.
**Fig. 4.** Mechanism of repression of TGF-β signaling by cytoplasmic SnoN. (a) Cytoplasmic localization of SnoN does not disrupt interaction with Smad proteins. Flag-tagged Smad2, Smad3, or Smad4 (F-Smads) were cotransfected with HA-tagged WT or mutant SnoN K30,31N in 293T cells. Levels of SnoN bound to Smad proteins were assessed by blotting the anti-Flag immunoprecipitates with anti-HA antibody. (c and d) Cytoplasmic SnoN sequesters Smad proteins in the cytoplasm and prevents TGF-β-induced nuclear translocation. Hep3B cells and NIH 3T3 cells (data not shown) were transfected with HA- or Flag-tagged SnoN proteins and treated with 200 pM TGF-β for 1 h, as indicated. Localization of endogenous Smad2 (c) and Smad4 (d) was determined by immunostaining with anti-Smad2 or anti-Smad4 antibodies, respectively. SnoN-transfected cells were identified by staining with anti-HA or anti-Flag, as indicated. (e) Sequestration of Smad2 by cytoplasmic SnoN depends on SnoN expression level. Low (0.25 μg) or high (1.0 μg) levels of SnoN (K30,31N) were transfected into NIH 3T3 cells. At 48 h after transfection, cells were treated with 200 pM TGF-β before immunostaining. Transfected SnoN K30,31N was detected with anti-HA antibody, and endogenous Smad2 was stained with anti-Smad2. White arrowheads indicate transfected cells. Nuclei were visualized with Hoechst dye.

**Fig. 3.** Mechanism of repression of TGF-β-mediated degradation. The increased repressive activity of cytoplasmic SnoN may result from a higher level of expression, because the steady-state level of SnoN K30,31N in infected cells was consistently higher than that of WT SnoN, especially in the presence of TGF-β (Fig. 3b and d). Therefore, we compared the half-life of cytoplasmic SnoN with that of WT SnoN in the absence or presence of Smad3 by using pulse-chase assays. In the absence of Smad3, cytoplasmic SnoN appeared to be slightly more stable than nuclear WT SnoN (Fig. 3c). However, in the presence of Smad3, the half-life of nuclear WT SnoN was reduced dramatically as shown in refs. 10, 13, 14, and 16, whereas that of SnoN K30,31N was unaffected (Fig. 3e). This finding indicates that cytoplasmic SnoN is resistant to Smad3-mediated degradation. Similarly, TGF-β treatment resulted in degradation of nuclear WT SnoN but had no effect on the level of SnoN K30,31N (Fig. 3f). These results suggest that degradation of SnoN probably occurs in the nucleus and that cytoplasmic SnoN is resistant to TGF-β-elicited degradation, resulting in a higher level of SnoN in the presence of TGF-β and, consequently, stronger repression of TGF-β signaling.

**Cytoplasmic SnoN Sequesters Smad Proteins in the Cytoplasm to Repress TGF-β Signaling.** The identified (8–11) mechanisms of repression of TGF-β signaling by SnoN occur largely in the nucleus. To determine how cytoplasmic SnoN represses TGF-β signaling, we examined the ability of cytoplasmic SnoN to bind to Smad proteins and affect their phosphorylation. In 293T cells transfected with Flag-Smad proteins together with HA-tagged WT or cytoplasmic SnoN, cytoplasmic SnoN bound to Smad2, Smad3, and Smad4 as efficiently as WT SnoN (Fig. 4a and b). Similarly, in stable cell lines expressing WT or K30,31N SnoN, no significant difference in the binding affinity of WT versus cytoplasmic SnoN for Smad4 was detected (data not shown). Cytoplasmic SnoN also did not affect receptor-mediated phosphorylation of the receptor-regulated Smad (R-Smad) proteins. In 293T cells transfected with a SnoN construct, phosphorylation of Smad2 by the active type I TGF-β receptor, TβRI (Alk5*), was unaffected by expression of either WT SnoN or SnoN K30,31N, suggesting that cytoplasmic SnoN does not antagonize TGF-β signaling by preventing R-Smad phosphorylation (Fig. 4b). Next, we examined whether cytoplasmic SnoN affects the nuclear translocation of the Smad proteins. In untransfected cells, endogenous Smad proteins are localized throughout the cell in the absence of TGF-β and concentrate in the nucleus upon TGF-β treatment (Fig. 4c and d, and data not shown). Overexpression of WT SnoN (nuclear) caused the Smad proteins to concentrate in the nucleus. When cytoplasmic SnoN (K30,31N) was overexpressed, all three Smad proteins were retained ex-
SnoN localization is nuclear in 3D cultures and cells that have withdrawn from the cell cycle. (a) SnoN is exclusively nuclear in differentiated 3D HMT-3522 S1 (S1) cultures. S1 cells were allowed to undergo morphological differentiation in 3D cultures for 10 days. Cryosections of 3D cultures were subjected to immunofluorescent staining as described in Methods. (b) SnoN localization throughout 3D morphological differentiation. S1 cells were allowed to differentiate in 3D cultures for various lengths of time. After the indicated number of days, 3D cellular structures were subjected to immunostaining for SnoN and Ki67, as described in Methods. (c) Cell-cycle withdrawal induces nuclear localization of SnoN. S1 cells were plated in complete medium for 3 days and then placed in medium lacking EGF (starvation) to induce withdrawal from the cell cycle. After 3 days, SnoN localization was visualized by immunofluorescent staining, and cells were costained with anti-Ki67 to confirm cell-cycle withdrawal. (d) ECM signaling has no effect on localization of SnoN. S1 cells were grown in complete medium for 3 days and then stimulated for an additional 3 days by the addition of reconstituted basement membrane. SnoN localization was then determined by immunofluorescent staining. (e) Cell-cycle arrest in G1 induces nuclear localization of SnoN. S1 cells were grown in complete medium for 3 days, and then placed in medium lacking EGF (starvation) or medium containing 10 μM either LY294002 (phosphatidylinositol 3-kinase inhibitor) or SP600125 (c-Jun N-terminal kinase inhibitor). After 3 days, SnoN localization was determined by immunostaining.

Intracellular Localization of SnoN Is Altered upon Cell Differentiation. Because SnoN can localize in both the cytoplasm and nucleus, we sought to determine how this localization is regulated. We had observed that localization of SnoN in normal mammary tissue exhibited a certain degree of heterogeneity (Fig. 1a) and wondered whether this heterogeneity is related to the differentiation states of cells. To test this hypothesis, we used a tissue culture differentiation system by using S1 mammary epithelial cells. When harvested from 2D culture and embedded in Matrigel (3D), S1 cells undergo morphological differentiation to form a multicellular acinus-like structure composed of 5–10 cells positioned in a polarized, spherical arrangement (25). After 10 days in 3D culture, the acinus-like structures formed by S1 cells were cryosectioned, and localization of SnoN was examined by immunofluorescence and compared with that of S1 cells cultured in 2D. Whereas SnoN in cells grown in 2D culture is distributed throughout the cytoplasm and nucleus, it is located exclusively in the nucleus after differentiation in 3D cultures (Fig. 5a). Thus, intracellular localization of SnoN is regulated during cell differentiation.

To determine when the shift to nuclear localization occurs during differentiation, we carried out a time-course study. S1 cells were embedded in 3D Matrigel. At different time points, cells were harvested and stained for SnoN and Ki67, which is a marker for proliferating cells. SnoN was distributed in both the cytoplasm and nucleus during the first 3 days of 3D culture, similar to what was observed in undifferentiated S1 cells in 2D culture (Fig. 5b and data not shown). By day 5, localization of SnoN shifted to the nucleus, and SnoN remained nuclear thereafter (Fig. 5b). Interestingly, the transition of SnoN localization from cytoplasmic and nuclear to exclusively nuclear correlated with the loss of Ki67 expression (Fig. 5b), suggesting that localization of SnoN may be related to cell-cycle withdrawal or arrest.

Morphological differentiation of S1 cells in 3D culture is induced by signals from the extracellular matrix (ECM) and accompanied later by withdrawal of cells from the cell cycle (25). To examine which of the two processes is responsible for the nuclear localization of SnoN, S1 cells were grown in medium lacking EGF to induce growth arrest or stimulated with reconstituted basement membrane to mimic ECM signaling. S1 cells cultured for 3 days in medium without EGF resulted in virtually complete withdrawal of cells from the cell cycle, as evidenced by the absence of staining for Ki67 (Fig. 5c). Interestingly, costaining with anti-SnoN revealed a marked shift in SnoN localization from predominantly cytoplasmic to predominantly nuclear localization of SnoN, with 85% of the cells exhibiting exclusively nuclear SnoN (Fig. 5c). In contrast, stimulation by ECM for 3 days did not affect the localization of SnoN (Fig. 5d).
5d). Therefore, withdrawal from the cell cycle appears to induce a shift from cytoplasmic to nuclear SnoN localization.

To determine whether, in addition to withdrawal from the cell cycle, cell-cycle arrest in G1 may alter SnoN localization, S1 cells in 2D culture were treated with pharmacological inhibitors of phosphatidylinositol 3-kinase (LY294002) or c-Jun N-terminal kinase (SP600125) for 3 days. Under these treatments, cells were arrested in G1, as confirmed by flow cytometry (data not shown), and SnoN was found to concentrate in the nucleus, similar to what was observed during cell-cycle withdrawal (Fig. 5e). Therefore, cell-cycle withdrawal and arrest both result in the accumulation of SnoN in the nucleus.

Discussion

In this article, we demonstrate differential regulation of SnoN activity in normal versus tumor tissues and cells at the level of intracellular localization. In normal tissues and cells, SnoN is localized predominantly in the cytoplasm and becomes nuclear during morphological differentiation upon withdrawal from the cell cycle or during cell-cycle arrest. When localized to the cytoplasm, SnoN still represses TGF-β signaling by sequestering the Smad proteins in the cytoplasm to prevent their nuclear translocation. Intracellular localization of Smad signaling occurs in response to TGF-β signaling and, therefore, appears to be more potent than nuclear SnoN in antagonizing TGF-β signaling.

Whereas SnoN localization in nontumorigenic cells is subject to regulation, the localization of SnoN in tumor cells appears constitutively nuclear. The regulatory processes linking SnoN localization and cell proliferation have likely been lost in tumor cells, leading to exclusively nuclear localization in tumor cells. This phenomenon is not unique to SnoN. Differential regulation of subcellular localization in normal and tumor cells has also been reported for the c-Abl tyrosine kinase (27). c-Abl is located in both the nucleus and cytoplasm, but nuclear c-Abl is active only in cycling cells, whereas cytoplasmic c-Abl functions in quiescent cells (28, 29). In contrast, in proliferating tumor cells, the transforming forms of Abl (v-Abl and Bcr-Abl) are exclusively cytoplasmic (30, 31). Therefore, cellular processes regulating the localization of a protooncoprotein in nonmalignant cells may be quite distinct from those that regulate localization of the cognate oncoprotein in tumor cells.

To our knowledge, the mechanisms that regulate the intracellular localization of SnoN have not been identified. We speculate that SnoN may localize to the cytoplasm through association with a cytoplasmic retention protein that masks the residues required for nuclear translocation (K30 and K31) in SnoN. This protein may be degraded or inactivated, allowing SnoN to migrate into the nucleus. Alternatively, SnoN may need the help of a nuclear translocation factor that recognizes sequences surrounding K30 and K31 in SnoN to facilitate its translocation into the nucleus. This translocation factor may be absent in normal cells but up-regulated in tumor cells or in normal cells undergoing differentiation.

Although both can repress TGF-β signaling, the cytoplasmic population of SnoN differs from the nuclear SnoN in the mechanism of repression and in its ability to undergo degradation in response to TGF-β. Cytoplasmic SnoN is resistant to TGF-β-induced degradation and inhibits Smad proteins through physical sequestration. Because a fraction of the Smad proteins may be sequestered in the cytoplasm, cells expressing cytoplasmic SnoN may pose a higher threshold for activation of TGF-β target genes as well as reduce the magnitude of Smad activities in the cells. Given that different expression levels and activities of various Smads are linked to cell responses to TGF-β, the duration of downstream signaling, which in turn affects the eventual signaling specificity (32, 33), this difference in the magnitude of TGF-β signaling due to differential localization of SnoN could result in activation of a separate set of target genes and influence the decision of cells to proliferate or differentiate. In tissues that undergo repeated periodic or chronic cycles of proliferation and differentiation, such as mammary gland or skin, this regulatory mechanism could have an important role in maintaining the appropriate functional state of these epithelial cells.

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